Alleviated mucosal and neuronal damage in a rat model of Crohn’s disease

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Abstract

AIM: To establish a rat model suitable to investigate the repetitive relapsing inflammations (RRI) characteristic to Crohn’s disease.

METHODS: Colitis was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). RRI were mimicked by repeating administrations of TNBS. Tissue samples were taken from control, once, twice and three times treated rats from the inflamed and adjacent non-inflamed colonic segments at different timepoints during the acute intestinal inflammation. The means of the ulcerated area were measured to evaluate the macroscopic mucosal damage. The density of myenteric neurons was determined on whole mounts by HuC/HuD immunohistochemistry. Heme oxygenase-1 (HO-1) expression was evaluated by molecular biological techniques.

RESULTS: TNBS-treated rats displayed severe colitis, but the mortality was negligible, and an increase of body weight was characteristic throughout the experimental period. The widespread loss of myenteric neurons, and marked but transient HO-1 up-regulation were demonstrated after the first TNBS administration. After repeated doses the length of the recovery time and extent of the ulcerous colonic segments were markedly decreased, and the neuronal loss was on a smaller scale and was limited to the inflamed area. HO-1 mRNA level was notably greater than after a single dose and overexpression was sustained throughout the timepoints examined. Nevertheless, the HO-1 protein up-regulation after the second TNBS treatment proved to be transient. Following the third treatment HO-1 protein expression could not be detected.

CONCLUSION: Experimentally provoked RRI may exert a protective preconditioning effect against the mucosal and neuronal damage. The persistent up-regulation of HO-1 mRNA expression may correlate with this.

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Key words: Crohn’s disease; Experimental rat model; Heme oxygenase-1; Myenteric neurons; Repetitive relapsing inflammation

Core tip: We report our first results derived from a newly developed rat model with chronic experimental colitis allowed us to modelling the recurring periods of recrudescence and remission in Crohn’s disease. Colitis was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Repetitive recurrent inflammations (RRI) were
mimicked by repeated administrations of TNBS. This study demonstrates for the first time that experimentally provoked RRI develop preconditioning 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 heme-oxygenase 1 messenger RNA expression.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of chronic intestinal inflammatory conditions. The major types of IBDs are Crohn’s disease (CD) and ulcerative colitis. CD is characterized by relapsing transmural inflammation that can affect any part of the gastrointestinal tract. Although the pathogenesis of CD is still unclear, the most widely accepted hypothesis is that an impairment of the mucosal barrier function due to a dysregulated immune response to an environmental factor can generate prolonged inflammation\[1,2]. The development of irreversible pathological alterations including strictureing and penetrating complications in response to the repetitive relapsing inflammations (RRI) characteristic of CD can indicate the transition from the early to the late disease which is dependent on the intensity rather than on the duration of the inflammation\[3].

The intestinal symptoms common among CD patients are often caused by intestinal motility abnormalities related to enteric neuropathy. The intestinal motor functions are regulated by the myenteric neurons. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation\[4]. In a previous study 40% loss of myenteric neurons was demonstrated in the inflamed segment of the colon four days following the induction of colitis in rats\[5]. Moreover, the complete loss of myenteric neurons was observed in the strictured region\[6]. Persistent alterations in neuronal signalling were documented even after the resolution of the colitis. The AH neurons, one electrophysiological type of enteric neurons that function as intrinsic afferent neurons, remained hyperexcitable eight weeks after the induction of colitis. In the same experiment larger amplitudes of fast excitatory postsynaptic potentials were measured in the S neurons as compared with the controls\[7]. Physiological disturbances are not restricted to the site of the inflammation. Suppression of noradrenaline release was observed in both the distal and the transverse colon and in the terminal ileum in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis\[8].

During the past two decades, experimental animal models of IBD have proved to be important tools for the detection of potential therapeutic agents and for the investigation of the pathogenesis. One of the most widely used haptinizing agents TNBS is used to induce colitis, resulting in mucosal inflammation mediated by a Th1 response. Nevertheless, in most cases the induction of acute necrotizing enterocolitis with a single high dose of TNBS was not able to generate the pathological alterations characteristic of CD and was accompanied by a high mortality rate\[9]. The repeated administration of TNBS in increasing doses resulted in chronic colitis and fibrosis in mice\[10] and in rats\[11] that most likely reflects the characteristics of the chronic phase of CD. Nevertheless, considerable levels of mortality, 25% and 18% respectively were still demonstrated.

Our aim was to establish a rat model of chronic colitis with the possible lowest mortality rate, suitable for the investigations of the long-term consequences of acute inflammation. In the present study the extent of mucosal and myenteric neuronal injuries were investigated in the acute phase of the experimentally mimicked RRI. Since increased expression of heme oxygenase-1 (HO-1) has been described in patients with CD\[12,13], it has been suggested that the activation of HO-1 may act as a natural defence to alleviate inflammation and tissue damage\[14-16]. Therefore, the quantitative changes in HO-1 mRNA and protein expression were also evaluated here.

MATERIALS AND METHODS

Animal model

All experiments were approved by from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Sprague-Dawley rats, weighing 200-220 g, kept on standard laboratory chow (Bioplan Kft., Hungary) and with free access to drinking water, were used throughout the experiments. They were housed in a restricted access room with controlled temperature (23 °C) and a light/dark (12 h:12 h) cycle. Colitis was induced locally with TNBS (Sigma-Aldrich, St. Louis, MO, United States; 10 mg) dissolved in 0.25 mL of 25% ethanol administered with a polyethylene cannula 8 cm proximal to the anus under pentobarbital anaesthesia (45 mg/kg, ip). The animals were deprived of food at 24 h before the induction of inflammation. Relapsing inflammations were mimicked by repeating the administration of TNBS with a two week-lag. The animals were randomly divided into control (n = 18), and once (n = 13), twice (n = 14) or three times (n = 11) TNBS-treated groups. The control animals received an enema of 0.25 mL of 0.9% saline. The rats were weighed weekly and monitored for activity, bloody diarrhoea and mortality.

Tissue handling

The animals were killed by cervical dislocation under
RNA was prepared from tissue homogenates as suggested by the manufacturer (Bioneer, Daejeon, South Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, United States). 2 μg of total RNA from HO-1 was transcribed in 15 μL of reaction mixture (1.2 μL dNTPs, 1.5 μL of Multiscribe Reverse Transcriptase (50 U/μL), 3 μL of RT Buffer, 3 μL of RT primer and 6.3 μL of nuclease-free water). All reactions were carried out for 10 min at 25 °C and then for 2 h at 37 °C in MyGenie32 Thermal Block (Bioneer, Daejeon, South Korea). qRT-PCR was performed in an Exicycler 96 (Bioneer, Daejeon, South Korea) in a total volume of 20 μL containing 10 μL of FastStart SYBR Green PCR Master Mix, 1 μL of specific primer (0.5 pmol/μL) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 °C to activate the Taq DNA polymerase. This was followed by 45 cycles of 15 s at 95 °C for denaturation, 45 s at 60 °C for annealing and 25 s at 72 °C for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM_012580.2 for HO-1 (forward: 5'-GTCAAGCACAGGGTGACAGA-3' and reverse: 5'-CTGCGAGCTCCTCAAACAGC-3') and reverse 5'-ACCTGTTTCATCATCATAATCAC-3') was used as a housekeeping gene to normalize expression data. The results were expressed as means ± SE.

Investigation of the quantitative properties of the myenteric neurons

Wholemount preparations were immunostained with the pan-neuronal marker HuC/HuD as described earlier.[18] Briefly, wholemounts were incubated overnight with anti-human neuronal protein HuC/HuD developed in mouse (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:2000) at 4 °C. Horseradish peroxidase activity was revealed by using 3, 3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:100). Peroxidase activity was incubated overnight at 4 °C in RNA Later (Qiagen, Venlo, The Netherlands). Those for Western blotting analysis were frozen immediately in liquid N2 and stored at -80 °C until use.

Quantification of HO-1 mRNA expression by qRT-PCR

Tissue samples were homogenized in AccuZol (Bioneer, Daejeon, South Korea) directly before qRT-PCR. Total RNA was prepared from tissue homogenates as suggested by the manufacturer (Bioneer, Daejeon, South Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, United States). 2 μg of total RNA from HO-1 was transcribed in 15 μL of reaction mixture (1.2 μL dNTPs, 1.5 μL of Multiscribe Reverse Transcriptase (50 U/μL), 3 μL of RT Buffer, 3 μL of RT primer and 6.3 μL of nuclease-free water). All reactions were carried out for 10 min at 25 °C and then for 2 h at 37 °C in MyGenie32 Thermal Block (Bioneer, Daejeon, South Korea). qRT-PCR was performed in an Exicycler 96 (Bioneer, Daejeon, South Korea) in a total volume of 20 μL containing 10 μL of FastStart SYBR Green PCR Master Mix, 1 μL of specific primer (0.5 pmol/μL) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 °C to activate the Taq DNA polymerase. This was followed by 45 cycles of 15 s at 95 °C for denaturation, 45 s at 60 °C for annealing and 25 s at 72 °C for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM_012580.2 for HO-1 (forward: 5'-GTCAAGCACAGGGTGACAGA-3' and reverse: 5'-CTGCGAGCTCCTCAAACAGC-3') and reverse 5'-ACCTGTTTCATCATCATAATCAC-3') was used as a housekeeping gene to normalize expression data. The results were expressed as means ± SE.

RESULTS

Macroscopic observations

Consistent with the findings of previous studies, the
TNBS-treated rats already displayed severe ulcerative intestinal inflammation associated with weakness and bloody diarrhoea on the first day after the induction of colitis with either single or repeated doses of TNBS. The mortality rate was negligible: only two animals died during the experiments. Independently of how many doses were administered, the symptoms always presented with the same severity, but the length of the recovery time decreased spectacularly after repeated treatments. Except for the first week of acute inflammation, when a gain in weight was not detected, a gradual increase in body weight was characteristic in all the rats throughout the experimental period (Table 1). The severe symptoms, like bloody diarrhoea accompanied by acute inflammation lasted for seven or eight days after the administration of a single dose of TNBS but were already resolved four or five days after repeated treatments. The accelerated mucosal healing after the repeated TNBS administrations was clearly demonstrated by the significant differences in the mean ulcerated areas on days two, four and eight after the induction of colitis (Figure 1). The mean area of the ulcerated colonic segment decreased significantly in all TNBS-treated groups between days two and eight following induction. Whereas, the area was still noteworthy eight days after the single TNBS administration, it was significantly reduced and hardly detectable after the second and third TNBS treatments. The extent of the ulcerated area was reduced significantly after the second dose of TNBS compared to that in the animals treated only once. Data are expressed as mean ± SE. *P < 0.01 vs once TNBS-treated group on day two. TNBS: 2,4,6-trinitrobenzenesulfonic acid.

controls (Figure 3). After the second and third TNBS treatments significant decreases in myenteric neuronal number were also observed first on day four after the induction of colitis, but in these cases the neuronal loss was less extensive (36% and 23%, respectively) and did not accelerate between days four and eight after the induction of colitis (Figure 3). Moreover, the decrease in myenteric neuronal density in the acute phase of the inflammation was not limited to the apparent inflamed area. Proximally and distally to the inflamed colonic segment a significant neuronal decrease was noticed four days after the first TNBS administration. However following the repeated doses of TNBS a significant loss of neurons in the colonic segments adjacent proximally and distally to the inflamed area was not detected (Figure 3).

Quantitative changes in HO-1 mRNA expression

The HO-1 mRNA expression was evaluated by qRT-PCR in all three colonic segments after single and repeated TNBS treatments. The HO-1 gene expression was markedly induced in the inflamed segment on day four after the first TNBS administration (Figure 4). However four days after the second treatment an approximately 50% higher HO-1 mRNA level was detected (Figure 4). The HO-1 mRNA expression then declined until day eight in the samples from the once TNBS-treated rats, and to a lesser extent also in the twice TNBS-treated rats, but it has never returned to the baseline level (Figure 4). Nevertheless, after the third treatment a nearly 45% increase in gene expression was measured between days four and eight and this high level of HO-1 mRNA expression was sustained even in the chronic phase of inflammation (not shown). Proximally to the inflamed colonic segment a mild increase of HO-1 mRNA expression was observed in the acute phase of the inflammation four days after the single and repeated TNBS doses, but in the distal

### Table 1  Weight characteristics of the four experimental groups of rats before the TNBS (initial) and eight days after the TNBS treatments (final)

|        | Body weight (g) ± SE |
|--------|----------------------|
|        | Initial              | Final               |
| Control| 201.5 ± 1.6          | 356 ± 20.9*         |
| 1 × TNBS| 238.3 ± 3.6          | 239 ± 10.9          |
| 2 × TNBS| 242.7 ± 3.3          | 319.3 ± 8.2*        |
| 3 × TNBS| 197.2 ± 3.5          | 376.5 ± 11.3*       |

*P < 0.01, initial vs final. TNBS: 2,4,6-trinitrobenzenesulfonic acid.
mucosal healing were salient features in the acute phases of inflammation after repeated TNBS treatment. However, regardless of the number of treatments long-range hyperaemia was always detected several days after the period of acute inflammation. This observation indicates that the post-inflammation remodelling of the vascular pattern is delayed in these rats. The delay in returning to the normal vascular pattern well after the mucosal healing is otherwise a regular problem in clinical practice.

We therefore consider that the rat model developed here will be suitable in future studies to reveal the molecular events behind vascular remodelling in acute intestinal inflammation and thereby help to open up new therapeutic avenues for the treatment of CD patients.

Although there are an appreciable number of unexplainable differences in the findings regarding CD-related alterations in the numbers of enteric nerve cells in human patients and animal models, the quantitative alterations of the ENS are now considered a hallmark of bowel inflammation. We therefore set out to establish whether the experimentally provoked RRI also alleviates the inflammatory damage in the ENS and reduces the rapid, significant and widespread loss of myenteric neurons demonstrated four and even more eight days after the administration of a single dose of TNBS. After the second and third TNBS treatments significant but less extensive decreases in the number of myenteric neurons were also observed first on day four post-induction, but further neuronal loss was never demonstrated. Examination of the intestinal segments adjacent proximally and distally to the inflamed area likewise significant decreases in neuronal density after the single dose of TNBS indicating the spreading of the neuronal injury outside the inflamed area. Literature data from surgical practice which indicate a neuronal pathway for the spreading of intestinal inflammation at the resection margins are in accordance with these findings. However, after the administration of repeated doses of TNBS the neuronal loss was strictly limited to the inflamed segments of the colon: no proximal or distal spread of the neuronal injury was noticed. Further long-range studies with the present model are now in progress to elucidate the pathogenetic role of the myenteric plexus in the spreading of CD.

These observations of alleviated mucosal and neuronal injury, accelerated mucosal healing and reduced and restricted neuronal cell loss clearly indicate the preconditioning effect of the experimentally provoked RRI. However, the accelerated recovery in the acute phase of inflammation might be misleading in early diagnosis of CD, and thus in avoiding the development of chronic complications. Therefore, studies on the long-term structural and functional consequences of this protective effect are currently in progress in our laboratory.

Since an increased expression of HO-1 was previously described in patients with CD as a crucial mediator of the mucosal defence, we expected changes in HO-1 gene expression behind the mucosal and neuronal defence demonstrated here. The HO-1 mRNA expression
was therefore evaluated by qRT-PCR after single and repeated TNBS treatments. Marked increase in HO-1 gene expression was already demonstrated on day four after administration of the single dose of TNBS and the expression was enhanced to a great extent after the second and even more so after the third treatment. There was a decline in HO-1 expression between days four and eight in samples from the once and twice TNBS-treated rats, although it never returned to the baseline level. However after the third treatment there was no decline at all, but rather a more enhanced expression of HO-1 mRNA was detected until the post-induction day eight. HO-1 mRNA down-regulation in adjacent non-inflamed sites at the same time led us to hypothesize that an early inactivation of the endogenous antioxidant defense system at the border-line of inflammation might contribute to the recurrence of CD proximal to the inflamed gut segments observed regularly after surgical resection. In parallel with the sustained up-regulation of HO-1 mRNA expression the increase in HO-1 protein level proved to be transient here and after the third treatment HO-1 protein expression could not be detected at all. Therefore, we suggest a post-transcriptional control mechanism for HO-1 expression in RRI. Recent stud-

Figure 3 Density of HuC/HuD-immunoreactive myenteric neurons in the colonic segments of control (C) (n = 12) and TNBS-treated rats four (n = 13) and eight (n = 14) days after colitis induction. In the TNBS-treated groups the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments were examined. Significant decrease in myenteric neuronal density was first detected on day four after each TNBS administration. When the rats were treated only once (1 × TNBS) the number of myenteric neurons decreased significantly in all three colonic segments, while after repeated treatments (2 × TNBS, 3 × TNBS), a significant decrease in neuronal number was demonstrated exclusively in the I segments. After the single dose of TNBS a further significant neuronal loss was detected until day eight post-induction, whereas after repeated treatments the number of myenteric neurons did not decrease further between days four and eight. Data are expressed as means ± SE. *P < 0.01 vs age-matched control groups; †P < 0.01 vs once TNBS-treated group on day four. TNBS: 2,4,6-trinitrobenzenesulfonic acid.

Figure 4 Relative mRNA expression of heme oxygenase-1 compared to the controls (n = 12) (C) in the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments four (n = 13) and eight (n = 14) days after TNBS treatments. Irrespective of the number of treatments (1 × TNBS, 2 × TNBS, 3 × TNBS), the HO-1 expression was always marked on day four in the I segments and a more limited expression was also detected in the P but not in the D colonic segments. After the first and second TNBS administrations the rate of HO-1 gene expression decreased, but never returned to baseline level up to day eight post-induction. Nevertheless, after the third TNBS treatment the high level of HO-1 expression was not merely sustained: a pronounced further increase was demonstrated until day eight post-induction. Data are expressed as means ± SD. TNBS: 2,4,6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.
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Figure 5  Protein expression of heme oxygenase-1 (32 kDa) in the inflamed segments of the colon four (n = 4) and eight (n = 5) days after TNBS treatments, compared to the controls (n = 4) (C). Four days after the first and second treatment (1 × TNBS, 2 × TNBS) elevated heme oxygenase-1 (HO-1) protein level was detected. Than the amounts of protein in both samples declined and eight days after the first treatment it reached already the baseline level. Following the third treatment (3 × TNBS) HO-1 protein expression could not be detected at all. TNBS: 2,4,6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.

with CD as a crucial mediator of the mucosal defence.

Peer review
The authors reported a modified model for CD that used repeated administrations of TNBS with a two-week-lag and concluded that experimentally provoked recurrent inflammation may exert a protective preconditioning effect against the mucosal and neuronal damage. The study is interesting, the experiments were well described and the results were clearly presented. In general the paper is well written.

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