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A novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin, ameliorates inflammatory colonic injury in mice

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Inflammatory bowel disease;
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Abstract

Background: In inflammatory bowel disease (IBD), gut inflammation is associated with the activation of nuclear factor kappa B (NF-κB), a key pro-inflammatory transcription factor.
Aim: To investigate the therapeutic potential of a novel, specific NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), we examined its effect on IBD using murine experimental colitis models.
Methods: The *in vitro* effect of DHMEQ was evaluated by inflammatory cytokine production and p65 immunostaining using HT-29 and RAW264.7 cells. The *in vivo* therapeutic effect of DHMEQ was studied in colitis induced by dextran sulphate sodium (DSS) and trinitrobenzenesulphonic acid (TNBS). In these, progression and severity of colitis was mainly assessed by the disease activity index (DAI), histopathology, cellular infiltration, and mRNA expression levels of pro-inflammatory cytokines in the colonic tissues.
Results: In RAW264.7 cells, DHMEQ significantly inhibited tumour necrosis factor (TNF)-α and interleukin (IL)-6 production induced by LPS in a dose-dependent manner by blocking the nuclear translocation of NF-κB. In addition, DHMEQ inhibited IL-8 production induced by LPS in HT-29 cells. DHMEQ significantly ameliorated DSS colitis as assessed by DAI scores, colonic oedema,

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and histological scores. Immunohistochemistry revealed that DHMEQ inhibited colonic infiltration of nuclear p65$^+$ cells, CD4$^+$ lymphocytes, and F4/80$^+$ macrophages. mRNA expression levels of the pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, IL-12p40, IL-17, and MCP-1 were also suppressed by DHMEQ administration. Furthermore, DHMEQ significantly ameliorated TNBS colitis as assessed by body-weight changes and histological scores.
Conclusion: DHMEQ ameliorated experimental colitis in mice. These results indicate that DHMEQ appears to be an attractive therapeutic agent for IBD.

1. Introduction

Inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing inflammatory disorders of the gastrointestinal tract that have a peak age of onset in the second to fourth decades of life. Pathogenesis of IBD involves a combination of genetic susceptibility, environmental triggers, immunological factors, and luminal microbial antigens.[1–4] Although the exact aetiologies causing IBD remain unknown, they are generally thought to result from an inappropriate and ongoing activation of the mucosal immune system against normal luminal flora. Both innate immunity responses, mainly mediated by monocytes/macrophages, and adaptive immunity responses launched by auto-reactive CD4$^+$ T cells have been postulated to play an important role in the initiation and progression of IBD.[5–7] Responding T cells exhibit a T helper type 1 (Th1) phenotype in CD and Th2 phenotype in UC.[1–4,6] Several studies have also shown that serum and mucosal interleukin (IL)-17 expressions were increased in IBD, particularly in UC.[8,9]

The transcription factor nuclear factor kappa B (NF-κB) consists of a homodimer or heterodimer of 2 subunits of the members of the NF-κB family: p65 (RelA), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), or NF-κB2 (p52 and its precursor p100),[10] and plays an essential role in inflammation, cellular stress control, and cell survival/death.[11,12] Activation of cells mediating immunity, such as macrophages, dendritic cells, or lymphocytes, is chiefly regulated by NF-κB activation.[11,13,14] Furthermore, expression of anti-apoptotic molecules and of various genes which encode pro-inflammatory mediators such as cytokines, adhesion molecules, and chemokines is NF-κB dependent.[10] Dysregulation of NF-κB activity has been implicated in numerous diseases including malignancies and chronic inflammatory disorders.[12,15] In past studies, it has been shown that NF-κB is up-regulated in the inflamed human colon of CD and UC patients and plays an important role at IBD onset in experimental colitis models.[14,16] Indeed, compounds related to 5-aminosalicylic acid (5-ASA), such as sulphasalazine and mesalamine, well-known conventional therapeutic drugs for IBDs, have been shown to exert therapeutic effect in part by suppression of NF-κB activation through inhibition of phosphorylation of IκBα.[17–19] However, these drugs are not specific for suppression of NF-κB activation; furthermore, problems related to their drug delivery exist, leading to their limited efficacy for IBDs.

Dehydroxymethylepoxyquinomicin (DHMEQ) is a low-molecular-weight derivative of the antibiotic epoxyquinomycin C.[20] This novel agent has been found to inhibit DNA binding and nuclear translocation of NF-κB by covalent binding to the specific cysteine residue of the NF-κB components p65, p50, RelB, and c-Rel, but not by impairing I-κB phosphorylation or degradation.[21,22] We and others have demonstrated that administration of DHMEQ prevented cardiac allograft rejection,[23] intestinal ischaemia–reperfusion injury,[24] rheumatoid arthritis,[25] and autoimmune uveoretinitis.[26] In this study, we examined the effect of DHMEQ on IBD using murine experimental colitis models.

2. Materials and methods

2.1. Cell culture

Murine macrophage-like cell line RAW264.7 and human colon adenocarcinoma cell line HT-29 were obtained from Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, MD, USA), respectively. The cells were cultured in RPMI 1640 culture media containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% foetal calf serum, and 50 μM 2-mercaptoethanol, and maintained at 37 °C in an incubator with 5% CO_2 and constant humidity.

2.2. Mice

Male C57BL/6 mice (age: 8 weeks, body weight: 22–24 g) and male BALB/c mice (age: 8 weeks, body weight: 24–26 g) were obtained from Japan SLC Inc. (Shizuoka, Japan). They were maintained under standard laboratory conditions. The experiments were approved by the Institutional Animal Care Committee, and were conducted following the guidelines of the animal care policy.

2.3. Reagents

DHMEQ was synthesised as described previously,[20,23] dissolved in dimethylsulfoxide (DMSO), and adjusted to 50 mg/mL. This stock solution was stored at −80 °C until use. For appropriate DHMEQ dose in the *in vivo* and *in vitro* experiments, the stock solution was dissolved in 0.5% carboxymethyl cellulose (CMC) solution or RPMI 1640 culture media described above. The final DMSO concentration was 4% *in vivo*, and ≤0.05% *in vitro*, respectively.

2.4. Measurement of cytokine release from cell lines

RAW264.7 (2×10^5 cells/well) and HT-29 (6×10^4 cells/well) cells in 96-well plates were incubated for 16 h. After pre-incubation, RAW264.7 cells were treated with DHMEQ for

2 h and stimulated with LPS 10 μg/mL for the desired periods of time. HT-29 cells were treated with DHMEQ for 1 h and stimulated with LPS 10 ng/mL. Supernatant was collected at 6 or 24 h after LPS stimulation and IL-6, IL-8, and tumour necrosis factor (TNF)-α levels in the supernatant were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

2.5. Immunocytochemistry of NF-κB in RAW264.7

RAW264.7 cells were incubated on micro slide glass for 72 h. After pre-culture, cells were treated with DHMEQ 10 μg/mL for 1 h and stimulated with LPS 10 μg/mL. Ten minutes after LPS stimulation, cells were fixed in 20% formaldehyde for 20 min. After fixing, cells were incubated with 1% H_2O_2 for 30 min, anti-p65 (Santa Cruz Biotechnology Inc., Tokyo, Japan) at 4 °C for 60 min, and treated by the EnVision plus method (K4002; Dako Ltd., Tokyo, Japan) for 30 min at room temperature, followed by visualisation with 3,3′-diaminobenzidine (DAB:K3466; Dako Ltd.) and counterstaining with haematoxylin.

2.6. Induction of colitis, treatments, and clinical assessment

2.6.1. Colitis induced by DSS

Male C57BL/6 mice (1 per cage) were given 3% dextran sulfate sodium (DSS: molecular weight = 36–50 kDa; MP Biomedicals Inc., Tokyo, Japan) in their drinking water for 5 days and thereafter provided with regular water for 5 days. Mice were intraperitoneally injected DHMEQ at a dose of 10, 20 or 40 mg/kg, or control vehicle (0.5% CMC containing with 4% DMSO) once or twice daily from day 0 to day 10. As a control therapeutic drug, 50 or 100 mg/kg of 5-ASA (Kyorin Pharmaceutical Co. Ltd., Tokyo, Japan) was intrarectally administered once per day. Body weight, stool bleeding, and stool consistency were monitored daily. Stool bleeding was assessed using Haemoccult Slide 5 Shionogi II (Shionogi & Co Ltd., Osaka, Japan). Animals were euthanised at time points and the large intestines without caecum were collected. These were weighed, measured, and evaluated for colonic oedema and microscopic damage. Severity of colitis was assessed by the disease activity index (DAI), colonic oedema (weight/length), and histological damage. The DAI was determined and scored in accordance with the method described previously.[27] Scores were calculated by grading on a scale of 0–4 the following parameters: change in weight (**0**: ≤1%, **1**: 1%–5%, **2**: 5%–10%, **3**: 10%–20%, **4**: >20%), stool bleeding (**0**: negative, **1**–**3**: haemoccult positive, **4**: gross bleeding), and stool consistency (**0**: normal, **1**: soft stools, **2**: loose stools, **3**: muddy stools, **4**: diarrhoea).

2.6.2. Colitis induced by TNBS

Male BALB/c mice (3 per cage) were lightly anaesthetised *via* inhalation of isoflurane, and were administered 150 μL containing 1.5 mg of trinitrobenzenesulphonic acid (TNBS; Sigma-Aldrich, Tokyo, Japan) diluted in 50% ethanol intrarectally *via* a 3.5-Fr catheter equipped with a 1-mL syringe. The catheter tip was inserted 4 cm proximal to the anal verge. To ensure proper distribution of TNBS within the entire colon and caecum, mice were kept in a vertical position for 30 s after intrarectal injection. Mice were given DHMEQ (15 mg/kg) or control vehicle (0.5% CMC containing 4% DMSO) *via* intraperitoneal injection twice daily from day 0 to day 4. The mice were euthanised on day 4 and their large intestines without caecum were collected. Progression and severity of colitis were assessed by body-weight change, colonic oedema (weight/length), macroscopic damage and histological damage of the colon. Macroscopic damage was evaluated and scored in a blinded manner as described previously,[28] according to the following criteria; **0**: normal appearance, **1**: focal hyperaemia, without ulcers, **2**: ulceration without hyperaemia or bowel wall thickening, **3**: ulceration with inflammation at one site, **4**: ulceration or inflammation at two or more sites, **5**: major sites of damage extending 1 cm along the length of the colon, **6**–**10**: when an area of damage extended 2 cm along the length of the colon, the score was increased by 1 for each additional cm of involvement.

2.7. Histological examination

2.7.1. Colitis induced by DSS

Specimens of whole colon without caecum were fixed in formalin and embedded in paraffin blocks. For histological examinations, 3-mm paraffin sections were stained with haematoxylin and eosin. Histological scoring of tissues was performed in a blinded manner by a skilled pathologist as described by Dieleman et al.[29] Grading index was as follows: inflammation severity (**0**: none, **1**: mild, **2**: moderate, **3**: severe), inflammation extent (**0**: none; **1**: mucosa, **2**: mucosa and submucosa, **3**: transmural), crypt damage (**0**: none, **1**: basal one-third damaged, **2**: basal two-thirds damaged, **3**: only surface epithelium intact, **4**: entire crypt and epithelium lost), and the percentage involvement in the ulcer or erosion (**1**: <1%, **2**: 1%–15%, **3**: 16%–30%, **4**: 31%–45%, **5**: 46%–100%). The sum of the first 3 scores (inflammation severity, inflammation extent, and crypt damage) was multiplied by the score of the percentage involvement.

2.7.2. Colitis induced by TNBS

Specimens of proximal colon (2.5 cm) were stained with haematoxylin and eosin. Histological scoring of tissues was performed as previously described.[30] The histological damage was categorised into 5 distinct groups, each being defined by particular levels of the following indexes; grade **0**: no signs of inflammation, grade **1**: very low level of leukocytic infiltration, grade **2**: low level of leukocytic infiltration, grade **3**: high level of leukocytic infiltration, high vascular density, and thickening of the colonic wall, grade **4**: transmural infiltrations, loss of goblet cells, high vascular density, and thickening of the colonic wall. Grading was performed in a double-blinded fashion by a skilled pathologist.

2.8. Immunohistochemistry

Three sections (distal, middle, proximal) of the colon were collected and frozen in the O.C.T. compound. For nuclear p65 staining, frozen tissue sections were cut, air dried, PFA fixed, and treated with 1% H_2O_2 for 30 min. Sections were incubated overnight at 4 °C with anti-p65 (Santa Cruz Biotechnology Inc.), and treated by the EnVision plus method (K4000; Dako Ltd.) for 30 min at room temperature, followed by visualisation

with 3,3′-diaminobenzidine (DAB:K3466; Dako Ltd.) and counterstaining with haematoxylin. To assess cellular infiltration, sections were fixed with acetone or PFA and incubated overnight at 4 °C with anti-CD4 (Santa Cruz Biotechnology Inc.), CD8 (Chemicon International Inc., Temecula, CA, USA), or F4/80 (AbD Serotec Ltd., Oxford, United Kingdom) antibodies, pre-treated with 1% H_2O_2 for 30 min. Sections were then treated with PBS, normal mouse serum, and anti-rat IgG for 30 min at room temperature and stained by the avidin–biotin complex method (PK4000; Vector Lab, Inc., Burlingame, USA), followed by visualisation with DAB (Dako Ltd.), and counterstaining with haematoxylin.

2.9. Real-time reverse-transcription polymerase chain reaction

Half of each mouse colon without caecum was snap frozen in liquid nitrogen and stored at −70 °C. Total RNA was extracted using Trizol (Invitrogen Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of 1 μg of total mRNA was performed at 37 °C using the Omniscript RT Kit (Qiagen K.K., Tokyo, Japan) with Oligo (dT) 20 primer (Toyobo Co Ltd., Osaka, Japan) and Protector RNase inhibitor (Roche Diagnostics K.K., Sapporo, Japan). Real-time PCR was performed on QuantiTect SYBR Green PCR Kit (Qiagen K.K.) with the LightCycler Carousel-Based System (Roche Diagnostics K.K.). PCR gene amplifications were performed using the primers listed in Table 1. Reactions were processed through 40 cycles at 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. Specificity of the resulting PCR products was confirmed by melting curves after each run, and data were analysed with Roche LightCycler data analysis software from absolute plasmid DNA standards. Levels of mRNA for each sample were normalised to GAPDH and quantified relative to untreated mice.

2.10. Statistical analysis

All data were expressed as means ± SEM, except for those of $p65^+$ cell counts, TNF-α, and IL-6 concentrations that were expressed as means ± SD. Multiple group analysis of DAI and colonic oedema in DSS colitis was performed using a one-way analysis of variance with a post-hoc Tukey's test. Comparison of 2 groups in all other data was analysed by Student's *t*-test. Differences were considered statistically significant if the *P* value was less than 0.05.

3. Results

3.1. DHMEQ suppresses pro-inflammatory cytokine secretion induced by LPS by blocking the nuclear translocation of NF-κB

Initially, to define the testing dose range of DHMEQ for *in vitro* use, a direct toxicity of the agent was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DHMEQ did not exert a toxicity on the intestinal epithelial cell line, HT-29 cells when supplemented at a dose ranging from 10 to 30 μg/mL (data not shown). To confirm the inhibitory effect of DHMEQ on the nuclear translocation of NF-κB, we treated RAW264.7 cells with DHMEQ at 10 μg/mL and examined the localisation of p65 after LPS stimulation. As shown in Fig. 1A and B, DHMEQ significantly inhibited the nuclear translocation of NF-κB. In RAW264.7 cells, LPS stimulation markedly increased IL-6 and TNF-α production in the culture supernatant, whereas the levels of these cytokines were reduced by DHMEQ in a dose-dependent manner (Fig. 1C and D). Likewise, DHMEQ inhibited IL-8 production in HT-29 cells (Fig. 1E).

3.2. DHMEQ ameliorates colitis induced by DSS

To determine the therapeutic potential of DHMEQ *in vivo*, we used a murine model of colitis induced by DSS. Colitis was induced by adding 3% DSS solution for 5 days followed by regular water for 5 days with DHMEQ or control vehicle. DHMEQ treatment at a dose up to 40 mg/kg/day did not exert a considerable effect, whereas, twice daily treatment at a dose of 20 mg/kg significantly reduced the severity of

Table 1 Primers used for PCR.

Gene name	Accession number		Sequence (5′→3′)	Product size (bp)
TNF-α	NM-013693	Forward	ACCCTCACACTCAGATCATC	188
		Reverse	GAGTAGACAAGGTACAACCC	
IL-1β	NM-008361	Forward	AGCTCATATGGGTCCGACAG	174
		Reverse	GGATGAGGACATGAGCACCT	
IL-6	NM-031168	Forward	CAAAGCCAGAGTCCTTCAGAG	143
		Reverse	GCCACTCCTTCTGTGACTCC	
IL-12-p40	NM-008352	Forward	AGGAGACAGAGGAGGGGTGT	111
		Reverse	AATAGCGATCCTGAGCTTGC	
MCP-1	NM-011333	Forward	TCCCAATGAGTAGGCTGGAG	126
		Reverse	TCTGGACCCATTCCTTCTTG	
IFN-γ	NM-008337	Forward	ATCTGGAGGAACTGGCAAAA	111
		Reverse	GTTGCTGATGGCCTGATTGT	
IL-17	NM-010552	Forward	CCAGGGAGAGCTTCATCTGT	117
		Reverse	CTTGGCCTCAGTGTTTGGAC	
GAPDH	NM-008084	Forward	TACACTGAGGACCAGGTTGT	137
		Reverse	CTGTAGCCGTATTCATTGTC	

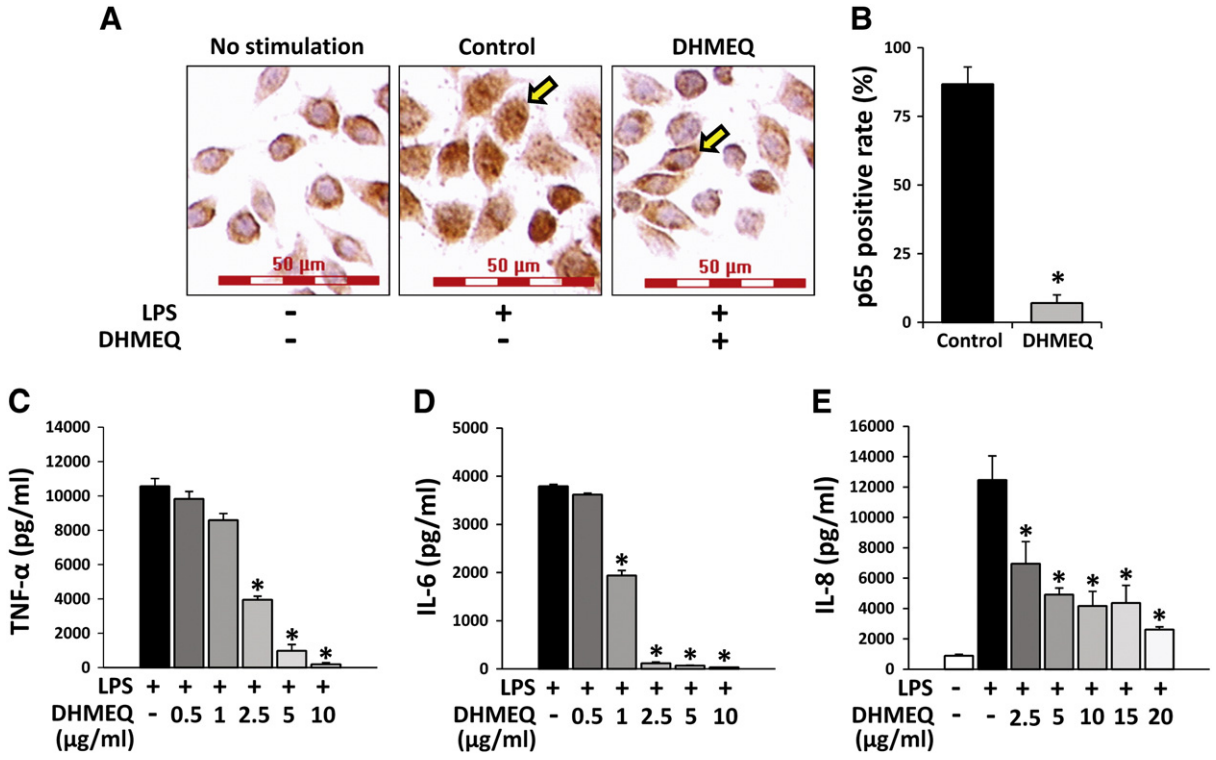

Figure 1 DHMEQ suppressed pro-inflammatory cytokine production induced by LPS by blocking the nuclear translocation of NF-κB. Localisation of NF-κB induced by LPS was studied in RAW264.7 cells. (A) Inhibition of p65 nuclear translocation in DHMEQ treatment is evident. (B) Nuclear cells positive for p65 were counted in different areas. HT-29 cells and RAW264.7 cells were incubated with the indicated concentrations of DHMEQ and LPS, respectively. Supernatants were harvested after 6 or 24 h and secreted cytokines were measured by ELISA. TNF-α (C) and IL-6 (D) production from RAW264.7, and IL-8 (E) production from HT-29 are evident. Data shown are means ± SD (n = 5). The asterisk indicates a statistically significant difference (*: $p<0.05$).

colitis (Fig. 2A). On day 5, control animals (given vehicle) presented a bloody, and loose stool, whilst mice treated with DHMEQ showed only occult bleeding and soft stool. The DAI score and colonic oedema, as assessed on day 10, were ameliorated by twice daily treatment with DHMEQ compared with controls (Fig. 2A and B). In contrast, 5-ASA treatment did not efficiently prevent progression of colitis induced by DSS (Fig. 2A and B).

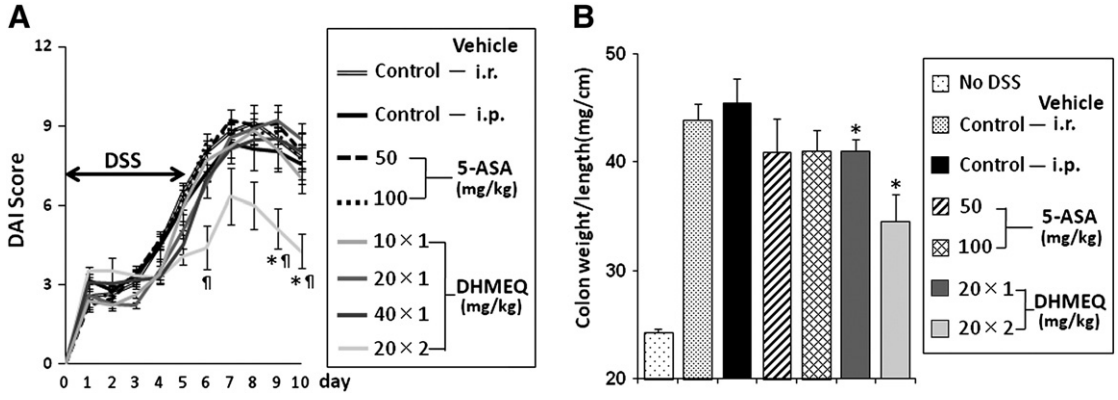

Figure 2 Effect of DHMEQ on colitis induced by DSS. C57BL/6 mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (10, 20 or 40 mg/kg/day) or vehicle was injected intraperitoneally once or twice daily. Comparison to 5-ASA, once daily treatment of 5-ASA (50 or 100 mg/kg) or vehicle was injected intrarectally. The effect on DAI (A) and oedema of the inflamed colon on day 10 (B) are evident. Data shown are representative of 3 independent experiments; means ± SE derived from 6 mice per group. The marks indicate a statistically significant difference (*: $p<0.05$ compared to control vehicle, ¶: $p<0.05$ compared to others).

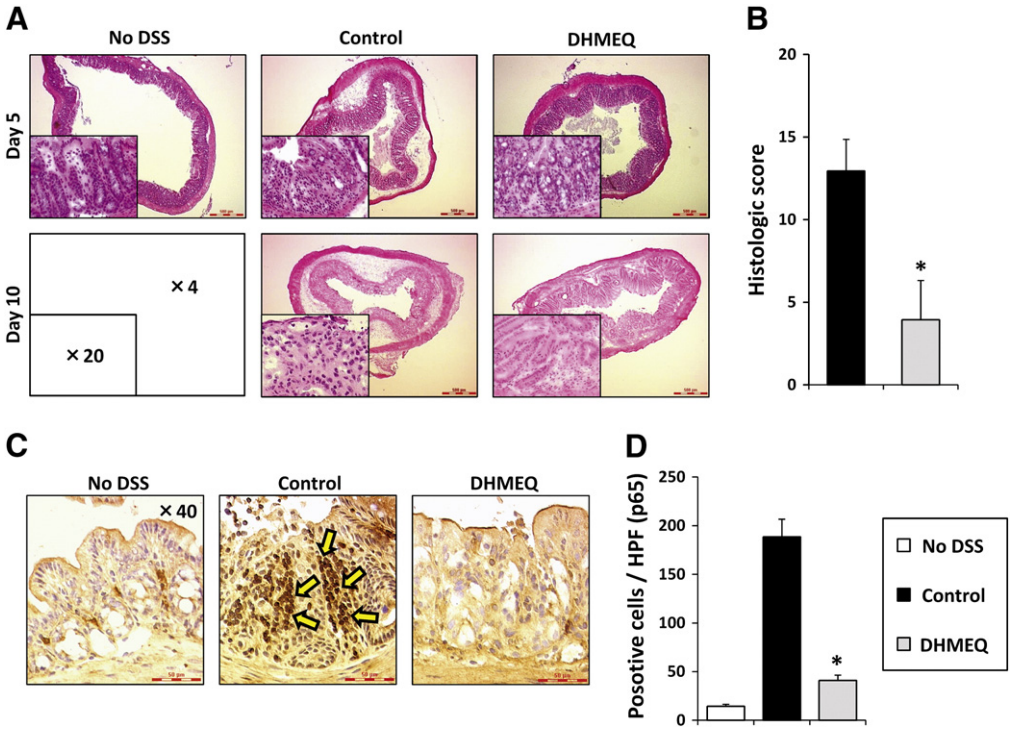

Figure 3 Effect of DHMEQ treatment on colonic tissue damage and NF-κB activity after induction of colitis induced by DSS. Mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. (A) Colonic specimens on days 5 and 10 that were treated twice daily with DHMEQ (20 mg/kg) or vehicle were stained with haematoxylin and eosin. (B) The improvement in histological scores of the inflamed colon with DHMEQ treatment is evident. (C) To analyse NF-κB activity, nuclear translocation of NF-κB in colonic tissues was determined by immunostaining for p65. (D) Cells positive for nuclear p65 were counted in different areas of the colon on day 10 in mice treated with DHMEQ, controls treated with vehicle, and untreated mice. Data shown are representative of 3 independent experiments; means ± SE, 6 mice per group. The asterisk indicates a statistically significant difference (*: $p<0.05$).

3.3. DHMEQ inhibits NF-κB activity of infiltrating cells after induction of colitis mediated by DSS

The severity of colonic inflammation and ulceration was further evaluated by histopathological examination. The colons obtained from controls showed marked infiltration of inflammatory cells, loss of crypts, reduction of goblet cells, focal ulcerations, extensive destruction of mucosal layer, and submucosal oedema. In contrast, the colons of the mice treated with DHMEQ showed only mild infiltration of inflammatory cells to the mucosa, minimal loss of crypts, and reduction of goblet cells as compared to the controls (Fig. 3A). The histopathological score assessed on day 10 revealed that the degree of colitis was significantly lower in the mice treated with DHMEQ than that of controls (Fig. 3B). To further analyse NF-κB activity in inflamed colonic tissues, p65 immunostaining was performed on day 10. In the controls, nuclear p65 was positive in the inflammatory cells and epithelial cells in crypt basements. In contrast, nuclear p65 expression of inflammatory cells was significantly reduced by DHMEQ treatment, and only the cytoplasm of epithelial cells became p65 positive (Fig. 3C and D).

3.4. DHMEQ prevents colonic leukocyte infiltration

In colitis induced by DSS, it is known that macrophages and lymphocytes infiltrate into the colonic mucosa.[31,32] We thus examined cell types of infiltrating cells by immunohistochemistry. On day 5, in the control colonic tissues, infiltration into the mucosa and submucosa was mainly by $F4/80^+$ macrophages and $CD4^+$ T lymphocytes and only a few $CD8^+$ T cells (Fig. 4A). These cellular infiltrates became more intense on day 10. Compared with controls, DHMEQ significantly suppressed infiltration of these cells. The amounts of F4/80 and $CD4^+$ cells were significantly lower in mice treated with DHMEQ relative to control vehicle (Fig. 4B). In addition, there was a tendency towards much less infiltration of $CD8^+$ cells in the colons of mice treated with DHMEQ compared with controls.

3.5. DHMEQ suppresses pro-inflammatory cytokine mRNA expressions after DSS treatment

To examine whether the protection from colitis induced by DSS in mice treated with DHMEQ was associated with a decrease in the production of inflammatory molecules, RNA was extracted from colonic specimens of vehicle and mice treated with DHMEQ, and analysed for the content of various inflammatory mediators. At the end of DSS exposure on day 5, IL-1β, IL-6, TNF-α, MCP-1, IL-12p40, and IL-17 mRNA levels were significantly increased in the colons obtained from the control mice (Fig. 5). On day 10, IL-1β, IL-6, and IL-17A transcripts were further up-regulated, MCP-1 did not change, and TNF-α and IL-12p40 mRNA levels decreased. The IFN-γ transcript increased not on day 5 but on day 10. In contrast,

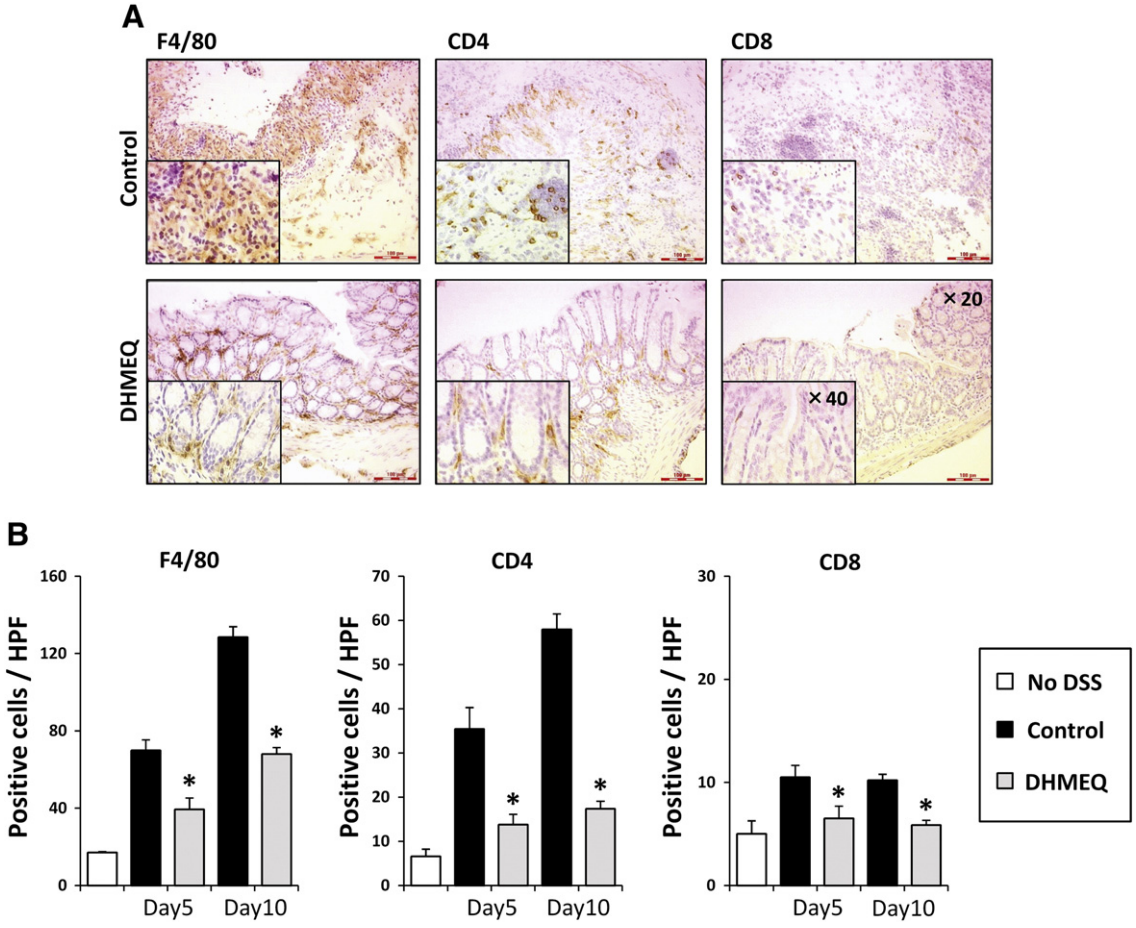

Figure 4 Protection of colitis induced by DSS in mice treated with DHMEQ is associated with a significant decrease in colonic infiltration of leukocytes. Mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (20 mg/kg) or vehicle was injected intraperitoneally twice daily. (A) For assessment of colonic cellular infiltration, frozen sections of colonic tissues from mice treated with DHMEQ and control mice treated with vehicle were stained with anti-CD4, anti-CD8, or anti-F4/80 antibodies. (B) Cells stained for anti-CD4, anti-CD8, or anti-F4/80 antibodies were counted in different areas of the colon on day 5 and day 10 after receiving DSS in mice treated with DHMEQ, controls, and untreated mice. Data are shown as the means ± SE with 4 mice on day 5, or 6 mice on day 10 per group. The asterisk indicates a statistically significant difference (*: $p<0.05$).

DHMEQ treatment significantly reduced IL-1β, IL-6, TNF-α, IL-12p40, and IL-17A mRNA levels in the colons on day 5 as compared to those of vehicle control mice (Fig. 5). A significant suppression by DHMEQ treatment of IL-1β, IL-6, and IL-17, as well as MCP-1 and IFN-γ mRNA levels in the colon was also noted on day 10 (Fig. 5).

3.6. Treatment with DHMEQ ameliorates colitis induced by TNBS

Finally, we examined the effect of DHMEQ on colitis induced by TNBS. Treatment with DHMEQ resulted in striking protection from colitis as assessed by body-weight change, colonic oedema (weight/length), and both macroscopic and histological damages of the colon (Fig. 6). Control mice exhibited progressive body-weight loss, a characteristic sign of severe intestinal inflammation after TNBS administration, whereas mice treated with DHMEQ showed significantly less body-weight loss (Fig. 6A). DHMEQ did not ameliorate colonic oedema (Fig. 6B). Macroscopic analysis of the colon, examined on day 4, showed marked bowel wall thickening, ulceration, and inflammation in the controls. DHMEQ administration significantly improved these damages of the colon as assessed by the macroscopic score (Fig. 6C). The severity of colonic inflammation and ulceration was evaluated further by histological examinations. On day 4, transmural inflammation characterised by infiltration of inflammatory cells, predominantly neutrophils and lymphocytes, was associated with ulcerations and loss of globule cells (Fig. 6D). DHMEQ administration improved these macroscopic injuries, and restored the histological appearance of the mucosa and submucosa (Fig. 6E).

4. Discussion

In this study, we examined the anti-inflammatory property of DHMEQ in intestinal epithelial cells (IECs) and macrophages *in vitro*. Regulation of mucosal immune responses to luminal antigens is known to involve IECs.[33] They function as antigen-presenting cells to different subsets of T cells and substantially contribute to the inflammatory processes in

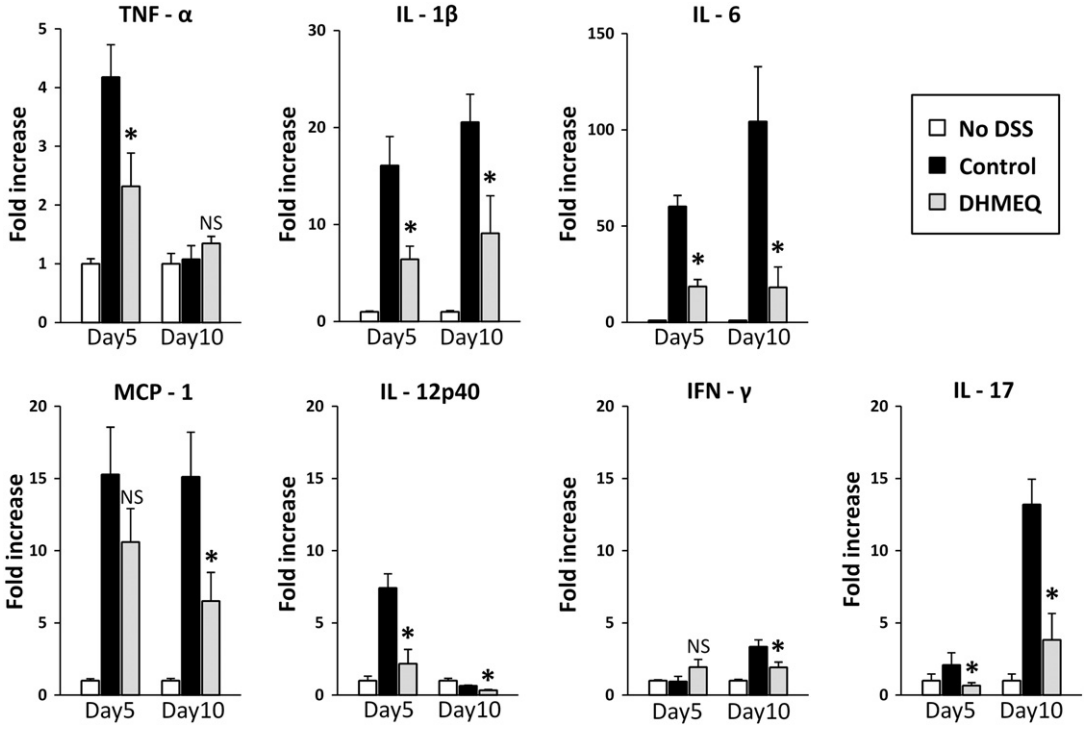

Figure 5 Protection of colitis induced by DSS in mice treated with DHMEQ is associated with a significant decrease in mRNA expression of pro-inflammatory cytokine. C57BL/6 mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (20 mg/kg) or vehicle was injected intraperitoneally twice daily. DHMEQ treatment (20 mg/kg), vehicle controls and no DSS mice were killed at day 5 and day 10. Colonic samples were analysed for content of the indicated molecules (TNF-α, IL-1β, IL-6, MCP-1, IL-12p40, IFN-γ, and IL-17) by real-time PCR. mRNA levels for each sample were normalised to GAPDH, then quantified relative to untreated mice. Data is shown as the means ± SE with 4 mice per group on day 5, or 6 mice per group on day 10. The asterisk indicates a statistically significant difference (*: $p<0.05$).

IBD by stimulating effector T cells and the release of IL-1, IL-6, IL-8, and TNF-α.[34,35] Of these cytokines, IL-8 is involved in the chemotaxis of neutrophils and T-cells, and it has been demonstrated that IECs overproduce IL-8 upon LPS or TNF-α stimulation *in vitro*.[36,37] In the present study, we demonstrated that DHMEQ suppressed IL-8 production induced by LPS by the HT-29 cell line.

In IBD, the intestinal lamina propria is associated with infiltration of mononuclear cells such as macrophages and lymphocytes. Macrophages, a major population of tissue-resident mononuclear phagocytes, play a key role in recognition and elimination of bacteria. Macrophages produce pro-inflammatory cytokines at the site of inflammation in response to activated Th1 cells, and induce tissue damage by enhancing mononuclear cell infiltration to the tissue.[2] In addition, dysfunction of macrophages is correlated with IBD incidence.[38] In fact, it has been reported that NOD2 mutant macrophages, which underlie the occurrence of intestinal inflammatory disease in a substantial subgroup of patients with CD, produce large amounts of IL-12 in response to stimulation with microbial components.[39,40] We have shown in the present study that DHMEQ inhibits the nuclear translocation of p65, and suppresses IL-6 and TNF-α production induced by LPS in the murine macrophage cell line RAW264.7.

To assess the *in vivo* efficacy of DHMEQ treatment on IBD, we utilised well-established models of murine colitis induced by DSS or TNBS. In the DSS colitis model, we examined the efficacy of DHMEQ at various doses. Based on our previous studies[23,24], DHMEQ was administered to animals *via* the i.p. route. Twice daily treatment with DHMEQ at 20 mg/kg/day markedly ameliorated disease activity related to colitis as assessed by DAI scores, colonic oedema, and histological damage. We also examined the efficacy of DHMEQ by intra-rectal administration in a preliminary study; however, this treatment did not show a clear effect on colitis (data not shown). In the current study, we further demonstrated the effect of DHMEQ in TNBS colitis model, which is a more aggressive model than the DSS colitis model,[41] as assessed by body-weight change, macroscopic damage, and histological damage. In addition, we compared the efficacy of DHMEQ on colitis with that of 5-ASA in order to evaluate the potential clinical utility. Previous publications reported that glucocorticoid steroids and 5-ASA are effective in preventing colitis in IBD animal models.[42,43] In comparison with 5-ASA, we found that DHMEQ was more potent than 5-ASA when examined in a DSS colitis model. This corroborates the previous finding that the NF-κB decoy, but not budesonide, ameliorated colitis in a DSS colitis model.[44] Besides the therapeutic potential of DHMEQ, no apparent side effects were noted in mice treated with DHMEQ under the current protocol. These data support our *in vitro* findings and confirm our hypothesis that inhibition of NF-κB by DHMEQ is an effective strategy for controlling colitis of IBD.

To understand the underlying mechanisms of protection mediated by DHMEQ from colitis, we investigated the colon

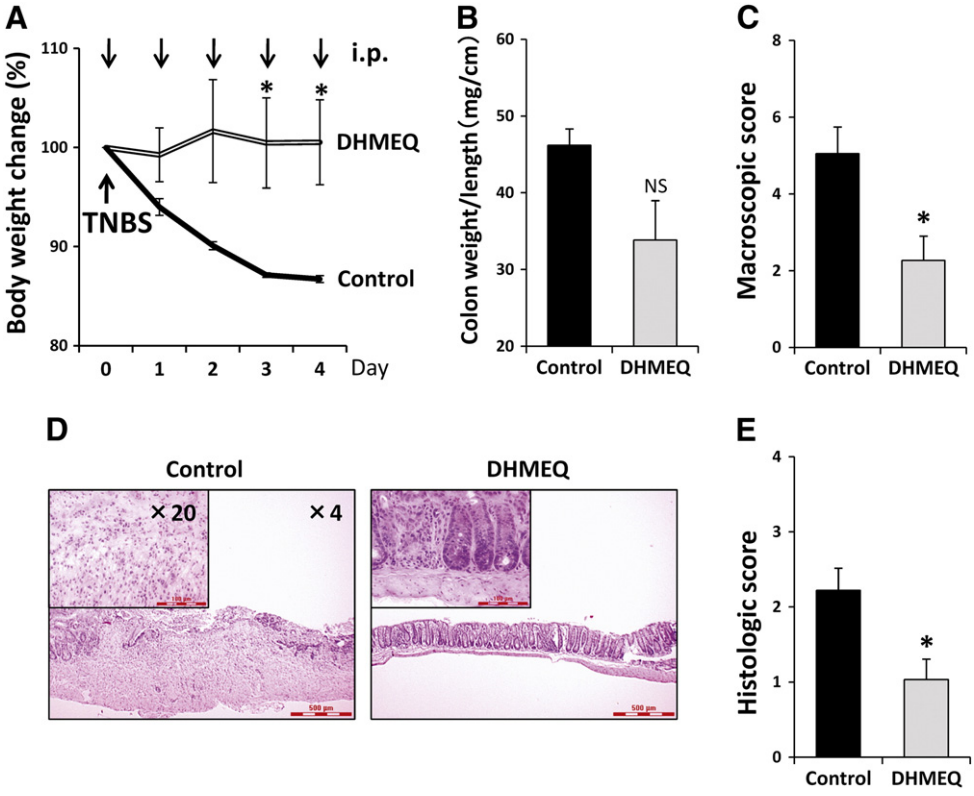

Figure 6 Treatment of colitis induced by TNBS by DHMEQ administration. Colitis was induced by rectal administration of TNBS on day 0. BALB/c mice were treated with DHMEQ or vehicle intraperitoneally twice daily. The mice were euthanised and the colons collected on day 4. (A) Body-weight curves, (B) oedema of the inflamed colons, (C) macroscopic scores of colonic tissue, (D) typical histological appearance of the colon, (F) histological scores of tissue specimens, are presented. The data shown are representative of 3 independent experiments; means ± SE for 6 mice per group. The asterisk indicates a statistically significant difference (*: $p<0.05$) compared with controls.

specimens of mice administered DSS by immunohistochemistry and real-time PCR analysis. Shortly after DSS administration, infiltration into the colon was mainly by F4/80$^+$ macrophages and CD4$^+$ T cells. This was consistent with previous reports.[31,32,45] Immunohistochemistry further revealed that many of these infiltrating cells were nuclear p65$^+$, whilst treatment with DHMEQ suppressed both cellular infiltrates and p65 expression. Corresponding to the findings by Ariga et al.[22] and our *in vitro* study, DHMEQ significantly inhibited pro-inflammatory cytokine production *in vivo* following DSS administration, as assessed by colonic mRNA expression of TNF-α, IL-1β, IL-6, and MCP-1. In conjunction with inflammation mediated by macrophages, dysregulation of T-cell response is also an important pathophysiological change in the development of IBD. In humans, cytokines associated with Th1 cells, such as IL-12 and IFN-γ, are increased in active CD patients.[6,7] Furthermore, recent study revealed that IL-17 expression in the mucosa and serum is up-regulated in active IBD patients.[8] Also, the importance of Th1/Th17 CD4$^+$ T cell response is known in chronic colitis and the delayed-phase of acute colitis induced by DSS in mice.[45–47] In the present study, DHMEQ not only suppressed infiltration of CD4$^+$ T cells in colonic mucosa, but also significantly decreased mRNA expression of molecules associated with IFN-γ, IL-12p40, and Th1/Th17 such as IL-6, IL-17, and MCP-1. These data corroborated the findings of Iwata et al., who have shown that amelioration of experimental autoimmune uveoretinitis by DHMEQ treatment was associated with inhibition of responses mediated by Th1/Th17.[26] Taken together, these data suggest that DHMEQ ameliorates colitis induced by DSS *via* suppression of macrophage and T-cell responses by blocking NF-κB activity.

Previous studies have shown that NF-κB inhibitors such as NF-κB decoy and NEMO-binding domain peptide, suppress pro-inflammatory cytokine production, and ameliorate experimental colitis.[31,44,48] In line with these reports, our present results with DHMEQ in colitis induced by both DSS and TNBS, indicate that inhibition of NF-κB activation would be a promising strategy for preventing gut inflammation. Although NF-κB decoy is anticipated for clinical applications in the area of dermatological external medicine, its molecular weight is high. In contrast, DHMEQ is a compound with low molecular weight,[20] and has shown specificity for NF-κB inhibition.[21] These characteristics of DHMEQ seem to have advantages for potential clinical applications.

In conclusion, our data provide evidence that a novel NF-κB inhibitor, DHMEQ, strongly ameliorates development of colitis. Although further studies confirming the safety and optimising treatment protocol of DHMEQ are necessary before clinical application, DHMEQ should be an attractive agent for the treatment of IBDs.

Conflict of interest statement

The authors have no conflict of interest to declare.

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