The influence of drug-resistant ulcerative colitis on the number of cocaine- and amphetamine-regulated transcript peptide-like immunoreactive (CART-LI) mucosal nerve fibres of the descending colon in children

Wpływ lekoopornego wrzodziejącego zapalenia jelita grubego na liczbę włókien nerwowych immunoreaktywnych wobec peptydu CART (transkryptu regulowanego przez kokainę i amfetaminę) w obrębie błony śluzowej okrężnicy zstępującej u dzieci

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Abstract

Introduction: Structures of the enteric nervous system (ENS) are able to change their functional or chemical phenotype as a result of adaptive responses to different physiological and pathological stimuli. One such factor, which changes the chemical coding of the ENS, is ulcerative colitis (UC). On the other hand the knowledge about the presence and functions of cocaine- and amphetamine-regulated transcript (CART) peptide as a neuromediator in the ENS is very limited, especially during pathological states.
Aim: Investigation of the changes in the number of CART-like immunoreactive (CART-LI) mucosal nerve fibres during drug-resistant ulcerative colitis in children.
Material and methods: The distribution pattern of CART-LI mucosal nerves was studied in bioptates from the descending colon using the immunofluorescence technique in 5 patients with UC (UC group) and 5 children (control – C group) hospitalized because of bleeding from lower sections of the gastrointestinal tract. In these patients colitis was excluded during endoscopic and histopathological examinations. Average age of patients was 14.2 years in the UC group and 11.4 years in the C group.
Results: A significant increase in the number of CART-LI mucosal nerve fibres was found in patients with UC (5.94 ±0.53 *per* observation field) in comparison to the control group (2.11 ±0.58)
Conclusions: Ulcerative colitis caused a visible increase in the number of CART-positive nerve fibres within the mucosal

Streszczenie

Wprowadzenie: Jelitowy układ nerwowy (*enteric nervous system* – ENS) jest zdolny do czynnościowych i neurochemicznych zmian pod wpływem różnorodnych czynników patologicznych. Jednym z nich jest wrzodziejące zapalenie jelita grubego, które znacznie zmienia kodowanie chemiczne w obrębie ENS. Z drugiej strony, wśród szerokiej gamy enterycznych neurotransmiterów, występujących w obrębie jelit, peptyd CART (transkrypt regulowany przez kokainę i amfetaminę) jest jednym z najmniej poznanych, szczególnie jeżeli chodzi o jego funkcje w przebiegu procesów patologicznych.
Cel: Celem badań była ocena zmiany gęstości CART-pozytywnych włókien nerwowych w obrębie błony śluzowej okrężnicy zstępującej podczas lekoopornego wrzodziejącego zapalenia jelita grubego u dzieci.
Materiał i metody: Ocenę gęstości włókien immunoreaktywnych wobec CART przeprowadzono przy użyciu badań immunohistochemicznych w bioptatach pochodzących z okrężnicy zstępującej. Próbki pobrano od 5 pacjentów cierpiących na wrzodziejące zapalenie jelita grubego (grupa UC). Grupę kontrolną (grupa C) stanowili pacjenci (n = 5) hospitalizowani z powodu krwawienia z dolnych odcinków przewodu pokarmowego, u których na podstawie badania endoskopowego i histopatologicznego wykluczono stan zapalny. Średni wiek pacjentów wynosił 14,2 roku w grupie UC i 11,4 roku w grupie C.
Wyniki: W błonie śluzowej okrężnicy zstępującej pacjentów cierpiących na wrzodziejące zapalenie jelita grubego zaobserwowano znaczne zwiększenie się liczby CART-pozytywnych

layer of the descending colon, which suggests the participation of this neuropeptide in pathological processes within the human GI tract.

włókien nerwowych (5,94 ±0,53 w polu widzenia) w porównaniu z grupą kontrolną (2,11 ±0,58).

Wnioski: Lekooporne wrzodziejące zapalenie jelita grubego powoduje wyraźny wzrost liczby CART-pozytywnych włókien nerwowych w błonie śluzowej okrężnicy zstępującej, co może sugerować udział tego peptydu w procesach patologicznych w obrębie przewodu pokarmowego człowieka.

Introduction

It is well known that the enteric nervous system (ENS) is involved in various pathological processes. Nerve structures of the ENS are able to change their structural, functional or chemical phenotype as a result of adaptive or reparative responses to a wide array of intestinal and extra-intestinal diseases [1-3, for review, see 4]. One such intestinal disease, which can induce significant changes in chemical coding of human ENS nerve structures, is ulcerative colitis (UC), belonging to the inflammatory bowel diseases (IBD), which constitutes a vital problem in present-day medicine because of its sustained course, severe complications, as well as high risk of cancer and intestinal resection [3, 5-7]. The aim of this study was to indicate the possible UC-induced changes in the number of colonic, mucosal cocaine- and amphetamine-regulated transcript peptide-like immunoreactive (CART-LI) nerve fibres in the descending colon of children.

It is necessary to remark that detailed functions of CART within the gastrointestinal (GI) tract have not yet been completely explained. This peptide, which was isolated for the first time in 1981 from the ovine hypothalamus [8], has been found within the enteric nervous system supplying different parts of the GI tract of the guinea pig, rat and pig [for review, see 9]. It may be involved in the inhibition of feeding [10], reduction of gastric acid secretion [11] and exacerbation of colonic motility [12] *via* regulatory circuits located within the central nervous system. The CART peptide has also been shown to reduce the amplitude of nitric oxide-induced relaxation of the large intestine [13]. Moreover, it is known that enteric CART-LI fibres and perikarya simultaneously contained vasoactive intestinal peptide (VIP), nitric oxide synthase (NOS) and/or calcitonin gene related peptide (CGRP) [13, 14], which may suggest neuroprotective, anti-inflammatory, sensory and/or motor functions of CART within the GI tract. The knowledge about the distribution and possible functions of CART within human ENS is highly fragmentary. So far only one study has reported on the presence of CART in the intestines of children aged below 4 years and the changes in enteric CART-like immunoreactivity during Hirschsprung's disease [15]. So this study, which for the first time analyses the influence of UC on the CART-like immunoreactivity in human colonic mucosal nerves, may contribute to a better understanding of the possible function of the CART peptide in pathological processes within the human intestine.

Material and methods

Ten children – patients of the Clinic of Paediatrics, Gastroenterology and Oncology of the Medical Academy in Gdansk – were included in the study. Among them 5 patients with diagnosed drug-resistant ulcerative colitis were assigned as an experimental group (UC group). The second, control group (C group, $n = 5$) constituted children who were hospitalized with symptoms of bleeding from lower sections of the GI tract, in whom IBD was excluded during endoscopic and histopathological examinations. Average age of patients was 14.2 years in the UC group and 11.4 years in the C group. In both studied groups, material for immunohistochemistry – fragments of mucosal layer of the descending colon – were obtained by biopsy during colonoscopy, which is the routine way of tissue collection for histological examinations. The fact of tissue collection for immunohistochemical study did not affect the decision of the surgeon concerning the number of bioptates collected from the patient. All procedures of the present study were performed in compliance with the instructions of the Bioethical Committee (decision NKEBN/642/2000) and are in accordance with the ethical standards of the Helsinki Declaration 1975 (revised 1983).

The fragments of mucosal layer were fixed by immersion for 20 min 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. Finally, bioptates were cut with a cryostat (–22°C) on 10 μm thick sections.

Cryostat sections were processed for routine single-labelling immunofluorescence. Briefly, after air-drying at room temperature (rt) for 45 min, 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 thiomersal in PBS for 1 h (rt).

Then, they were incubated (overnight; rt, in humid chamber) with antiserum directed towards CART (rabbit monoclonal, Phoenix Pharmaceuticals, USA, 1 : 16 000). The complex of primary antiserum bound to the appropriate antigen was visualized by incubation (1 h, rt) with species-specific secondary antiserum conjugated to biotin (Jackson ImmunoResearch, USA, 1 : 800) and the latter antibody was then visualized by a streptavidin-CY3 complex (Jackson, 1 : 8000, 1 h, rt). Each step of the immunolabelling was followed by rinsing of the sections with PBS (3 × 10 min, pH 7.4).

Semi-quantitative evaluation of the density of the CART-immunostained nerve terminals within the mucosal layer was based on counting all terminals immunoreactive to CART *per* observation field (magnification × 400) under an Olympus BX51 microscope equipped with epi-fluorescence and an appropriate filter set. Nerve profiles were counted in 3 observation fields of 10 sections *per* patient (in total: in 30 observation fields *per* patient) and obtained data were pooled and presented as the mean. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results

Presence of CART-immunoreactivity was observed in the mucosal nerve fibres of all bioptates studied. In the control group the number of these fibres in particular patients fluctuated between 1 and 3.63 *per* observation field (Table I). Clear differences between individual observation fields and between patients were observed. In some fields CART-LI fibres were very rare or they were not observed at all, but in other ones such fibres were relatively thick. All CART-positive fibres in the control group were rather delicate and on average 2.11 ±0.58 fibres per observation field were observed (Figure 1A).

Ulcerative colitis caused a clear increase in the number of CART-LI mucosal fibres (Table I). The number of these fibres in the UC group always exceeded 4.5 *per* observation field and varied from 4.83 to 7.93 in particular patients. Moreover, in the UC group fields without CART-positive fibres were not observed and the fibres were often larger and more visible in comparison with the control group (Figure 1B). The average number of CART-positive mucosal processes in patients with UC was 5.94 ±0.53 *per* observation field.

Moreover, both in the control group and in patients with UC no relation between age and density of CART-IR fibres was observed (Table I).

Discussion

The results of this study demonstrate that the CART-LI nerve fibres are present in the mucosal layer of the human descending colon under physiological conditions, but the density of such fibres is not high. This is in accordance with previous observations in humans [15] as well as in other mammals, including the pig, which is the optimal laboratory animal in studies on the GI tract because of the well-known similarity between the human and porcine ENS [16]. The number of mucosal CART-LI nerve fibres observed during this study in the control group is generally similar to that observed for fibres within porcine colonic mucosa [17] and congruent with previous studies in humans [15].

Table I. The number of CART-IR mucosal nerve fibres in the descending colon in the control group (C group) and in patients with drug-resistant ulcerative colitis (UC group)
Tabela I. *Liczba CART-pozytywnych włókien nerwowych w obrębie błony śluzowej okrężnicy zstępującej w grupie kontrolnej (grupa C) i u pacjentów cierpiących na lekooporne wrzodziejące zapalenie jelita grubego (grupa UC)*

	C group					UC group				
Patient number	1	2	3	4	5	6	7	8	9	10
Age of the patient [years]	12	14	16	15	14	9	12	12	10	14
Total number of CART-positive fibres (*per* 30 fields)	109	30	34	42	102	159	238	145	178	172
Number of CART-LI processes *per* observation field	3.63	1.00	1.13	1.40	3.40	5.30	7.93	4.83	5.93	5.73
Average number of CART-LI fibres *per* observation field ± SEM	2.11 ±0.58					5.94 ±0.53				

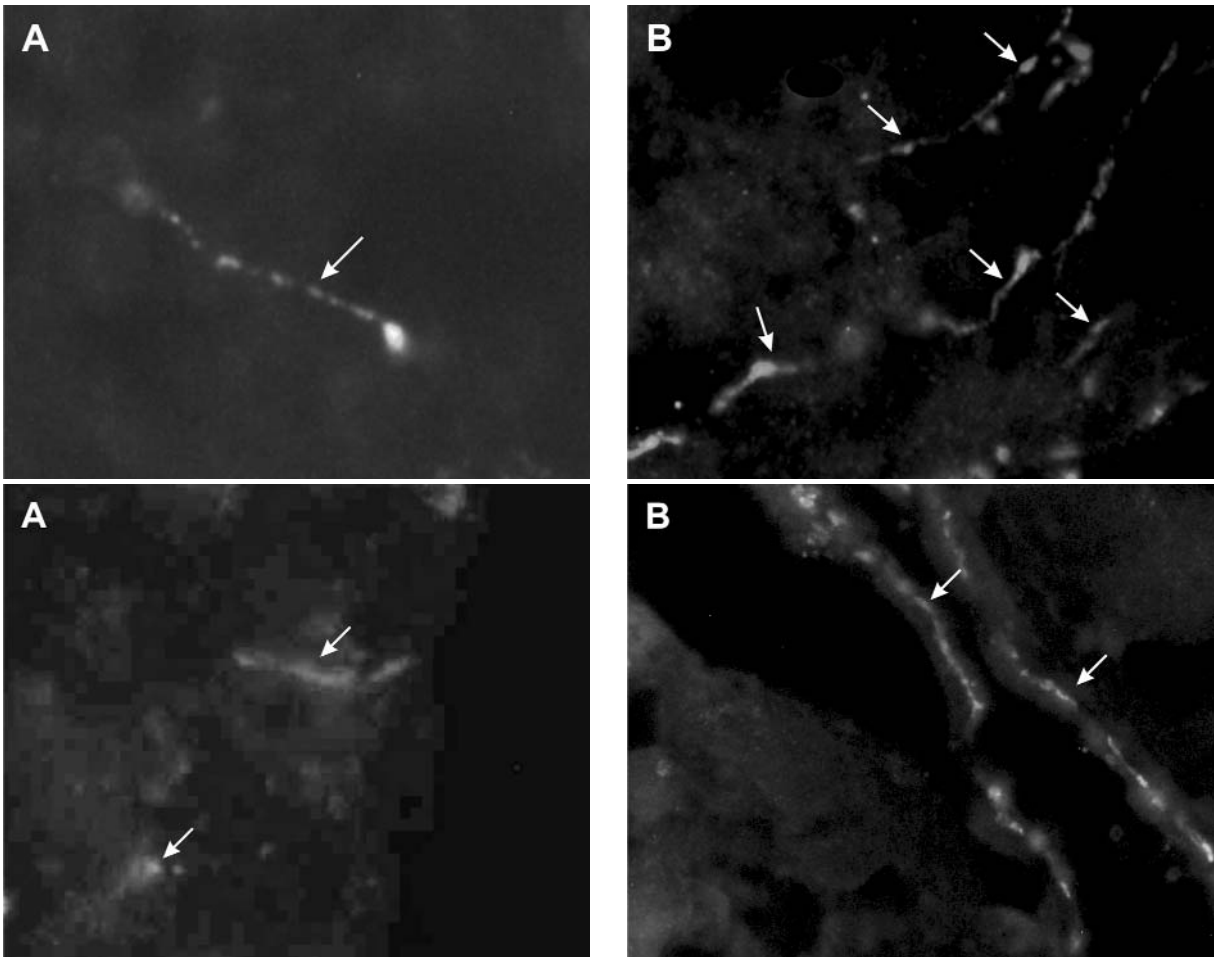

Fig. 1. Distribution pattern of nerve fibres (arrows) immunostained for CART within the mucosal layer of descending colon in children under physiological conditions (A) and during drug-resistant ulcerative colitis (B). Magnification 400×

Ryc. 1. *Dystrybucja włókien nerwowych (strzałki) immunoreaktywnych wobec CART w obrębie błony śluzowej okrężnicy zstępującej u dzieci w warunkach fizjologicznych (A) i podczas lekoopornego wrzodziejącego zapalenia okrężnicy* (*B*). *Powiększenie 400*×

Moreover, we present the first report on the fact that UC, a pathological state known to affect ENS immunoreactivity [3, 5], may also induce changes in the number of CART-positive mucosal fibres within the human descending colon. The clear increase of such fibres in patients with UC, observed in this study, may suggest that CART may be involved in the regulatory processes under pathological states in the human GI tract.

So far very little is known about the particular functions of CART within the human ENS. All observations with only one exception [15] have been performed in animals. CART was described as a reducer of colonic motility [12] and an inhibitor of gastric acid secretion by cholinergic pathways [11] as well as an inhibitor of nitric oxide-mediated relaxation in the colon *in vitro* [13]. Previous investigations concerning the human GI tract [15] showed the presence of CART in the intestine of children aged 2-41 months and changes in the CART-like immunoreactivity of ENS during Hirschsprung's disease. Previous observations together with the results of the present study indicate a role of CART in the regulation of pathological mechanisms in the descending colon. Moreover, the increase of CART-like immunoreactivity in mucosal layers during UC may reflect a role of the CART-LI processes in regulation of intestinal secretion, but to date the functions of CART in intestinal pathology still remain unknown. Additionally, the co-localisation of CART and better known gastrointestinal neuromediators such as VIP, CGRP and NOS in the same nerve structures of the ENS described in both human [15] and other

mammals [for review, see 9] may suggest the similar function of these substances. VIP and NO are known as neuroprotective and anti-inflammatory factors [18] and CGRP has been reported to function as a sensory neurotransmitter [19] and a protective agent in experimental colitis [20]. Thus CART might also be involved in promotion of the survival of enteric neurons and their protection in intestinal disorders, which is supported by data showing that CART is able to promote the survival of rodent enteric neurons *in vitro* [13].

Moreover, revealed differences in the CART-like immunoreactivity between particular patients observed in the present study, both under physiological conditions and during drug-resistant UC, suggest that the CART-like immunoreactivity within mucosal fibres in the human descending colon might be affected by other, still unknown, regulatory factors.

To summarise, the results obtained in this study may indicate a role of CART in the function of the human colonic mucosa, not only in physiological conditions, but also under pathological states such as drug-resistant colitis. However, further studies are needed to elucidate the exact role of CART in physiology and pathology of the human GI tract.

Conclusions

Ulcerative colitis caused a clear increase in the number of CART-positive nerve fibres within the mucosal layer, which suggests the participation of this neuropeptide in the pathological processes within the human GI tract.

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