Dietary medium-chain triglycerides prevent chemically induced experimental colitis in rats

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The effects of dietary medium-chain triglycerides (MCTs) on experimental colitis induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS) were investigated in rats. Male Wistar rats were given an intracolonic injection of TNBS and were then fed liquid diets containing MCTs or corn oil (AIN93) as controls. Serum and tissue samples were collected 1 week after TNBS enema. The severity of colitis was evaluated pathologically, and tissue myeloperoxidase (MPO) activity was measured. Furthermore, messenger RNA (mRNA) and protein levels for inflammatory cytokines and a chemokine were assessed by reverse-transcription polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. In another set of experiments, the protein expression of Toll-like receptor (TLR)-4 in the colon was measured 1 week after feeding of liquid diets. To investigate the effects of MCTs on macrophages, RAW246.7 macrophages were incubated with media containing albumin conjugated with MCT or linoleic acid, which is the major component of corn oil. Then, the production of tumor necrosis factor-α (TNF-α) was measured. Dietary MCTs blunted significantly the protein levels of TLR-4 in the colon. Furthermore, the expression of TLR-4 was significantly blunted in RAW264.7 cells incubated with MCTs compared with cells incubated with linoleic acid. Induction of interleukin 1β (IL-1β), TNF-α, and macrophage inflammatory protein-2 (MIP-2) in the colon was attenuated by dietary MCT. Furthermore, MPO activities in the colonic tissue were significantly blunted in animals fed the MCT diets compared with those fed the control diets. As a result, dietary MCTs improved chemically induced colitis significantly. MCTs most likely are useful for the therapy of inflammatory bowel disease as an anti-inflammatory immunomodulating nutrient. (Translational Research 2010;155:131–141)

Abbreviations: ANOVA = analysis of variance; BSA = bovine serum albumin; DMEM = Dulbecco's modified Eagle medium; ELISA = enzyme-linked immunosorbent assay; FBS = fetal bovine serum; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IBD = inflammatory bowel disease; IL = interleukin; MCT = medium-chain triglyceride; MIP = macrophage inflammatory protein; MPO = myeloperoxidase; mRNA = messenger RNA; RT-PCR = reverse-transcription polymerase chain reaction; TLR = Toll-like receptor; TNBS = 2,4,6-trinitrobenzene sulphonic acid; TNF-α = tumor necrosis factor-α

nflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the gastrointestinal tract that are of unknown origin.[1] IBDs are characterized by an infiltration of neutrophils into the colon accompanied by necrosis of epithelial cells and ulceration. Although the exact pathogenesis of IBDs is poorly understood, evidence indicates that it involves interaction between the innate and acquired immune system, genetic

AT A GLANCE COMMENTARY

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Background

In the current study, dietary supplementation of medium-chain triglycerides (MCTs) protect against 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis, which is an animal model of Crohn's disease. Because MCT is a general nutrient in a variety of types of enteral nutrition, the feasibility of a therapeutic approach for Crohn's disease using an MCT-rich enteral diet is promising.

Translational Significance

In conclusion, MCTs as immunomodulating nutrients are most likely useful for the treatment and maintenance of the remission of Crohn's disease.

susceptibility, and bacterial flora.[2,3] General therapies for IBDs include glucocorticosteroids and sulfasalazine; however, the beneficial effects are still limited.[1,4] Immunosuppressive drugs also have been applied to control severe illness, although they have serious complications and toxic side effects.[3] Alternatively, nutrition is also a major factor affecting the intestinal environment, and therefore, diets have been implicated in the pathogenesis of IBDs.[5,6] The role of nutrition in the management of IBDs has received increasing attention. Consequently, various dietary measures have been recommended as adjuvant, if not primary, therapies.[7]

It was reported that a daily supplement of medium-chain triglycerides (MCTs) prevented increases in intestinal and hepatic injury after administration of endotoxin.[5,8] In those studies, MCTs inhibited the activation of the hepatic macrophage by inhibition of the expression of CD14.[8] It was reported that intestinal macrophages activated by internal endotoxin are involved in the mechanism of Crohn's disease.[9] These findings support the hypothesis that MCTs are also effective against Crohn's disease; however, the effect of MCTs on IBD has not yet been elucidated. Therefore, the specific purpose of this study was to evaluate the effect of dietary MCTs on chemically induced experimental colitis induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS) in rats as a model of human Crohn's disease.[10]

MATERIALS AND METHODS

TNBS-induced colitis model. Male Wistar rats weighing 200 g were obtained from Japan SLC Inc. (Shizuoka, Japan). Rats were housed in a clean, temperature-controlled environment with a 12-h light-dark cycle and were given free access to regular laboratory chow diet and water for several days. All animals received humane care, and the study protocols were approved by the Committee of Laboratory Animals at University of Yamanashi according to institutional guidelines. The powder diet without oil (AIN-93) was purchased from Oriental Yeast (Tokyo, Japan). Rats under light ether anesthesia were given a single enema of TNBS solution (50 mg in 50% of ethanol/rat; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 50% of ethanol via a catheter inserted 8 cm from the anus.[11] Then rats were fed liquid diets containing either corn oil (control diets; control diet group) or MCTs (MCT diets; trioctanoin 8:0; Nihon-Yushi Co. Ltd., Tokyo, Japan; MCT diet group) (Table I). To avoid essential fatty acid deficiency, corn oil (5% of total fat) was supplemented in the MCT diets.[12] The diets were given freely throughout the observation periods, and symptomatic parameters (body weight and severity of diarrhea) were recorded. To evaluate the severity of diarrhea, the following scores were used: 0, no diarrhea; 1, mild diarrhea; 2, severe, watery diarrhea. Animals under light ether anesthesia were killed by exsanguination from the aorta 7 days after TNBS treatment to obtain blood and tissue specimens. Tissue specimens were kept at 80°C until assayed. The colonic tissue specimen was then fixed in buffered formalin and embedded in paraffin, and the tissue sections were stained by hematoxylin and eosin. Colonic inflammation in the histology specimen was assessed using a modification of the pathologic grading system of Macpherson and Pfeiffer[13] as follows: grade 0 = normal findings; grade 1 = mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and edema, punctate mucosal erosions often associated with capillary proliferation, muscularis mucosae intact; grade 2 = grade 1 changes involving 50% of the specimen; grade 3 = prominent inflammatory infiltrate and edema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa, rare inflammatory cells invading the muscularis propriae but without muscle necrosis; grade 4 = grade 3 changes involving 50% of the specimen; grade 5, extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extends deeply into the muscularis propria; and grade 6 = grade 5 changes involving 50% of the specimen. All scoring was performed by the same individual under blind conditions to prevent observer's bias.

Measurement of myeloperoxidase activity. Tissue myeloperoxidase (MPO) activity was determined by a standard enzymatic procedure as previously described by Krawisz et al[14] with slight modifications. Briefly, each tissue specimen (about 100 mg) was homogenized

Table I. Components of control and MCT-containing diets

	g/kg	
	Control diet	MCT diet
Casein	200.0	200.0
L-cystine	3.0	3.0
Corn starch	397.49	397.49
Maltodextrin	132.0	132.0
Corn oil	70.0	3.5
Medium-chain triglycerides	0.0	66.5
Cellulose	50.0	50.0
Mineral mix, AIN-93 G-MX	35.0	35.0
Vitamin mix, AIN-93-VX	1.0	1.0
Choline bitartrate	2.5	2.5
Ethoxyquin	0.01	0.01

in buffer (0.5% hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer, pH 6.0) for 90 s on ice. Then tissue homogenate was sonicated for 10 s and underwent 3 cycles of freeze-thaw ($-70\,^\circ$C/$37\,^\circ$C). The samples were centrifuged at 20,000 rpm for 20 min at $4\,^\circ$C, and the supernatant was collected. Samples (100 mL) were added to 2.9 mL of 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL O-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the kinetics of absorbance at 460 nm was measured using a spectrophotometer at $25\,^\circ$C. Protein concentration of the supernatant was determined using a Bradford assay kit (Bio-Rad Laboratories, Hercules, Calif) for calibration, and values were standardized using MPO purified from human leukocytes (Sigma Chemical Co., St. Louis, Mo).

Endotoxin assay. Blood was collected via the aorta 7 days after TNBS treatment in pyrogen-free heparinized syringes and was centrifuged at 1200 rpm for 10 min. The plasma was stored at $-80\,^\circ$C in pyrogen-free glass tubes until assay using a Limulus Amebocyte Lysate test kit (Kinetic-QCL; BioWhittaker, Walkersville, Md).[15,16]

Media preparation. Nonisotopic octanoate (Nihon-Yushi Co. Ltd) or linoleic acid (Sigma Chemical Co.) was dissolved in anhydrous ethanol to a concentration of 100 mmol/L.[17] The stock fatty acid was then added to 40 g/L bovine serum albumin (BSA) to a final concentration of 1.0 mmol/L and incubated at $37\,^\circ$C for 30 min to allow binding of the fatty acid to BSA solution. The fatty acid/BSA mixture was then added to Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) to the final concentration of 100 mmol/L.

Cell culture. Mouse RAW264.7 macrophages were cultured in DMEM containing 100 mmol/L linoleic acid or octanoate supplemented with 10% heat-inacti-

vated FBS, 100 U/mL ampicillin, and 100 mg/mL streptomycin in 5% CO_2 at $37\,^\circ$C for 7 days. The culture media was changed every 2 days. RAW264.7 cells were treated with lipopolysaccharide (10 ng/mL, from *Escherichia coli* 0111: B4; Sigma Chemical Co.) for up to 6 h before harvesting culture media and whole-cell lysate, and the samples were kept at $-80\,^\circ$C until assayed.

RNA preparation and measurement of the messenger RNA (mRNA) expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and macrophage inflammatory protein-2 (MIP-2) by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). Tissues from the colon were collected 7 days after intracolonic injection of TNBS. The tissues were stored at $-80\,^\circ$C until assays were performed. The mRNA expression of TNF-α, IL-1β, and MIP-2 was assessed by semiquantitative RT-PCR. Total RNA was isolated using an RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and it was used for the PCR assay to detect mRNA expression. The reverse transcription of total RNA (2 mg) was performed in a final volume of 100 mL containing $1 \times$ TaqMan RT buffer, 5.5 mmol/L $MgCl_2$, 500 mmol/L each dNTP, 2.5 mmol/L random hexamers, 0.4 U/μL RNase inhibitor, and 1.25 U/μL multiscribe reverse transcriptase. The primer sets for TNF-α IL-1β, MIP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) contained the sequences detailed below[18,19]:

TNF-α sense (5'-ATGAGCACAGAAAGCAT-GATG-3')
 antisense (5'-TACAGGCTTGTCACTCGAATT-3')
 IL-1β sense (5'-GGGCGCTTCAAGGCATAA-3')
 antisense (5'-CAGCACGAGGCATTTTTGTT-3')
 MIP-2 sense (5'-CAGAGCTTGAGTGTGACG-3')
 antisense (5'-TCGTACCTGATGTGCCTC-3')
 GAPDH sense (5'-TGAAGGTCGGAGTCAACG-GATTTGGT-3')
 antisense (5'-CATGTGGGCCATGAGGTCCAC-CAC-3').

Aliquots (5 μL) of synthesized complementary DNA were added to 45 μL PCR mix containing 5 μL of $10\times$ PCR buffer, 1 μL of each deoxynucleotide (1 mmol/L each), 0.5 μL of sense and antisense primers (0.15 mmol/L), and 0.25 μL of DNA polymerase (Gene Amp PCR kit; Perkin Elmer Cetus, Norwalk, Conn). The reaction mixture was covered with a wax gem (Perkin Elmer Cetus), and amplification was initiated by 1 min of denaturation at $94\,^\circ$C for 1 cycle, followed by multiple (20 to 35) cycles at $94\,^\circ$C for 30 s, $55\,^\circ$C for 30 s, and $72\,^\circ$C for 1 min using a GeneAmp PCR system 9800 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, samples were incubated for 7 min at $72\,^\circ$C.

Control   **MCT**
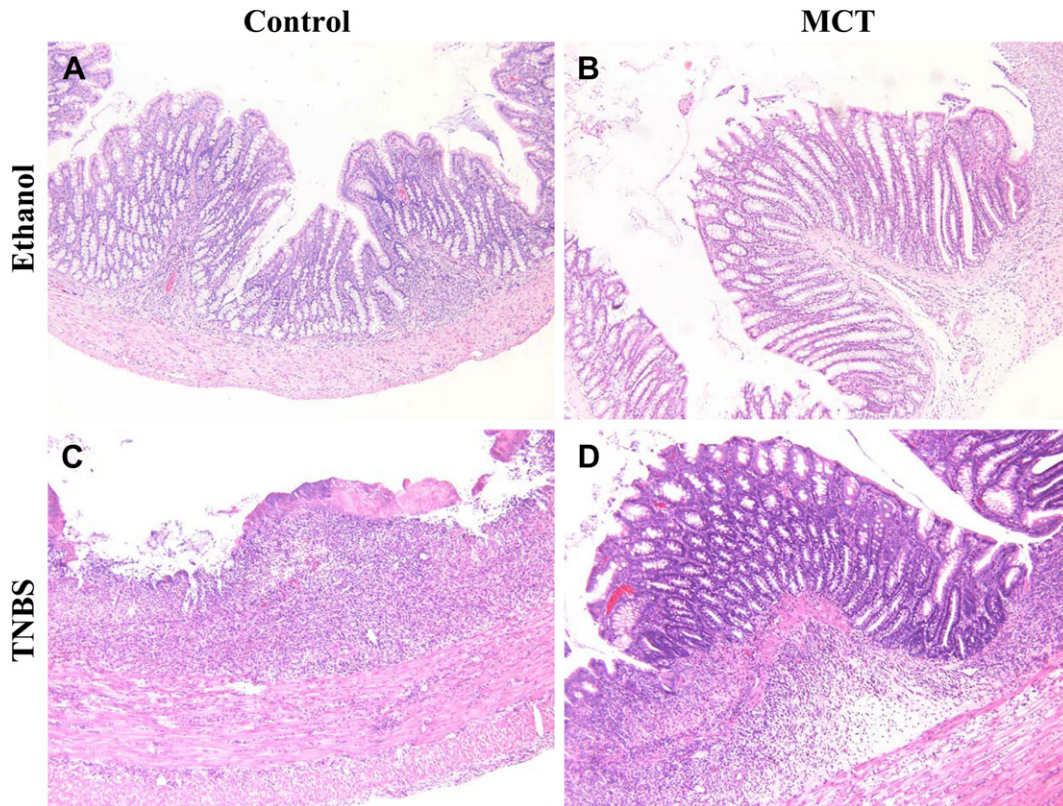
Fig 1. Pathologic changes in the colon. Representative microphotographs of colonic tissues from the control diet-fed animals treated with an ethanol vehicle (**A**) or TNBS after 7 days (**C**). The MCT diet-fed animals treated with an ethanol vehicle (**B**) or TNBS after 7 days (**D**) are shown. Original magnification: × 400. Control, animals fed the control diets; MCT, animals fed the MCT diets.

The amplified PCR products were subjected to electrophoresis at 100 V through a 2% agarose gel (GIBCO Laboratories Life Technologies, Carlsbad, Calif) for about 30 min. The agarose gels were stained with 0.5 mg/mL ethidium bromide Tris-borate-ethylene diaminetetraacetic acid buffer (ICN, Costa Mesa, Calif) and photographed with type 55 Polaroid positive/negative film. A densitometric analysis of the captured image was performed on a Macintosh computer using National Institutes of Health image 1.54 analysis software. The area under the curve was normalized for GAPDH content.

Preparation of samples for Western blotting analysis of Toll-like receptor (TLR)-4. RAW cells were lysed in immunoprecipitation lysis buffer containing 50 mmol/L hydroxyethyl piperazine ethanesulfonic acid (pH 7.9), 250 mmol/L NaCl, 20 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, and 1:100 Protease Inhibitor Set III (Calbiochem, Darmstadt, Germany).

The tissues were washed free of their contents using cold phosphate buffered solution, blotted on filter paper, and weighed. A crude particulate fraction of the intestine was prepared according to the method of Perez et al[20]

with some modifications. Briefly, the organ was homogenized using a Caframo homogenizer (Wiarton, Ontario). Ten volumes of cold buffer (pH 7.4) were used containing 10 mmol/L N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES, Sigma Chemical), 0.2 mol/L sucrose, 1 mmol/L $MgCl_2$, 12.5 mmol/L benzamidine and 21 mmol/L leupeptin. A small sample of homogenate was kept to assay the protein concentration. The remainder was centrifuged at $1000 \times g$ for 10 min at $4°C$, after which the supernatant was ultracentrifuged at $100,000 \times g$ for 60 min at $4°C$. The resulting pellet was resuspended in TES-sucrose buffer containing 10 mL/L Triton X-100, 12.5 mmol/L benzamidine, 21 mmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (Boehringer Mannheim Canada, Laval, Quebec, Canada). This procedure yielded a crude particulate fraction rather than a pure membrane fraction. Protein concentration was determined using a colorimetric assay Bio-Rad DC protein assay, and samples were stored at $-80°C$.

Western blotting of TLR-4 in the gut. Samples of the colon crude particulate fractions and the cell line were diluted in non-reducing Laemmli buffer. Extracted proteins (55 μg) were separated by 100 g/L sodium-dodecyl sulfate-
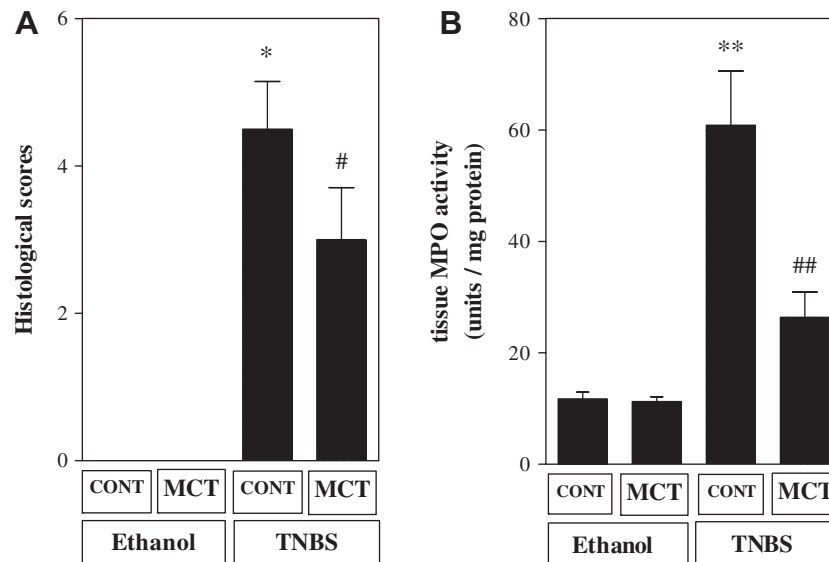
Fig 2. Pathologic score and tissue MPO activity in the colon after TNBS treatment. Pathologic changes were scored (**A**), and MPO activity in the colonic tissue 7 days after treatment with ethanol or TNBS was determined (**B**) as described in the Materials and Methods. Data represent mean ± SEM ($n = 8$). MCT, animals fed the MCT diets; Control, animals fed the control diets. *$P < 0.05$ compared with the control diet-fed animals treated with ethanol vehicle; and #$P < 0.05$ compared with the control diet-fed animals treated with TNBS by the Mann-Whitney rank sum test. **$P < 0.05$ compared with the control diet-fed animals treated with ethanol vehicle. ##$P < 0.05$ compared with the control diet-fed animals treated with TNBS by ANOVA with Bonferroni's post-hoc test.

polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Tris-buffered saline-Tween 20 containing 50 g/L skim milk and probed with polyclonal goat anti-rat TLR-4 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, Calif) followed by horseradish peroxidase-conjugated secondary antibody when appropriate. The membranes were then incubated with a chemiluminescent substrate (ECL reagent; Amersham Life Science, Buckinghamshire, UK) and exposed to X-OMAT film (Eastman Kodak Company, Rochester, NY). A densitometric analysis of the image was performed on a Macintosh computer using NIH image 1.54 analysis software (Bethesda, Md).

Enzyme-linked immunosorbent assay (ELISA). The colonic tissue was homogenized in cold phosphate-buffered saline using a Polytron-type homogenizer. The tissue homogenate was then centrifuged at 20,000 rpm for 20 min at 4 °C to obtain the supernatant. The total protein concentrations of the tissue supernatant and whole-cell lysate were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories) for calibration, and protein concentrations of IL-1β, TNF-α, and MIP-2 in the tissue homogenate and culture media were determined using ELISA kits (IL-1β and TNF-α: R&D Systems Inc.; MIP-2: Immuno-Biological Laboratories Inc., Gumma, Japan) according to the manufacturer's instructions.

Statistical analysis. The data were expressed as mean ± SEM. Statistical differences between mean values were analyzed by analysis of variance (ANOVA) with Bonferroni's post-hoc test. A P-value less than 0.05 was considered significant.

RESULTS

Effects of MCTs on pathophysiologic changes after TNBS enema. There were no significant differences in food intake throughout all experiments in each group (the control diet group, 64 ± 13 ml/day; and the MCT diet group, 62 ± 15 ml/day). Thus, anorexia was not observed throughout all experiments in each group.

First, the symptomatic parameters, including body weight loss and diarrhea caused by colitis after an intracolonic injection of TNBS, were investigated. In the control and the MCT diet groups, rats treated with ethanol enema gained body weight for 1 weeks; the average increment reached 4.2 ± 0.5 g and 4.1 ± 0.3 g per day, respectively ($n = 8$). However, in the control diet group, rats treated with a single intracolonic TNBS injection did not gain or lose body weight; the average increment reached 0.3 ± 0.1 g for 7 days after enema (data not shown). In contrast, the MCT diets prevented the loss of body weight caused by TNBS, the average increment reaching 2.0 ± 0.2 g in the same period ($n = 8$, $P < 0.05$ compared with the control diet-fed animals treated with TNBS by the Mann-Whitney rank sum test).
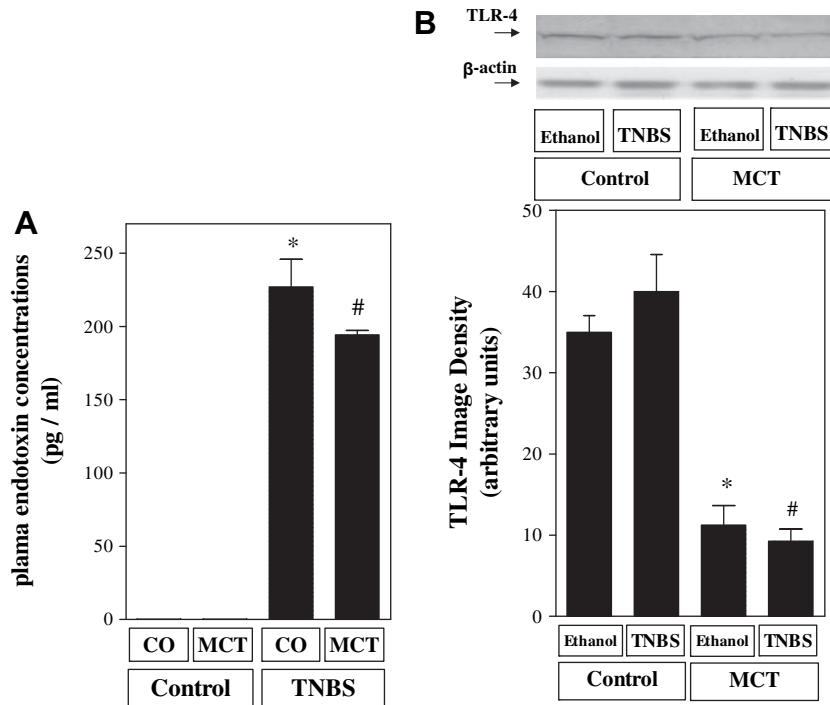
Fig 3. Plasma endotoxin concentration and protein expression of TLR-4 in the colon after TNBS treatment. Plasma endotoxin concentrations (**A**) and protein levels of TLR-4 in colon tissues (**B**) after treatment with ethanol or TNBS were determined as described in Materials and Methods. Data represent mean $\pm$ SEM ($n = 8$). MCT, animals fed the MCT diets; Control, animals fed the control diets. *$P < 0.05$ compared with the control diet-fed animals treated with ethanol vehicle. #$P < 0.05$ compared with the control diet-fed animals treated with TNBS by ANOVA with Bonferroni's post-hoc test.

Furthermore, the severity of diarrhea after the TNBS treatment was scored according to the scale described in the Materials and Methods section. In the control and the MCT diet groups, no animals treated with ethanol showed diarrhea (data not shown) ($n = 8$ for each group, average score: 0 ± 0 in both the 2 groups at day 3). In contrast, TNBS caused severe diarrhea, which peaked around day 3, and the average score at the end of the period reached the levels of 1.6 ± 0.7 ($n = 8$) in the control diet group as expected. In comparison, the grade of diarrhea was improved significantly in animals fed the MCT diets, with an average score of 0.8 ± 0.2 ($n = 8$, p <0.05 compared with the control diet-fed animals treated with TNBS by the Mann-Whitney rank sum test).

The effect of MCTs on histopathologic changes and MPO activity in the colon after TNBS treatment. The severity of colonic inflammation and ulceration was evaluated by histopathologic observations (Fig 1). As shown in Fig 1, ulcer formation and massive transluminal infiltration of inflammatory cells, predominantly polymorphonuclear neutrophils, were observed 7 days after intracolonic injection of TNBS in the control diet group. In contrast, ulceration was mild and inflammatory cells were localized in the mucosa, and the submucosal area

in animals fed the MCT diets. These histologic findings were graded using criteria described detail in the Materials and Methods section. In the control diet group, histologic scores were about 4.5 seven days after TNBS enema (Fig 2, A). Dietary MCTs also improved microscopic scores significantly by about 30%.

Effects of MCTs on MPO activity in the colon after TNBS enema. To evaluate inflammation in the colon, MPO activities in the distal colonic tissue without ulceration were measured by a standard enzymatic assay (Fig 2, B). MPO activities were about 10 units/mg protein 7 days after ethanol enema. This activity increased significantly nearly 6-fold after TNBS enema in the control diet group. In contrast, this increase was significantly blunted by about 60% by dietary MCTs.

Effects on MCTs on plasma endotoxin levels. Plasma endotoxin levels were minimal after ethanol enema (Fig 3, A). In contrast, endotoxin levels were significantly elevated to about 200 pg/mL in the control diet group. Although values in the MCT diet group were lower than those in the control diet group, no significant differences were observed between the 2 groups.

Effects of MCTs on expression of TLR-4 in the colon. Western blotting analyses of TLR-4 were performed on colonic tissue samples (Fig 3, B). In the
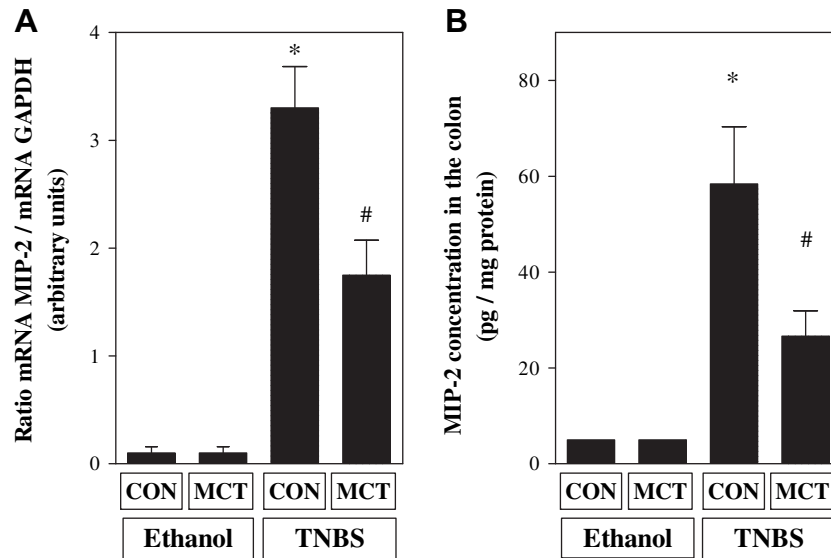
A

B

C

D
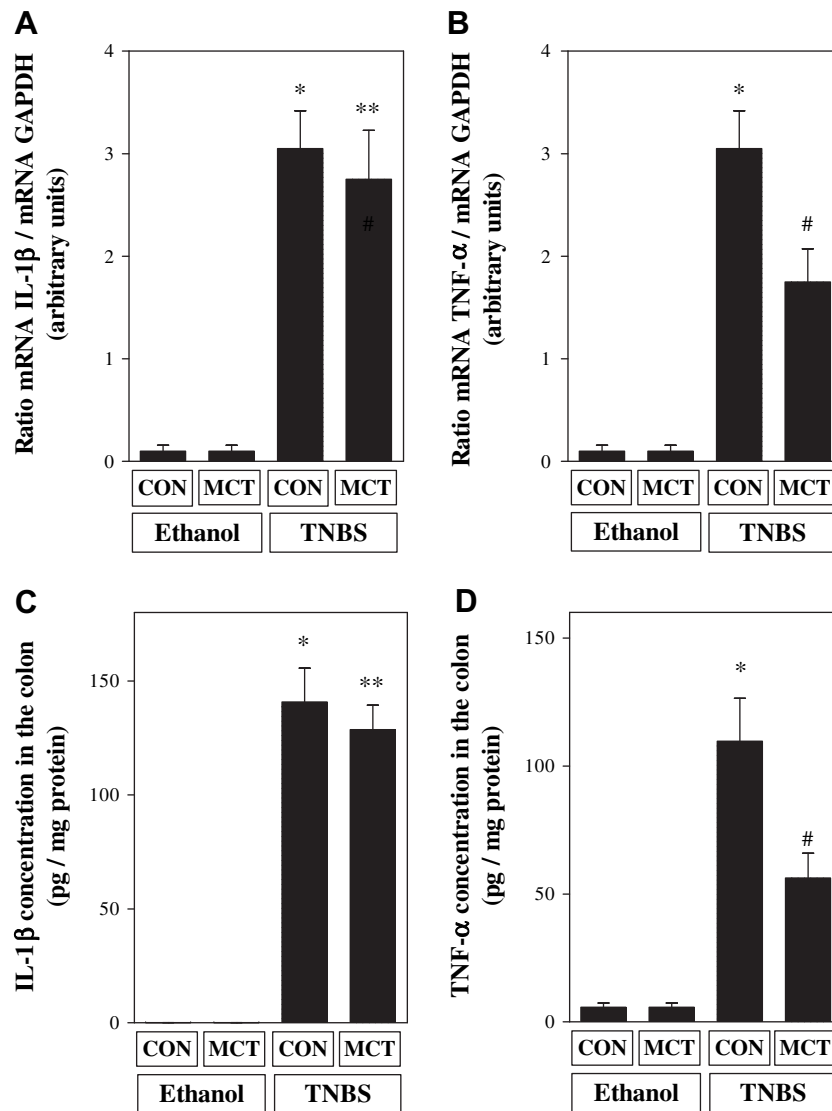
Fig 4. Expression of TNF-α and IL-1β mRNA and protein levels in the colon after TNBS treatment. The mRNA expression of TNF-α and IL-1β in the colonic tissue 7 days after treatment with ethanol or TNBS was measured as described in Materials and Methods. Data from densitometric analysis for TNF-α (**A**) and IL-1β (**B**) are plotted. TNF-α (**C**) and IL-1β (**D**) protein levels in the colonic tissue 7 days after treatment with ethanol vehicle or TNBS were measured by ELISA. The data represent mean $\pm$ SEM ($n = 8$). MCT, animals fed the MCT diets; Control, animals fed the control diets. *$P < 0.05$ compared with the control diet-fed animals treated with ethanol vehicle. **$P < 0.05$ compared with the MCT diet-fed animals treated with an ethanol vehicle. #$P < 0.05$ compared with the control diet-fed animals treated with TNBS by ANOVA with Bonferroni's post-hoc test.

control diet group, protein expression of TLR-4 was detected in rats after ethanol enema, and this expression was significantly blunted about 60% by dietary MCTs. After TNBS enema, protein expression of TLR-4 increased slightly in the control diet group. Importantly, the expression was also significantly blunted by about 60% in the colon in rats fed the MCT diets for 7 days.

Effects of MCTs on the mRNA expression of TNF-α and IL-1β in the colon. The mRNA expression of TNF-α and IL-1β was assessed in the colon from rats fed the control

diets or the MCT diets (Fig 4). Because IL-1β has been characterized as a pivotal proinflammatory cytokine produced from both inflammatory cells and mucosal epithelial cells during colonic inflammation,[21] steady-state mRNA levels of IL-1β was measured in the colonic tissue without ulceration using RT-PCR. As shown in Fig 4, the mRNA expression of IL-1β in the colonic tissue was minimal in both the control and MCT diet groups after ethanol enema (Fig 4, *A*). In contrast, the expression increased significantly about 10-fold 7 days after TNBS

Fig 5. Expression of MIP-2 mRNA and protein levels in the colon after TNBS treatment. The mRNA expression of MIP-2 was measured by RT-PCR (**A**). Protein levels of MIP-2 in the colonic tissue 7 days after treatment with ethanol or TNBS were measured by ELISA (**B**). MCT, animals fed the MCT diets; Control, animals fed the control diets. Data represent mean $\pm$ SEM ($n = 8$). *$P < 0.05$ compared with the control diet-fed animals treated with an ethanol vehicle; and #$P < 0.05$ compared with the control diet-fed animals treated with TNBS by ANOVA with Bonferroni's post-hoc test.

enema in the control diet group. This TNBS-induced increase in the mRNA expression of IL-1β was not changed in the MCT diet group compared with the control diet group.

Furthermore, TNF-α, which is a key proinflammatory cytokine produced predominantly from macrophages, also was measured in the colon (Fig 4, *B*). In the control diet group, the mRNA expression of TNF-α was elevated nearly 10-fold 7 days after TNBS enema. This increase was also blunted significantly by about 40% by dietary MCTs.

To confirm these results, tissue protein levels of IL-1β and TNF-α were measured by ELISA. As shown in Fig 4, the protein levels of both IL-1β and TNF-α were increased significantly 7 days after TNBS enema in the control diet group. On the one hand, the increases in IL-1β were not changed in the MCT diet group (Fig 4, *C*). On the other hand, increases in TNF-α levels caused by TNBS were significantly blunted by about 55% in the MCT diet group (Fig 4, *D*), which is consistent with results of the mRNA expression. Collectively, these findings indicated that MCTs prevented colonic inflammation caused by TNBS at least in part by preventing expression of the proinflammatory cytokine TNF-α.

Effects of MCTs on the mRNA expression of MIP-2 in the colon. After ethanol enema, the mRNA expression of MIP-2 was minimal in the colon in rats fed the control diets or the MCT diets (Fig 5, *A*). After TNBS enema,

the mRNA expression significantly increased in rats fed the control, and this increase was significantly blunted by about 50% by dietary MCTs.

To confirm these results, the protein levels of MIP-2 were measured by ELISA. As shown in Fig 5, *B*, protein levels of MIP-2 were increased significantly 7 days after TNBS enema in the control diet group. In contrast, the increases in MIP-2 levels caused by TNBS were significantly blunted by about 55% in the MCT diet group. Collectively, these findings indicated that MCTs prevented colonic inflammation caused by TNBS by preventing production of this inflammatory chemokine.

Effects of MCT on the mRNA expression of inflammatory cytokines and protein levels of TLR-4 in RAW264.7 macrophages. To elucidate the site of action in the gut of inhibition of protein levels of TLR-4 and cytokines by dietary MCTs, the effect of MCTs on induction of TLR-4, TNF-α, and IL-1β in macrophages was evaluated by *in vitro* experiments using the RAW264.7 cell. In RAW264.7 cells incubated with BSA, the protein expression of TLR-4 was observed in cells incubated with or without LPS (Fig 6). This expression did not changed in cells incubated with linoleic acid. However, the expression was significantly inhibited by MCTs.

Lipopolysaccharide (10 ng/mL)-induced increases in protein levels of TNF-α and IL-1β in the media were blunted significantly when cells were incubated with MCTs at concentrations of 1 mmol/L (Fig 7). These findings support the hypothesis that MCTs inhibit
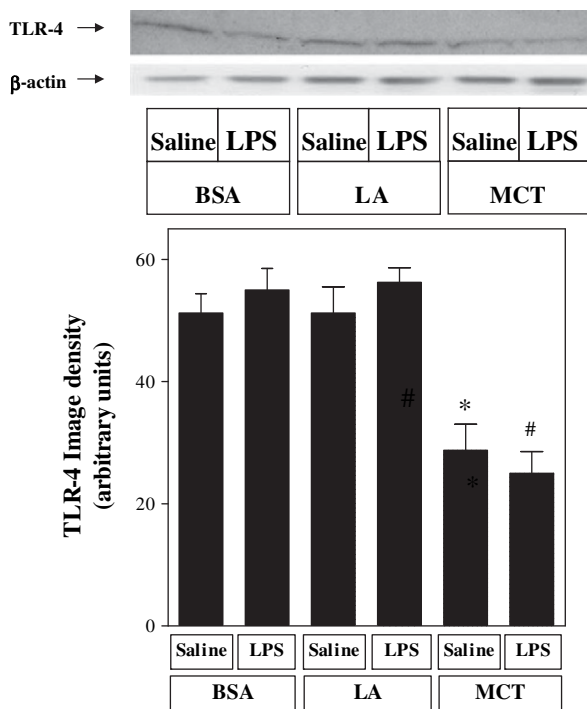
Fig 6. Effect of fatty acids on the expression of protein levels of TLR-4 in RAW246.7 macrophages *in vitro*. Protein levels of TLR-4 in RAW246.7 macrophages incubated with MCTs or linoleic acid for 7 days were measured by Western blotting. (**A**) Representative photographs of specific bands for TLR-4 are shown. The data represent mean ± SEM ($n = 8$). LPS, lipopolysaccharide; LA, linoleic acid. *$P < 0.05$ compared with cells treated with linoleic acid and saline. #$P < 0.05$ compared with cells treated with linoleic acid and LPS by ANOVA with Bonferroni's post-hoc test.
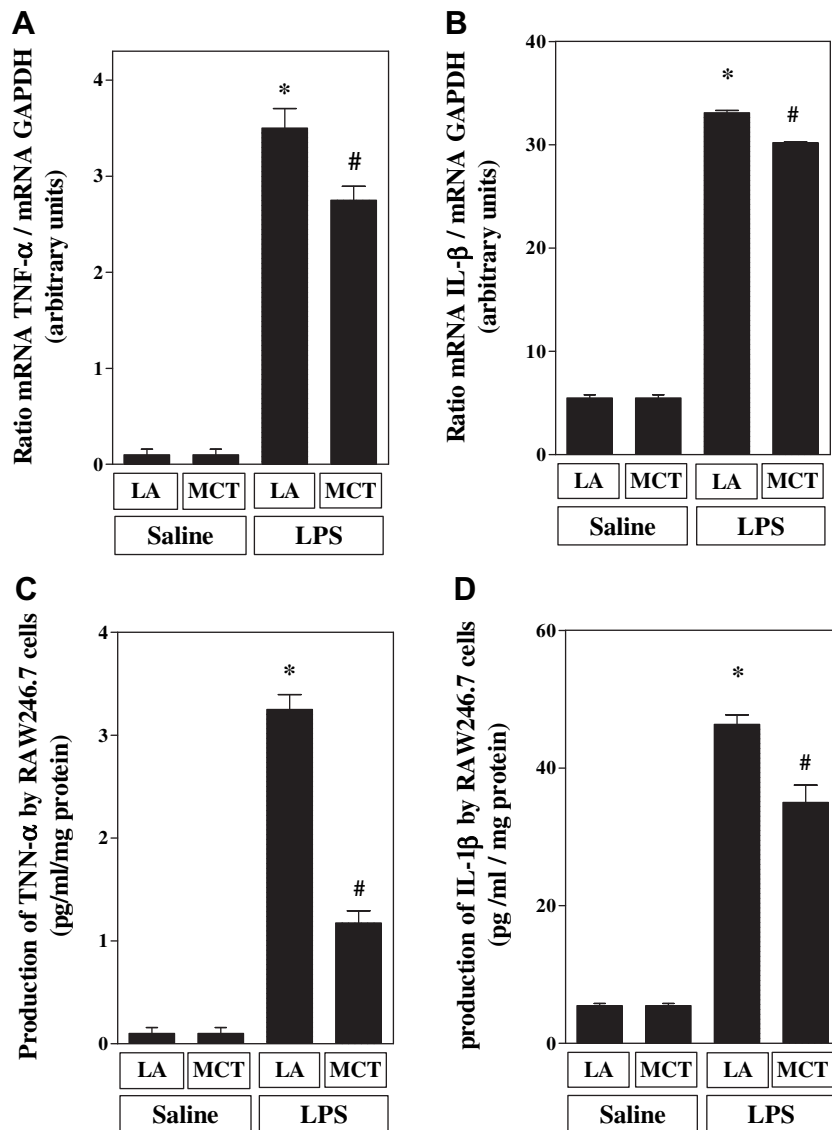
production of inflammatory cytokines and chemokines from macrophages in a direct manner, thereby ameliorating experimental colitis in the rat.

DISCUSSION

Medium-chain triglycerides have been shown to be protective against various types of organ injuries, including endotoxin-induced hepatic and intestinal injuries,[8] as well as alcohol-induced liver damage.[22] In the current study, treatment with dietary MCTs prevented colitis induced by TNBS, which is consistent with the previous report.[23,24] These results demonstrate the protective effect of MCTs against inflammation in the colon.[25,26] Induction of proinflammatory cytokines TNF-α and a chemokine MIP-2 in the colonic tissue was decreased dramatically by dietary MCTs (Figs 4 and 5), which suggests a potential clinical value in terms of therapeutic application for IBDs. TNBS-induced colitis exhibits clinical, histologic, and microscopic similarities to Crohn's disease, and the course of colonic injury has been well characterized.[27] Recent studies have reported that the proinflammatory cytokine TNF-α plays a pivotal

role in the inflammatory cascade,[28] because a neutralizing antibody for TNF-α prevents TNBS-induced colitis in mice.[29] In the current study, dietary MCTs prevented the induction of TNF-α in the colonic tissue (Fig 7), which indicates that MCTs most likely improve colonic injury by preventing the production of TNF-α from activated macrophages in the colon. Indeed, MCTs blunt the increases in serum TNF-α levels after LPS injection in the rat,[8] and MCTs inhibit TNF-α production by isolated Kupffer cells, which are resident hepatic macrophages, in a direct manner.[8] The mechanism of the protective effect of MCTs has been investigated. Tissue macrophages have been reported to express the endotoxin receptor TLR-4. Furthermore, endotoxin, its receptor TLR-4, and tissue macrophages are involved in the mechanism of the Crohn's disease.[20,30] In the current study, dietary MCTs significantly blunted protein levels of TLR-4 in the colon (Fig 3), whereas plasma endotoxin levels were not significantly different between the 2 groups (Fig 3). Furthermore, in RAW264.7 macrophages, protein levels of TLR-4 were significantly blunted by treatment with MCTs *in vitro* (Fig 6). Consequently, the mRNA expression and production of TNF-α and IL-1β were significantly blunted by MCTs in RAW264.7 macrophages (Fig 7). Therefore, it is speculated that the mechanisms by which MCTs prevent TNBS-induced colitis involve inhibition of production of TNF-α from tissue macrophages and the expression of the TLR-4 in the colon. These results support the hypothesis that resident tissue macrophages are the cell types responsible for the protective effects of MCTs. Moreover, TNBS-induced colitis is characterized as the inflammation of colonic tissue because of the severe infiltration of inflammatory cells, predominantly neutrophils.[27] Recently, it was reported that chemokines such as IL-8 play a pivotal role in the accumulation of neutrophils into inflammatory foci in TNBS-induced colitis.[31] In this study, therefore, mRNA levels and protein levels of MIP-2, rat homologs of IL-8, were measured in the colonic tissue after TNBS treatment (Fig 5). Interestingly, dietary MCTs significantly prevented increases in mRNA/protein levels of MIP-2 in the colon after TNBS enema. This result is explained in part by downregulation of TNF-α in animals fed the MCT diets because TNF-α is a key proinflammatory cytokine that induces MIP-2. Collectively, it also is hypothesized that MCTs prevent both the accumulation and activation of neutrophils, thereby attenuating neutrophil-dominant inflammation in the colonic tissue caused by TNBS.

Clinical implications. Therapeutic strategies for Crohn's disease target the specific inhibition of the inflammatory cytokine cascade. Recent trials of infliximab and CDP571, which are neutralizing antibodies for TNF-α, for the treatment of Crohn's disease have

Fig 7. Effect of fatty acids on the mRNA expression and production of inflammatory cytokines by RAW246.7 macrophages in vitro. The mRNA expression of TNF-α (**A**) and IL-1β (**B**), and production of TNF-α (**C**) and IL-1β (**D**) by RAW246.7 macrophages incubated with medium-chain triglycerides or linoleic acid for 7 days were measured by ELISA. Data represent mean ± SEM ($n = 6$). LPS, lipopolysaccharide; LA, linoleic acid. *$P < 0.05$ compared with cells treated with LA and saline. #$P < 0.05$ compared with cells treated with LA and LPS by ANOVA with Bonferroni's post-hoc test.

demonstrated efficacy in the induction and maintenance of remission in luminal and fistulizing Crohn's disease both in adults and children.[32,33] Furthermore, etanercept, which is a soluble TNF receptor:Fc fusion protein, has been used in the treatment of Crohn's disease.[34] Evidence from these clinical trials indicates that inhibition of TNF-α is pivotal for the remission of inflammation in patients who suffer from Crohn's disease.[35] In the current study, MCTs inhibited the production of inflammatory cytokines and chemokines in the colonic tissue, as well as the production of those mediators by activated

macrophages and the accumulation of activated neutrophils into the colon, which ameliorated colonic injury. These findings clearly indicate that dietary supplementation of MCTs protects against TNBS-induced colitis, which is an animal model of Crohn's disease. Because MCT is a general nutrient in a variety of types of enteral nutrition,[36] the feasibility of a therapeutic approach for Crohn's disease using an MCT-rich enteral diet is promising. In conclusion, MCTs as immunomodulating nutrients most likely are useful for the treatment and maintenance of the remission of Crohn's disease.

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