Enteroendocrine cells, stem cells and differentiation progenitors in rats with TNBS-induced colitis

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Abstract. Patients with inflammatory bowel disease (IBD), as well as animal models of human IBD have abnormal enteroendocrine cells. The present study aimed to identify the possible mechanisms underlying these abnormalities. For this purpose, 40 male Wistar rats were divided into 4 groups as follows: the control group, the group with trinitrobenzene sulfonic acid (TNBS)-induced colitis with no treatment (TNBS group), the group with TNBS-induced colitis treated with 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G; an activator protein-1 inhibitor) (DTCM-G group), and the group with TNBS-induced colitis treated with dehydroxymethylepoxyquinomicin (DHMEQ; a nuclear factor-κB inhibitor) treatment (DHMEQ group). Three days following the administration of TNBS, the rats were treated as follows: those in the control and TNBS groups received 0.5 ml of the vehicle [0.5% carboxymethyl cellulose (CMC)], those in the DTCM-G group received DTCM-G at 20 mg/kg body weight in 0.5% CMC, and those in the DHMEQ group received DHMEQ at 15 mg/kg body weight in 0.5% CMC. All injections were administered intraperitoneally twice daily for 5 days. The rats were then sacrificed, and tissue samples were taken from the colon. The tissue sections were stained with hemotoxylin-eosin and immunostained for chromogranin A (CgA), serotonin, peptide YY (PYY), oxyntomodulin, pancreatic polypeptide (PP), somatostatin, Musashi1 (Msi1), Math1, Neurogenin3 (Neurog3) and NeuroD1. The staining was quantified using image analysis software. The densities of CgA-, PYY-, PP-, Msi1-, Neurog3- and NeuroD1-positive cells were significantly lower in the TNBS group than those in the control group, while those of serotonin-, oxyntomodulin- and somatostatin-positive cells were significantly higher in the TNBS group than those in the control group. Treatment with either DTCM-G or DHMEQ restored the densities of enteroendocrine cells, stem cells and their progenitors to normal levels. It was thus concluded that the abnormalities in enteroendocrine cells and stem cells and their differentiation progenitors may be caused by certain signaling substances produced under inflammatory processes, resulting in changes in hormone expression in enteroendocrine cells. These substances may also interfere with the colonogenic activity and the differentiation of the stem-cell secretory lineage into mature enteroendocrine cells.

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Introduction

The large intestine contains five types of enteroendocrine cells: namely serotonin-, polypeptide YY (PYY)-, oxyntomodulin (enteroglucagon)-, pancreatic polypeptide (PP)- and somatostatin-producing cells (1). In addition, chromogranin A (CgA) is expressed by all enteroendocrine cells and is used as a common marker for them (2). The interaction between enteroendocrine and immune cells during inflammation was recently discussed, and this interaction is thought to play a pivotal role in the inflammatory process (3).

Patients with inflammatory bowel disease (IBD), as well as animal models of human IBD have been shown to have abnormal enteroendocrine cells (4-24). The nature of the changes in enteroendocrine cells differs between ulcerative colitis (UC), Crohn's disease (CD) and microscopic colitis (4-23). The mechanisms underlying such abnormalities are not yet known. However, a recent study using an animal model of human UC, namely dextran sulfate sodium-induced colitis, found that the abnormalities in enteroendocrine cells strongly correlated with the abnormal differentiation progeny of stem cells (25). It has been suggested that the abnormalities in the enteroendocrine cells in this animal model are caused by an abnormal stem cell differentiation progeny toward enteroendocrine cells (25).

Trinitrobenzene sulfonic acid (TNBS)-induced colitis in experimental animals is commonly used as an animal model of human CD (26). The enteroendocrine cells in this animal

model have been reported to be abnormal (27). The treatment colitis with activator protein 1 (AP-1) and nuclear factor-κB inhibitors, which are potent anti-inflammatory agents, has been shown to restore enteroendocrine cells to normal levels (27).

The aim of the present study was to determine whether the changes in the densities of enteroendocrine cells in TNBS-induced colitis involve stem cell differentiation and/or the cellular expression of enteroendocrine cell hormones.

Materials and methods

Rats. A total of 40 male Wistar rats (Hannover GALAS; Taconic Farms Inc., Lille Skensved, Denmark) with a mean body weight of 200 g (range, 160-250 g) were housed in Macrolon III cages with water and food available *ad libitum*. They were fed a standard diet (B&K Universal, Nittedal, Norway) and were kept at a temperature of 18-22˚C, a relative humidity of 50-60%, and under a 12/12-h light/dark cycle. The rats were allowed to acclimatize to the conditions in the animal house for at least 7 days prior to being used in the experiments. The rats were divided into the following 4 groups containing 10 animals in each: i) the control group; ii) the group with TNBS-induced colitis with no treatment (TNBS group); iii) the group with TNBS-induced colitis treated with 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G; an activator protein-1 inhibitor) (DTCM-G group); and iv) the group with TNBS-induced colitis treated with dehydroxymethylepoxyquinomicin (DHMEQ; a nuclear factor-κB inhibitor) (DHMEQ group).

This study was performed in accordance with the Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (86/609/EEC), in compliance with the Helsinki Declaration. The Local Ethics Committee for Experimental Animals at the University of Bergen (Bergen, Norway) approved the study.

Use of TNBS to induce colitis. Colitis was induced in the rats in the TNBS, DTCM-G and DHMEQ groups as previously described (28) using a single dose of TNBS (Sigma-Aldrich Logistik, Steinheim, Germany). The animals were anesthetized with isoflurane (Schering-Plough Pharmaceuticals, North Wales, PA, USA), and TNBS was administered into the colon at 8 cm from the anal margin (25 mg/animal in 50% ethanol solution; 0.5 ml/rat), followed by 2 ml of air. TNBS was administered via an 8.5-cm-long, 2.5-mm-diameter round-tipped Teflon feeding tube (AngTheo, Lidingö, Sweden). The animals were kept in the prone position with their hind legs raised for approximately 3 min following the administration of TNBS. They were supervised until recovery and then monitored daily. The rats in the control group were subjected to the same procedure as the rats in the TNBS group, except that 0.9% saline was introduced into the colon instead of TNBS.

Treatment with DTCM-G and DHMEQ. Three days following the administration of TNBS, the rats were treated as follows: those in the control and TNBS groups received 0.5 ml of the vehicle [0.5% carboxymethyl cellulose (CMC)], those in the DTCM-G group received DTCM-G at 20 mg/kg body weight in 0.5% CMC, and those in the DHMEQ group received DHMEQ at 15 mg/kg body weight in 0.5% CMC. All injections were administered intraperitoneally twice daily for 5 days. The synthesis of DTCM-G and DHMEQ is described in detail elsewhere (29-34). Animals exhibiting signs of pain were administered a subcutaneous injection of 1 ml of a 0.3-g/ml Temgesic solution (Merck Pharmaceuticals, Kenilworth, NJ, USA). At the end of the experiments, the animals were sacrificed by the inhalation of CO_2 and tissue samples were obtained from the colon.

Histopathological and immunohistochemical examinations. The colonic tissues were fixed in 4% buffered paraformaldehyde overnight, embedded in paraffin, and cut into 5-μm-thick sections, which were stained with hematoxylin and eosin (ThermoFischer Scientific, Waltham. MA, USA). Inflammation was evaluated using the scoring system of Hunter *et al* (35). The sections were also immunostained using the ultraView Universal DAB Detection kit (version 1.02.0018) and the BenchMark Ultra IHC/ISH staining module (both from Venata Medical Systems, Basel, Switzerland). The sections were incubated with the primary antibodies for 32 min at 37˚C. Details of the primary antibodies used are presented in Table I.

Morphometry. The endocrine cells were quantified using image analysis software (version 1.7, cellSens; Olympus, Tokyo, Japan). The numbers of endocrine, and Musashi (Msi1)-, Math1-, Neurogenin3 (Neurog3)- and NeuroD1-positive cells were counted manually. The area of epithelial cells was determined manually by drawing an enclosed region using the computer mouse.

The densities of endocrine cells were expressed as the number of immunoreactive endocrine cells per square millimeter of epithelium, the density of Msi1 cells was expressed as the number of immunoreactive cells per crypt, and the densities of Math1, Neurog3 and NeuroD1 cells were expressed as the number of immunoreactive cells per field. Quantification was performed in 10 randomly chosen microscopic fields using a X40 objective. The measurements were made by the same individual (M.E.-S.) who was blind to the identities of the slides.

Statistical analysis. Differences between the control, TNBS, DTCM-G and DHMEQ groups were analyzed using the Kruskal-Wallis non-parametric test, with Dunn's test as a post-test. The correlations between abnormalities in the densities of PYY/oxyntomodulin-, CgA/serotonin- and PP/somatostatin-positive cells were determined using the non-parametric Spearman correlation test. The data are presented as the mean ± SEM values. Probability values of $P<0.05$ were considered to indicate statistically significant differences.

Results

Two animals died spontaneously in the TNBS group. There were no deaths in the other 3 groups.

Histopathological and immunohistochemical examinations. The histopathological inflammation scores were 6.4±1.1, 1.8±1.2 and 2.3±0.9 in the TNBS, DTCM-G and DHMEQ groups, respectively (Kruskal-Wallis test, P=0.002). Dunn's test showed that the scores differed between the TNBS group, and the DTCM-G and DHMEQ groups (P=0.04 and 0.02, respectively) (data not shown).

Table I. Primary antibodies used in immunohistochemical staining.

Antibodies raised against	Source	Code no.	Working dilution	Type of antibody	Detects
N-terminal of purified CgA	Dako (Glostrup, Denmark)	M869	1:1,000	Monoclonal, raised in mouse	CgA
Serotonin	Dako	5HT-209	1:1,500	Monoclonal, raised in mouse	Serotonin
PYY	Alpha-Diagnostica (San Antonio, TX, USA)	PYY 11A	1:1,000	Polyclonal, raised in rabbit	PYY
Porcine glicentin/glucagon	Acris antibodies (Herford, Germany)	BP508	1:800	Polyclonal, raised in rabbit	Oxyntomodulin (enteroglucagon)
Synthetic human PP	Diagnostic Biosystems (Pleasanton, CA, USA)	#114	1:400	Polyclonal, raised in rabbit	PP
Synthetic human somatostatin	Dako	A566	1:200	Polyclonal, raised in rabbit	Somatostatin
Residues 5-21 [APQP GLASPDSPHDPCK] of the human, mouse and rat Msi1	Novus Biologicals Europe (Abingdon, UK)	NB100-1759	1:100	Polyclonal, raised in rabbit	Msi1
Sy\nthetic peptide surrounding amino acid 190 of human Math1	BioVision (Milpitas, CA, USA)	3658-100	1:50	Polyclonal, raised in rabbit	Math1
KLH-conjugated synthetic peptide between 40-69 amino acids from the N-terminal region of human Neurog3	ThermoFisher Scientific (Oslo, Norway)	BT-B56180	1:50	Polyclonal, raised in rabbit	Neurog3
Recombinant full-length human NeuroD1	Nordic BioSite (Täby, Sweden)	PA5-11893	1:100	Polyclonal, raised in rabbit	NeuroD1

CgA, chromogranin A; Msi, Musashi1; Neurog3, Neurogenin3; PYY, peptide YY; PP, pancreatic polypeptide.

CgA-, serotonin-, PYY-, oxyntomodulin-, PP-, somatostatin-, Msi1-, Math1-, Neurog3- and NeuroD1-positive cells were found in all the colonic tissues from the rats in all groups. The CgA-, serotonin-, PYY-, oxyntomodulin-, PP- and somatostatin-positive cells were located mostly in the crypts of Lieberkühn. Msi1-positive cells were found exclusively in the crypts of Lieberkühn. Msi1-positive cells in rats with colitis tended to accumulate at the margins of deep ulcers. Math1-, Neurog3- and NeuroD1-positive cells were observed in the crypts and alongside the gland of Lieberkühn (Figs. 2 and 6).

Morphometry. The results of the quantification of the different types of endocrine cells, stem cells and differentiation progenitors in all 4 experimental groups are summarized in Tables II and III.

The Kruskal-Wallis test showed that there were significant differences between the experimental groups regarding both PYY- and oxyntomodulin-positive cells (P=0.0003 and 0.001, respectively). Whereas the density of PYY-positive cells was significantly reduced in the TNBS group relative to the controls, the density of oxyntomodulin-positive cells was significantly increased (P<0.0001 for in both) (Figs. 1 and 2). The density of PYY-positive cells inversely correlated with the density of oxyntomodulin-positive cells (r=-0.7, P=0.04).

The densities of CgA- and serotonin-positive cells differed significantly between the control, TNBS, DTCM-G and DHMEQ groups (P=0.04 and 0.006, respectively). In the TNBS group, the density of CgA-positive cells was significantly reduced (P=0.02) and that of serotonin-positive cells was increased (P=0.004) (Fig. 3). The density of CgA-positive cells inversely correlated with the density of serotonin-positive cells (r=-0.7, P=0.03).

The Kruskal-Wallis test showed that there were significant differences in both the PP-positive and somatostatin-positive cell densities between the control and experimental groups (P=0.002 and 0.01, respectively). While the density of PP-positive cells was reduced in the TNBS group relative to controls (P=0.001) (Fig. 4), that of somatostatin-positive cells

Table II. Densities of colonic enteroendocrine cells in the 4 experimental groups.

Endocrine cell type	Controls	TNBS	DTCM-G	DHMEQ
CgA-positive	111.8±17.9	51.0±21.1[a]	104.0±14.6	107.6±17.1
Serotonin-positive	40.6±6.6	62.6±7.5[b]	39.6±6.2	37.0±5.4
PYY-positive	87.3±2.7	14.1±3.0[c]	83.8±4.0	83.9±2.6
Oxyntomodulin-positive	44.8±3.7	78.3±6.8[c]	48.3±4.4	49.3±3.8
PP-positive	58.0±3.5	31.8±7.5[c]	69.3±6.2	60.4±4.4
Somatostatin-positive	43.6±3.2	69.9±7.8[b]	40.5±3.2	43.7±5.1

Data are the mean ± SEM values. TNBS, trinitrobenzene sulfonic acid; DTCM-G, 3-[(dodecylthiocarbonyl)-methyl]-glutarimide; DHMEQ, dehydroxymethylepoxyquinomicin; PP, pancreatic polypeptide; PYY, peptide YY; CgA, chromogranin A. [a]P<0.05, [b]P<0.01 and [c]P<0.001 vs. controls.

Table III. Densities of colonic stem cells and differentiation progenitors in the 4 experimental groups.

Cell type	Controls	TNBS	DTCM-G	DHMEQ
Msi1-positive	4.8±0.5	1.9±0.3[b]	4.9±0.5	4.5±0.5
Math1-positive	72.2±8.5	98.8±12.7	97.2±9.3	98.3±12.7
Neurog3-positive	70.9±11.2	43.6±3.8[a]	106.0±12.6	79.5±12.0
NeuroD1-positive	68.8±10.4	44.5±7.2[a]	107.5±11.7	81.0±12.4

Data are the mean ± SEM values. TNBS, trinitrobenzene sulfonic acid; DTCM-G, 3-[(dodecylthiocarbonyl)-methyl]-glutarimide; DHMEQ, dehydroxymethylepoxyquinomicin; Msi1, Musashi1; Neurog3, Neurogenin3. [a]P<0.05 and [b]P<0.01 vs. controls.

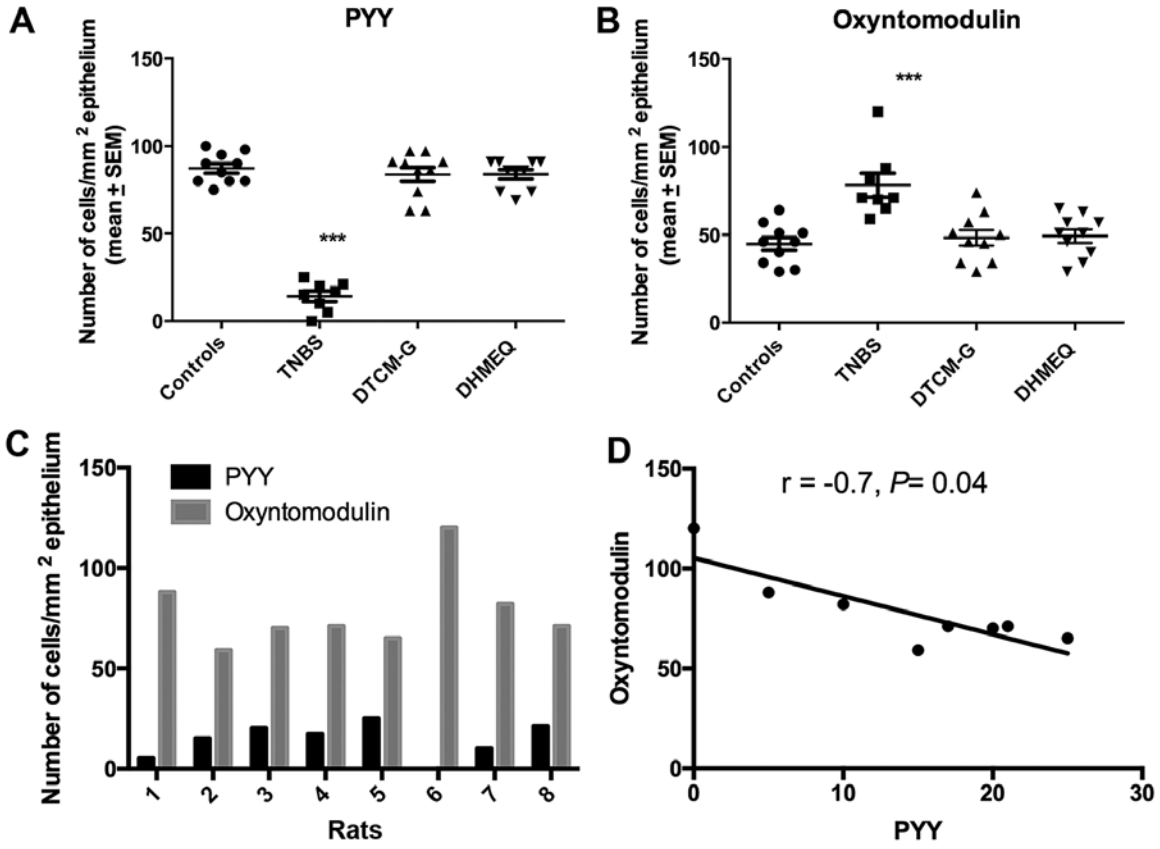

Figure 1. Densities of (A) peptide YY (PYY)-positive cells and (B) oxyntomodulin-positive cells in the control, trinitrobenzene sulfonic acid (TNBS), 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G), and dehydroxymethylepoxyquinomicin (DHMEQ) groups. (C) Densities of PYY-positive and oxyntomodulin-positive cells in each rat of the TNBS group and (D) their correlation. ***P<0.001 compared to controls.

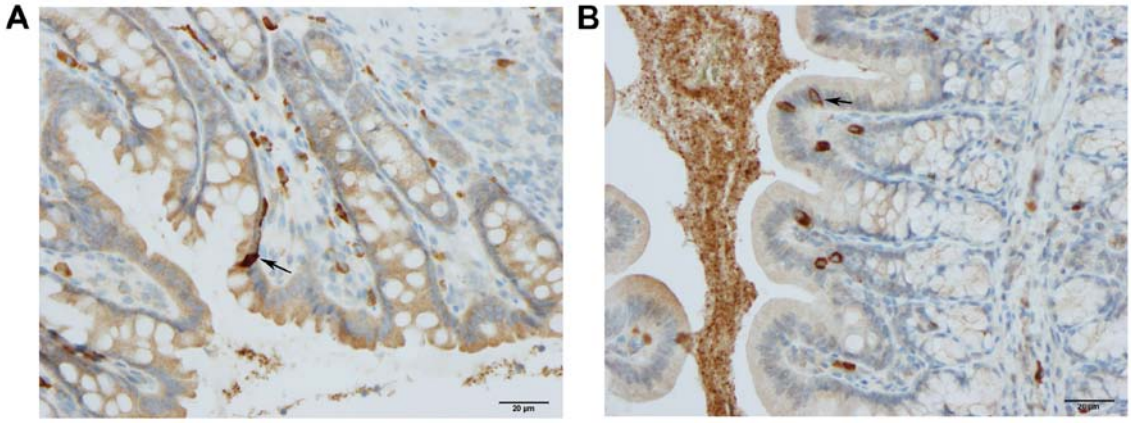

Figure 2. (A) Peptide YY (PYY)-positive cells and (B) oxyntomodulin-positive cells in a rat of the trinitrobenzene sulfonic acid (TNBS) group.

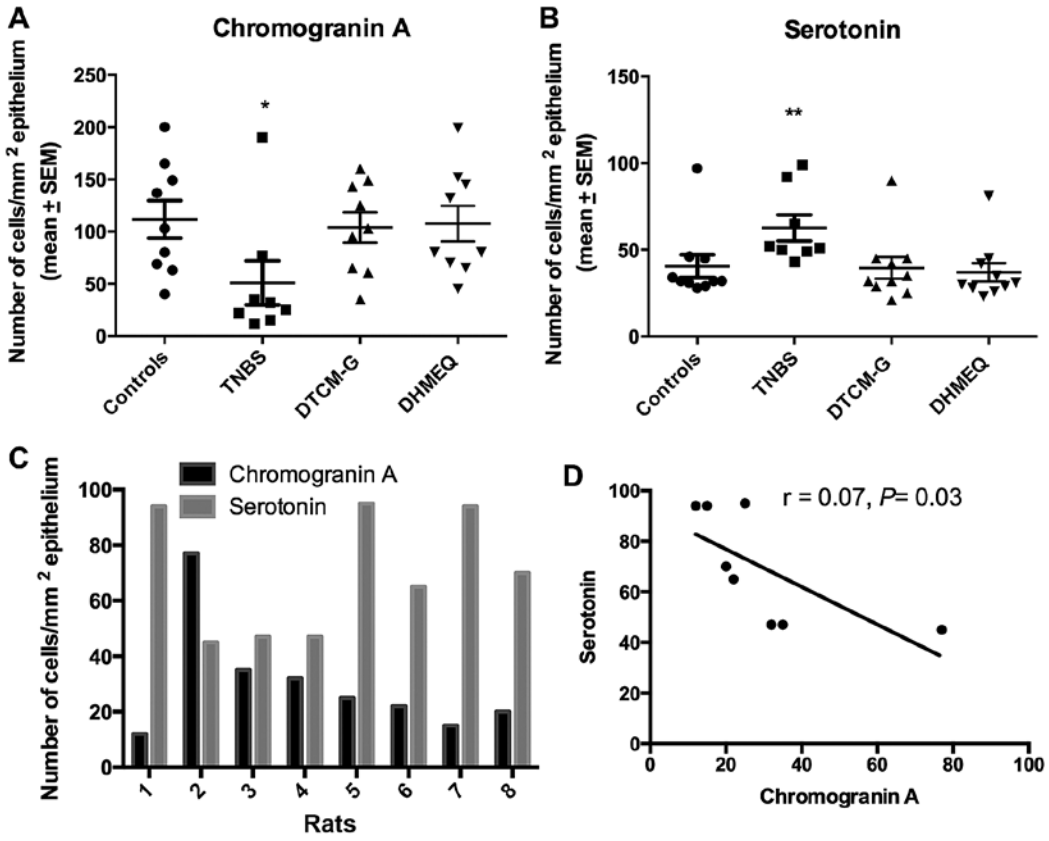

Figure 3. Densities of (A) chromogranin A (CgA)-positive cells and (B) serotonin-positive cells in the control, trinitrobenzene sulfonic acid (TNBS), 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G), and dehydroxymethylepoxyquinomicin (DHMEQ) groups. (C) Densities of CgA-positive and serotonin-positive cells in each rat of the trinitrobenzene sulfonic acid (TNBS) group and (D) their correlation. *P<0.05 and **P<0.01 compared to controls.

was increased (P=0.006). The density of PP-positive cells inversely correlated with the density of serotonin-positive cells (r=-0.8, P=0.004).

The Kruskal-Wallis test showed that there were significant differences in the densities of Msi1-, Neurog3- and NeuroD1-positive cells, but not in those of Math1-positive cells (P=0.0008, 0.006, 0.003 and 0.2, respectively). The densities of Msi1-, Neurog3- and NeuroD1-positive cells were reduced relative to the controls (P=0.0004, 0.04 and 0.03, respectively), whereas the density of Math1-positive cells was not (P=0.1) (Figs. 5 and 6).

Discussion

The interaction between enteroendocrine cells and immune cells has been recently debated, and it is believed that such an interaction plays an important role in the pathophysiology of IBD (3,36-40). Enteroendocrine cells in the same animal model for human CD studied herein have previously been reported to be abnormal (27). The mechanisms underlying these abnormalities however, are unknown.

It is well known that two hormones can be localized in the same enteroendocrine cell, namely glucagon-like

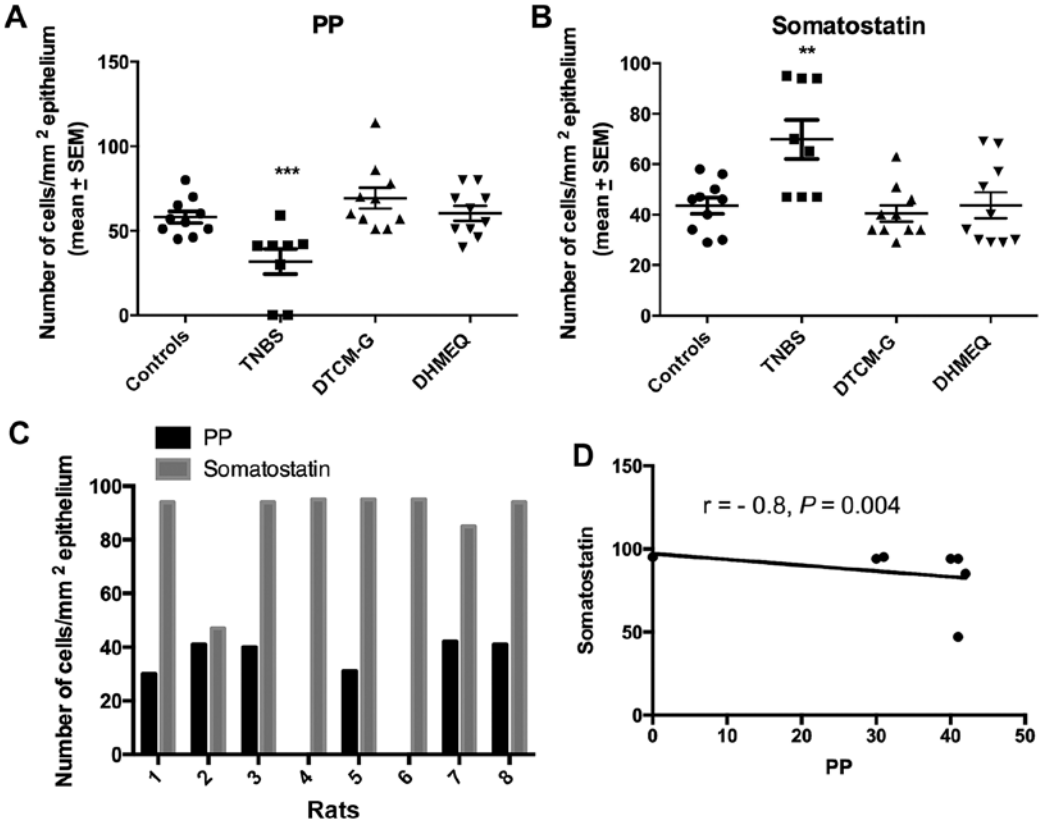

Figure 4. Densities of (A) pancreatic polypeptide (PP)-positive cells and (B) somatostatin-positive cells in the control, trinitrobenzene sulfonic acid (TNBS), 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G), and dehydroxymethylepoxyquinomicin (DHMEQ) groups. (C) Densities of PP-positive and somatostatin-positive cells in each rat of the TNBS group and (D) their correlation. **P<0.01 and ***P<0.001 compared to controls.

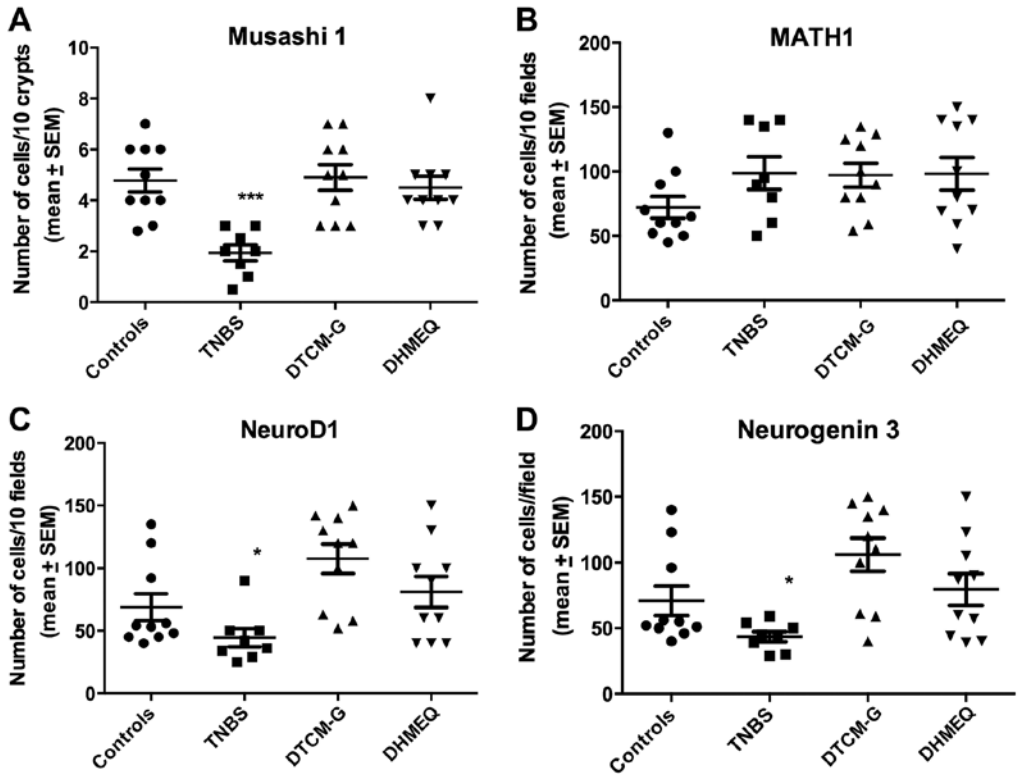

Figure 5. Densities of (A) Musashi1 (Msi1)-positive, (B) Math1-positive, (C) Neurogenin3 (Neurog3)-positive, and (D) NeuroD1-positive cells in the colon of rats in the control, trinitrobenzene sulfonic acid (TNBS), 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G), and dehydroxymethylepoxyquinomicin (DHMEQ) groups. *P<0.05 and ***P<0.001 compared to controls.

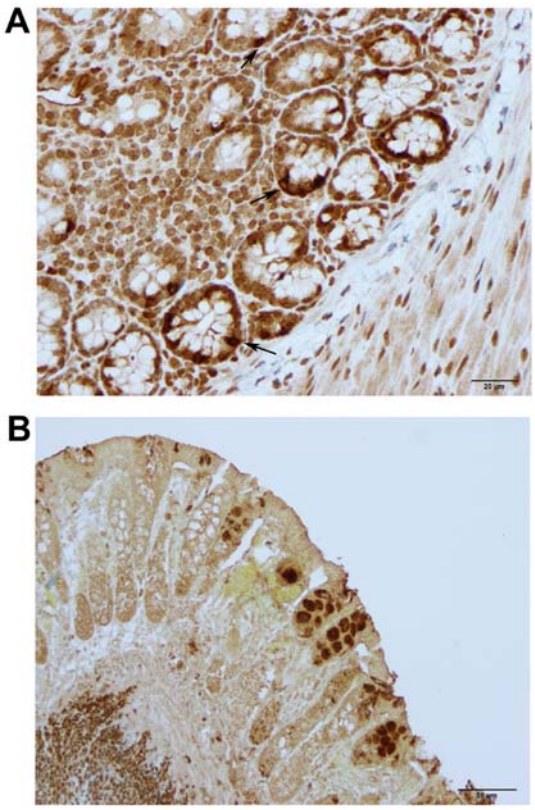

Figure 6. Musashi1 (Msi1)-positive cells (arrows) in (A) a control rat and (B) in a rat with trinitrobenzene sulfonic acid (TNBS)-induced colitis.

peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) in the small intestine, and PYY and oxyntomodulin in the distal small and large intestines (41-44). Recent studies have further demonstrated that mature enteroendocrine cells are capable of expressing up to 7 different hormones (45-47). In the present study, the increase in the oxyntomodulin-positive cell density was accompanied by a decrease in the PYY-positive cell density, with a significant inverse correlation. Similar observations were found concerning the CgA/serotonin-positive and PP/somatostatin-positive cell densities. It is reasonable to assume that the inflammatory process affects enteroendocrine cells so that they 'switch off' the expression of a certain hormone and 'switch on' the expression of another.

The intestine contains 4 to 6 stem cells per crypt, which either divide into new stem cells (self-renewal; clonogeny) or differentiate into all types of epithelial cells (differentiation progeny) (48-59). The differentiation progeny includes two lineages: secretory and absorptive. The secretory lineage gives rise to goblet, endocrine and Paneth cells, while the absorptive lineage gives rise to absorptive enterocytes (48-59). Msi1 is a transcription factor expressed by both intestinal stem cells and their early progeny (60-63). Math1 is expressed in the secretory lineage by an early progenitor, and mutant ($Math1^{-/-}$) mice have no secretory cells (64). Neurog3 is expressed by an early progenitor in the secretory lineage, which directs the differentiation of secretory progenitors into endocrine cells (65). Transgenic mice ($Neurog3^{-/-}$) express normal densities of goblet and Paneth cells, but no enteroendocrine cells at all (65-67). NeuroD1 is expressed by progenitors derived from Neurog3 progenitors (68,69). Mice deficient in NeuroD1 do not have a certain subgroup of enteroendocrine cells (66,70).

In this study, the density of Msi1-immunoreactive cells in TNBS-induced colitis was reduced relative to the controls, indicating that the clonogenic activity of the stem cells is affected by inflammation. On the other hand, the density of Math1-immunoreactive cells did not differ between the group with TNBS-induced colitis and the controls, suggesting that inflammation does not interfere with the early secretory lineage differentiation. The present observation that the densities of both Neurog3- and NeuroD1-positive cells were lower in rats with TNBS-induced colitis than in the controls may indicate a decease in the differentiation of stem cells into enteroendocrine cells.

The reduction in enteroendocrine cells observed in this study following the induction of colitis by TNBS seems to be caused by i) the 'switching on' and 'switching off' of the expression of certain hormones by enteroendocrine cells, and ii) decreases in the clonogenic activity of the stem cell and in the differentiation into enteroendocrine cells from stem cell progenitors. It may be speculated that the inflammatory processes trigger certain signaling substances that cause certain enteroendocrine cells to change their hormone expression. These substances may also affect the colonogenic activity and the differentiation of the stem cell secretory lineage into mature enteroendocrine cells.

The 'switching on and off' of the expression of hormones of enteroendocrine cells must occur on a timescale of minutes or hours, and stem cells differentiate into mature intestinal cells in 2-3 days (61). This explains why changes in the densities of enteroendocrine cells, stem cells and differentiation progeny to enteroendocrine cells could be detected 3 days after the induction of colitis using TNBS, and that the treatment of colitis for 5 days with anti-inflammatory agents restored their densities to normal levels.

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