Abnormal differentiation of stem cells into enteroendocrine cells in rats with DSS-induced colitis

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Received August 20, 2016; Accepted December 5, 2016

DOI: 10.3892/mmr.2017.6266

Abstract. The present study aimed to determine whether there is an association between abnormalities in enteroendocrine cells in dextran sulfate sodium (DSS)-induced colitis and the clonogenic and/or proliferative activities of stem cells. A total of 48 male Wistar rats were divided into four groups. Animals in the control group were provided with normal drinking water, whereas DSS colitis was induced in the remaining three groups. The rats with DSS-induced colitis were randomized into the following three groups: i) DSS group, which received 0.5 ml 0.5% carboxymethyl cellulose (CMC; vehicle); ii) DSS-G group, which was treated with 3-[(dodecylthiocarbonyl)-methyl]-glutarimide at 20 mg/kg body weight in 0.5% CMC; and iii) DSS-Q group, which was treated with dehydroxymethylepoxyquinomicin at 15 mg/kg body weight in 0.5% CMC. Treatments were administered intraperitoneally twice daily for 5 days in all groups. Subsequently, tissue samples from the colon were stained with hematoxylin-eosin, or immunostained for chromogranin A (CGA), Musashi 1 (Ms1), Math-1, neurogenin 3 (Neurog3) and neurogenic differentiation DI (NeuroDI). The densities of CGA, Ms1, Math-1, Neurog3- and NeuroDI-immunoreactive cells were determined. DTCM-G, and DHMEQ ameliorated the inflammation in DSS-induced colitis. The density of CGA, Neurog3- and NeuroDI-immunoreactive cells was significantly higher in the DSS group compared with in the control group, and the density of CGA cells was correlated with the densities of Neurog3- and NeuroDI-immunoreactive cells. There were no significant differences in the densities of Ms1- and Math-1-immunoreactive cells among the four experimental groups. The elevated densities of enteroendocrine cells detected in DSS-induced colitis may be due to the increased differentiation of early enteroendocrine progenitors during secretory lineage. It is probable that the DSS-induced inflammatory processes trigger certain signaling pathways, which control differentiation of the stem-cell secretory lineage into mature enteroendocrine cells.

Introduction

Inflammatory bowel disease (IBD) is a chronic disease that consists of ulcerative colitis (UC) and Crohn's disease (1-4). The clinical course of IBD varies markedly, from frequent relapses, to chronic active disease, to years of complete remission (5). At present, the etiology of IBD is not completely understood (6-8). There are at least five distinct types of enteroendocrine cell in the large intestine, which are arranged between the epithelial cells lining the intestinal lumen (9,10). These cells regulate intestinal motility, secretion and absorption, as well as visceral sensitivity, local immune defense, cell proliferation and appetite (9,11-26). The enteroendocrine cells in the large intestine are abnormal in patients with IBD and in animal models of IBD (24,27-42). Interactions between the hormones secreted by the large intestine enteroendocrine cells and the immune system have previously been debated, and it has been speculated that these interactions serve a critical role in the pathophysiology of IBD (43-45).

The cause of abnormalities in the large intestine enteroendocrine cells in IBD is not currently known. Abnormal intestinal enteroendocrine cells have been reported in congenital malabsorptive diarrhea alongside mutated transcription factor Neurogenin 3 (Neurog3), and in mutant mice with ablation of Neurog3 (46). The present study aimed to investigate whether the abnormalities observed in intestinal enteroendocrine cells in dextran sulfate sodium (DSS)-induced colitis are associated with abnormalities in the clonogenic and/or proliferative activities of stem cells (47). Furthermore, it was investigated whether the alterations in enteroendocrine cells and stem cells may be restored by treatment with two anti-inflammatory agents: 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G) and dehydroxymethylepoxyquinomicin (DHMEQ). These agents have been demonstrated to exert potent anti-inflammatory activity in animal models (48,49).
Materials and methods

Rats. A total of 48 male Wistar rats (6 weeks old; Hannover GALAS; Taconic Europe A/S, Lille Skensved, Denmark) with a mean body weight of 290 g (range, 238-385 g) were housed in Macrolon III cages with ad libitum access to food and water. The rats were fed a standard diet (B&K Universal Limited, Hull, UK), and were maintained under the following conditions: Temperature between 20 and 22°C, relative humidity between 50 and 60%, and 12/12-h light/dark cycle.

The animals were allowed to acclimate in the animal house for ≥1 week prior to experimentation, and were then divided into 4 groups, each containing 12 rats. Rats in the control group were provided with normal drinking water for 7 days, whereas colitis was induced in the other three groups using DSS, as previously described (50,51). Briefly, the rats were provided with distilled drinking water containing 5% DSS (40 kD; TDB Consultancy AB, Uppsala, Sweden) for 7 days. The rats with DSS-induced colitis were randomized into the following three groups: i) DSS group, which received 0.5 ml 0.5% carboxymethyl cellulose (CMC; vehicle); ii) DSS-G group, which was treated with DTCM-G at 20 mg/kg body weight in 0.5% CMC; and iii) DSS-Q group, which was treated with DHMEQ at 15 mg/kg body weight in 0.5% CMC. Treatments were administered intraperitoneally twice daily for 5 days in all groups.

Histopathology and immunohistochemistry. The tissue samples were fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut into 5 mm sections. The sections were stained with hematoxylin and eosin, or immunostained using the ultraView Universal DAB Detection kit (version 1.02.0018; Ventana Medical Systems, Inc., Tucson, AZ, USA) and the BenchMark Ultra IHC/ISH staining module (Ventana Medical Systems, Inc.). The sections were incubated with the following primary antibodies for 32 min at 37°C: Monoclonal mouse anti-N-terminus of purified chromogranin A (CgA; 1:1,500; cat. no. M869; Dako Denmark A/S, Glostrup, Denmark); polyclonal rabbit anti-residues 5-21 [APQPGLASPDHPDCK] of the human, mouse and rat Musashi 1 (Msi1) protein (1:100; cat. no. NB100-1759; R&D Systems Europe, Abingdon, UK); polyclonal rabbit anti-synthetic peptide surrounding amino acid 190 of human Math-1 (1:50; code no. 3658-100; BioVision, Inc., Milpitas, CA, USA); polyclonal rabbit anti-KLH-conjugated synthetic peptide between 40-69 amino acids from the N-terminal region of human Neurog3 (1:50; cat. no. PA5-11893, Thermo Fisher, Oslo, Norway); and polyclonal rabbit anti-recombinant full-length human neurogenic differentiation D1 (NeuroD1; 1:50; cat. no. PA5-47381; Thermo Fisher). All of these antibodies detect antigens in humans and rats.

Quantification. The number of CgA-, Msi1-, Math-1-, Neurog3- and NeuroD1-immunoreactive cells, the number of crypts, and the area containing epithelial cells were counted in ten randomly selected microscopic fields using a light microscope (BX 43). Measurements were performed using cellSens imaging software (version 1.7; Olympus Corporation, Tokyo, Japan). This morphometric method has previously been validated (56). The number of immunoreactive cells and crypts in each field were counted manually by pointing and clicking the computer mouse, whereas the area of epithelial cells was determined by manual drawing using the computer mouse. A x40 objective was used, for which each frame (field) on the monitor represented a tissue area of 0.035 mm². The density of CgA was expressed as the number of immunoreactive endocrine cells per square millimeter of epithelium, the density of Msi1 was expressed as the number of immunoreactive cells per crypt, and the densities of Math-1, Neurog3 and NeuroD1 were expressed as the number of immunoreactive cells per field. Immunostained sections were coded, and measurements were performed by the same individual (M.E-S.), who was blinded to the identity of the sections.

Statistical analysis. The Kruskal-Wallis nonparametric test and Dunn's post hoc test were used to compare between the control, DSS, DSS-G and DSS-Q groups. Correlations between abnormalities/alterations in the densities of CgA-, Neurog3-, and NeuroD1-immunoreactive cells were determined using the nonparametric Spearman correlation test. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

The colon samples collected from rats in the control, DSS-G and DSS-Q groups appeared histopathologically normal; however, in the DSS group, disturbed mucosal architecture, crypt abscesses, edema, bleeding and immune cell infiltration were observed (Fig. 1).

CgA immunostaining. CgA-immunoreactive cells were detected in crypts and alongside the gland of Lieberkühn. The cell densities in the control, DSS, DSS-G and DSS-Q groups were 131.7±20.4, 319.1±32.0, 123.9±22.6 and 141.3±14.3 cells/mm² epithelium, respectively (Kruskal-Wallis test, P<0.0001; Figs. 2 and 3). Dunn's test indicated that the density of CgA-immunoreactive cells was significantly higher in the DSS group compared with in the control group (P<0.0001). The densities of CgA in DSS-G and DSS-Q did not differ from that of controls (P=0.9, and 0.1, respectively). The CgA-immunoreactive cell density was correlated with the densities of Neurog3- and NeuroD1-immunoreactive cells (r=0.8; P=0.006 for both).

Msi1 immunostaining. Msi1-immunoreactive cells were observed exclusively in the crypts of the gland of Lieberkühn.
The cell densities in the control, DSS, DSS-G and DSS-Q groups were 4.9±0.5, 4.8±0.5, 5.1±0.6 and 5.0±0.6 cells/crypt, respectively (Kruskal-Wallis test, P=0.98; Fig. 4).

Math-1 immunostaining. Math-1-immunoreactive cells were observed in the crypts and alongside the gland of Lieberkühn. The cell densities in the control, DSS, DSS-G and DSS-Q groups were 80.2±10.4, 101.6±10.7, 99.1±8.3 and 100.1±11.3 cells/field, respectively (Kruskal-Wallis test, P=0.41; Fig. 4).

Neurog3 immunostaining. Neurog3-immunoreactive cells were detected in the crypts and alongside the gland of Lieberkühn (Figs. 4 and 5). The cell densities were 79.1±11.1, 223.1±36.0, 103.8±12.4 and 77.3±10.7 cells/field in the control, DSS, DSS-G and DSS-Q groups, respectively (Kruskal-Wallis test, P=0.002). The Neurog3-immunoreactive cell density was significantly higher in the DSS group compared with in the control group (Dunn's test: P=0.0002; Fig. 4C). There was no statistically significant difference between controls and DSS-G and DSS-Q regarding Neurog3 cell density (P=0.1, and 0.7, respectively).

NeuroD1 immunostaining. Similar to Neurog3, NeuroD1-immunoreactive cells were observed in the crypts and alongside the gland of Lieberkühn. The cell densities were 73.3±10.7, 217.3±24.4, 105.8±11.8 and 79.1±10.7 cells/field in the control, DSS, DSS-G and DSS-Q groups, respectively (Kruskal-Wallis test, P=0.0001). The density of NeuroD1-immunoreactive cells was significantly higher in the DSS group compared with in the control group (Dunn's test, P=0.0002; Fig. 4). The densities of NeuroD1 in DSS-G, and DSS-Q did not differ from that of controls (P=0.07, and 0.9, respectively).

Discussion
CgA is a general marker for enteroendocrine cells (57). In the present study, the density of CgA-immunoreactive cells
in the large intestine was significantly elevated in rats with DSS-induced colitis, which is in agreement with previously reported observations (47). DSS-induced colitis is an animal model that is very similar, but not identical, to human UC (58).
The density of CgA-immunoreactive cells in the large intestine has also been reported to be higher in patients with UC compared with in healthy subjects (27). The intestine contains between 4 and 6 stem cells per crypt, and these cells exhibit two types of activity: i) Dividing into new stem cells (self-renewal, clonogeny) and ii) differentiating into all types of epithelial cell (differentiation) (59-71). The differentiating stem cell progeny includes two lineages: Secretory and absorptive. The secretory lineage gives rise to goblet, endocrine and Paneth cells, whereas the absorptive lineage gives rise to absorptive enterocytes (59-71). Msi1 is a transcription factor expressed by intestinal stem cells and their early progeny (71-74). In the present study, the density of Msi1-immunoreactive cells did not differ between rats in the DSS group and those in the control group, thus indicating that the clonogenic activity of the stem cells was not affected by inflammation.

Math-1 is expressed by an early progenitor in the secretory lineage, and Math<sup>-/-</sup> mice lack secretory cells (75). The present study indicated that the density of Math-1-immunoreactive cells did not significantly differ between rats in the DSS group and those in the control group, thus indicating that the clonogenic activity of the stem cells was not affected by inflammation.

Neurog3 is expressed in endocrine progenitor cells, which direct the differentiation of secretory progenitors into endocrine cells (46). Neurog3<sup>-/-</sup> mice possess normal densities of goblet and Paneth cells; however, they possess no pancreatic endocrine or enteroendocrine cells (46,76,77). NeuroD1 is a transcription factor that is expressed by cells derived from Neurog3 progenitors (78,79). Mice deficient in NeuroD1 do not possess a subgroup of enteroendocrine cells (46,80). In the present study, the densities of Neurog3- and NeuroD1-immunoreactive cells were higher in DSS-induced rats compared with in control rats. Furthermore, this elevation was strongly correlated with the increased CgA-immunoreactive cell density. This finding provided evidence to suggest that the increased density of enteroendocrine cells observed following DSS-induced colitis may be caused by an increase in the differentiation of early enteroendocrine progenitors during the secretory lineage. Intestinal stem cell proliferation is regulated by numerous signaling pathways (71). It is probable that the DSS-induced inflammatory processes trigger certain signaling pathways, which control the differentiation of the stem-cell secretory lineage into mature enteroendocrine cells.

The present study confirmed the findings of previous studies, that DTCM-G and DHME exhibit potent anti-inflammatory activity in animal models of UC (48,49). Stem cells differentiate rapidly into mature intestinal cells; this process typically takes 2-3 days (72). This may explain why, in the present study, treating rats with DSS-induced colitis with the anti-inflammatory agents DTCM-G and DHME for only 5 days restored the densities of CgA, Neurog3- and NeuroD1-immunoreactive cells to those of the control group. The rapid proliferation and differentiation of epithelial cells are disturbed by inflammation, which causes impairment in epithelial barrier function (81-84). Polyphenols, which is quite different from DTCM-G and DHME, exert a protective effect on epithelial cells and consequently suppress the inflammatory response (81-83).
In conclusion, the present study demonstrated that the elevated densities of enteroendocrine cells detected in DSS-induced colitis are probably due to increased differentiation of early enteroendocrine progenitors during the secretory lineage. It is likely that inflammatory processes trigger certain signaling pathways that control differentiation of the stem-cell secretory lineage into mature enteroendocrine cells. In addition, this process appears to be responsive to short-term anti-inflammatory treatment. It is probable that stem cell transplantation may be an effective treatment for patients with IBD, that have not responded to current available treatment.

Acknowledgements

The present study was supported by grants from Helse-Fonna (grant no. 40415) and Helse-Vest (grant no. 911978), Norway.

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