Effect of a novel water-soluble vitamin E derivative as a cure for TNBS-induced colitis in rats

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Abstract. Lipid peroxidation mediated by oxygen free radicals plays an important role in the pathogenesis of inflammatory bowel disease (IBD). Vitamin E is a lipid-soluble antioxidant and is generally considered to protect against lipid peroxidation of the cell membrane and to scavenge singlet oxygen and superoxide anion radical. Therefore, vitamin E or its derivatives are expected to have particular application for patients suffering from IBD. The aim of this study was to investigate the anti-oxidative effects of the water-soluble vitamin E derivative, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetra-methylchroman-6-ol(TMG), on the therapy of experimental colitis in rats. Colitis was induced in male Wistar rats weighing 200 g using an enema of trinitrobenzene sulfonic acid (TNBS) dissolved in 50% ethanol; TMG dissolved in physiological saline was injected intra-peritoneally every day from 24 h after the enema of TNBS. The damage score, wet weight of the colon, and increase in body weight were estimated 1 week after the enema of TNBS. Thiobarbituric acid-reactive substances (TBA-RS), an index of lipid peroxidation, and tissue-associated myelo-peroxidase (MPO) activity in the colonic mucosa were measured 1 week after the induction of colitis. As a result, increase in body weight was inhibited by the induction of colitis, although the inhibition was reduced in the group treated with TMG. The damage score, wet weight, TBA-RS and MPO activity were increased significantly in the colitis group; however, they were inhibited by the administration of TMG. These results suggest that TMG is effective for the treatment of colitis in rats induced by TNBS. In the future, TMG could be a new therapeutic agent for IBD.

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Introduction

It has been proposed that lipid peroxidation mediated by oxygen free radicals plays an important role in the pathogenesis of inflammatory bowel disease (IBD) (1-3), such as Crohn's disease and ulcerative colitis (UC). In rats, the experimental colitis, induced by intracolonic injection of 2,4,6-trinitro-benzene sulfonic acid (TNBS), exhibits clinical, histological, and macroscopic similarities to human IBD, and it is useful for studying the pathophysiology of human IBD (4). We have shown that lipid peroxides are increased significantly in the colonic mucosa (5), and treatment with superoxide dismutase (SOD) improves the colitis score (6). These reports suggested that inhibition of lipid peroxidation or scavenging of oxygen free radicals produces valuable preventive and therapeutic strategies for IBD. Vitamin E is a major lipophilic antioxidant and its excellent antioxidant activity is well recognized (7-10). It has also been reported that vitamin E protects membrane lipids from peroxidation (11,12) and scavenges not only chain-carrying peroxyl radicals but also singlet oxygen (13,14) and superoxide anion radical (15,16). Therefore, vitamin E or its derivatives may be expected to reduce the development of tissue injury in IBD. Murase *et al* (17) succeeded in the synthesis of a novel water-soluble vitamin E derivative, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetra-methylchroman-6-ol (TMG) (Fig. 1), by α-glucosidase-catalyzed transglycosylation using an aqueous-organic solvent system. They also reported its antioxidative activity to be equal to that of α-tocopherol or ascorbic acid and elucidated its excellent water-solubility (>1000 mg/ml). The aim of this study was to investigate the antioxidative effects of the water-soluble vitamin E derivative for the cure of experimental colitis in rats.

Materials and methods

Reagents. All chemicals were prepared immediately before use. TMG was a gift from CCI Pharmacy Co., Ltd. (Gifu, Japan). Trinitrobenzene sulfonic acid (TNBS), thiobarbituric acid (TBA) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

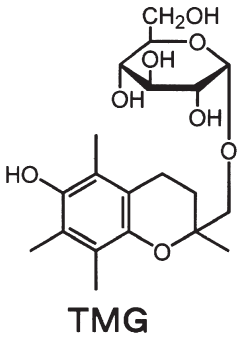

Figure 1. Structures of 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG).

1,1,3,3-Tetramethoxy propane was obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals used were of reagent grade.

Animals. Male Wistar rats, weighing 190-210 g were obtained from Keari Co. Ltd. (Osaka, Japan). They were housed at 22˚C in a controlled environment with 12 h of artificial light per day. They were fasted for 48 h before the experiment but had free access to drinking water. All experimental procedures described below were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

Induction of colitis. TNBS-induced colitis was established using the method of Morris *et al* (19). In brief, rats were lightly anesthetized with Pentobarbital following a 48-h fast, then a rubber catheter (OD 2 mm) was inserted via the anus so that the tip was 8 cm proximal to the anus. TNBS dissolved in 50% ethanol (120 mg/ml) was instilled into the lumen of the colon through the catheter (0.25 ml in volume). Following instillation of TNBS at 30 mg per rat, the anus was occluded with a clip for 1 h.

Design of treatment. All rats were randomized into groups receiving different concentrations of TMG or only the physiological saline vehicle. TMG was dissolved in physiological saline. Each test drug was administered by intra-peritoneal injection once a day from 24 h after TNBS administration (day 1) until day 7.

Evaluation of colonic damage. After the induction of colitis, the rats were weighed on days 0 and 7 to evaluate the effect on general nutrition. Rats were killed on day 7 and the distal colon was removed and opened by longitudinal incision. As indices of inflammation, damage was estimated macroscopically as the sum of the mucosal score and the serosal score. The mucosal score was rated on a 6-point scale (0-5) according to the criteria established by Morris *et al* (18). The serosal score was rated on a 4-point scale (0-3) according to the criteria established by Yoshida *et al* (19) (Table I). The degree of colitis was evaluated by an independent observer, without previous knowledge of the treatment. For histological evaluation, formalin-fixed tissue was stained with hematoxylin and eosin (H&E) and evaluated light microscopically by a pathologist unaware of the experimental conditions.

Assessment of colonic inflammation. The wet weight of the colon was determined. As an index of lipid peroxidation, the concentration of thiobarbituric acid-reactive substances (TBA-RS) was measured using the method of Ohkawa *et al* (20). Colonic mucosa was scraped from the deeper layers using two glass slides and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a Teflon Potter-Elvehjem homogenizer. TBA-RS in mucosal homogenates was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-Tetramethoxy propane as a standard. The protein concentration in the colonic mucosal homogenates was measured using the method of Lowly *et al*

Table I. Criteria for the damage score of the colon.[a]

Score	Gross morphology
Mucosal score	
0	No damage
1	Localized hyperemia, but no ulcers
2	Linear ulcers with no significant inflammation
3	Linear ulcers with inflammation at one site
4	Two or more sites of ulceration and/or inflammation
5	Two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon
Serosal score	
0	No adhesion
1	Adhesion <5 mm along the length of the colon at one site
2	Two or more major sites of adhesion <5 mm and/or one site of adhesion extending 5-10 mm along the length of the colon
3	Two or more major sites of adhesion extending 5-10 mm or one site of adhesion extending >10 mm along the length of the colon

[a]Moriss *et al* (18)/Yoshida *et al* (19).

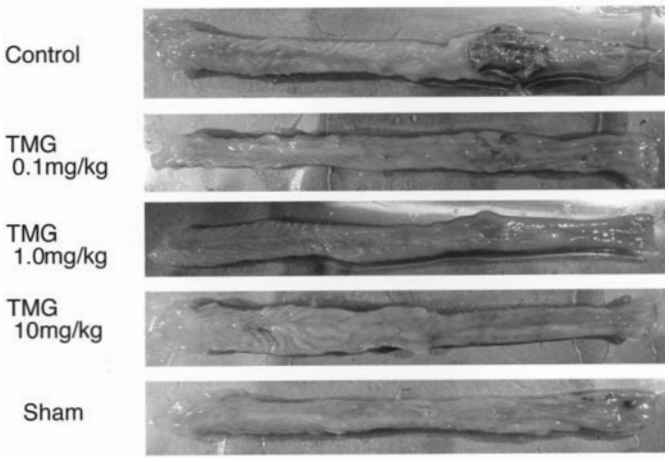

Figure 2. Severe colitis was produced with hyperemia, edema, thickening, ulceration and necrosis in the control group. These changes were reduced in the TMG-treated groups at doses of 1.0 and 10 mg/kg.

(21). As an index of neutrophil accumulation, tissue-associated myeloperoxidase (MPO) activity was determined using a modification of the method of Grisham *et al* (22). Mucosal homogenate (2 ml) was centrifuged at 20,000 x g for 15 min at 4˚C to pellet insoluble cellular debris. The supernatant was used for the measurement of colonic cytokine concentration. The pellet then was rehomogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000 x g for 15 min at 4˚C, and supernatants were saved. MPO activity was assessed by measuring H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine and it was expressed as units per mg protein. One unit of enzyme activity was defined as the amount of myeloperoxidase that caused a change in absorbance of 1.0/min at 655 nm and 25˚C.

Measurement of cytokines in colonic mucosa. The content of tumor necrosis factor (TNF)-α in colonic mucosa was determined by enzyme-linked immunosorbent assay (ELISA) using a rat tumor TNF-α ELISA kit (BioSource International, Inc., Camarilla, California, USA). Furthermore, the content of cytokine-induced neutrophil chemoattractants-1 (CINC-1) in colon mucosa was determined by ELISA using a Rat GRO/CINC-1 ELISA kit (Immuno Biological Laboratories, Gunma, Japan) according to the manufacturer's instructions.

Statistical analysis. Colonic damage scores are presented as scatter plots. Differences between groups were compared by analysis of variance (Kruskal-Wallis test) followed by the Mann-Whitney U-test, a nonparametric test. Data on body weight, wet weight of the colon, TBA-RS, MPO activity, the content of TNF-α and CINC-1 were presented as the mean ± SEM, and were compared using an analysis of variance (ANOVA) followed by Fischer's protected least significant difference test (Fischer's PLSD). A value of $p<0.05$ was considered significant.

Results

Effect of TMG on TNBS-induced colonic injury. In the rats exposed to TNBS, macroscopic findings of the colon demonstrated severe colitis with hyperemia, edema, thickening, ulceration and necrosis. In contrast, the rats treated with TMG at doses of 1.0 and 10 mg/kg showed smaller erosions with mild edema in the colon (Fig. 2). As a result, the Colonic damage score showed significant increase by TNBS administration. The increase in damage score was significantly decreased by the treatment with TMG (Fig. 4). The therapeutic

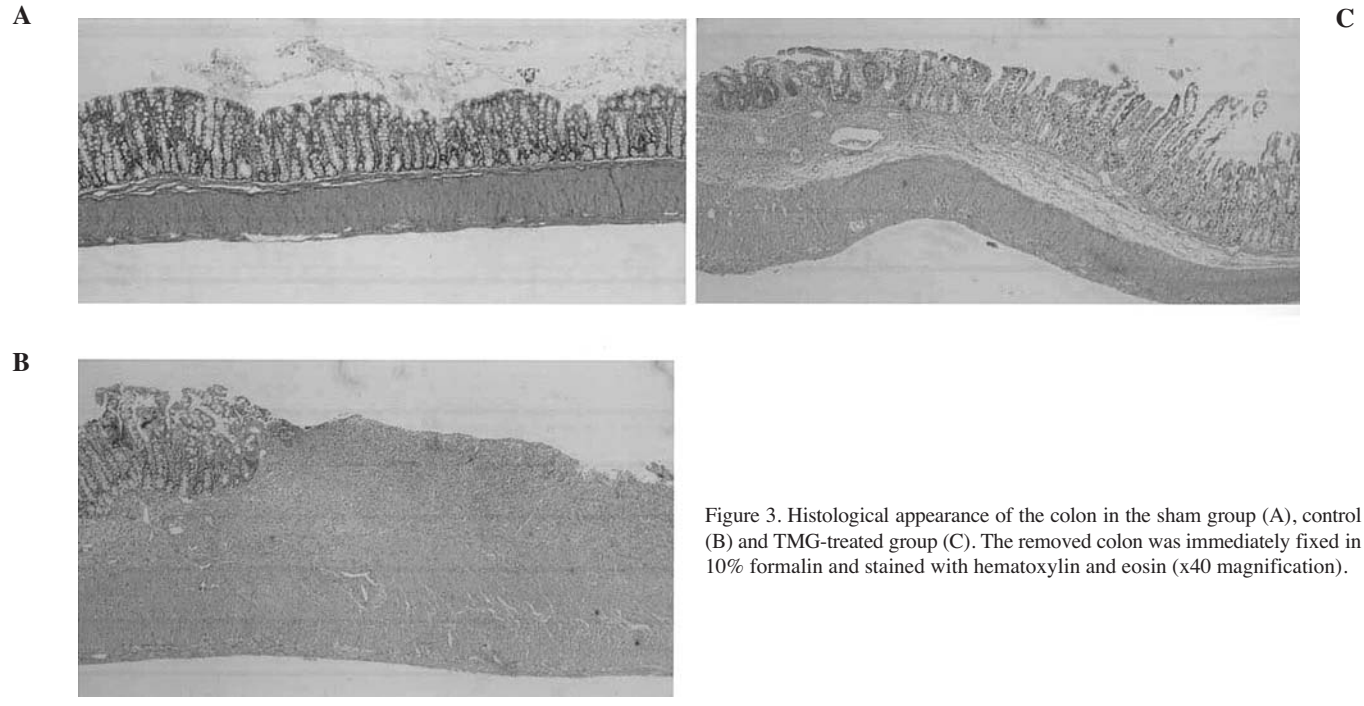

Figure 3. Histological appearance of the colon in the sham group (A), control (B) and TMG-treated group (C). The removed colon was immediately fixed in 10% formalin and stained with hematoxylin and eosin (x40 magnification).

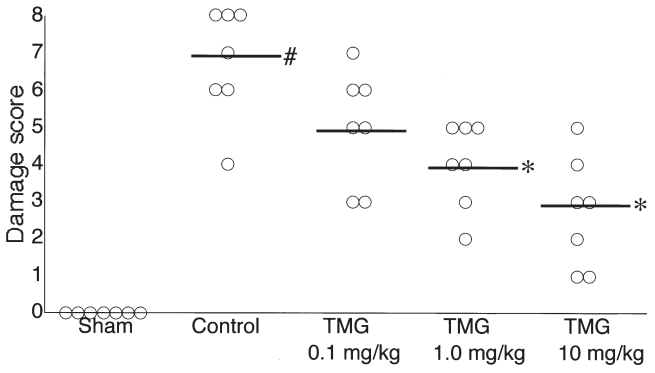

Figure 4. Effect of TMG on colonic damage score. TMG solution was administered through intraperitonial injection daily for 6 days after TNBS administration. Rats in the control group and the sham group received injection of vehicle (physiological saline) instead of TMG. Rats in the sham group received an enema of physiological saline instead of TNBS. Data are expressed as a scatter plot. #p<0.05 when compared to the sham group, *p<0.05 when compared to the TNBS group.

effect of TMG was also confirmed by histology. Fig. 3 shows typical histological features in the sham group, control group and TMG-treated group (10 mg/kg). Administration of TNBS showed a marked thickness of the colon wall with the infiltration and aggregation of numerous inflammatory cells having prominent lymphocytes in clusters forming lymph follicle. These changes extended transmurally with small noncaseating granulomas distributed throughout. In contrast, the rats treated with TMG at doses of 1.0 and 10 mg/kg showed only mild and moderate degrees of infiltration of inflammatory cells and slight mural thickening in the colonic mucosa. Moreover, the TNBS colitis group developed symptoms of acute colitis with increased diarrhea and loss of body weight. However, TMG-treated groups showed significantly less inhibition of weight gain compared with the control group (Fig. 5A).

Effect of TMG on TNBS-induced colonic inflammation. Colonic wet weight significantly increased in the control group. The increase in colonic wet weight was significantly decreased by treatment with TMG at doses of 1.0 and 10 mg/kg (Fig. 5B).

Effect of TMG on TBA-reactive substances in colonic mucosa. The extent of lipid peroxidation was determined by measuring TBA-RS in the colonic mucosa. TBA-reactive substances markedly increased in the control group. This increase was significantly inhibited in all groups treated with TMG (Fig. 6A).

Effect of TMG on MPO activity in colonic mucosa. The neutrophil accumulation was evaluated by measuring the MPO activity in the colonic mucosa. The MPO activity markedly increased in the control group. This increase was significantly inhibited by treatment with TMG at a dose of 10 mg/kg (Fig. 6B).

Effect of TMG on protein content of inflammatory cytokines in colonic mucosa. The content of both mucosal CINC-1 and TNF-α markedly increased in the control group. These increases in the levels of CINC-1 and TNF-α were significantly inhibited by treatment with TMG at a dose of 10 mg/kg (Fig. 6C and D).

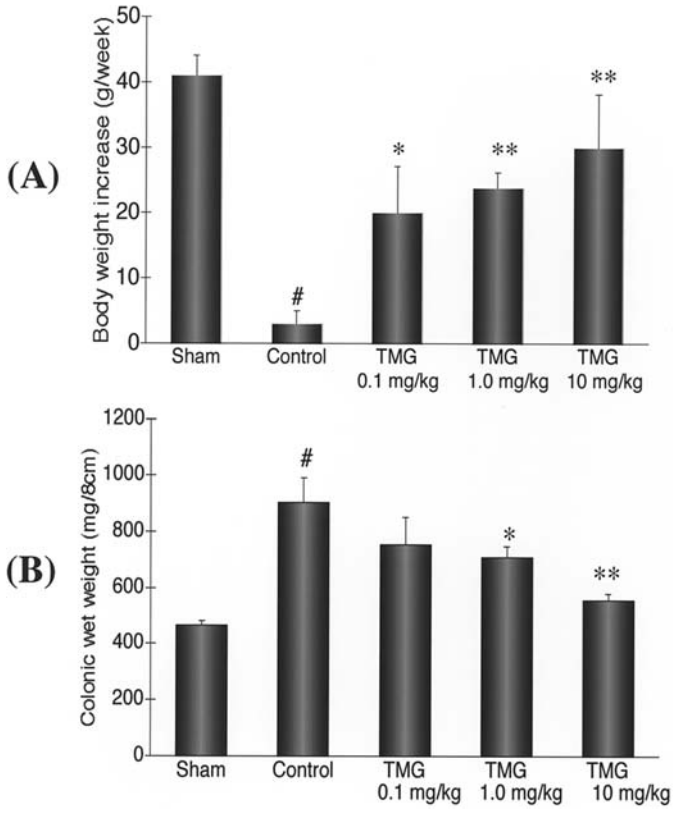

Figure 5. The changes in (A) increased body weight and (B) wet weight of colon mucosa. Data are expressed as the mean ± SE of 7 rats. #p<0.05 when compared to the sham group, *p<0.05 when compared to the TNBS group, **p<0.01 when compared to the TNBS group.

Discussion

The present study demonstrated that TMG, a novel water-soluble vitamin E derivative had a therapeutic effect on TNBS-induced colonic injury in rats. In this study, colonic injury was assessed by several methods including body weight, histology, the damage score, and colonic wet weight. In each assessment, the TMG treatment significantly inhibited colonic injury in TNBS-treated rats. An important observation of the present study was that TBA reactive substances, an index of lipid peroxidation, significantly increased in the colonic mucosa after TNBS administration and this increase was significantly inhibited by treatment with TMG. It has been recognized that oxygen radical-mediated lipid peroxidation plays an important role in the pathogenesis of gastrointestinal mucosal injuries (23-26). Our previous study (19) showed that TMG prevented colonic mucosal injury induced by TNBS in rats, suggesting that induction of lipid peroxidation is an early and critical event in the experimental model of IBD. In addition, the generated reactive oxygen species (ROS) has been implicated in the formation of the TNBS-induced colitis model (5,27) as well as human IBD (28). Although TNBS itself produces ROS in its oxidative metabolism process (29), the majority of ROS in the colonic mucosa is thought to be released by monocytes and macrophages (30), which are markedly increased in the colon during chronic inflammation. There are some major lines of evidence that implicate ROS as a mediator of TNBS-induced mucosal injury: i) this injury was significantly attenuated by a free radical scavenger such as Mn-SOD or Zinc-carnoise

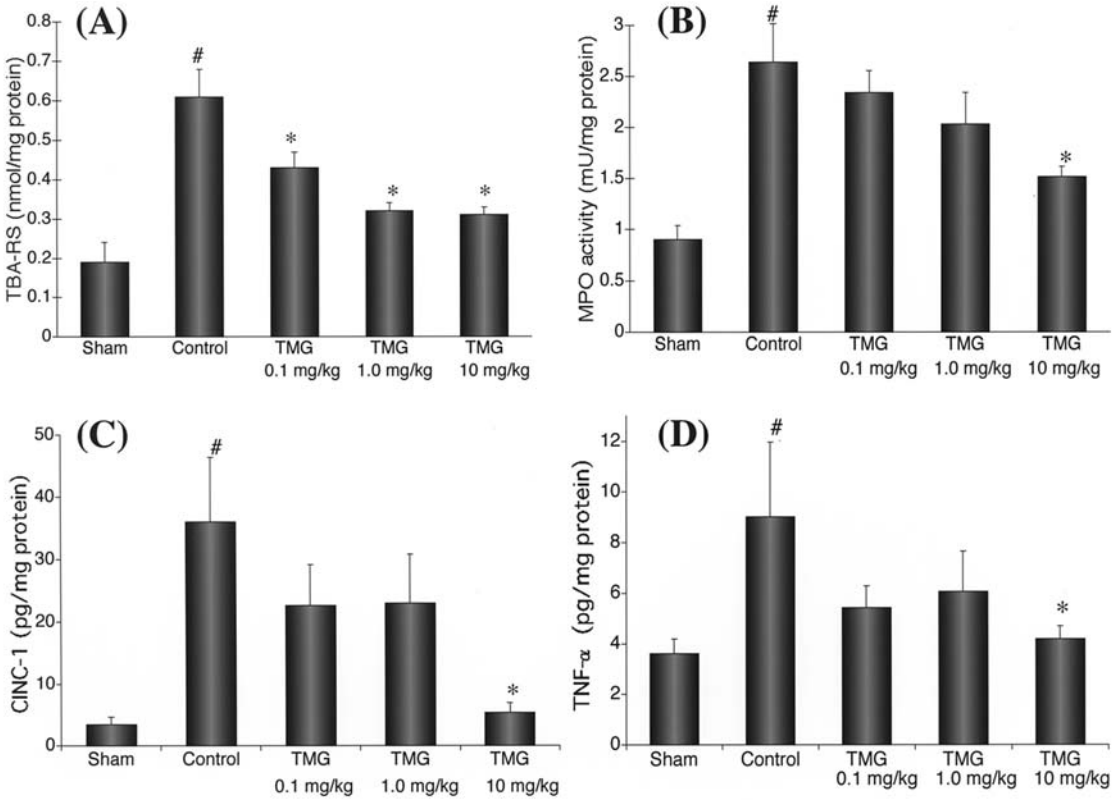

Figure 6. The changes in (A) thiobarbituric acid-reactive substances (TBA-RS), (B) myeloperoxidase (MPO) activity, (C) CINC-1 and (D) TNF-α in colonic mucosa. Data are expressed as the mean ± SE of 7 rats. #p<0.05 when compared to the sham group, *p<0.05 when compared to the TNBS group.

chelate compound (5,6); ii) oxypurinol, an inhibitor of xanthine oxidoreductase, significantly ameliorated TNBS-induced mucosal injury (27); and iii) colonic mucosal α-Tocopherol, an internal antioxidative agent, was significantly decreased after TNBS administration (19). Our data suggested that TMG inhibited lipid peroxidation in the colonic mucosa and reduced colonic inflammation. The effect of TMG could be associated with scavenging of hydroxy radical, which is the only ROS able to initiate lipid peroxidation. The effect of TMG could also be associated with the intra-membrane termination of lipid peroxidation.

Recent studies demonstrated that the adhesion molecules are involved in the migration of inflammatory cells towards the inflamed area and play a crucial role in the development of TNBS-induced colitis. It has been reported that the expression of adhesion molecule mRNA increased dramatically after the induction of colon inflammation by TNBS (31). Our previous study showed that anti-intercellular adhesion molecule (ICAM)-1 antibody inhibited the colonic injury treated with TNBS (32). Furthermore, our present study showed that MPO activity, an index of tissue-associated neutrophil accumulation, significantly increased in the colonic mucosa after TNBS administration and this increase was significantly inhibited by treatment with TMG. This result indicated that TMG can inhibit the overexpression of neutrophil or endothelial cell adhesion molecules by quenching TNBS-induced ROS and, subsequently, neutrophil infiltration into mucosal tissue can be inhibited.

Additionally, the present study showed that TMG reduced the content of inflammatory cytokines (CINC-1 and TNF-α) in colonic mucosa treated with TNBS. It appeared that inflammatory cytokines were produced both from neutrophils themselves and from endothelial cells stimulated by adhered neutrophils. In our previous study, TMG reduced the content of both TNF-α and CINC-2ß in gastric mucosa induced by ischemic-reperfusion in rats (33). We suggest that TMG inhibits cytokine production through scavenging ROS derived from the xanthine-anthine oxidase system, vascular endothelial cells or neutrophils.

Vitamin E is recognized as the major lipid soluble anti-oxidant preventing oxidative attack of membrane lipids and other membrane compounds (7-10). α-tocopherol, a major constituent of vitamin E, is known as a radical-scavenging antioxidant. Previous study reported that α-tocopherol scavenges not only chain-carrying peroxyl radicals (8,10) but also singlet oxygen (34) and superoxide anion radical. However, vitamin E is lipophilic and, to be absorbed into tissue, oral vitamin E must be absorbed at microvilli of the small intestine independently or by interaction with chylo-microns, then carried to the liver by the lymphatics system, distributed among lipoproteins, and transported to other organs (35). TMG is a synthetic vitamin E derivative with a chromanol ring, responsible for radical-scavenging activity, like α-tocopherol, and has excellent antioxidative activity, equal to that of α-tocopherol (17). In addition, TMG acquires high water-solubility by replacement of the long phytyl side chain of α-tocopherol with a glucosyl group, which enables it to be carried rapidly to the organs and supplied to tissue (17), and acts as a powerful antioxidant by scavenging both the chain-initiating radicals and chain-propagating radicals generated

either in the lipid or in the aqueous phase (36). Therefore, TMG has many advantages as an atioxidant in comparison with α-tocopherol.

In summary, the results of the present study demonstrated that TMG inhibited lipid peroxidation and reduced the development of colon inflammation induced by TNBS in rats. In addition, the anti-inflammatory effect seems to be related to the impairment of neutrophil function and cytokine production in colon mucosa. This investigation suggests that TMG has a potential to become a new therapeutic agent for IBD.

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