Galanin – immunoreactive nerve fibers in the mucosal layer of the canine gastrointestinal tract during inflammatory bowel disease

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Abstract

The effect of inflammatory bowel disease (IBD) on the density of galanin - immunoreactive (GAL-IR) nerve fibers was determined in the mucosa of canine duodenum, jejunum, and descending colon. Fiber density was evaluated by a single immunofluorescence method in biopsy specimens obtained from healthy dogs and patients with variable severity of the disease. The density of GAL-IR nerve fibers was determined by the semi-quantitative method by counting fibers in the field of view (0.1 mm²). Fiber density was higher in dogs with moderate and severe IBD than in healthy animals. The results of the study suggest that GAL present in intestinal nerve fibers could play a role in the pathogenesis and development of canine IBD.

Keywords: dogs, inflammatory bowel disease, digestive tract, nervous system, galanin, immunohistochemistry.

Introduction

Canine inflammatory bowel disease (IBD) is a group of intestinal disorders characterised by a cellular infiltration of the lamina propria of the intestinal mucosa (1, 28). The causes of IBD in humans and animals remain unknown despite extensive research into the aetiopathogenesis of the disease. Numerous authors have postulated that excessive sensitivity of gut-associated lymphoid tissue (GALT) to intestinal antigens plays a key role in the development of IBD. The sensitivity disrupts immune processes in intestinal lymphatic tissues and leads to biochemical and psychosomatic disorders. Other etiological factors include genetic traits, infections, parasites, hypoxia, stress, impaired mucosal permeability, food allergies, and adverse side-effects of certain drugs. Regardless of the etiological agent, an inflammatory cascade is triggered to release chemical mediators that damage tissues, and chemotactic factors are induced to stimulate the migration of infiltrating cells to the intestinal basement membrane (16, 20, 26). According to recent research, substances that act as neurotransmitters and/or neuromodulators within the enteric nervous system (ENS) play an important role in intestinal inflammatory processes (10, 11, 29). Galanin (GAL) is one of such substances (9, 17, 22, 24).

Galanin is a peptide composed of 29 (in human 30) amino acids, which arises from the cleavage of a 123-amino acid protein known as preprogalanin. Galanin sequence in mammals shows a very high degree of homology, which amounts over 85% among rat, mouse, swine, cattle, and human, where the first 15 amino acids from the N-terminus of GAL are identical (5). Galanin acts by three types of G-protein coupled receptors (GPCRs): GAL-1R in the central nervous system (CNS), GAL-R2 in the CNS and peripheral tissues, and GAL-R3 in peripheral tissues. GAL-2R mediates smooth muscle contractions in the small intestine (30). Previous studies have shown that galanin is widely distributed in the central and peripheral nervous systems (5, 24). It is also known that GAL is present in the digestive tract of numerous mammals, including human (9, 17, 24), and can play various functions in regulation of intestinal action. These functions depend on the species and part of the...
digestive tract. For example GAL induces the contraction of the muscles in rat ileum (7), while in the canine pylorus and ileum it displays relaxatory activity (8). Moreover, galanin regulates the secretion of gastric acid and pancreatic enzymes, as well as modulates ion channels to control the secretion of other neurotransmitters (13, 25, 27). It should be pointed out that it refers to the neuroprotective functions of GAL in the enteric nervous system. Admittedly, galanin can act as a neuronal survival-promoting factor within some parts of the peripheral and central nervous systems (15), as well as play an important roles during experimentally-induced colitis (23), suggesting its neuroprotective role within the enteric nervous system. On the other hand, previous in vitro investigations on synthetic galanin have not demonstrated such effect within porcine and ovine cell cultures of the ENS (3, 4). Studies of IBD in humans indicated an increase in the activity of GAL-R1 receptors in the colon of patients with ulcerative colitis (UC) and Crohn's disease (CD) (6). So far, changes in the expression of GAL-immunoreactive nerve fibers in dogs with IBD have not been investigated.

The objective of this study was to determine the density of GAL-positive nerve fibers in the mucosa of different sections of the gastrointestinal tract under physiological conditions and in patients with variable severity of IBD. The results would contribute to our knowledge about the function of GAL and the role of various pathological processes in IBD.

**Material and Methods**

The experiment was performed on 28 German shepherd hybrid dogs of both sexes, with body weight of 15 to 25 kg, aged 6 to 10 years. The control group consisted of seven healthy dogs qualified for the experiment based on the results of IBD screening tests conducted in a dog shelter in Olsztyn. The experimental groups comprised patients of the Veterinary Clinic of the University of Warmia and Mazury in Olsztyn. Dogs from all groups were qualified for the experiment on the basis of clinical, laboratory, and endoscopic examination results as well as histopathology of duodenal, jejunal, and colonic mucosa.

All animals with suspected IBD were subjected to biochemical, radiological, para-sitolological, bacteriological, and mycological stool tests and provocation trials to exclude other diseases associated with chronic diarrhoea. Animals diagnosed with IBD were divided into the following groups, based on their CIBDAI scores (16):

Group I – mild IBD, CIBDAI score – 4-5 points, histopathological score “+”, seven dogs;

Group II – moderate IBD, CIBDAI score – 6-8 points, histopathological score “++”, seven dogs;

Group III – severe IBD, CIBDAI score – 10-16 points, histopathological score “+++”, seven dogs.

Specimens for immunohistochemical analyses were obtained from experimental and control animals during gastroscopic or colonoscopic examinations 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). The obtained biopsy specimens (three from every section of the gastrointestinal tract) were fixed by immersion in 4% buffered paraformaldehyde solution for 15 min. The specimens were rinsed in phosphate solution (pH 7.4) for 3 d, with daily buffer replacement. They were transferred to 18% saccharose solution in phosphate buffer and stored at 4°C. An immunohistochemical staining was performed by the immunofluorescence method described by Gorkowski (12) on 10 micrometer frozen tissue sections cut in the Microm cryostat (HM525, Walldorf, Germany) and placed on gelatin coated slides. The sections were dried for 45 min at room temperature and rinsed three times in buffered NaCl solution (PBS, 0.1 mol, pH 7.4) for 15 min. They were incubated in blocking solution containing 10% goat serum, 0.1% bovine serum albumin (BSA), 0.01% NaN₃, Triton X-100, and thimerosal in PBS for 1 h, and then stored in a humidity chamber at room temperature. GAL antibodies (Rabbit, Peninsula, San Carlos, USA, cat no. RIN7153, working dilution 1:4000) were applied to the specimens after repeated rinsing in NaCl solution (PBS, 0.1 mol, pH 7.4). The sections were incubated overnight with the primary antibody, rinsed three times, and incubated with specific secondary antibody conjugated to Alexa Fluor 594 (Donkey, Invitrogen USA, working dilution 1:1000) for 1 h at room temperature. After repeated rinsing, the specimens were mounted on slides with glycerol solution and PBS (1:2; pH 7.4) and covered with coverslips. Stained sections were evaluated under the Olympus BX51 fluorescence microscope equipped with filters. Mucosal fibers were subjected to a semi-quantitative evaluation by counting the number of GAL-immunoreactive fibers in the field of view (0.1 mm²). Fibers were counted in four fields of view in three sections of every biopsy specimen from the duodenum, jejunum, and colon. In every patient, a total of 36 fields of view in every part of the intestine were evaluated per substance. The evaluated fields were separated by a minimum distance of 100 µm to avoid repeated counts. Obtained data were pooled and presented as a mean ± standard deviation (SD). Microphotographs were captured with a digital camera in the Analysis 3.0 application. The specificity of the applied sera was verified in a pre-absorption test. A synthetic peptide was added in the amount of 2 to 10 µg, to 100 µL of the antibody solution prepared in the working concentration, and the mixture was incubated for 24 h at 4°C and used to stain sections of the small and large intestines. The
tissues were incubated with the prepared antibodies to eliminate specific staining.

The significance of differences between groups was determined by the Kruskal-Wallis test at $P \leq 0.05$ (significant) and $P \leq 0.01$ (highly significant). The results were processed using the Statistica 9.1 software (StatSoft, Inc.).

Results

GAL-positive nerves were observed in all segments of the digestive tract both in control animals and in all dogs suffering from IBD. In control animals, the number of processes immunoreactive to GAL was relatively evenly distributed in each part of the intestine, and amounted to $5.3 \pm 1.9$, $5.2 \pm 2.2$, and $4.2 \pm 1.2$ per observation field in duodenal, jejunal, and colonic mucosa respectively. In dogs suffering from mild IBD (group I), a minor decrease in the number of mucosal GAL-immunoreactive fibers was observed in all segments of the digestive tract (Fig. 1) and in these patients the values in question were determined at $3.4 \pm 1.6$, $3.6 \pm 1.8$, and $3.2 \pm 0.7$ in the duodenum, jejunum, and descending colon respectively. It should be pointed out that no statistically significant differences were observed between control animals and group I.

A higher number of GAL-immunoreactive fibers were reported in duodenal, jejunal, and colonic mucosa in dogs suffering from moderate and severe IBD (groups II and III) compared to controls and group I (Fig. 1). In group II, the number of GAL-immunoreactive fibers per observation field amounted to $13.1 \pm 0.4$ in the duodenum, $14.7 \pm 1.2$ in jejunum, and $9.6 \pm 1.3$ in descending colon, and in animals affected with severe IBD (group III) these values amounted to $11.7 \pm 1.6$, $14.8 \pm 3.2$, and $8.5 \pm 2.9$ respectively. Highly significant differences in the number of GAL-immunoreactive mucosal nerves per observation field in all intestinal segments were observed between animals of group I vs. patients of groups II and III.

Highly significant differences in the number of GAL-immunoreactive fibers in duodenal, jejunal, and colonic mucosa were noted between animals of groups II and I, whereas the number of the described processes in duodenal and jejunal mucosa differed significantly between group II and control animals. Highly significant differences in the number of GAL-immunoreactive fibers in duodenal, jejunal, and colonic mucosa were observed between group III and group I patients. The obtained results are summarised in Table 1 and the density of GAL-immunoreactive intestinal mucosal fibers in healthy dogs and patients with IBD of varying severity is presented in Figs 2-4.
Table 1. The number of mucosal GAL-immunoreactive nerve fibers per observation field in the duodenum, jejunum, and descending colon in control group (C) and in dogs suffering from mild (Group I), moderate (Group II), and severe (Group III) IBD.

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>5.3 ± 1.9 (c)</td>
<td>3.4 ± 1.6 (CD)</td>
<td>13.1 ± 0.4 (aB)</td>
<td>11.7 ± 1.6 (B)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>5.2 ± 2.2 (c)</td>
<td>3.6 ± 1.8 (CD)</td>
<td>14.7 ± 1.2 (aB)</td>
<td>14.8 ± 3.2 (B)</td>
</tr>
<tr>
<td>Colon</td>
<td>4.2 ± 1.2</td>
<td>3.2 ± 0.7 (a)</td>
<td>9.6 ± 1.3 (B)</td>
<td>8.5 ± 2.9 (B)</td>
</tr>
</tbody>
</table>

\(a\) – significantly different from control group
\(b\) – significantly different from group I
\(c\) – significantly different from group II
\(d\) – significantly different from group III

Kruskal-Wallis test
\(P < 0.05\) – lowercase letters
\(P < 0.01\) – uppercase letters

Fig. 2. Distribution of GAL-immunoreactive fibers in canine duodenal mucosa: A – control group, B – group I, C – group II, D – group III (400×).

Fig. 3. Distribution of GAL-immunoreactive fibers in canine jejunal mucosa: A – group C, B – group I, C – group II, D – group III (400×).
Discussion

Galanin can exert both stimulatory and inhibitory effects on peristalsis in the muscular layer of gastric and intestinal mucosa. In dogs, galanin induces relaxation of jejunal smooth muscle cells (8). It inhibits the secretion of gastric mucosa cells and hormones - glucagon and insulin, regulates electrolyte transport across gastric mucosa, and probably demonstrates the neuroprotective effects (14, 17, 19). Benya et al. (6) observed an increase in the expression of the GAL-1R receptor and identified galanin as a secretagogue (factor stimulating enterocyte secretion). According to the cited authors, an intensified expression of GAL-1R receptors in various inflammatory states, including UC and CD, plays an important role in the pathogenesis of infectious diarrhoea. In their study on GAL-immunoreactive fibers in children suffering from ulcerative colitis, Kamińska et al. (17) reported a significant decrease in the concentrations of GAL and other neurotransmitters with protective functions, including vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP).

In our study, highly significant differences in the number of GAL-immunoreactive nerves were observed between group I vs. groups II and III in all sections of the duodenum, jejunum, and colon. Significant differences were noted between control and group II in the duodenum and jejunum. An increase in the density of GAL-immunoreactive fibers in moderate and severe IBD (Fig. 2) could be attributed to the neuroprotective effects of galanin, which protects intestinal nervous tissues against the adverse consequences of inflammation.

The role of galanin in diarrhoea can probably be attributed to the intensified expression of the GAL-1R receptor and the increased secretion of CT in inflammation (6, 22, 23). Matkowskyj et al. (23) observed in mice with induced colonic inflammation that fluid secretion in individuals genetically deprived of the GAL-1R receptor was half that noted in genetically unmodified animals. The above results could explain the higher frequency of bowel movements and watery consistency of faeces in group II and III animals. Marrero et al. (21) used galanin antibodies to reduce fluid secretions in the mouse colon, which validates the hypothesis that diarrhoea is correlated with GAL-1R expression in IBD.

Galanin could also participate in the nociceptive process in IBD. Kerr et al. (18) described the effects of endogenous galanin on central neuropathic pain after damage to peripheral tissues. Those findings could explain a decrease in activity levels, general deterioration in well-being and abdominal sensitivity to touch in group III patients.

To sum up, the obtained results strongly suggest the functions of GAL in pathological processes during IBD, although many aspects of these activities are still unknown. One of them is a slight decrease in number of GAL – positive nerves in dogs suffering from mild IBD, which can be caused by the reduction of neuronal transport of galanin from perikarya to nerve endings in the initial stage of the disease. It should be pointed out that all changes in GAL-immunoreactivity observed during the present study can result from primary (mechanisms connected with IBD-induced changes in the intestine) or secondary (for example pain) actions of the described disease and may arise from the changes at the transcriptional, translational, or metabolic and transport level. So, the observed changes can be connected with the involvement of GAL in the conducting of pain stimuli and/or regulation of fluid secretion (5, 25, 27). Moreover, an increase in GAL-immunoreactive nervous structures within the gastrointestinal tract observed during the present study and previous investigations (9) suggests the neuroprotective function of this peptide. Admittedly previous in vitro studies (3, 4) have not confirmed the direct neurotrophic properties of GAL within the ENS, but it cannot be excluded that this substance can act indirectly on enteric neurons via other neuromediators and/or neuromodulators. Nevertheless, the explanation of the exact functions of galanin during IBD requires further studies.

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References


