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Original article

The effect of budesonide on the expression of Ki-67 and PCNA and the apoptotic index in dogs with inflammatory bowel disease

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Abstract

The aim of this study was to determine the effect of budesonide on the expression of Ki-67 and PCNA proliferative antigens and the apoptotic index in the course of inflammatory bowel disease (IBD) and to evaluate the applicability of these markers in monitoring IBD treatment in dogs. The experiment was performed on 28 dogs of different breeds and both sexes, with body weight of 6 to 20 kg, aged 6 to 10 years. The animals diagnosed with IBD were divided into four groups of 7 dogs each, including three experimental groups characterized by various severity of IBD and a control group. The dogs from the experimental groups were administered budesonide (Entocort, Astra-Zeneca, Sweden) in daily doses depending on body weight of animal – 1.0 mg (6-10 kg), 1.5 mg (11-15 kg) or 2.0 mg (16-20 kg) and control group dogs were orally administered empty gelatin capsules (placebo) for 30 days. The expression of Ki-67 and PCNA antigens was determined immunohistochemically, and the apoptotic index was expressed as the number of TUNEL-positive lamina propria cells in duodenal, jejunal and colonic mucosa before and after 30 days of budesonide therapy. The results of the study point to the limited applicability of Ki-67 and PCNA proliferation markers and high applicability of the apoptotic index in monitoring IBD progression and treatment in dogs. Budesonide exerted significant anti-apoptotic effects in canine patients with various severity of IBD, which indicates that next-generation glucocorticosteroids can be effectively used in the treatment of gastrointestinal diseases characterized by high values of the apoptotic index, including IBD.

Key words: dogs, inflammatory bowel disease, budesonide, Ki-67, PCNA, apoptotic index

Introduction

Canine inflammatory bowel disease (IBD) is a syndrome etiology of which has not yet been fully elucidated. Possible causes of IBD include environmental factors, bacterial infections, genetic predispositions, side effects of selected drugs, and disorders of the intestinal immune system (Fiocchi 1998, Jergens 2002, Allenspach and Gaschen 2003, German et al. 2003, Bhatia and Tandon 2005, Jergens and Simpson 2012). Some IBD therapies rely on next-generation glucocorticosteroids which exert anti-inflamma-

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tory effects by inhibiting the release of inflammatory response mediators and reactions involving cytokines (Angelucci et al. 2008, Rychlik et al. 2016). In dogs, chronic IBD can be alleviated by regulating the activity of T cells and proinflammatory/anti-inflammatory cytokines which participate in immune response in the gastrointestinal tract. Research into the effects of glucocorticosteroids on the differentiation, proliferation and apoptosis of intestinal epithelial cells contributes to the treatment of IBD.

Digestive system cells proliferate rapidly. Continuously exfoliating intestinal epithelial cells undergo rapid proliferation and apoptosis. The Ki-67 antigen and the proliferating cell nuclear antigen (PCNA) are widely used as biological markers of cell proliferation. The above markers complement conventional histopathological analyses and provide detailed information for prognosis and treatment, in particular in neoplastic diseases (Cunningham et al. 1997, Molino et al. 1997, Suwa et al. 1997, Viberti et al. 1997, Van Diest et al. 1998).

Apoptosis is a physiological process that plays an important role in the achievement and maintenance of tissue and organ homeostasis, butit is also involved in various pathological processes (Elmore 2007). Apoptosis is disrupted in many diseases, including in Crohn's disease (CD) and ulcerative colitis (UC) in humans (Sipos et al. 2002, Di Sabatino et al. 2003).

Research investigating the effect of next-generation glucocorticosteroids on the apoptosis and function of intestinal epithelium could contribute to the prevention and treatment of IBD with various severity (Jung et al. 2001). Dandrieux et al. (2008) evaluated the effect of glucocorticosteroids on lymphocyte apoptosis in dogs with mild, moderate and severe IBD and observed a highly significant decrease in lymphocyte apoptosis after 8 weeks of treatment with prednisolone, administered at 1 mg/kg BW every 12 hours. The reported decrease in the apoptotic index was correlated with a decrease in Canine Inflammatory Bowel Disease Activity Index (CIBDAI) scores (Jergens et al. 2003). Prednisolone treatment did not induce histopathological changes in the examined dogs.

In dogs, histopathological confirmation of IBD based on an analysis of tissue samples collected during endoscopy is not easy; however, a correlation between CIBDAI scores and the results of histopathological analyses of small intestinal mucosa was noted in dogs with IBD (Rychlik et al. 2012). Foci of dysplasia transforming into cancer may be observed during IBD progression. The identification of dysplastic foci in intestinal mucosal samples collected for histopathological analysis contributes to the course control of IBD before and after treatment (Riddell et al. 1983, Matkowskyj et al. 2013). For this reason, efforts are being attempted to find effective markers for detecting dysplastic foci in IBD. Immunohistochemical methods are used to identify proliferation markers such as Ki-67 and PCNA (Kullmann et al. 1996, Noffsinger et al. 1996).

There is evidence to indicate that lack of balance between apoptosis and proliferation of intestinal epithelial cells plays a key role in IBD. In humans, IBD leads to an increase in the apoptotic index and the proliferative index of lamina propria cells in the intestinal mucosa (Sipos et al. 2002, 2005). The apoptotic index is also elevated in active ulcerative colitis (UC) (Yukawa et al. 2002).

There is a general scarcity of published data on the expression of Ki-67 and PCNA proliferative antigens in dogs undergoing IBD treatment. The aim of this study was to determine the effect of budesonide on the expression of Ki-67 and PCNA and the apoptotic index in IBD and to evaluate the applicability of these markers in monitoring IBD treatment in canine patients.

Materials and Methods

Animals

The experiment was performed on 28 dogs of different breeds and both sexes, with body weight of 6 to 20 kg, aged 6 to 10 years. The dogs were patients of the Veterinary Polyclinic of the University of Warmia and Mazury in Olsztyn. The experiment was approved by the Local Ethics Committee for Animal Experimentation in Olsztyn (Resolution No. 47/2009/DTN of 24 June 2009). The animals presented symptoms of IBD, including chronic diarrhea and vomiting of various severity and frequency.

Procedures

The patients were qualified for the experiment based on the results of clinical, laboratory, endoscopic and histopathological examinations of mucosal tissue samples collected from the duodenum, jejunum and colon. Stool samples were subjected to parasitological, bacteriological and mycological analyses. All dogs had valid certificates of vaccination against contagious diseases (parvovirosis, canine distemper, adenovirus infection, leptospirosis, rabies). The concentration of canine pancreas-specific lipase (spec cPL) was determined in the SNAP spec cPL test (IDEXX), and the levels of pancreas-specific enzymes trypsin and trypsinogen (trypsin-like immunoreactivity, TLI) were de-

termined in the chemiluminescence enzyme immunoassay (CLIA) to rule out other gastrointestinal disorders involving diarrhea (acute and chronic pancreatitis, exocrine pancreatic insufficiency). Systemic disorders and diseases affecting other organs with secondary gastrointestinal symptoms were ruled out during additional laboratory and imaging tests. Antibiotic-responsive enteropathy (ARE) was ruled out by orally administering tetracycline for 4 weeks. A 6-week food challenge test (Royal Canin Hypoallergenic feed) was conducted to rule out food-responsive enteropathy (FRE). The final diagnosis of IBD and its particular degrees – mild, moderate and severe, was done based on endoscopic examination and histopathological analysis of intestinal musosa sections. The severity of IBD was evaluated according to CIBDAI and histopathological scores (Jergens et al. 2003, Day et al. 2008).

Groups

Animals diagnosed with IBD were divided into four groups of 7 dogs each, including three experimental groups with various severity of the disease and a control group. The dogs from experimental groups I (mild IBD, CIBDAI score of 4-5 points, histopathological score +), II (moderate IBD, CIBDAI score of 6-8 points, histopathological score ++) and III (severe IBD, CIBDAI score of 10-16 points, histopathological score +++) were orally administered budesonide (Entocort, Astra-Zeneca, Sweden), a synthetic glucocorticosteroid with potent local anti-inflammatory and antiallergic activity, in daily doses of 1.0 mg/animal (6-10 kg BW), 1.5 mg/animal (11-15 kg BW) or 2.0 mg/animal (16-20 kg BW) for 30 days.The control group comprised dogs with severe IBD, CIBDAI score of 9-12 points and histopathological score +++ which were orally administered an empty gelatin capsule (placebo) for 30 days. Enrollment of dogs for study groups was done according to the rules presented in former paper (Rychlik et al. 2012). In the experimental groups, budesonide doses and the duration of treatment were similar to those reported in other studies (Tumulty et al. 2004, Stroup et al. 2006, Pietra et al. 2013).

Immunohistochemical analysis and the apoptotic index

Tissue samples for immunohistochemical (IHC) analyses and determinations of the apoptotic index were obtained from all dogs (experimental and control) during gastroscopy or colonoscopy with the use of FB-24U-1 biopsy forceps with a diameter of 2.5 mm and FB-50U-1 biopsy forceps with a diameter of 3.7 mm (Olympus). IHC and apoptosis assays of lamina propria cells from duodenal, jejunal and colonic mucosa were performed before (day 0) and after (day 30) budesonide therapy. The assays were conducted at the Department of Pathological Anatomy of the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn.

Determination of Ki-67 and PCNA expression

The expression of Ki-67 and PCNA was determined according to standard IHC methods. Primary mouse monoclonal antibodies diluted 1:75 (clone MIB-1) (DAKO) and 1:200 (clone PC10) (DAKO) were used. The average number of cells with a positive color reaction was determined in microscopic specimens.

Apoptosis assay

Apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Gavrieli et al. 1992) with the use of the TACS 2TdT-DAB In Situ Apoptosis Detection Kit (Trevigen, USA). The apoptotic index was expressed as the number of TUNEL-positive cells that were stained brown.

Statistical analysis

The significance of differences between groups before and after treatment were determined with the use of Duncan's *post hoc* test at $p \leq 0.05$ (significant) and $p \leq 0.01$ (highly significant) in the Statistica 9.1 program (StatSoft Inc.).

Results

The administration of budesonide to dogs with various severity of IBD (mild, moderate, severe) for 30 days influenced the expression of Ki-67 and PCNA, and the apoptotic index of glands and villi in small and large intestinal mucosa. The observed changes in expression were non-uniform, and they were manifested by an increase or a decrease in the number of Ki-67-positive and PCNA-positive cells in patients with mild, moderate and severe IBD before and after budesonide treatment. In all experimental groups, the administration of budesonide for 30 days induced

Table 1. Average number of Ki-67-positive and PCNA-positive cells and mean values of the apoptotic index in duodenal, jejunal and colonic mucosa of dogs with mild IBD (group I) before and after budesonide treatment.

Substance			Duodenum		Jejunum		Colon	
			Group I before treatment	Group I after treatment	Group I before treatment	Group I after treatment	Group I before treatment	Group I after treatment
Ki-67	Glands	$\bar{x}$ SD	2.98571 ± 3.84596	16.34286** ± 11.54496	10.19286 ± 15.95432	10.30143 ± 8.51417	1.00000 ± 1.411559	6.99286** ± 7.02166
	Intestinal villi	$\bar{x}$ SD	2.38571 ± 1.50048	5.27857** ± 2.33949	2.77857 ± 2.63895	2.83571 ± 3.28326	na	na
PCNA	Glands	$\bar{x}$ SD	13.62857 ± 10.27906	18.78571** ± 13.22415	4.54286 ± 5.25130	8.22143** ± 6.44566	5.10000 ± 2.632648	10.43571** ± 9.16932
	Intestinal villi	$\bar{x}$ SD	21.64571 ± 16.78328	30.15000** ± 13.99402	43.26429 ± 22.34556	28.22857** ± 9.97676	na	na
Apoptosis	Glands	$\bar{x}$ SD	11.20000 ± 8.13368	6.28571** ± 6.23362	14.97143 ± 16.63878	6.94286** ± 7.47437	10.59571 ± 15.51699	1.66857** ± 0.84848
	Intestinal villi	$\bar{x}$ SD	8.26429 ± 6.29826	3.24286** ± 2.87409	23.62143 ± 15.55543	3.23571** ± 1.48513	na	na

$\bar{x}$ – mean value
SD – standard deviation
na – not analyzed
* significance at p≤0.05 – significant
** significance at p≤0.01 – highly significant

Table 2. Average number of Ki-67-positive and PCNA-positive cells and mean values of the apoptotic index in duodenal, jejunal and colonic mucosa of dogs with moderate IBD (group II) before and after budesonide treatment.

Substance			Duodenum		Jejunum		Colon	
			Group II before treatment	Group II after treatment	Group II before treatment	Group II after treatment	Group II before treatment	Group I after treatment
Ki-67	Glands	$\bar{x}$ SD	15.30000 ± 4.81940	6.40143** ± 3.88120	28.15714 ± 9.27934	19.09143** ± 13.78039	6.96714 ± 3.02694	2.28429** ± 3.32301
	Intestinal villi	$\bar{x}$ SD	2.94286 ± 1.11484	3.81429** ± 1.76463	2.88571 ± 3.60344	4.05714** ± 3.475489	na	na
PCNA	Glands	$\bar{x}$ SD	43.25714 ± 22.60817	33.68571** ± 13.29466	21.97143 ± 10.01294	21.60000 ± 9.90404	6.49857 ± 4.04469	20.30714** ± 12.32404
	Intestinal villi	$\bar{x}$ SD	48.12857 ± 16.12593	29.84286** ± 22.54853	25.50714 ± 14.08890	20.28571** ± 6.35280	na	na
Apoptosis	Glands	$\bar{x}$ SD	22.54286 ± 18.96258	22.21429 ± 19.42193	7.83571 ± 17.55313	3.88000** ± 7.65777	12.10000 ± 18.08198	15.89429** ± 22.25256
	Intestinal villi	$\bar{x}$ SD	11.41714 ± 20.30583	9.53571** ± 15.85622	22.32143 ± 15.52927	15.42143** ± 13.31878	na	na

$\bar{x}$ – mean value
SD – standard deviation
na – not analyzed
* significance at p≤0.05 – significant
** significance at p≤0.01 – highly significant

a regular and highly significant decrease in the apoptotic index of small intestinal villi. A highly significant decrease in the apoptotic index of glands in small and large intestinal mucosa was also noted in dogs with mild and severe IBD, and in jejunal mucosa in animals with moderate IBD. The only exception was a highly significant increase in the apoptotic index in the glands of colonic mucosa, whereas no changes in this index were observed in duodenal glands in patients with moderate IBD. No significant changes

Table 3. Average number of Ki-67-positive and PCNA-positive cells and mean values of the apoptotic index in duodenal, jejunal and colonic mucosa of dogs with severe IBD (group III) before and after budesonide treatment.

Substance			Duodenum		Jejunum		Colon	
			Group III before treatment	Group III after treatment	Group III before treatment	Group III after treatment	Group III before treatment	Group III after treatment
Ki-67	Glands	$\bar{x}$ SD	6.29714 ± 3.53057	19.88571** ± 14.75959	8.50571 ± 10.0127	15.76429** ± 14.63998	1.87143 ± 1.74925	3.33143** ± 2.41945
	Intestinal villi	$\bar{x}$ SD	8.78143 ± 10.62624	7.30143** ± 9.37273	2.33571 ± 1.43432	1.89286** ± 1.21738	na	na
PCNA	Glands	$\bar{x}$ SD	15.87143 ± 12.01897	33.97857** ± 16.73663	18.22857 ± 11.68796	21.29286** ± 18.19135	14.67000 ± 9.26224	15.18000 ± 9.49197
	Intestinal villi	$\bar{x}$ SD	19.21429 ± 16.57643	21.75571** ± 8.53124	23.57143 ± 12.04225	29.17143** ± 12.14355	na	na
Apoptosis	Glands	$\bar{x}$ SD	18.90000 ± 20.73984	8.57143** ± 4.46979	16.21429 ± 18.57619	7.21429** ± 7.83229	12.02000 ± 15.76200	8.61000** ± 17.76048
	Intestinal villi	$\bar{x}$ SD	17.11143 ± 19.22428	15.72857** ± 11.00530	11.98857 ± 14.84442	7.92143** ± 6.96766	na	na

$\bar{x}$ – mean value
SD – standard deviation
na – not analyzed
* significance at $p \leq 0.05$ – significant
** significance at $p \leq 0.01$ – highly significant

Table 4. Average number of Ki-67-positive and PCNA-positive cells and mean values of the apoptotic index in duodenal, jejunal and colonic mucosa of dogs with severe IBD (group IV) before and after the administration of placebo.

Substance			Duodenum		Jejunum		Colon	
			Group IV before treatment	Group IV after treatment	Group IV before treatment	Group IV after treatment	Group IV before treatment	Group IV after treatment
Ki-67	Glands	$\bar{x}$ SD	6.37237 ± 3.55867	6.39623 ± 3.58773	8.52652 ± 10.03560	8.63671 ± 10.12590	1.92118 ± 1.81653	1.98264 ± 1.85448
	Intestinal villi	$\bar{x}$ SD	8.94561 ± 10.85231	8.99813 ± 10.91371	2.56811 ± 1.56873	2.76822 ± 1.60275	na	na
PCNA	Glands	$\bar{x}$ SD	15.98251 ± 12.64821	16.01622 ± 12.93651	18.45723 ± 11.97256	18.68167 ± 11.99628	14.81000 ± 9.51294	14.95000 ± 9.56327
	Intestinal villi	$\bar{x}$ SD	19.56829 ± 16.67275	19.96522 ± 16.81265	23.58451 ± 13.05634	23.67812 ± 13.34812	na	na
Apoptosis	Glands	$\bar{x}$ SD	19.20000 ± 20.99351	19.52116 ± 21.01523	16.41413 ± 18.69217	16.76825 ± 18.76582	12.07000 ± 15.78100	12.09000 ± 15.91300
	Intestinal villi	$\bar{x}$ SD	18.65132 ± 19.56822	18.91327 ± 19.81732	11.99431 ± 14.96512	11.99832 ± 14.98461	na	na

$\bar{x}$ – mean value
SD – standard deviation
na – not analyzed

in the expression of Ki-67 and PCNA, or the apoptotic index were noted in control group dogs with severe IBD which received placebo for 30 days.

The average number of Ki-67-positive and PCNA-positive cells and the mean values of the apoptotic index in duodenal, jejunal and colonic mucosa of dogs with mild, moderate and severe IBD before and after budesonide treatment and in the control group before and after placebo administration are presented in Tables 1-4.

Discussion

The results of this study revealed considerable differences in the expression of Ki-67 and PCNA antigens and apoptotic index values in the examined sections of the gastrointestinal tract in dogs with various severity of IBD before budesonide treatment. Steady change trends in the number of Ki-67-positive and PCNA-positive cells in intestinal glands and/or villi in duodenal, jejunal and/or colonic mucosa were not observed in dogs with mild, moderate and severe IBD after 30 days of budesonide therapy. In dogs with severe IBD, the administration of placebo for 30 days did not influence the expression of Ki-67 and PCNA in intestinal villi and mucosal glands in the examined sections of the gastrointestinal tract.

There is a general scarcity of studies concerning evaluation of the effectiveness of next-generation glucocorticosteroids in the treatment of canine IBD based on analyses of Ki-67 and PCNA proliferative antigens. The identification of proliferative markers that are significantly correlated with the patients' clinical responses to IBD treatment and the determination of molecular prognostic factors could significantly contribute to the effectiveness of treatment in patients with various severity of IBD, including cases resistant to glucocorticosteroids (steroid resistance).

In human medicine, the effectiveness of cancer treatments has been studied by numerous authors based on selected proliferative markers. The effectiveness of treatments targeting inflammatory disorders in humans, such as UC or CD, has not been investigated based on intestinal analyses of proliferative markers. Numerous studies have demonstrated correlations between high values of Ki-67 and PCNA indices in tumor cells before chemotherapy and tumors' sensitivity to cytostatic treatment (Chang et al. 2000, Cleator et al. 2002). The expression of Ki-67 and PCNA antigens in sensitive cells decreased after such treatment (Bottini et al. 2001, Cleator et al. 2002). According to research, increased expression of Ki-67 and PCNA in tumor cells and the presence of tumor protein p53 (apoptotic factor) are associated with a decrease in progression free survival and overall survival (Gonzalez-Angulo et al. 2005, Urruticoechea et al. 2005). In canine IBD, the changes in Ki-67 and PCNA are not evenly distributed in the examined intestinal segments, and no clear increasing or decreasing trends are observed. This suggests that these proliferative antigens are not highly effective predictors of responses to budesonide treatment in dogs with various severity of IBD. However, the study of Carrasco et al. (2015) showed usefulness of histology followed by Ki-67 index determination and finally PCR to improve accuracy of distinguishing intestinal lymphoma from IBD in dogs.

Many gastrointestinal disorders, including IBD, are associated with increased secretion of proinflammatory cytokines (IFN-γ, TNF-α) which intensify epithelial cell apoptosis and increase the permeability of intestinal epithelium (Li et al. 2008). Budesonide inhibits the synthesis of proinflammatory cytokines and epithelial cell apoptosis in the small intestine and the colon. In this study, a highly significant decrease in the values of the apoptotic index was observed in duodenal and jejunal villi in all experimental groups after budesonide treatment. A highly significant decrease in the values of the apoptotic index was also noted in small intestinal glands in all experimental groups, and in colonic glands in patients with mild and severe IBD after 30 days of budesonide therapy. Apoptotic index values did not change in the control group receiving placebo for 30 days.

The results of this study indicate that Ki-67 and PCNA proliferative antigens determined in IHC analyses are not highly reliable predictors of canine IBD. In contrast, the apoptotic index is useful for monitoring IBD course and treatment in dogs. Budesonide exerted highly significant anti-apoptotic effects on dogs with various severity of IBD, which suggests that next-generation glucocorticosteroids can be effectively used to treat gastrointestinal diseases characterized by high values of the apoptotic index, including IBD. Budesonide treatment could be controversial in patients with alimentary tract tumors due to the drug's protective effects that could inhibit apoptosis and contribute to further tumor growth. According to some authors, IBD may be difficult to differentiate from lymphoma based on histopathological changes in intestinal mucosa (Simpson and Jergens 2011). Further research on a larger population is required to determine or confirm the applicability of selected markers for monitoring next-generation glucocorticosteroid therapy in canine IBD.

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