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1,4-Dihydroxy-2-naphthoic acid from *Propionibacterium freudenreichii* reduces inflammation in interleukin-10-deficient mice with colitis by suppressing macrophage-derived proinflammatory cytokines

Yoshikiyo Okada, Yoshikazu Tsuzuki, Kazuyuki Narimatsu, Hirokazu Sato, Toshihide Ueda, Hideaki Hozumi, Shingo Sato, Ryota Hokari, Chie Kurihara, Shunsuke Komoto, Chikako Watanabe, Kengo Tomita, Atsushi Kawaguchi, Shigeaki Nagao, and Soichiro Miura[1]

Department of Internal Medicine, National Defense Medical College, Tokorozawa City, Saitama, Japan

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ABSTRACT

The anti-inflammatory mechanism of prebiotics has recently been shown to have an impact on the host immune system. DHNA from *Propionibacterium freudenreichii* is known to promote the proliferation of *Bifidobacterium* and can ameliorate colitis, although its mode of action remains unknown. In this study, we investigated whether DHNA attenuates inflammation in piroxicam-treated IL-10$^{-/-}$ mice, particularly focusing on the changes of the host immune mechanism. DHNA was administered to IL-10$^{-/-}$ mice with colitis, and the expression of adhesion molecules and mRNA levels of proinflammatory cytokines were determined. DHNA pretreatment attenuated the piroxicam-induced histological changes. The increased F4/80-positive cell infiltration and VCAM-1 expression were decreased by DHNA administration. The increased mRNA levels of proinflammatory cytokines were also suppressed by DHNA. In in vitro experiments, increased mRNA levels of proinflammatory cytokines after endotoxin exposure were decreased significantly by DHNA pretreatment in RAW264.7, a macrophage cell line, and IL-10$^{-/-}$ mice BMMs, whereas the expression of VCAM-1 in bEnd.3 cells, a endothelial cell line, was not affected. Taken together, these findings suggest that administration of DHNA is useful for the treatment of colitis in piroxicam-treated IL-10$^{-/-}$ mice and that attenuation of colitis by DHNA may partly be a result of its direct action on intestinal macrophages to inhibit proinflammatory cytokine production. *J. Leukoc. Biol.* 94: 473–480; 2013.

Introduction

The major IBDs, CD and UC, are chronic intestinal diseases that are characterized by persistent inflammation of the intestinal tissue. Under germ-free conditions, chronic colitis does not develop in IBD animal models, such as IL-10$^{-/-}$ [1], TCR-α mice [2], and senescence-accelerated mice P1/Yit [3]. Furthermore, intestinal inflammation has been suggested to disturb the delicate balance between the intestinal microbiota and host immune system in patients with IBD [4].

Recently, prebiotic therapy, or control of the intestinal microbiota, has been performed in IBD clinical trials and experimental animal models. Prebiotics are generally defined as indigestible substances that selectively stimulate the activity of beneficial bacteria in the gut to improve host health [5–7]. Although these studies have focused mainly on the modulation of the enteric environment and beneficial bacteria, the effects of prebiotics on the host immune system have been reported recently in animal models and human clinical trials [8–10].

A cell-free filtrate of *P. freudenreichii* culture was reported to have a selective stimulating effect on bifidobacterial growth, and DHNA, an intermediate metabolite of menaquinone biosynthesis, was found to be the main component that promoted the specific in vitro proliferation of the genus *Bifidobacterium* [11, 12]. We reported previously that DHNA exerts anti-inflammatory effects in a murine dextran sulfate sodium colitis model by suppressing lymphocyte homing through the reduction of cell adhesion molecule expression [13]. However, the exact process or sequence of

Abbreviations: bEnd.3= brain endothelial cell line, BMM=bone marrow-derived macrophage, CD=Crohn's disease, DHNA=1,4-dihydroxy-2-naphthoic acid, IBD=inflammatory bowel disease, IL-10$^{-/-}$=IL-10-knockout, MAdCAM-1=mucosal addressin cell adhesion molecule-1, PSGL-1=P-selectin glycoprotein ligand-1, qPCR=quantitative PCR, UC=ulcerative colitis

1. Correspondence: Dept. of Internal Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa City, Saitama 359-8513, Japan. E-mail: miura@ndmc.ac.jp

how DHNA exerts anti-inflammatory effects by modulating the mucosal immune system remains unknown. Innate immune cells, such as monocytes differentiated into tissue macrophages, also migrate to the mucosa of inflamed bowels and play an important role in the development of mucosal inflammation and ulceration [14]. Therefore, knowing whether DHNA affects the migration of an innate cell population and whether it affects the cross-talk between innate immunity and acquired immunity would be important.

IL-10$^{-/-}$ mice develop spontaneously a chronic, T cell-mediated, transmural colitis that shares many features with human CD. However, as a result of the inconsistency in the development of spontaneous colitis in IL-10$^{-/-}$ mice, Berg et al. [15] described the rapid development of colitis in IL-10$^{-/-}$ mice treated with piroxicam, a NSAID. These mice developed severe colonic inflammation within 2 weeks of NSAID treatment, which was similar to the spontaneous IBD that developed in 3-month-old IL-10$^{-/-}$ mice. NSAID-induced colitis is histologically quite similar to spontaneous colitis in IL-10$^{-/-}$ mice and is characterized by marked infiltration of the colon with CD4^{+} T cells and macrophages [15].

Therefore, this study aimed to determine whether oral administration of DHNA attenuates colonic mucosal inflammation, particularly monocyte migration and cytokine production, in IL-10$^{-/-}$ mice fed a diet containing 200 g · kg^{-1} piroxicam to generate a mouse CD model of colitis. Additionally, a monocyte/macrophage cell line RAW264.7, IL-10$^{-/-}$ mice BMMs, and a mouse brain endothelial cell line bEnd.3 were stimulated with LPS to determine whether DHNA influences immune or endothelial cells directly.

MATERIALS AND METHODS

Animals and experimental protocol

Five-week-old female IL-10$^{-/-}$ mice with a C57BL/6 background were used in this study. The mice were housed under specific, pathogen-free conditions, and their care and use were in accordance with the guidelines of the National Defense Medical College. The study protocol was approved by the Animal Ethics Committee of the National Defense Medical College (No. 05083). DHNA, purchased from Wako (Osaka, Japan), was dissolved in dimethyl sulfoxide (0.5 ml) and added to a vehicle (distilled water; with 1% ascorbic acid, w/v).

DHNA (2.0 mg/kg) or vehicle (control) was administered to the mice for 14 days in freely available drinking water. Thereafter, the drinking water was changed to water without DHNA, and colitis was induced in the IL-10$^{-/-}$ mice by feeding them powdered rodent chow (CE-2; Clea Japan, Tokyo, Japan) containing NSAID (200 ppm piroxicam; Sigma-Aldrich, St. Louis, MO, USA). Each experimental group contained 10 mice. After 7 days, mice were killed. The colon was removed for further experiments after death (experimental period, 21 days).

Assessment of colonic damage and immunohistochemistry for inflammatory cells and adhesion molecules

A segment of the proximal colon was removed from the sacrificed mice, fixed in 10% buffered formalin, and embedded in paraffin. Next, 4-μm longitudinal sections were stained with H&E. Histological damage was assessed by crypt scoring, as described by Cooper et al. [16]: Grade 0, intact crypt; Grade 1, loss of the basal one-third of the crypt; Grade 2, loss of the basal two-third of the crypt; Grade 3, loss of the entire crypt, with an intact surface epithelium; and Grade 4, loss of the entire crypt and surface epithelium. The histological damage score was assessed in each segment and averaged in proportion to the length of the muscularis mucosa.

Another section of the removed colon was fixed for 12 h at 4°C in periodate-lysine-PFA. Subsequently, the tissues were washed and dehydrated for 12 h with PBS, containing 10%, 15%, or 20% sucrose. After fixation, the tissues were embedded in Tissue-Tek OCT compound (Sakura Fineteck, Tokyo, Japan) and frozen in liquid nitrogen. Next, 7 μm sections of frozen tissue were cut using a cryostat. Immunohistochemistry was performed using labeled streptavidin-biotin. Primary antibodies for immunostaining were mAb that react with MAdCAM-1 (MECA367, rat IgG2a), VCAM-1 (429, rat IgG2a), PSGL-1 (2PH2, rat IgG2a), β7-integrin (M293, rat IgG2a), and CD4 (L3T4, rat IgG2a). These antibodies were obtained from PharMingen (San Diego, CA, USA). F4/80 (CI:A3-1, rat IgG2b) was used as a macrophage marker (AbD Serotec, Oxford, UK). Isotype-matched IgG was used as a negative control. The tissues were treated with biotinylated goat anti-rat IgG (PharMingen) and visualized using streptavidin-fluorescein isothiocyanate. The VCAM-1- and MAdCAM-1-positive regions in the tissue sections were quantified as positive area/mm of the muscularis mucosa. The number of inflammatory cells of the muscularis mucosa was expressed/mm.

In vitro experiments

The mouse monocyte/macrophage cell line RAW 264.7 and the mouse brain endothelial cell line bEnd.3 were used in this experiment. In addition to these cell lines, BMMs from IL-10$^{-/-}$ mice were used. BM cells were isolated from the femurs of IL-10$^{-/-}$ mice. After separation of BM cells, BMMs were obtained by culture of BM cells in M-CSF (20 ng/ml) for 7 days.

All cells were maintained in DMEM (Sigma-Aldrich), supplemented with 10% FBS (Sigma-Aldrich) and 4 mM l-glutamine (Sigma-Aldrich) at 37°C in the presence of 5% CO_2. For all experiments, cells were seeded at 1 × 10^{6}/ml in a six-well culture plate and cultured for 24 h; they were then exposed to 10^{-4} M DHNA for 24 h preincubation. Preliminary experiments confirmed that over 8 h preincubation time was required for the induction of a DHNA-mediated anti-inflammatory response in LPS-exposed RAW264.7 cells. Subsequently, the cells were stimulated with LPS (1 μg/ml, serotype B55:O5; *Escherichia coli*; Sigma-Aldrich) and DHNA for 8 h. After incubation, the cells were harvested, and the mRNA levels of cytokines were determined by real-time qPCR.

Real-time qPCR

Colonic tissues, RAW264.7 cells, BMMs, or bEnd.3 cells were homogenized in the lysis buffer included in an RNeasy Mini kit (Qiagen, Hilden, Germany). After isolation, total RNA (1 μg) was used for RT, performed using a QuantiTect Reverse Transcription Kit (Qiagen). TaqMan PCR amplifications were performed using cDNA samples with TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). TaqMan probes and primers for IL-1β, IL-6, TNF-α, IL-12p40, IL-23p19, IL-17A, IFN-γ, VCAM-1, MAdCAM-1, and 18SrRNA were developed as TaqMan gene expression assays by Life Technologies. Target mRNA was analyzed using the comparative cycle threshold method of relative quantification, with an 18S rRNA calibrator sample isolated from untreated IL-10$^{-/-}$ mice as an internal control. All samples were assayed in duplicate.

Statistical analysis

Parametric data were statistically analyzed using Student's *t*-test, and nonparametric data were statistically analyzed using the Mann-Whitney *U*-test. All results are expressed as the mean ± SEM from 10 animals or five experiments. A significant difference was defined as $P < 0.05$.

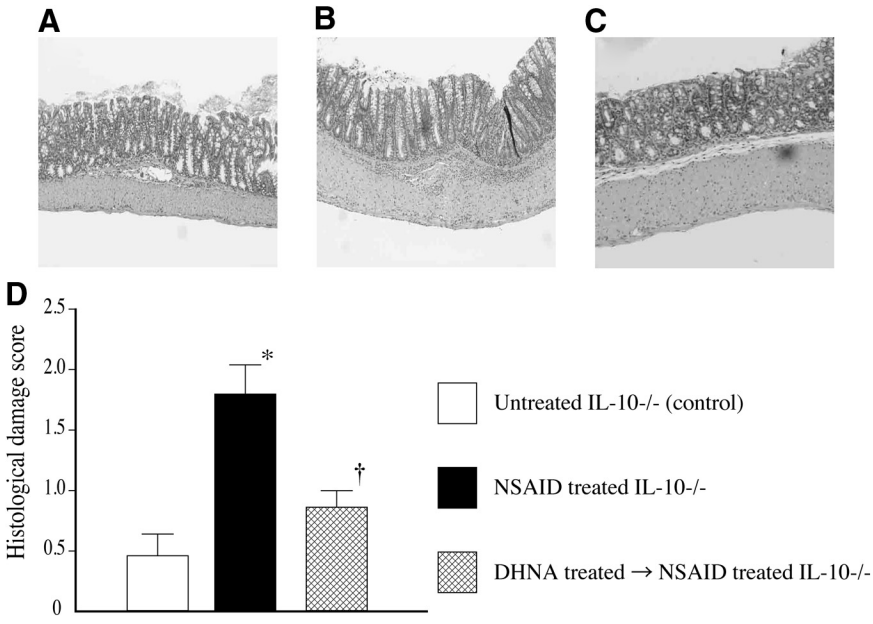

Figure 1. Effect of DHNA administration on acute NSAID-induced colitis in IL-10$^{-/-}$ mice (H&E staining, ×100). (A) Proximal colon from 8-week-old control (untreated) IL-10$^{-/-}$ mice. Very mild colitis was observed. (B) Proximal colon from 8-week-old IL-10$^{-/-}$ mice treated with NSAID (piroxicam) for 7 days (200 ppm piroxicam in the diet for 7 days). Progressive inflammation, manifested as goblet cell depletion; marked cell infiltration; and erosion were observed. (C) Proximal colon from the DHNA treatment group (2.0 mg/kg). Remarkable suppression of cell infiltration and erosions was observed. (D) Effect of DHNA administration on histological damage score in 8-week-old IL-10$^{-/-}$ mice treated with NSAID (piroxicam) for 7 days (200 ppm piroxicam in the diet for 7 days). Histological damage score was determined using paraffin sections of the proximal colon. The crypt scoring system (0–4 scale) was used with H&E staining (Grade 0, intact crypt; Grade 1, loss of the basal one-third of the crypt; Grade 2, loss of the basal two-thirds of the crypt; Grade 3, loss of the entire crypt with an intact surface epithelium; Grade 4, loss of the entire crypt and surface epithelium). *$P < 0.05$ versus the control group (untreated IL-10$^{-/-}$); †$P < 0.05$ versus the NSAID-treated IL-10$^{-/-}$ group. Results are expressed as the mean (±SEM; n=10).

RESULTS

Colitis in NSAID-treated IL-10$^{-/-}$ mice is attenuated by DHNA pretreatment

Initial colonic inflammation evaluation in the untreated control IL-10$^{-/-}$ mice at 8 weeks of age revealed minimal cellular infiltration in the lamina propria of the proximal colon and a histological damage score of 0.45 ± 0.18 (**Fig. 1A** and **D**). NSAID treatment resulted in the development of marked colitis in these mice. Goblet cell depletion, muscle layer thickening, prominent cellular infiltration, and erosion in the proximal colon were noted (Fig. 1B). The histological damage scores were significantly higher in the treated mice than in the untreated IL-10$^{-/-}$ mice (Fig. 1D). In the DHNA prevention group, leukocyte infiltration to the lamina propria and submucosal layer of the proximal colon was notably decreased (Fig. 1C). The damage score of the DHNA prevention group was significantly lower than that of the NSAID-treated IL-10$^{-/-}$ group (Fig. 1D).

The number of F4/80-positive cells and expression of VCAM-1 in the NSAID-treated IL-10$^{-/-}$ mice colonic tissue are decreased by DHNA pretreatment

Figure 2 shows the number of infiltrating cells and their subpopulations in the colonic tissue of the NSAID- and DHNA-treated IL-10$^{-/-}$ groups.

In the NSAID-treated group, significant increases in infiltrating CD4- and F4/80-positive cells were noted in the colon compared with those in the untreated IL-10$^{-/-}$ group. The β7- and PSGL-1-positive cells were also higher in the proximal colon of the NSAID-treated group. These increased numbers of CD4- and β7-positive cells were not significantly attenuated by DHNA treatment. However, a noteworthy finding was that the number of infiltrating F4/80-positive cells was remarkably reduced by DHNA treatment. **Figure 3** shows the area of the colonic mucosa that had adhesion molecules. As we have reported previously, macrophage VCAM-1/T lymphocyte-MadCAM-1-adhesive systems play critical roles in the induction of inflammation [17, 18]. VCAM-1 expression was significantly higher in the proximal colonic tissue of the NSAID-treated group than in the untreated IL-10$^{-/-}$ group (Fig. 3A). The VCAM-1 induced by NSAID treatment was significantly inhibited almost to control levels by DHNA treatment. Similarly, MAdCAM-1 expression was significantly higher in the proximal

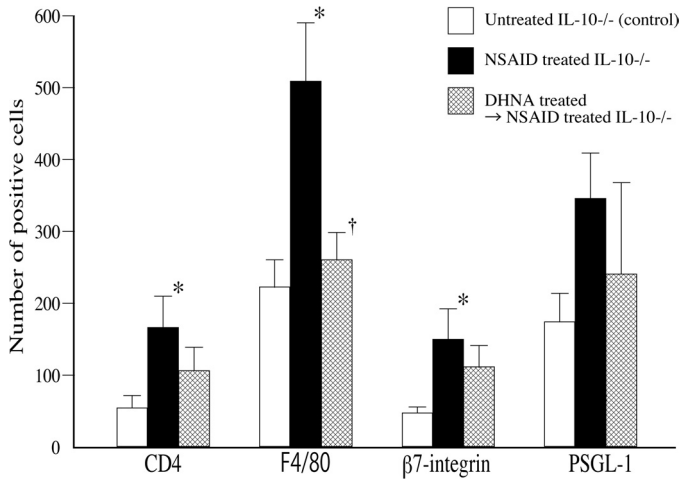

Figure 2. Effect of DHNA administration on the number of CD4-, F4/80-, β7-integrin-, and PSGL-1-positive cells in the colonic mucosa. The number of cells is expressed as positive cells/mm muscularis mucosa. *$P < 0.05$ versus the control group (untreated IL-10$^{-/-}$); †$P < 0.05$ versus the NSAID-treated IL-10$^{-/-}$ group. Results are expressed as the mean (±SEM; n=10).

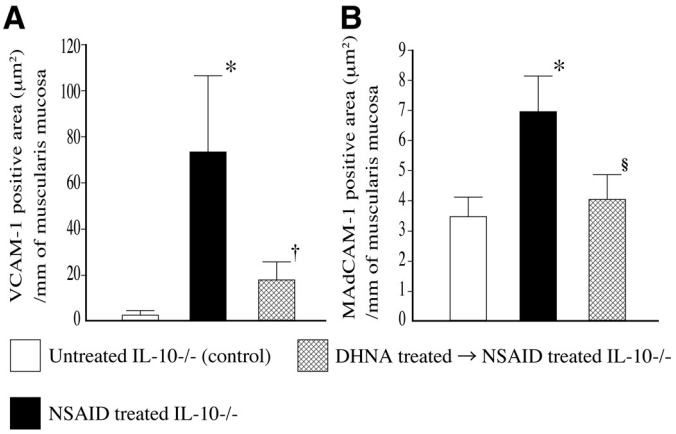

Figure 3. Effect of DHNA administration on the expression of VCAM-1 and MAdCAM-1 in the colonic mucosa. The expression was measured using ImageJ and expressed as area/mm of the muscularis mucosa. *$P < 0.05$ versus the control group (untreated IL-$10^{-/-}$); †$P = 0.054$ versus the NSAID-treated IL-$10^{-/-}$ group; §$P = 0.051$ versus the NSAID-treated IL-$10^{-/-}$ group. Results are expressed as the mean (±SEM; n=10).

colonic tissue of the NSAID-treated group than in the untreated IL-$10^{-/-}$ group (Fig. 3B). However, DHNA treatment did not lead to significant inhibition of MAdCAM-1 expression in the NSAID-treated IL-$10^{-/-}$ group (P=0.051).

mRNA levels of proinflammatory cytokines in the NSAID-treated IL-$10^{-/-}$ mice colonic tissue are down-regulated by DHNA pretreatment

Figure 4 shows the mRNA levels of various cytokines in the colonic tissue, as determined by real-time qPCR.

The mRNA expression levels of IL-1β, IL-6, and IL-17A were significantly higher in the NSAID-treated group than in the untreated IL-$10^{-/-}$ group. DHNA treatment significantly prevented the increase in the expression levels of these mRNAs in the proximal colonic tissue (Fig. 4A, B, and F).

The mRNA levels of TNF-α and IFN-γ were elevated in the NSAID-treated group, although not at significant levels, compared with those in the untreated IL-$10^{-/-}$ group; DHNA treatment inhibited the increase of TNF-α expression in the proximal colonic tissue (Fig. 4C and G).

The mRNA level of IL-12p40 was higher in the NSAID-treated group than in the untreated IL-$10^{-/-}$ group; however, DHNA treatment did not affect the mRNA expression of IL-12p40 in the proximal colonic tissue (Fig. 4D). On the other hand, a significant increase in the mRNA expression level of IL-23p19 in the proximal colonic tissue, induced by NSAID treatment, was significantly repressed by DHNA treatment (Fig. 4E).

The mRNA expression level of MCP-1, a key mediator of monocyte migration, was significantly higher in the NSAID-treated group than in the untreated IL-$10^{-/-}$ group; however, its expression was not affected by DHNA treatment (Fig. 4H).

DHNA treatment in vitro decreases mRNA expression of proinflammatory cytokines in macrophages but not mRNA expression of adhesion molecules in endothelial cells

To investigate whether DHNA directly influences the activity of immune or endothelial cells during inflammation, we performed an in vitro assay, in which the mouse monocyte/macrophage cell line RAW 264.7, BMMs isolated from IL-$10^{-/-}$ mice, and the mouse brain vascular endothelial cell line bEnd.3 were stimulated by LPS. The mRNA expression levels of all proinflammatory cytokines were significantly higher in the LPS-stimulated RAW 264.7 cells and BMMs than in the control cells (**Figs. 5** and **6**). The mRNA levels of IL-6 and IL-23p19 in the LPS-stimulated RAW 264.7 cells and BMMs were suppressed significantly by DHNA treatment (Figs. 5B and E

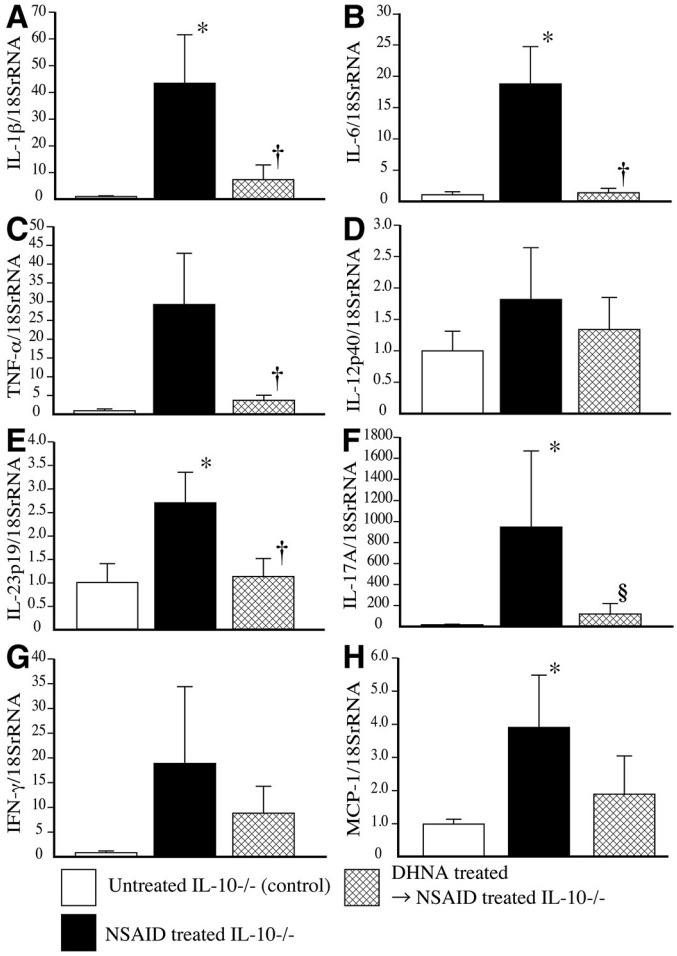

Figure 4. The mRNA expression of inflammatory cytokines in the proximal colon of mice with NSAID-induced (piroxicam, 200 ppm) colitis and the attenuating effect of DHNA. IL-1β (A), IL-6 (B), TNF-α (C), IL-12p40 (D), IL-23p19 (E), IL-17A (F), IFN-γ (G), and MCP-1 (H). The mRNA levels were determined by real-time qPCR. The columns represent the average ratio of cytokines and 18S rRNA from 10 mice. *$P < 0.05$ versus the control group (untreated IL-$10^{-/-}$); †$P < 0.05$ versus the NSAID-treated IL-$10^{-/-}$ group; §P=0.057 versus the NSAID-treated IL-$10^{-/-}$ group. Results are expressed as the mean (±SEM; n=10).

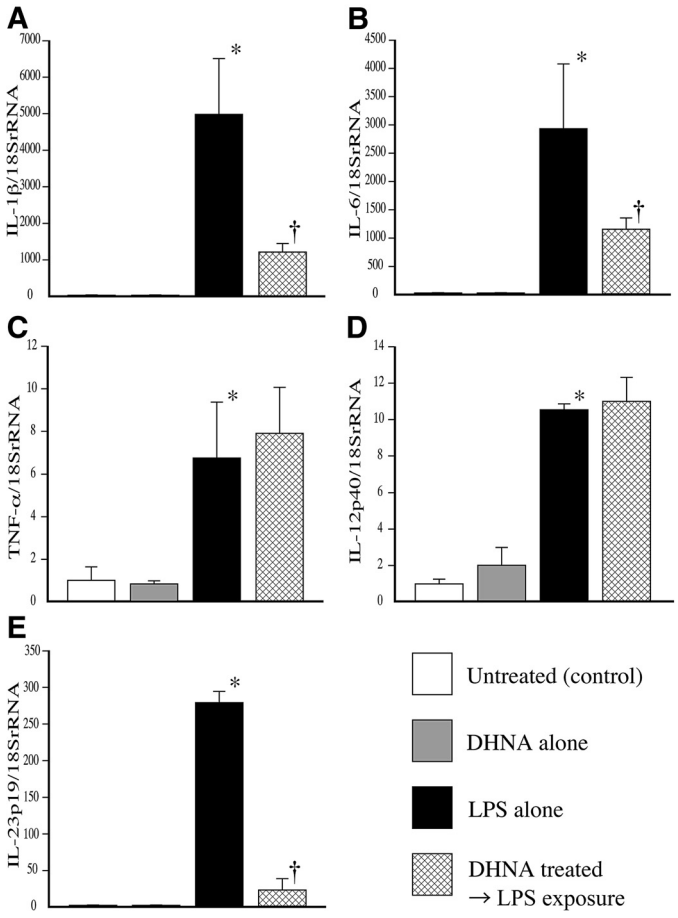

Figure 5. The mRNA expression of various cytokines in RAW264.7 cells stimulated by LPS (10 μg/ml) and the attenuating effect of DHNA (10^{-4} M). IL-1β (A), IL-6 (B), TNF-α (C), IL-12p40 (D), and IL-23p19 (E). The mRNA levels were determined by real-time qPCR. The columns represent the average ratio of cytokines and 18S rRNA from 10 different experiments. *$P < 0.05$ versus the control group (unstimulated); †$P < 0.05$ versus the LPS-stimulated RAW264.7 cells. Results are expressed as the mean (±SEM; n=10).

and 6B and E). The mRNA expression levels of TNF-α and IL-12p40 were also increased significantly in the LPS-stimulated RAW 264.7 cells; however, DHNA treatment did not prevent the LPS-induced increase in these cytokines (Fig. 6C and D). In the case of BMMs, the suppressive effects by DHNA treatment were not observed on mRNA expression levels of IL-1β (Fig. 6A).

The effects of DHNA on the mRNA expression of the two types of adhesion molecules in bEnd.3 cells are shown in **Fig. 7**. The mRNA expression levels of VACM-1 and MAdCAM-1 were significantly higher in the LPS-stimulated bEnd.3 cells, and DHNA treatment did not affect their expression levels.

DISCUSSION

NSAID-treated IL-$10^{-/-}$ mice were used to evaluate the anti-inflammatory effects of DHNA on colitis. NSAID-treated IL-$10^{-/-}$ mice develop lesions and immune responses, such as inflammatory cytokine production, that are quite similar to the spontaneous colitis, noted in IL-$10^{-/-}$ mice. Both models are characterized by predominant mononuclear infiltration in the lamina propria, with areas of transmural inflammation [15]. However, NSAID-treated IL-$10^{-/-}$ models are characterized by consistent and rapid development of colitis that is suitable for the evaluation of various anti-inflammatory drugs. The control, untreated IL-$10^{-/-}$ mice did not show significant colonic inflammation at the tested time-points (at 8 weeks).

DHNA significantly attenuated the rapid development of colonic inflammation in NSAID-treated IL-$10^{-/-}$ mice with colitis by decreasing the disease activity index and the numbers of infiltrating cells in the colonic tissue. In the present study, subpopulations of inflammatory infiltrates in the lamina propria and submucosa were analyzed using immunohistochemical staining. The inflammatory infiltrates predominantly

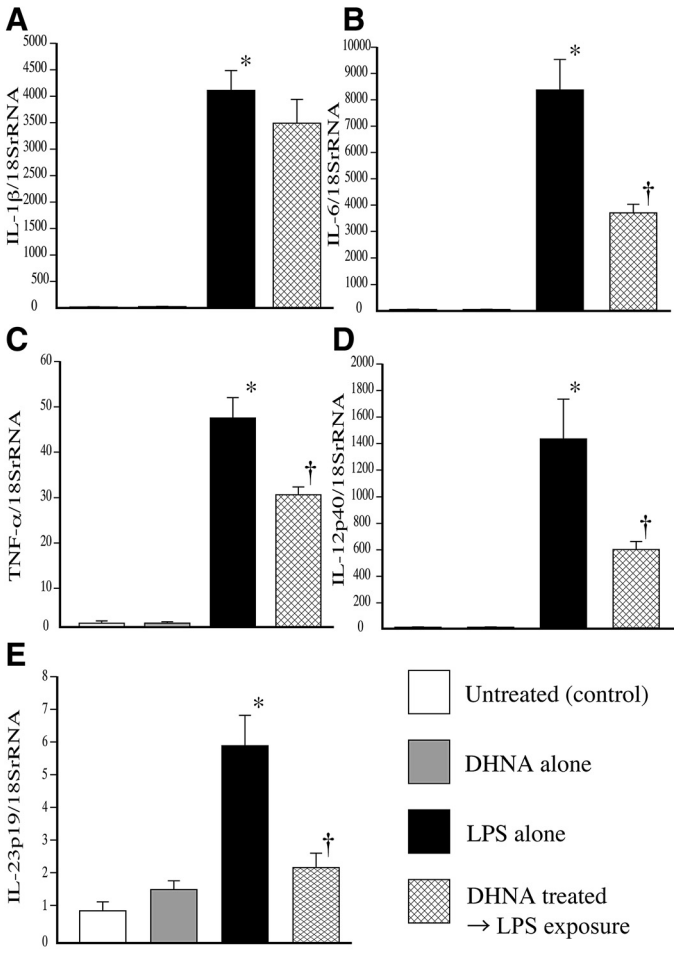

Figure 6. The mRNA expression of various cytokines in BMMs isolated from IL-$10^{-/-}$ mice, stimulated by LPS (10 μg/ml) and the attenuating effect of DHNA (10^{-4} M). IL-1β (A), IL-6 (B), TNF-α (C), IL-12p40 (D), and IL-23p19 (E). The mRNA levels were determined by real-time qPCR. The columns represent the average ratio of cytokines and 18S rRNA from 10 different experiments. *$P <$ 0.05 versus the control group (unstimulated); †$P < 0.05$ versus the LPS-stimulated RAW264.7 cells. Results are expressed as the mean (±SEM; n=10).

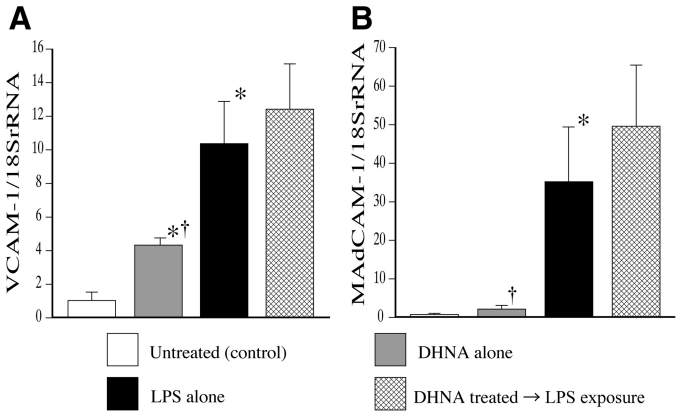

Figure 7. The mRNA expression of various adhesion molecules in bEnd.3 cells stimulated by LPS (10 μg/ml) and the attenuating effect of DHNA (10^{-4} M). VCAM-1 (A) and MAdCAM-1 (B) mRNA levels were determined by real-time qPCR. The columns represent the average ratio of adhesion molecules and 18S rRNA from 10 different experiments. *$P < 0.05$ versus the control group (unstimulated); †$P < 0.05$ versus the LPS-stimulated bEnd3 cells. Results are expressed as the mean (±SEM; n=10).

consisted of mononuclear cells, although small numbers of neutrophils and eosinophils were also found. A significant increase in F4/80-positive cells was particularly noted in the NSAID-treated IL-$10^{-/-}$ group (Fig. 2), although the numbers of CD4- or β7-integrin-positive lymphocytes were also increased. F4/80 is considered the best macrophage marker [19], and an increase in F4/80-positive cells might suggest an increase in monocyte migration to the inflamed colonic tissue. An increase in the level of the chemokine MCP-1 in the colonic tissue might also suggest monocyte migration. Resident intestinal macrophages generally do not express the LPS coreceptor CD14; however, a substantial proportion of mucosal macrophages expresses CD14 in patients with IBD. Studies on macrophage accumulation in inflamed intestinal mucosa have shown that endothelial cells in the mucosal microvessels of CD patients have high levels of CD34, a ligand that promotes the rolling of L-selectin-positive monocytes [20]. In this study, F4/80-positive cells were significantly lower in the DHNA-treated group than in the NSAID-treated IL-$10^{-/-}$ group; however, infiltration of CD4- or β7-integrin-positive lymphocytes was not suppressed by DHNA (Fig. 2). The first step of monocyte migration is mediated by P-selectin and its counterpart, PSGL-1, whereas the second step involves the adhesion molecules classified into the Ig superfamily VCAM-1 on the endothelium [21, 22]. Our recent antibody-blocking studies have shown that PSLG-1, P-selectin, and VCAM-1 promote $CD14^+$ monocyte rolling and adherence in the intestinal mucosa, particularly the ileal mucosa, in a mouse model of spontaneous ileitis [17]. Thus, attenuation of the increased influx of monocytes in the colonic mucosa of NSAID-treated IL-$10^{-/-}$ mice might be closely related to the decrease in VCAM-1 expression by DHNA administration. The regulation of adhesion molecules or their counterparts is as an important strategy for treating IBD. Indeed, antibody drugs, such as Natalizumab, which blocks the binding of integrins α4β1 and α4β7 to VCAM-1 and MAdCAM-1, have been used to treat IBD [23, 24]. However, many recent studies have shown adverse effects of antibody drugs, resulting from the development of neutralizing antibodies against the drugs themselves [25]. DHNA treatment might be safer and more advantageous than antibody treatment if it can successfully inhibit the overexpression of adhesion molecules in the inflamed mucosa of IBD patients.

Because of the rapid development of colonic inflammation in NSAID-treated IL-$10^{-/-}$ mice, there was a significant production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, possibly as a result of the loss of anti-inflammatory activity of IL-10 and PGs. DHNA administration also decreased mRNA levels of these proinflammatory cytokines in NSAID-treated IL-$10^{-/-}$ mice, concomitantly with the attenuation of colonic mucosal damage (Figs. 1 and 4). Examination of biopsy specimens of CD and UC patients has revealed that IL-1β, IL-6, and TNF-α, expressed mostly in the lamina propria at sites that are rich in macrophages and showing abundant staining of VCAMs [26, 27], showed that TNF-α and IL-1 sequentially induce the expression of endothelial ICAM-1 and VCAM-1. These findings suggest that DHNA-induced decreases in IL-1β, IL-6, and TNF-α expression might be important for inhibiting the expression of adhesion molecules, such as VCAM-1 and MAdCAM-1. Furthermore, the expression levels of IL-23p19 and IL-17A, but not those of IL-12p40 and IFN-γ, were significantly lower in the DHNA-treated IL-$10^{-/-}$ mice than in the NSAID-treated IL-$10^{-/-}$ mice (Fig. 4). IL-23 and IL-12 are expressed mainly by cells of the innate immune system, such as macrophages or DCs, although their biological properties are clearly distinct. IL-23 is a key mediator of intestinal inflammation and required for the accumulation and function of Th17 cells, which produce IL-17A, a powerful proinflammatory cytokine found in various types of colitis. In contrast, IL-12 facilitates the differentiation of IFN-γ-secreting Th1 cells, leading to the sustenance of chronic inflammatory disorders of colitis [28, 29]. Mixed Th1/Th17 responses are believed to mediate CD as well as colitis in NSAID-treated IL-$10^{-/-}$ mice [30, 31]. DHNA might preferentially influence the activity of intestinal innate-immunity cells, such as macrophages, to a greater extent than that of adaptive immune cells and suppress the function of Th17 cells by reducing IL-23 expression induced by intestinal macrophages.

Originally, DHNA is known to promote the proliferation of *Bifidobacterium* spp. in vitro [12]; in our preliminary study, a significant increase in the number of CFUs on TOS-propionate agar in the cecal content was noted in vivo in DHNA-treated IL-$10^{-/-}$ mice compared with that in untreated and NSAID-treated IL-$10^{-/-}$ mice (data not shown), suggesting an increase in *Bifidobacterium* spp. However, the findings of the current study suggest that besides its prebiotic action, DHNA could attenuate colitis in NSAID-treated IL-$10^{-/-}$ mice by influencing the host gut immune system. Namely, DHNA was speculated to decrease the production of various proinflammatory cytokines and expression of adhesion molecules by acting

directly on macrophages or endothelial cells. Hence, an in vitro assay was performed, in which the monocyte/macrophage cell line RAW264.7, BMMs isolated from IL-10$^{-/-}$ mice, and the brain endothelial cell line bEnd.3 were stimulated by LPS to mimic an inflammatory condition, and the effect of DHNA exposure was determined. In the RAW264.7 cells and BMMs, the expression levels of IL-6 and IL-23p19, which increased upon LPS stimulation, were decreased significantly by DHNA pretreatment (Figs. 5 and 6). DHNA has a naphthoquinone skeleton similar to vitamin K2 [12], and both DHNA and vitamin K2 have been reported to suppress osteoclast differentiation and formation [32, 33]. Hence, we speculated that DHNA enters macrophages by a similar mechanism as that of vitamin K2, possibly by binding to the lipoprotein receptor, followed by receptor-mediated endocytosis [34].

DHNA also affects the responsiveness of RAW 264.7 cells to bacterial products other than LPS. In our preliminary study, we found that DHNA also significantly suppressed the mRNA levels of IL-6 in RAW 264.7 cells, which were stimulated by the bacterial lipoprotein palmitoyl-3-cysteine-serine-lysine-4. Moreover, the inhibitory effect of DHNA may not be a result of LPS tolerance in RAW 264.7 cells, as we found that DHNA treatment showed a significant attenuating effect on IL-6 mRNA expression, even when it was administered after the start of LPS exposure (data not shown).

On the other hand, the mRNA levels of VCAM-1 and MAdCAM-1 in the LPS-stimulated bEnd.3 cells were not affected by DHNA pretreatment (Fig. 7). These results suggest that the anti-inflammatory effect of DHNA might result from a direct action on macrophages, leading to decreased production of proinflammatory cytokines. The reduction of the expression of adhesion molecules, such as VCAM-1, in the vascular endothelial cells by DHNA treatment appears to be the secondary outcome of the reduction of proinflammatory cytokines released by macrophages.

In conclusion, our study showed that oral DHNA administration improved the histological score and inflammatory cell infiltration in NSAID-treated IL-10$^{-/-}$ mice and thus, might be considered a useful treatment strategy for colitis in this mouse model. Our findings also suggested an important anti-inflammatory action of DHNA, that is, suppression of proinflammatory cytokine production from macrophages and inhibition of further migration of monocytes to the inflamed colonic mucosa. The results of the present study suggest that DHNA might be an effective bioactive substance that can modulate directly the host immune system in human IBD.

AUTHORSHIP

Y.O. and S. M. participated in research design. Y.O. and Y.T. conducted experiments. Y.O., Y.T., and R.H. contributed new reagents or analytic tools. Y.O., Y.T., K.N., H.S., T.U., H.H., S.S., R.H., C.K., S.K., C.W., K.T., A.K., and S.N. performed data analysis. Y.O., Y.T., and S.M. wrote or contributed to the writing of the manuscript.

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DISCLOSURES

There are no conflicts of interest to declare.

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KEY WORDS:
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