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SOMATOSTATIN AS AN ACTIVE SUBSTANCE OF ENTEROENDOCRINE CELLS IN THE CANINE DIGESTIVE TRACT IN PHYSIOLOGICAL CONDITIONS AND DURING INFLAMMATORY BOWEL DISEASE

S. GONKOWSKI[1], A. RYCHLIK[2] and J. CALKA[1]

[1]Department of Clinical Physiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland; [2]Department of Clinical Diagnostics, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland

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The aim of the present investigation is to examine the changes in the number of somatostatin–like immunoreactive (SOM-LI) enteroendocrine cells in various parts of the canine gastrointestinal (GI) tract during canine inflammatory bowel disease (IBD). The distribution of SOM-LI enteroendocrine cells was studied using the double-labeling immunofluorescence technique with antisera against chromogranin A (CgA; used here as a marker of enteroendocrine cells) and somatostatin (SOM). Evaluation of the number of CgA-positive cells, which also contained SOM in the mucosal layer of canine stomach, duodenum, jejunum and descending colon was based on the counting of such cells per unit area (0.1 mm^2). In physiological conditions, the number of SOM-LI enteroendocrine cells has been shown to constitute 5.30±2.07 in the stomach, 2.23±0.56 in the duodenum, 1.86±0.48 in the jejunum and 1.19±0.36 in the descending colon. Canine IBD caused an increase in the number of cells studied in the stomach (to 9.55±1.46) and the jejunum (to 3.84±1.16), while the changes observed in the duodenum and the descending colon have not been statistically significant. The obtained results suggest that SOM-LI enteroendocrine cells, as well as somatostatin, may be involved in pathological processes during canine IBD. Moreover, this study can be treated as the first step of application of SOM and/or its analogues in the treatment of canine IBD in the future.

Canine idiopathic inflammatory bowel disease (IBD) is a heterogeneous group of chronic or recurrent pathological processes within the gastrointestinal (GI) tract, mainly characterized by non-specific symptoms such as loss of appetite, vomiting, diarrhea and weight loss (1, 2). The pathogenesis of this disease remains not exactly understood and one of such problems is the participation of enteroendocrine cells and somatostatin (SOM) in pathological processes associated with canine IBD.

It is well-known that the GI tract is the largest endocrine organ in the body, which contains various classes of enteroendocrine cells distributed in the glands and epithelial cells (3), containing several biological active factors (4-7) and playing multiple roles, such as the control of peristaltic movements, secretion of gastric and intestinal glands, blood flow, regulation of feeding behavior as well as adaptive and reparative processes within the GI tract (3, 4, 8, 9).

Somatostatin is one of the substances, which is widely distributed in the gastrointestinal tract,

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Mailing address: Dr Slawomir Gonkowski,
Department of Clinical Physiology,
Faculty of Veterinary Medicine,
University of Warmia and Mazury,
Oczapowskiego Str 13, 10-957 Olsztyn, Poland
Tel.: +48 895234376 fax: +48 89 523 38 77
e-mail: slawomir.gonkowski@uwm.edu.pl

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both in the enteric nervous system and in the enteroendocrine cells (7, 10, 11). It is a well-known inhibitory factor, which reduces gastric acid release and the secretion of many gut hormones (12), as well as decreasing intestinal motility, blood flow in the GI tract and food intake (13, 14). SOM is also known as an important anti-inflammatory and anti-nociceptive agent which can reduce lymphocyte proliferation as well as the production of immunoglobulins and pro-inflammatory cytokines (15).

Therefore, the aim of the present investigation is to describe SOM-LI enteroendocrine cells in the mucosal layer of various segments of the canine digestive tract in physiological conditions as well as during canine IBD, which can produce a more complete understanding of the pathological processes of this disease.

MATERIALS AND METHODS

The present study was carried out on twelve half-breed male dogs of similar ages and weights (6-18 kg body weight, approximately 8-10 years old) divided into two experimental groups: control (C group; n=6), which contained healthy animals, and dogs with inflammatory bowel diseases [inflammatory (I); n=6]. Control animals were enrolled in this study during screening veterinary check-ups for IBD in kennels in Olsztyn and Ilawa (Poland). Dogs with IBD were qualified also from these kennels or were the patients of Veterinary Polyclinic, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland). All experiments were performed in compliance with the instructions of the Local Ethics Committee in Olsztyn (Poland) (decision number 51/2008), with special attention paid to minimizing any stress reaction.

Histopathological examinations (performed at the Laboratory of Histopathology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland) and other complex veterinary diagnostic investigations excluded any pathological states in control dogs, whereas the diagnosis of IBD in animals within group I was made based on appropriate clinical signs which are considered to be characteristic for this disease (1, 11). These symptoms included a history of clinical findings of chronic small and large intestinal diarrhea (80% of patients) and/or chronic emesis (95% of patients) as well as weight loss for a duration of at least 3 weeks, the exclusion of other chronic diseases by complete blood count, serum biochemistry, serum trypsin-like immunoreactivity (TLI), Spec cPL (canine pancreas – specific lipase) test, urinalysis, parasitic and bacterial analyses of fecal samples, abdominal ultrasonography and the presence of lymphoplasmacytic and/or eosinophilic inflammation on histopathological review of biopsies from the gastrointestinal tract. Moreover, 30% of the patients had poor coat quality. The final diagnosis was made by endoscopic (panendoscopy and colonoscopy) and histopathological examinations. Lymphocytic-plasmacytic gastroenteritis was diagnosed in all dogs of the IBD group. After tissue collection for the present study, according to the WSAVA gastrointestinal section diagnostic protocol, the animals were given fenbendazole, then oxytetracycline and hypoallergic feed for 6 weeks in order to confirmat IBD and exclusion of parasitical, infectious and food sensitivity disorders. All dogs with IBD used during the present investigation were scored based on the Canine Chronic Enteropathy Clinical Activity Index (CCECAI) to assess the severity of the disease (1), where the clinical severity is categorized by the total CCECAI score as follows: clinically insignificant (score 0–3), mild (score 4–5), moderate (score 6–8), severe (score 9–11), or very severe (score ≥12). In all animals of the I group, the severity of IBD was severe or very severe (above 9 points in CCECAI scale) and signs of the disease were present in all segments of the GI tract studied.

Three samples of the mucosal layer from the same parts of the gastric fundus, duodenum, the proximal part of jejunum and the descending colon were taken from all dogs during standard pan-endoscopy and colonoscopy using a video-endoscope and Olympus CF-Q165L (working length 1680, diameter 12.8 mm) and biopsy forceps FB-24U-1 (diameter 2.5 mm) or FB- 50U-1 (diameter 3.7 mm).

Biopsies of particular parts of the digestive tract were fixed by immersion for twenty minutes in 4% buffered paraformaldehyde (pH 7.4) prepared *ex tempore*, rinsed in phosphate buffer (0.1 M, pH 7.4, at 4° C) for 72 h and transferred into 18% phosphate-buffered sucrose, where they were kept at 4°C until sectioning (at least for 5 days). Finally, biopsies were cut with a cryostat (Microm type HM525, Walldorf, Germany) in -22°C into 10-µm thick sections.

The cryostat sections were processed for routine double-labeling immunofluorescence as described previously by Gonkowski et al. (16). Briefly, after air-drying at room temperature (rt) for 45 min, the sections were incubated with a blocking solution containing 10% normal goat serum, 0.1% bovine serum albumin, 0.01% NaN_3, Triton X-100 and thimerozal in PBS for 1 h (rt). They were then incubated (overnight; rt, in a humid chamber) with a mixture of primary antisera raised in different species and directed towards chromogranin A (CgA, mouse monoclonal, Abcam, Cambridge, UK,

used here as a marker of enteroendocrine cells according to previous studies (9, working dilution 1:1000) and somatostatin (SOM, rat monoclonal, AbD Serotec, Oxford, UK; working dilution 1:100). Complexes of primary antisera bound to appropriate antigens were visualized by incubation (1 h, rt) with species-specific secondary antisera conjugated to alexa fluor 594 or alexa fluor 488 (both from Invitrogen, USA, in a working dilution of 1:1000). Each step of immuno-labeling was followed by rinsing the sections with PBS (3x10 min, pH 7.4). The standard specificity tests of antibodies were carried out, i.e. pre-absorption of the neuropeptide antisera with appropriate antigen and omission and replacement of the primary antisera by non-immune sera were performed to test the antibodies and specificity of the method. The pre-absorption was performed as follows: biopsies of the digestive tract were incubated with "working" dilutions of primary antibodies directed towards CgA or SOM, which had been pre-absorbed for 18 h at 37°C with 20 μg of human SOM (Hölzel Diagnostika GmbH, Köln, Germany) or human CgA (Abcam, Cambridge, UK), respectively. This procedure, as well as omission and replacement of primary antisera by non-immune sera, completely eliminated specific stainings.

The evaluation of the number of the SOM-LI enteroendocrine cells within the mucosal layer of the particular fragment of the digestive tract was based on counting all cells immunoreactive both to chromogranin A and SOM per observation field (0.1 mm^2) under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. Cells were counted in 4 observation fields of 3 sections from 3 biopsies per animal (i.e. in 36 observation fields per dog) within the particular fragments of the digestive tract. To prevent double counting, the studied sections were located at least 100 μm apart from each other. The obtained data were pooled and presented as mean ±SD (standard deviation). All pictures were captured by a digital camera connected to a PC. Statistical analysis was carried out with Student's t-test (Statistica 9.0 StatSoft Inc, Tusla, Oclahoma, USA) and the differences were considered as statistically significant at $p<0.05$.

RESULTS

During the present study, SOM–positive enteroendocrine cells in the mucosal layer were observed within all segments of canine digestive tract studied both in physiological conditions and during IBD (Table I; Fig. 1; Fig. 2). In control dogs, the number of such cells fluctuated from 5.30±2.07 in the stomach (Fig. 1A) to 1.19±0.36 within the descending colon (Fig. 2CI) and clearly backward declines. Moreover, significant differences in the number of SOM-LI enteroendocrine cells were noted between particular control dogs, especially in the stomach, where the average quantity of such cells amounted from 3.31 to 8.88 per observation field.

Inflammatory bowel disease caused changes in the number of SOM-LI enteroendocrine cells, but not in all parts of digestive tract studied. Namely, an increase in the number of such cells was observed in the stomach (Table I; Fig. 1) (where these modifications were the most visible) and the jejunum (Table I; Fig. 2 B), whereas the changes within the duodenum were not statistically significant (Table 1; Fig. 2 A,C). Differences between the particular dogs in group I, as with the control animals, were also noted and their intensity was highest in the stomach, where the average number of SOM-LI enteroendocrine cells ranged from 7.97 to 12.00. In contrast to the control group, the backward decline in the number of SOM–positive enteroendocrine cells was less visible, because statistically significant

Table I. *Somatostatin – like immunoreactive enteroendocrine cells in the mucosal layer of the canine digestive tract under physiological conditions (C group) and during inflammatory bowel disease (I group).*

Part of the digestive tract	C group	I group
Stomach	5.30±2.07[a]	9.55±1.46[b]
Duodenum	2.23±0.56[c]	2.69±0.83[c]
Jejunum	1.86±0.48[d]	3.84±1.16[e]
Descending colon	1.19±0.36[e]	0.99±0.12[e]

Statistically significant data ($p<0.05$) between C and I groups, as well as between parts of the digestive tract, are marked by different letters, and non-significant data are marked by the same letters.

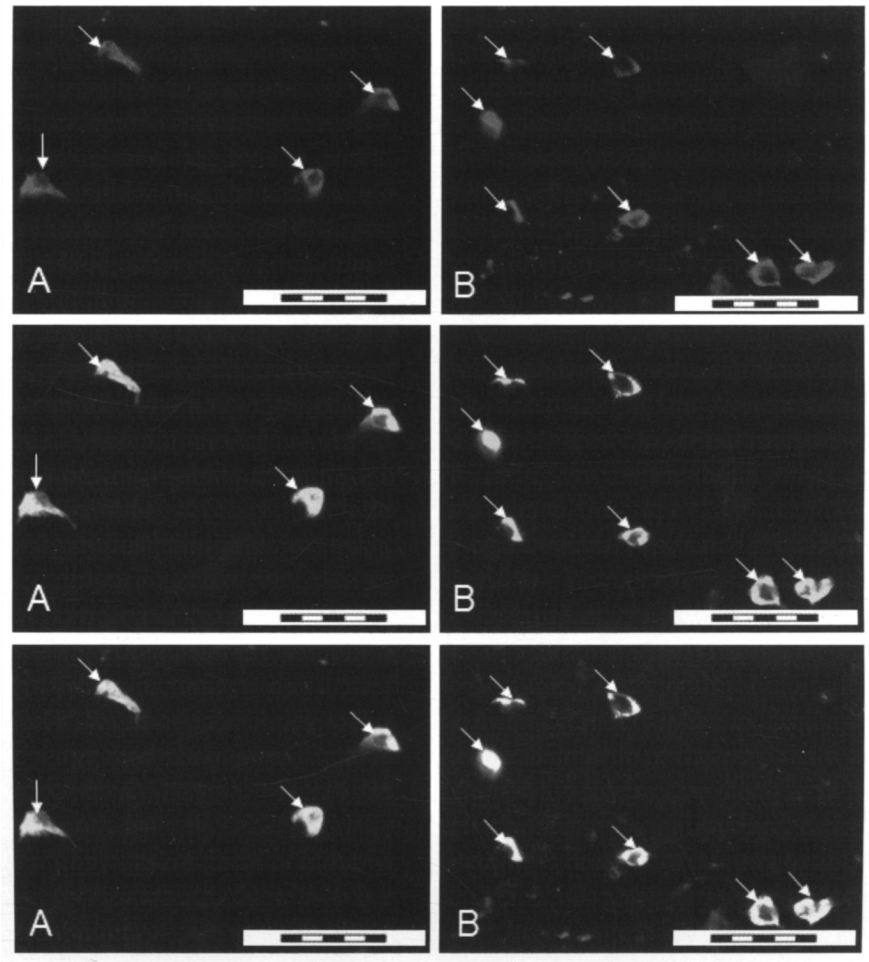

Fig. 1. *Distribution pattern of enteroendocrine cells in the mucosal layer of the canine stomach immunostained for chromogranin A (top line) and somatostatin (central line) in physiological conditions (**A**) and during inflammatory bowel disease (**B**). Co-localization of both substances is indicated with arrows. Bottom line of pictures shows overlap of both stainings. Scale bar 40 μm.*

changes were not observed between the duodenum and the jejunum (Table I).

DISCUSSION

The obtained results, showing that canine IBD causes an increase in the number of SOM-positive enteroendocrine cells in certain segments of GI tract, suggest the influence of these diseases on gastrointestinal endocrine structures and/or involving of SOM in inflammatory processes. The increase in the number of SOM-LI enteroendocrine cells, on the one hand, may arise from the augmentation of SOM synthesis as an adaptive process in the GI tract while, on the other hand, it may indicate an inhibition of SOM release from mucosal endocrine cells. Previous studies on Crohn's disease in humans, where unchanged number of SOM-positive cells accompanied by augmentation of soluble somatostatin level was studied (17), seem to support the first theory mentioned above. However, observed changes are difficult to explain and may be the result of primary or secondary (pain reaction) actions of pathogenic factors and take place in the transcriptional, translational or metabolic level. Moreover, previous studies show different changes in SOM–LI enteroendocrine cells according to various pathological conditions (4, 18-21) and these

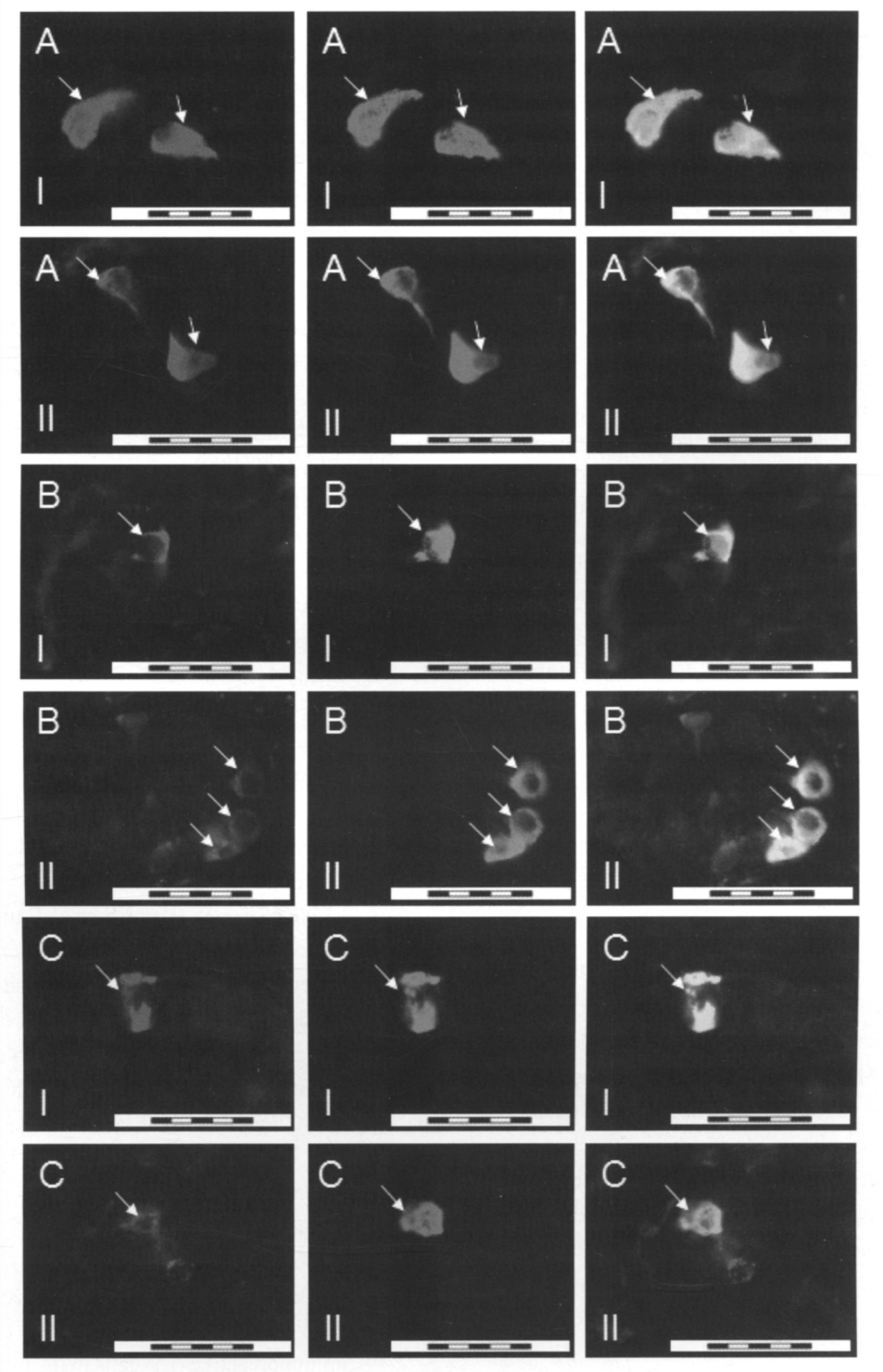

Fig. 2. *Distribution pattern of enteroendocrine cells in the mucosal layer of the canine intestine (A – duodenum, B – jejunum, C – descending colon) immunostained for chromogranin A (left column) and somatostatin (central column) in physiological conditions (I) and during inflammatory bowel disease (II). Co-localization of both substances is indicated with arrows. Right column of pictures shows overlap of both stainings. Scale bar 20 μm.*

discrepancies probably result both from interspecies differences and characters of pathological factors studied.

The involvement of SOM in pathological processes is most likely connected with its functions as an anti-inflammatory agent, which down-regulates pro-inflammatory cytokine expression and release (22), lymphocyte proliferation and immunoglobulin production (15) and plays an important role in the reduction of nociception (23).

To sum up, the results obtained in this study may suggest the participation of SOM-LI gastrointestinal enteroendocrine cells in processes connected with canine IBD, which is probably connected with functions of SOM as an anti-inflammatory factor. But confirmation of these suppositions requires further systematic, detail *in vivo* and *in vitro* investigations.

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