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Report

Methotrexate Induces Hyperplasia of Enterochromaffin Cells in Mouse Jejunum

Takuji Machida,*[,a] Airi Tanaka,[a] Megumi Kobayashi,[a] Kohei Imai,[a] Masahiko Hirafuji,[a,b] and Kenji Iizuka[a]

[a]*Department of Pharmacological Sciences, School of Pharmaceutical Sciences, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan;* [b]*Present address: School of Health Sciences, Iryo Sosei University, 5-5-1 Chuodai Iino, Iwaki, Fukushima 970-8551, Japan*

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5-Hydroxytryptamine (5-HT) is synthesized by L-tryptophan hydroxylase (TPH) and is stored mainly in enterochromaffin cells of the mucosal epithelium. We previously reported that administration of methotrexate, an anticancer agent, to rats caused hyperplasia of enterochromaffin cells, and nitric oxide (NO) might be involved in the underlying mechanism. The aim of this study was to clarify the effect of methotrexate on hyperplasia of enterochromaffin cells in mice. C57BL/6J mice were intraperitoneally injected with methotrexate or saline as a control. Methotrexate caused an increase in the number of TPH-expressing cells (i.e., enterochromaffin cells) in the jejunum. Methotrexate also increased inducible, but not constitutive, NOS mRNA expression. Our results indicate that methotrexate potentiates 5-HT synthesis in mice, as we previously found in rats.

Key words methotrexate, intestine, 5-hydroxytriptamine, inducible nitric oxide synthase

INTRODUCTION

The use of high doses of methotrexate, an anti-cancer drug, is associated with gastrointestinal damage, which leads to malabsorption, diarrhea, and weight loss.[1,2] In addition, methotrexate causes moderate emesis in humans and experimental animal models.[3,4] 5-Hydroxytryptamine (5-HT) has important roles in the development of chemotherapy-induced nausea and vomiting as well as improving reduced motor function. 5-HT is synthesized by tryptophan hydroxylase (TPH), the rate-limiting enzyme of 5-HT formation, and is stored mainly in enterochromaffin cells of the mucosal epithelium.[5] We previously reported that a single administration of methotrexate to rats caused pica, which is analogous to emesis, and potentiated intestinal 5-HT synthesis associated with enterochromaffin cell hyperplasia.[4] We also found that nitric oxide (NO) produced by inducible NO synthase (iNOS) plays a critical role in the methotrexate-induced hyperplasia of enterochromaffin cells in the intestine.[6]

The C57BL/6 mouse was the first mouse strain whose genome was fully sequenced in 2002. This strain is widely used as physiological or pathophysiological models for *in vivo* experiments and is also often applied to generate transgenic or knockout mouse models, including NOS knockout mice.[7] To further elucidate the role of NO in methotrexate-regulated hyperplasia of enterochromaffin cells in the intestine, it is necessary to perform experiments using NOS knockout mice in addition to pharmacological studies with NOS inhibitors. Therefore, as a preliminary study, we investigated whether the NO-dependent effect of methotrexate on enterochromaffin cell hyperplasia is also found in C57BL/6J mice. Our results show that methotrexate significantly induces hyperplasia of enterochromaffin cells and iNOS expression in mouse intestine, and the effects of methotrexate are similar to those previously found in rats.[4,6]

MATERIALS AND METHODS

Materials Methotrexate was obtained from Pfizer Co., Ltd. (Tokyo, Japan). The other reagents used in this study were of special grade and were purchased from local suppliers unless otherwise noted.

Animals Male C57BL/6J mice weighing 20–25 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed under constant conditions at a room temperature of 22 ± 2°C and humidity of 50 ± 10% with a regular 12-h light (08:00–20:00)-dark (20:00–08:00) cycle and free access to water and food. The animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of the Health Sciences University of Hokkaido.

Drug Treatment The mice were intraperitoneally injected with methotrexate at a dose of 200 mg/kg at Day 0 and 100 mg/kg at Day 1. The dosage of methotrexate used in this study was determined from the study of de Koning *et al*.[8] At 24 h after the second methotrexate injection (i.e., Day 2), the mice were euthanized by exsanguination under light anesthesia using isoflurane. Control mice were injected with equivalent volumes of physiological saline. Jejunum tissues were dissected in approximately 3-cm long segments and frozen rapidly in liquid nitrogen and stored until further analysis.

Immunohistochemical Analysis Fresh ileal tissues were also fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin for immunohistochemical analysis. After deparaffinization, the specimens were allowed to react with an anti-TPH polyclonal antibody (1:400; Abcam,

*To whom correspondence should be addressed. e-mail: tmachida@hoku-iryo-u.ac.jp

Table 1. Oligonucleotide Sequences of Primers Used for RT-PCR

Gene name	Forward	Reverse
TPH1	5' TTGGGCTGTGCAAACAAGATG 3'	5' TGTTTACAGGCAATCTTGGGATCA 3'
iNOS	5' TGTCTGCAGCAGCACTTGGATCA 3'	5' AACTTCGGAAGGGAGCAATG 3'
nNOS	5′ CTCACCCCGTCCTTTGAGTA 3′	5' GGTCGCTTTGACTCTCTTGG 3'
eNOS	5′ CAACGCTACCACGAGGACATT 3′	5′ CTCCTGCAAAGAAAAGCTCTGG 3'
IL-1β	5′ TCGAGCCCTGGAAGACCCACATCTG 3′	5′ GTTGTTCCTCTTCCAAGGTGTTTGCCTTAT 3′
TNF-α	5′ GTGATCGGTCCCAACAAGGA 3′	5′ AGGGTCTGGGCCATGGAA 3′
GAPDH	5′ AGGTCGGTGTGAACGGATTTG 3′	5' TGTAGACCATGTAGTTGAGGTCA 3′

Abbreviations: TPH1, tryptophan hydroxylase 1; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Cambridge, UK) diluted in Tris-buffered saline at room temperature for 30 min. The number of anti-TPH antibody-positive cells located in the epithelial cell line of the villus and crypt was counted under a light microscope and presented as the number of enterochromaffin cells per villus, as previously described.[4)]

Real-Time RT-PCR The expression of mRNA isolated from the intestine was analyzed by real-time RT-PCR with total RNA. The primer sets are shown in Table 1. The PCR products were calculated relative to glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analysis Statistical analysis of the results was performed using either the Mann–Whitney *U*-test or two-way analysis of variance with a *post hoc* Tukey's test for multiple comparisons. *P*-values < 0.05 were considered significant.

RESULTS

We first evaluated the effect of methotrexate on body weight. As shown in Fig. 1, the weight gain observed in the controls was significantly suppressed by the administration of methotrexate. Next, the effects of methotrexate on the number of TPH-expressing enterochromaffin cells in the intestinal mucosa were determined by immunohistochemical analysis. In the control intestinal tissue, nearly all enterochromaffin cells were sparsely located in the epithelial cell layer of the villi as well as in the crypts (Fig. 2A). Methotrexate increased the number of enterochromaffin cells in the intestinal mucosa, particularly in the crypts, at 48 h after the first injection (Fig. 2A and B). TPH1 mRNA expression was not significantly changed by methotrexate administration at 48 h after the first injection (Fig. 2C).

Next, we investigated the effect of methotrexate on NOS mRNA expression. As shown in Fig. 3A–C, iNOS, but not neuronal NOS (nNOS) or endothelial NOS (eNOS), mRNA expression was significantly increased by methotrexate administration. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are representative cytokines involved in the induction of iNOS and cyclooxygenase-2 after administration of methotrexate.[9,10)] Therefore, we investigated the effect of methotrexate on their mRNA expression in the intestine. Although there was no significant effect, IL-1β and TNF-α mRNA expression tended to increase following methotrexate administration (Fig. 3D and E). Thus, the gastrointestinal inflammation induced by methotrexate administration may affect iNOS mRNA expression, although no obvious morphological changes were observed following methotrexate administration (Fig. 2A).

DISCUSSION

In the present study, we found that methotrexate significantly induced hyperplasia of enterochromaffin cells and iNOS, but not constitutive NOS, expression in mouse intestine, which has also been observed in rat intestine after single administration of 50 mg/kg methotrexate.[4,6)]

Enterochromaffin cells originate from pluripotent stem cells in the depth of the crypts and migrate upward to the surface of the villi.[11)] Thus, our results indicate that methotrexate caused a transient stimulation of intestinal stem cells to generate fully differentiated cell types within 48 h after the first injection. Since there was no significant effect on TPH1 mRNA expression by methotrexate, it is possible that TPH1 mRNA expression was transiently increased by methotrexate but had returned to the control level by 48 h after the first injection.

Because the hyperplasia of enterochromaffin cells induced by single administration of methotrexate is blocked by the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in rat intestine,[6)] endogenous NO plays a critical role in the hyperplasia. Interestingly, L-NAME administration further promotes hyperplasia along with tissue injury when methotrexate is administered repeatedly with an equivalent final total dose of 50 mg/kg (i.e., 12.5 mg/kg/d for 4 d) to rats.[12,13)] Therefore, the role of NO in the change of 5-HT synthesis is different between the single and repetitive administration of methotrexate, and its control is presumed to be complicated. The protocol for the administration of methotrexate to mice used in this study is generally in agreement with the results for single administration to rats.

A recent study showed that intraperitoneal administration of methotrexate to BALB/c mice increased TNF-α and IL-1β

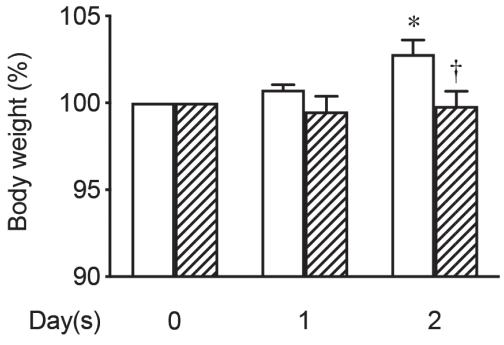

Fig. 1. Effect of Methotrexate on Body Weight in Mice

Body weight is expressed as a percentage with 100% representing Day 0. Open columns, control; hatched columns, methotrexate. Each column represents the mean ± S.E. (n = 6). *p < 0.05 versus Day 0, †p < 0.05 versus control (Day 2)

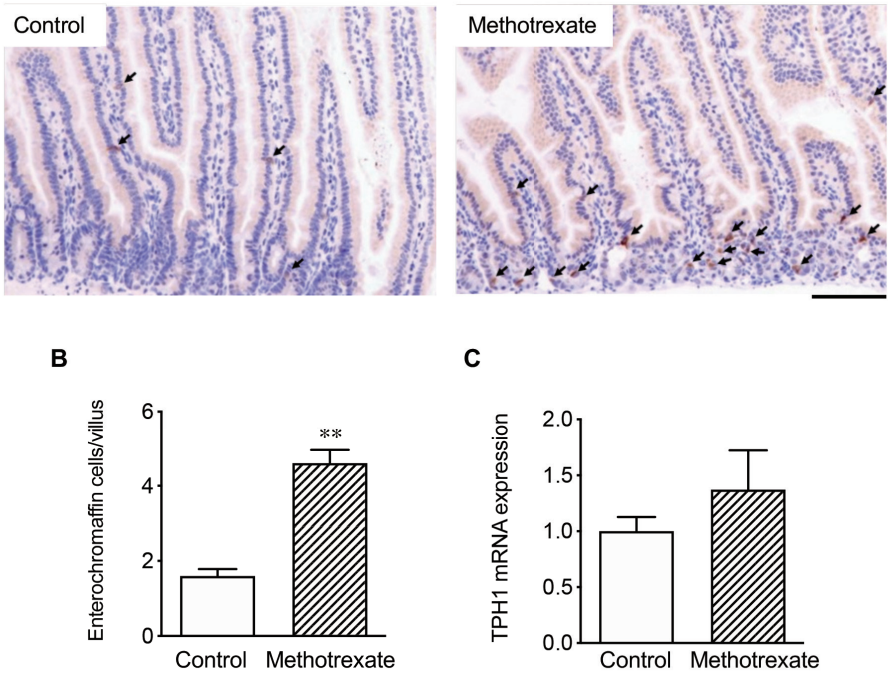

Fig. 2. Effect of Methotrexate on TPH Expression in Mouse Jejunum Tissue

A. Jejunum tissues were dissected and fixed with 4% paraformaldehyde for immunohistochemical examination with an anti-TPH antibody; scale bar = 100 μm. B. The number of anti-TPH antibody-positive cells in the ileal mucosa (arrows in A). C. TPH1 mRNA expression. Each column represents the mean ± S.E. (n = 6). $^{**}p < 0.01$ versus control

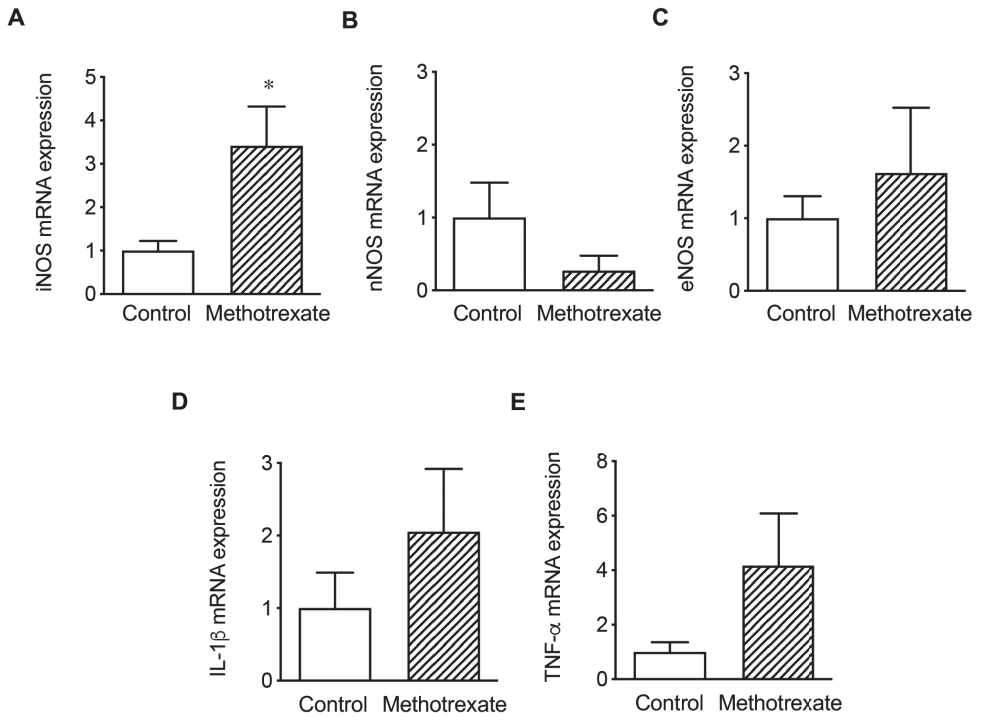

Fig. 3. Effects of Methotrexate on NOS, IL-1β, and TNF-α mRNA Expression in Mouse Jejunum Tissue

Each column represents the mean ± S.E. (n = 5–6 for A and E; n = 6 for B–D). $^{*}p < 0.05$ versus control

mRNA expression in the jejunum as well as the pro-inflammatory M1 macrophage/anti-inflammatory M2 macrophage ratio in mesenteric lymph nodes by changing intestinal microbial components.[14] Moreover, dietary restriction has been reported to protect the function of intestinal stem cells and diminish the intestinal toxicity induced by intraperitoneal administration of methotrexate via the global regulation of gut microbiota in C57BL/6 mice.[15] Therefore, it is speculated that the changes of microbiota induced by methotrexate have an important role in NOS expression as well as the hyperplasia of entero-

chromaffin cells. Further studies are required to determine the precise mechanisms of iNOS regulation and the relationship between iNOS expression and 5-HT synthesis and metabolism.

In conclusion, the results of this study show that methotrexate induces hyperplasia of enterochromaffin cells accompanied with increased iNOS expression in mouse jejunum, similar to previous findings in rats. It will be possible to clarify the detailed mechanisms of the role of NO in intestinal 5-HT synthesis and metabolism by using NOS knockout mice in addition to pharmacological studies in future investigations.

Conflict of interest The authors declare no conflict of interest.

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