PHD THESIS

STRUCTURAL AND MOLECULAR CHANGES OF THE ENTERIC NERVOUS SYSTEM IN RATS WITH CROHN'S DISEASE

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Introduction

Digestive functions are regulated by a complex neuronal network, the enteric nervous system. The enteric neurons, the interstitial cells of Cajal and the glial cells organized into two major ganglionated plexuses and direct the processes of absorption, secretion and peristalsis. The motor functions are regulated by the myenteric plexus located between the circular and longitudinal layers of the muscularis externa.

Inflammatory bowel diseases (IBDs) are the group of chronic intestinal inflammatory conditions. Nowadays IBDs are becoming more common in Western societies particularly among young people. Crohn's disease (CD) is a multifactorial, relapsing disorder with chronic inflammation involving all layers of the gut wall. The development of irreversible pathological alterations including stricturing complications in response to the repetitive relapsing inflammations characteristic of CD leads to partial or total intestinal obstruction with potentially life-threatening consequences. Nevertheless, the pathogenesis of strictures is still unclear and no direct therapeutic strategies exist to effectively prevent or reverse this condition.

Over the past two decades, experimental animal models of IBDs have proven to be important tools for detecting potential therapeutic agents and investigating pathogenesis. One of the most widely used Crohn-like model obtained by local application of the haptinizing agent 2,4,6-trinitrobenzene sulfonic acid (TNBS). TNBS is dissolved in ethanol which disrupts the mucosal barrier and facilitates TNBS infiltration into the gut wall. Therefore, the formation of trinitrophenyl-haptenated colonic proteins leads to the induction of an unbalanced Th1-cell response that resembles CD. In general, TNBS-induced colitis is used for the investigation of the acute effects of bowel inflammation. Nevertheless, there is not yet a suitable experimental animal model that allow to investigate the chronic consequences of CD.

The intestinal symptoms common among CD patients are often caused by intestinal motility abnormalities related to myenteric neuropathy. The evidence suggests that both the structure and function of the enteric nervous system are altered substantially by intestinal inflammation. A 40% loss of intrinsic neurons was earlier demonstrated in the inflamed segment of the colon four days following the induction of colitis in rats. Moreover, the complete loss of myenteric neurons was observed in

the strictured region. Although there have been numerous studies of the enteric nervous system in inflammation, the structural and molecular changes to the enteric nervous system under the repetitive relapsing inflammations have not been studied yet.

After colitis induction immune cells like T-lymphocytes, antigen-presenting dendritic cells and macrophages produce multiple cytokines. The anti-inflammatory cytokine, transforming growth factor-beta (TGF-beta) is produced by the suppressor T-cells. TGF-beta 2 promotes excessive deposition of the extracellular matrix proteins that leads to scarring. In contrast, TGF-beta 3 is the most effective in inducing re-epithelialisation and reduces fibrosis. Previous studies have shown that the intestinal mucosa of CD patients expressed significantly higher levels of bioactive TGF-beta 2, however the expression of TGF-beta 3 was significantly lower compared to the controls.

Heme oxigenase-1 (HO-1) is inducible by a variety of oxidative stress and is known to play an important role in the protection of tissues from oxidative damage. Matrix metalloproteinases (MMPs) are involved in the remodelling of extracellular matrix under physiological and pathological conditions, their function is regulated by specific endogenous tissue inhibitor of metalloproteinases (TIMP1). MMP9, also known as gelatinase B is responsible for the degradation of type IV and V collagens.

Aims

We aimed to establish a rat model of chronic colitis suitable to investigate the structural and molecular changes in the whole thickness of the gut wall throughout a 120 day experimental period. We focused on the development of intestinal strictures. Repeated administration of TNBS in 25% ethanol was applied to mimic repetitive recurrent inflammations. Between the 2^{nd} and 8^{th} day the acute, while between 60^{th} and 120^{th} day the chronic consequences of colitis were investigated by light- and electron microscopic morphometry, immunohistochemistry and molecular biology.

- What are the main parameters of a rat model with chronic colitis, which suitable to investigate the intestinal strictures characteristic to Crohn's disease?
- How acute intestinal inflammation influences the quantitative properties of the myenteric neurons?
- How chronic intestinal inflammation influences the quantitative properties of myenteric neurons?
- Are there any structural components within the gut wall, which have a special role in the stricture formation?
- How the acute and chronic inflammation influences the mRNA expression of inflammatory markers and enzymes?

Materials and methods

Adult male rats weighing 200-220 g were used throughout the experiments. Colitis was induced locally with TNBS dissolved in 25% ethanol, administered with a polyethylene cannula 8 cm proximal to the anus. Repetitive relapsing inflammations were mimicked by repeating administrations of TNBS with a two-week-lag. The animals were randomly divided into control, once, twice and three times TNBS-treated groups. The control animals received an enema of saline. The rats were weighed weekly and monitored for activity, bloody diarrhoea and mortality. The animals were killed by cervical dislocation in different time points (2, 4, 8, 60, 90 and 120 days) after the TNBS treatments. The gut segments were cut along the mesentery and pinched flat. Digital photographs were taken to evaluate the macroscopic mucosal damage and the extent of the ulceration was measured.

Tissue samples were taken from the inflamed segment, and also proximal and distal to the inflamed segment of the colon for morphological and molecular studies. Since the originally inflamed segments could be recognized macroscopically also after mucosal healing, tissue samples were taken from all three colonic segments also in the chronic phase of the inflammation. Whole-mount preparations were processed for light microscopic immunohistochemistry using HuC/HuD as a pan-neuronal marker. The Plexus Pattern Analysis software developed earlier in our laboratory was applied to count the number, and Image J was used to measure the area of myenteric neuronal soma.

Tissue homogenates from intestinal samples derived from control and TNBS-treated rats were used to study the mRNA expression of HO-1, TGF-beta 2, TGF-beta 3, MMP9 and TIMP1 by quantitative real-time polymerase chain reaction.

Light- and transmission electron microscopic morphometry was used to investigate the ultrastructure of intestinal strictures developed in the chronic phase of the inflammation after the third TNBS treatment. The width of the tight junctions between adjacent epithelial cells, the thickness of lamina muscularis mucosae and external muscle layers of the gut wall were determined. The expansion of extracellular matrix in the circular muscle layer was evaluated from the distance between intestinal

smooth muscle cells. The induction of apoptosis was confirmed in the myenteric ganglia and their microenvironment by quantitative post-embedding immunohistochemistry using primary antibody against anti-caspase-9 as proapoptotic marker.

Data from TNBS-treated rats were always compared to the age-matched controls. The statistical analysis was performed by using one-way ANOVA and Newman-Keuls test.

Results

TNBS-treated rats displayed severe ulcerative intestinal inflammation, but the mortality was negligible, and a gradual increase of body weight was characteristic in all the rats throughout the experimental period. Alleviated macroscopic mucosal damage and also accelerated mucosal healing were salient in the acute phases of inflammation after repeated doses. Strictures first appeared 60 days after the TNBS treatments and the frequency of strictures increased until day 120^{th}.

According to previous data, significant decrease in the number of myenteric neurons was first demonstrated 4 days after the administration of a single dose of TNBS, and further significant decrease was demonstrated until day 8^{th}. Moreover, the decrease in neuronal density was not limited to the apparent inflamed area. Proximal and distal to the inflamed colonic segment significant neuronal loss with smaller scale was also noticed 4 days after the first TNBS administration. However, after applying repeated doses of TNBS, neuronal loss was strictly limited to the inflamed segments of the colon, and did not accelerated between 4^{th} and 8^{th} days. Shrinkage of neuronal cell bodies was always demonstrated right after completed of the neuronal loss, on the 8^{th} day after the first, and already on the 4^{th} day after the repeated TNBS doses.

Nevertheless, 8 days after the treatments further neuronal loss was not detected, myenteric neuronal numbers were equal with the aged-matched controls. In the chronic phase, the restoration of impaired morphology of myenteric neurons was observed, which was speeded up after repeated treatments.

Transmission electron microscopy showed complete re-epithelialization in the strictured region. The width of tight junctions between adjacent enterocytes did not change compared to controls. Morphometrical analyzes revealed significant thickening of the lamina muscularis mucosae and the external muscle layers on the 90^{th} day. While until the 120^{th} day further significant thickening was measured in the external longitudinal and circular muscle layers of the gut wall, the thickness of lamina muscularis mucosae was similar to the controls. On the day 90^{th} myenteric ganglia and interstitial cells of Cajal were seemingly intact, while on the day 120^{th} the morphological signs of apoptosis were frequently seen and excess matrix deposition

was recorded between intestinal smooth muscle cells. The induction of apoptosis was also confirmed by caspase-9 post-embedding immunohistochemistry on the 90th day in the smooth muscle cells and on the 120th day also in myenteric ganglia.

Marked increase in the gene expression of the HO-1 was demonstrated in the inflamed colonic segment already after the 4th day of the single dose of TNBS administration. Overexpression was observed in a great extent after the second and even more after the third treatment, and high level of HO-1 mRNA was sustained even in the chronic phase of inflammation. While induction of HO-1 was also detected adjacent proximally to inflamed segment in the acute phase of the inflammation, gene repression was demonstrated in the non-inflamed sites of the colon in the chronic phase. CD-like expression profile of the TGF-beta isoforms was showed after repeated TNBS doses, TGF-beta 2, but not TGF-beta 3 mRNA was up-regulated in the inflamed and also in the non-inflamed sites. MMP9 was up-regulated after the third TNBS treatment in all three colonic segments. Nevertheless, down-regulation of the TIMP1 was detected in the inflamed segment at the same time.

Discussion

In conclusion, we established a rat model suitable to investigate the acute and also the chronic consequences of intestinal inflammation characteristic to CD. The expression pattern of TGF-beta isoforms was characteristic to CD and strictures developed in the chronic phase of the inflammation.

Since alleviated macroscopic mucosal damage was observed after repeated TNBS doses, we hypothesize that repetitive inflammations develop pre-conditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury. Decreased severity of gut inflammation after repeated TNBS treatments might be associated with the persistent up-regulation of HO-1 expression.

Nevertheless, after repeated TNBS treatments the chronic complications aggravated. We assumed that thickening of smooth muscle layers of the gut wall and the formation of strictures was not accompanied by the impairment of mucosal barrier function or myenteric neuropathy. Because of the altered expression of MMP/TIMP1 excess deposition of extracellular matrix was observed among the smooth muscle cells within the circular muscle layer. As a result, smooth muscle cells and myenteric ganglia shoved off from each other, which might resulted in a deficient innervation.

Publications

Thesis related publications

P. Talapka, LI. Nagy, A. Pál, M. Z. Poles, M. Bagyánszki, LG. Puskás, É. Fekete, N. Bódi (2014) Alleviated mucosal and neuronal damage at the acute phase of recurrent inflammation in a rat model of Crohn's disease. (submitted)

P. Talapka, N. Bódi, I. Battonyai, É. Fekete, M. Bagyánszki (2011) Subcellular distribution of nitric oxide synthase isoforms in the rat duodenum. World Journal of Gastroenterology 17(8); 1026-1029. **IF: 2.574**

N. Bódi, I. Battonyai, **P. Talapka**, É. Fekete, M. Bagyánszki (2009) Spatial pattern analysis of nitrergic neurons in the myenteric plexus of the duodenum of different mammalian species. Acta Biologica Hungarica 60(4); 347-358. **IF: 0.551**

Other publications

N. Bódi, **P. Talapka**, M. Z. Poles, E. Hermesz, Zs. Jancsó, Z. Katarova, F. Izbéki, T. Wittmann, É. Fekete, M. Bagyánszki (2012) Gut region-specific diabetic damage to the capillary endothelium adjacent to the myenteric plexus. Microcirculation 19(4); 316-326. **IF: 2.763**

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Impact factor of full papers: 12.445

Abstracts published in conference proceedings

N. Bódi, **P. Talapka**, M. Bagyánszki, A. I. Rosztóczy, É. Fekete, F. Izbéki, T. Wittmann (2010) The role of structural capillary damage in the loss of nitrergic myenteric neurons in a rat model of diabetes mellitus. 18th United European Gastroenterology Week, Barcelona, Spain. Gut 59 (Suppl III) A91 **IF: 10.732**

N. Bódi, **P. Talapka**, M. Bagyánszki, A. Rosztóczy, É. Fekete, T. Wittmann, F. Izbéki (2010) Intestinal endothelial dysfunction may have a role in the development of myenteric nerve damage in a rat model of diabetes mellitus. Magyar Gasztroenterológiai Társaság 52. Nagygyűlése, Tihany, Magyarország. Z Gastroenterol. 48:598-599 **IF: 1.131**

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R. Á. Nagy, N. Bódi, **P. Talapka**, M. Bagyánszki, É. Fekete, T. Wittmann, F. Izbéki (2011) Effects of acute and chronic quinolinic acid treatment on gastrointestinal motility in mice. 19th United European Gastroenterology Week, Stockholm, Sweden. Gut 60 (Suppl 3) A313 **IF: 10.732**

N. Bódi, **P. Talapka**, Zs. Jancsó, M. Z. Poles, E. Hermesz, M. Bagyánszki, É. Fekete, F. Izbéki, T. Wittmann (2012) Gut region-specific differences in the cellular and subcellular distribution of nitric oxide synthase isoforms after chronic ethanol exposure in rats. 20th United European Gastroenterology Week, Amsterdam, The Netherlands (Az előadás angol nyelvű kivonata megjelent: Gut Supplement No III Vol 61, A304) **IF: 10.732**

P. Talapka, L. I. Nagy, M. Poles, L. G. Puskás, É. Fekete, M. Bagyánszki, N. Bódi (2012) Aligned alteration of enteric neurons, smooth muscle cells and inflammatory markers involved in stricture formation in a rat model of Crohn's disease. 20th United European Gastroenterology Week, Amsterdam, The Netherlands (Az előadás angol nyelvű kivonata megjelent: Gut Supplement No III Vol 61, A156) **IF: 10.732**