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 preincubation, 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	

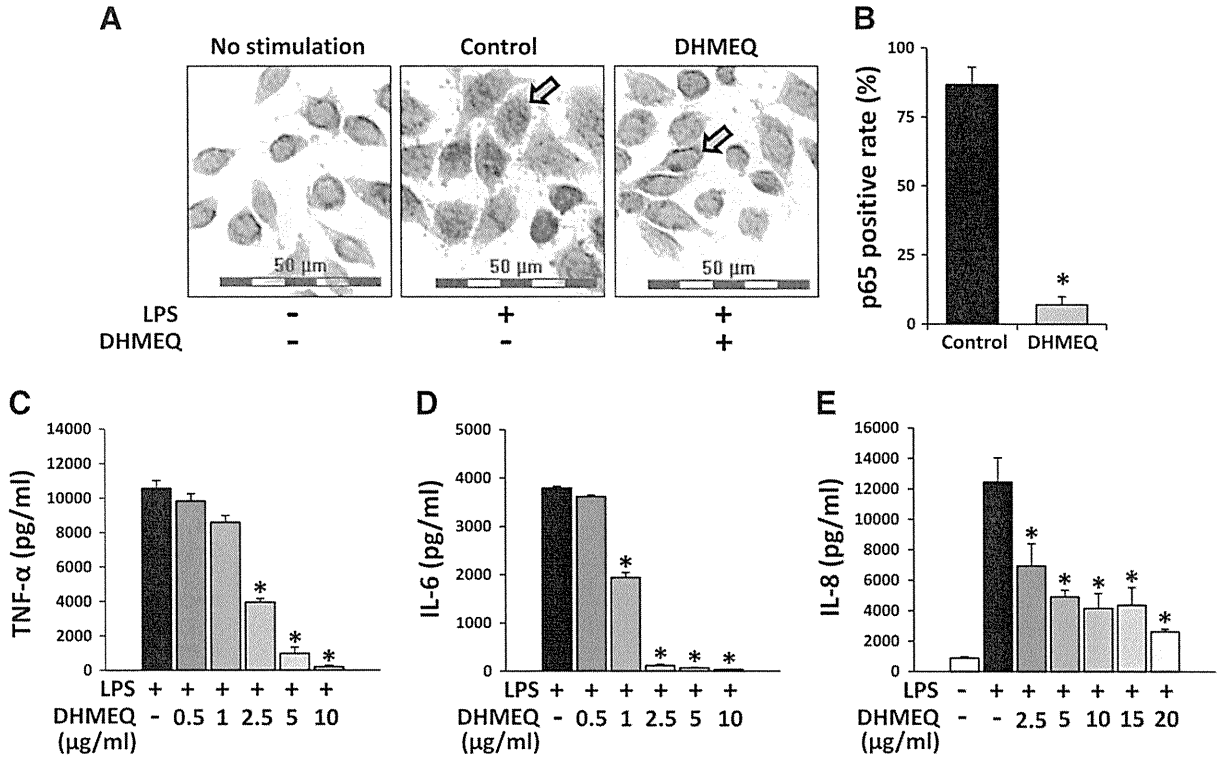

Figure 1 DHMEQ suppressed pro-inflammatory cytokine production induced by LPS by blocking the nuclear translocation of NF-κB. Localisation of NF-κB induced by LPS was studied in RAW264.7 cells. (A) Inhibition of p65 nuclear translocation in DHMEQ treatment is evident. (B) Nuclear cells positive for p65 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).

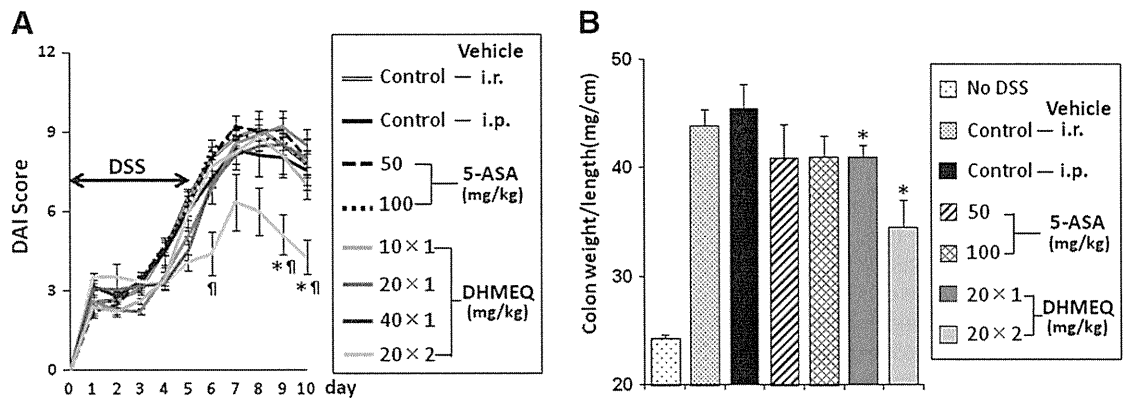

Figure 2 Effect of DHMEQ on colitis induced by DSS. C57BL/6 mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (10, 20 or 40 mg/kg/day) or vehicle was injected intraperitoneally once or twice daily. Comparison to 5-ASA, once daily treatment of 5-ASA (50 or 100 mg/kg) 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).

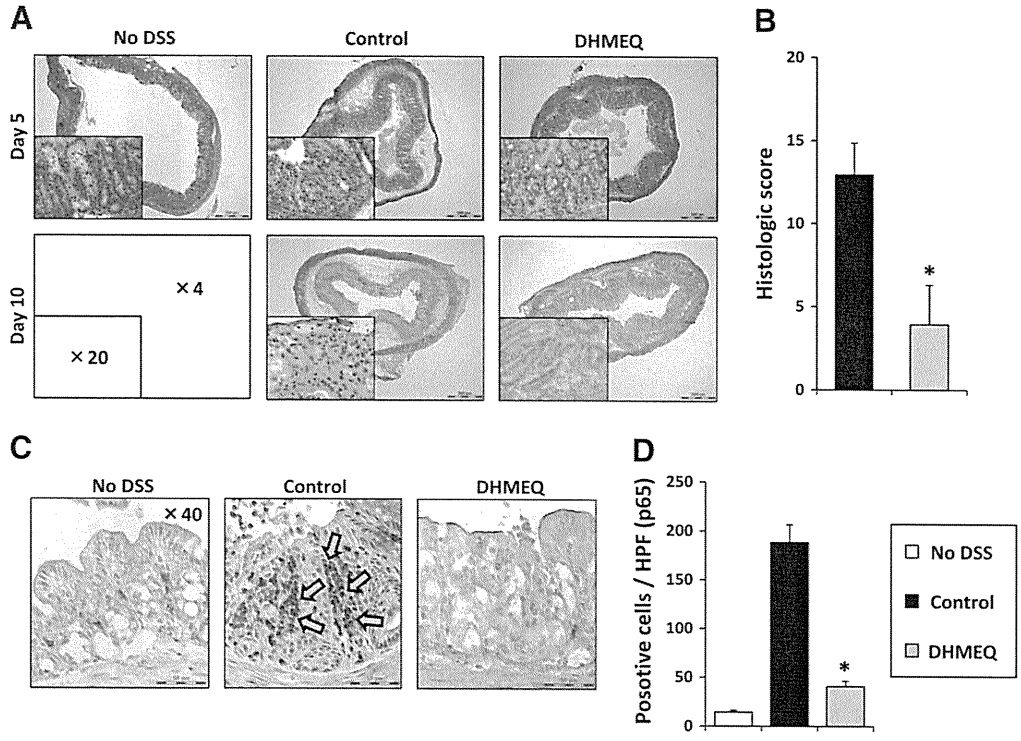

Figure 3 Effect of DHMEQ treatment on colonic tissue damage and NF-κB activity after induction of colitis induced by DSS. Mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. (A) Colonic specimens on days 5 and 10 that were treated twice daily with DHMEQ (20 mg/kg) or vehicle 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,

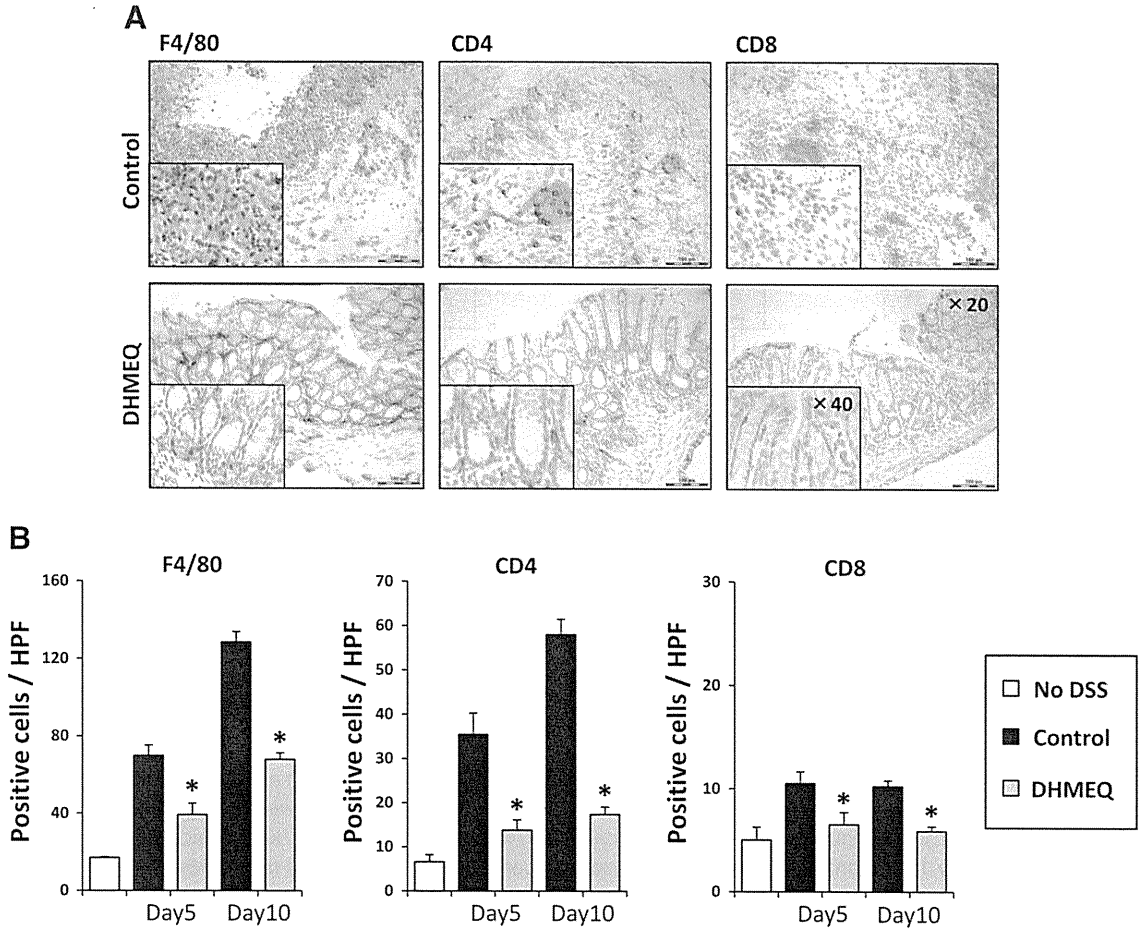

Figure 4 Protection of colitis induced by DSS in mice treated with DHMEQ is associated with a significant decrease in colonic infiltration of leukocytes. Mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (20 mg/kg) or vehicle was injected intraperitoneally twice 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

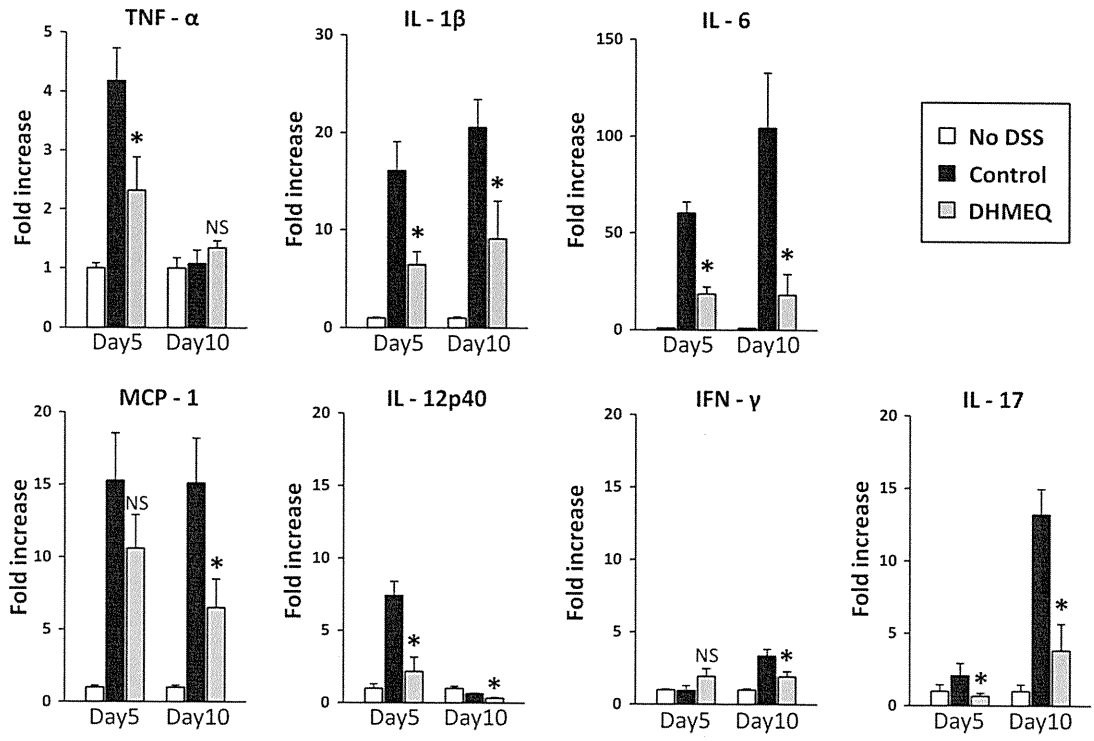

Figure 5 Protection of colitis induced by DSS in mice treated with DHMEQ is associated with a significant decrease in mRNA expression of pro-inflammatory cytokine. C57BL/6 mice were given 3% DSS solution for 5 days, followed by regular water for 5 days. DHMEQ (20 mg/kg) or vehicle was injected intraperitoneally 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

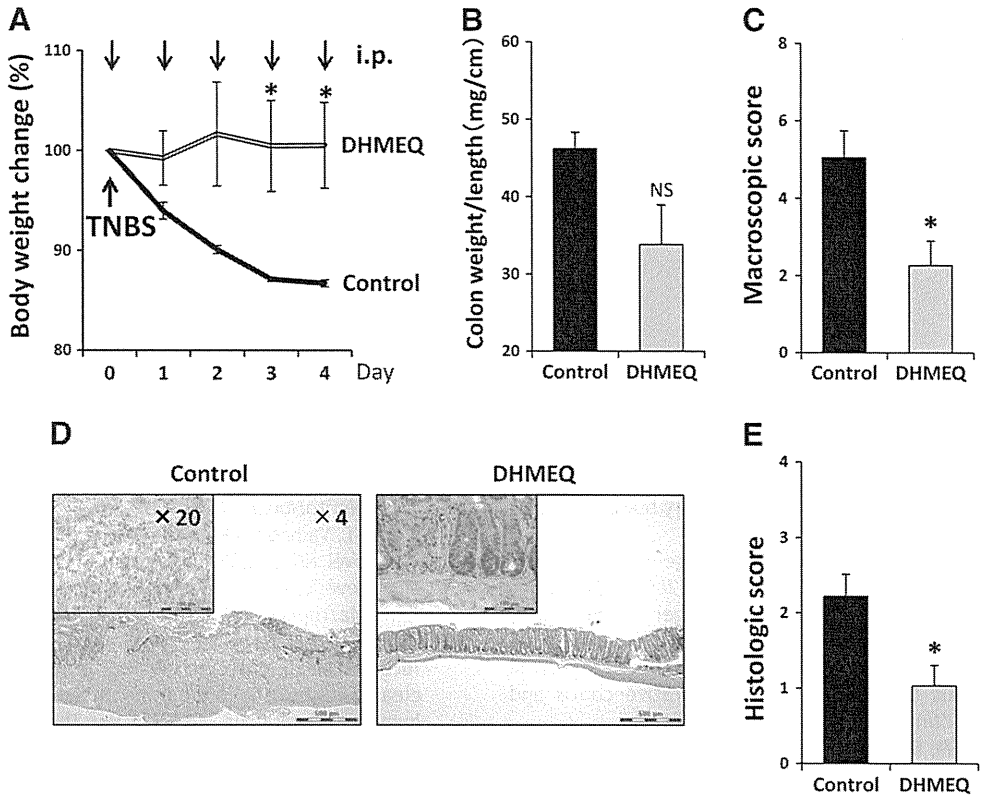

Figure 6 Treatment of colitis induced by TNBS by DHMEQ administration. Colitis was induced by rectal administration of TNBS on day 0. BALB/c mice were treated with DHMEQ or vehicle intraperitoneally twice daily. The mice were euthanised and the colons collected on day 4. (A) Body-weight curves, (B) oedema 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.

Acknowledgements

This study was in part supported by funding from the Promotion of Fundamental Studies in Health Sciences from the National Institute of Biomedical Innovation (NIBIO), and Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS), Japan.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i4.340

World J Gastroenterol 2012 January 28; 18(4): 340-348
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

α-fetoprotein, vascular endothelial growth factor receptor-1 and early recurrence of hepatoma

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Supported by Japan Society for the Promotion of Science KAKENHI 21390369 [Grant-in-Aid for Science Research (B)]
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Received: July 1, 2011 Revised: August 25, 2011
Accepted: August 31, 2011
Published online: January 28, 2012

Abstract

AIM: To investigate whether α-fetoprotein (AFP) and vascular endothelial growth factor receptor (VEGFR)-1 correlate with early recurrence of hepatoma/hepatocellular carcinoma (HCC).

METHODS: From 2000 to 2005, 114 consecutive patients with HCC underwent primary curative hepatectomy. The mean age was 60.7 (8.7) years and 94 patients were male. The median follow-up period was 71.2 mo (range: 43-100 mo). Immediately prior to commencing laparotomy, 5 mL bone marrow was aspirated from the sternum and collected in citrate-coated test tubes. The initial 2 mL of bone marrow aspirate was discarded in each case. AFP mRNA and VEGFR-1 mRNA in the bone marrow and peripheral blood (BM- and PH-AFP mRNA and BM- and PH-VEGFR-1 mRNA, respectively) were measured by real-time quantitative reverse transcription polymerase chain reaction. As normal controls, VEGFR-1 mRNA in the bone marrow and peripheral blood was also measured in 11 living liver donors. These data were evaluated for any correlation with early recurrence, comparing clinical and pathological outcomes.

RESULTS: The cut-off value of the BM-AFP mRNA and PH-AFP mRNA level in patients with HCC was set at 1.92×10^{-7} and zero, respectively, based on data from the controls. A total of 34 (29.8%) and six (5.4%) patients were positive for BM-AFP mRNA and PH-AFP mRNA, respectively. The BM-VEGFR-1 mRNA levels in all HCC patients were higher than those in the normal controls, and this was the case also for PH-VEGFR-1mRNA. The 25-percentile values for the BM- and PH-VEGFR-1 mRNA in HCC patients were used as the cut-off values for assigning the patients into two groups based on these transcript levels. The High group for BM- VEGFR-1 mRNA contained 81 (71.1%) HCC cases and the Low group was assigned 33 (28.9%) patients. These numbers for PH-VEGFR-1mRNA were 78 (75.0%) and 26 (25.0%), respectively. HCC recurred in 80 patients; in the remnant liver in 48 cases, in the remnant liver and remote tissue in 20, and in the remote tissue alone in 12. BM-AFP mRNA-positive cases showed a significantly higher rate of early recurrence (within 1 year of surgical treatment) compared with BM-AFP mRNA-negative patients ($P = 0.0091$). Patients were classified into four groups according to the level/status of their BM-VEGFR-1 and BM-AFP mRNA as follows: group A ($n = 23$), BM-VEGFR-1/BM-AFP mRNA = low/negative; group B ($n = 57$) high/negative; group C ($n = 10$) low/positive; group D ($n = 24$), high/positive. This classification was found to correlate with a recurrence of this

disease within 1 year (P = 0.0228). The disease-free survival curve of group A was significantly better than that of groups B, C or D (P = 0.0437, P = 0.0325, P = 0.0225). No other classification (i.e., PH-VEGF-R1/BM-AFP, BM-VEGF-R1/PH-AFP, and PH-VEGF-R1/PH-AFP mRNA) showed such a correlation.

CONCLUSION: The evaluation of BM-AFP and BM-VEGFR-1 mRNA in patients with HCC may be a valuable predictor of disease recurrence following curative resection.

Key words: α-fetoprotein; Vascular endothelial growth factor receptor-1; mRNA; Early recurrence; Hepatocellular carcinoma

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Kamiyama T, Takahashi M, Nakanishi K, Yokoo H, Kamachi H, Kobayashi N, Ozaki M, Todo S. α-fetoprotein, vascular endothelial growth factor receptor-1 and early recurrence of hepatoma. *World J Gastroenterol* 2012; 18(4): 340-348 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i4/340.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i4.340

INTRODUCTION

Various factors are thought to contribute to hepatocellular carcinoma (HCC) recurrence, which commonly results in death, including multicentric carcinogenesis in the remnant liver due to an underlying hepatitis-B-virus- or hepatitis-C-virus-induced liver cirrhosis[1], hematogenic spread, or micrometastasis of HCC cells prior to surgery or during hepatectomy by manipulation of the liver[2]. Recently, using various molecular biological markers, the detection of malignant cells in the systemic circulation and bone marrow has become possible and the presence of these cells has been found to correlate with the clinical outcome[3-8]. We have also reported from our laboratory that the detection of HCC cells in the bone marrow by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of α-fetoprotein (AFP) mRNA before curative hepatectomy correlates with HCC recurrence and patient survival outcomes. Although early recurrence within 1 year of curative resection for HCC is one of the most important factors affecting the prognosis and clinical outcomes[9,10], the relationship between early recurrence and disseminated cancer cells has not yet been evaluated.

It has been recently hypothesized that metastasis is dependent on both isolated cancer cells and the host response. Kaplan *et al*[11] have reported that bone-marrow-derived hematopoietic progenitor cells that express vascular endothelial growth factor receptor (VEGFR)-1 migrate to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of tumor cells both *in vitro* and *in vivo*. Moreover, it has been reported that the simultaneous presence of isolated tumor cells and VEGFR-1 expression at pre-metastatic sites is clinically significant for disease progression in gastric cancer[12]. With regard to HCC however, there has been no study to date of the association between isolated cancer cells and the expression of VEGFR-1.

In our present study, we examined whether the expression of AFP mRNA and VEGFR-1 in the bone marrow and peripheral blood, detected by sensitive real-time quantitative RT-PCR, could predict early recurrence in consecutive HCC patients who had undergone a curative hepatic resection.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Board of the Hokkaido University, School of Advanced Medicine. Informed consent was obtained from each patient in accordance with the Ethics Committee Guidelines at our institution.

From July 2000 to June 2005, 114 consecutive patients underwent primary curative hepatectomy at the First Department of Surgery, Hokkaido University Hospital. The mean age was 60.7 (8.7) years and 94 patients were male. The Child-Pugh staging was A in 110 patients and B in four. Patients were discharged from the hospital at an average of 17.5 (7.1) d after surgery. They were followed up at 3-mo intervals by computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography (US) and laboratory tests for AFP, lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and protein induced by vitamin K absence or antagonist-Ⅱ (PIVKA-Ⅱ). The median follow-up period was 71.2 mo (range: 43 mo-100 mo).

As normal controls, VEGFR-1 mRNA in the bone marrow and peripheral blood was also measured in 11 living liver donors. The cut-off value for AFP mRNA/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the bone marrow and peripheral blood was set as described in our previous study[13].

Sample collections

Immediately prior to commencing the laparotomy, 5 mL bone marrow was aspirated from the sternum and collected in citrate-coated test tubes. The initial 2 mL of the bone marrow aspirate was discarded in each case.

RNA isolation and reverse transcription

Bone marrow samples were prepared for the measurement of total RNA using a Blood RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol with minor modifications. Briefly, 5 mL bone marrow cells were mixed with 25 mL Reagent buffer erythrocyte lysis (EL). They were then cooled on

ice for 15 min, centrifuged, and the cell pellets were collected. The pellets were suspended in 1.35 mL buffer and applied to the reagent columns, and then washed twice with reagent buffer containing ethanol. Total RNA was eluted with RNase-free water. These bone marrow RNA samples were stored at -80 ℃ until use. cDNA was generated from 1 μg total RNA using Moloney murine leukemia virus reverse transcriptase (SuperScript Ⅱ, Life Technologies, Carlsbad, CA, United States), plus 20 pmol/L each dNTP and 10 pmol/L oligo dT primers in a 20-μL final reaction volume at 42 ℃ for 1 h. This was followed by heating at 99 ℃ for 5 min.

Real-time quantitative RT-PCR

A LightCycler PCR and detection system (Roche Diagnostics, Mannheim, Germany) was used for amplification. Online quantification real-time RT-PCR was then performed in glass capillaries according to the manufacturer's protocol. The cDNA was amplified in a 20-μL PCR reaction mixture containing each dNTP (with dUTP instead of dTTP), 1 × PCR buffer, specific primers, and magnesium chloride.

For the detection of AFP, two adjacent oligonucleotide probes were used: the LightCycler Red 640 fluorophore, hAFP-LCR; (5'-CTTGCACACAAAAGCCCACTCCA-3') and a fluorophore labeled at the 3'-end with fluorescein, hAFP-FITC; (5'-TCGATCCCACTTTTCCAAGTT-3') (Nihon Gene Research Laboratories, Sendai, Japan). The sense and antisense primers (kindly supplied by Dr. Hiroaki Nagano at Osaka University) used for the amplification of AFP were as follows: 5'-TGCAGCCAAAGTGAAGAGGGAAGA-3') (hAFP-s) and 5'-CATAGCGAGCAGCCCAAAGAAGAA-3' (hAFP-As). The RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 62 ℃ for 15 s, and 72 ℃ for 15 s. The final cycle was followed by a 10-min extension step at 40 ℃.

For the detection of VEGFR-1, two adjacent oligonucleotide probes were used: hVEGFR-1-LCR; 5'-TTCCGTGTCCCCACTGCCAA-3' and hVEGFR-1-FITC; 5'-GGGAAGCTCACTGGCATGGC-3'. The sense and antisense primers for the amplification of VEGFR-1 were as follows: 5'-TCATGAATGTTTCCCTGCAA-3' (h VEGFR-1-S) and 5'-GGAGGTATGGTGCTTCCTGA-3' (h VEGFR-1-As). These primers were designed using sequences described in a previous report[14]. RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 60 ℃ for 10 s, and 72 ℃ for 16 s. The final cycle was followed by a 10-min extension step at 40 ℃.

For the detection of GAPDH as an internal control, two adjacent oligonucleotide probes were used: hGAPDH-LCR; 5'-TTCCGTGTCCCCACTGCCAA-3' and hGAPDH-FITC; 5'-GGGAAGCTCACTGGCATGGC-3'. The sense and antisense primers for the amplification of GAPDH were as follows: 5'-GCCTCCTGCACCACCAACTG-3' (hGAPDH-S) and 5'-CGACGCCTGCTTCACCACCTTCT-3' (hGAPDH-As). The RT-PCR amplification was carried out for one cycle of 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 10 s, 60 ℃ for 10 s and 72 ℃ for 16 s. The final cycle was followed by a 10-min extension step at 40 ℃.

Quantification analysis

Quantification data were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. In this analysis, the background fluorescence was removed by setting a noise band. The crossing point for the calculation of amplified PCR products was set by the intersection of the best-fit line through the log-linear lesion and the noise band. The standard curve was a plot of the "crossing point" versus the copy number of DNA fragments inserted into the cloning vector.

Statistical analysis

Cumulative survival and disease-free survival (DFS) rates were computed according to the Kaplan-Meier method and compared between groups using the Breslow-Gehan-Wilcoxon test. The Cox proportional hazards model was used for multivariate analysis. Statistical analyses using standard tests (χ^2, t test) were performed where appropriate. Significance was defined as $P < 0.05$. Statistical analyses were performed using StatView 5.0 Windows (SAS Institute Inc., Cary, NY, United States).

RESULTS

Analysis of AFP mRNA levels in bone marrow

The mean AFP mRNA/GAPDH ratio in the bone marrow (BM-AFP mRNA) of HCC patients, as determined by real-time quantitative RT-PCR, was 3469.27×10^{-7} (range: 0-$348\,526.19 \times 10^{-7}$). The cut-off value of the BM-AFP mRNA level was set at 1.92×10^{-7} (with reference to a previous report)[13]. The HCC patients were then divided into two groups according to this cut-off value. Accordingly, 80 patients (70.2%) were found to be negative for BM-AFP mRNA and 34 patients (29.8%), assigned to the "High" group, were positive for this transcript.

Expression of AFP mRNA in peripheral blood

No AFP mRNA was detectable in the peripheral blood of the control patients, therefore, the cut-off value for AFP mRNA/GAPDH in the peripheral blood (PH-AFP mRNA) was set at zero. Accordingly, six patients (5.4%) were found to be positive and 105 (94.6%) were negative for AFP mRNA. Due to some sampling loss, peripheral blood samples were unavailable for three patients.

Expression of VEGFR-1 mRNA in bone marrow

The mean VEGFR-1 mRNA/GAPDH in the bone marrow (BM-VEGFR-1 mRNA) of normal controls, again determined by real-time quantitative RT-PCR measurements, was 0.1497×10^{-3} (range: 0.0212×10^{-3} to 0.3213×10^{-3}). The mean BM-VEGFR-1 mRNA level in the HCC patients was 3.8474×10^{-3} (range: 0.3481×10^{-3} to

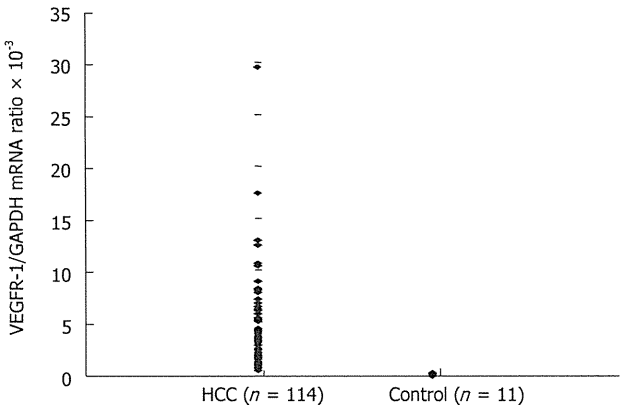

Figure 1 Expression of vascular endothelial growth factor receptor-1 mRNA in bone marrow detected by real-time quantitative reverse transcription polymerase chain reaction. The mean VEGFR-1/GAPDH mRNA ratio in the bone marrow (BM-VEGFR-1 mRNA) of normal controls was 0.1497 × 10^{-3} (range: 0.0212 × 10^{-3}-0.3213 × 10^{-3}). The mean BM-VEGFR-1 mRNA level in HCC patients was measured at 3.8474 × 10^{-3} (range: 0.3481 × 10^{-3}-29.5885 × 10^{-3}). HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptor; BM: Bone marrow; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

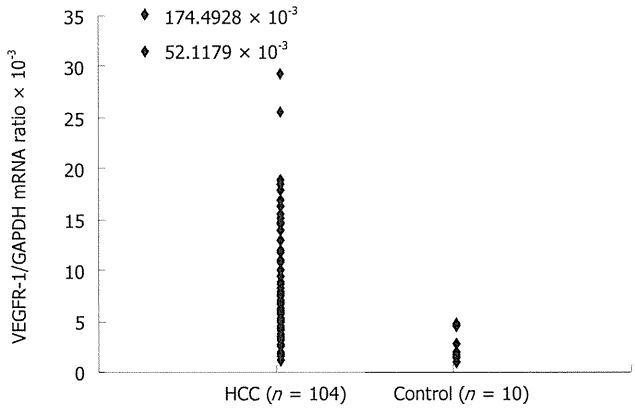

Figure 2 Expression of vascular endothelial growth factor receptor-1 mRNA in peripheral blood detected by real-time quantitative real-time quantitative reverse transcription polymerase chain reaction. The mean VEGFR-1/GAPDH mRNA ratio in the peripheral blood (PH-VEGFR-1 mRNA) of normal controls was 2.4944 × 10^{-3} (range: 1.0730 × 10^{-3}-4.6958 × 10^{-3}). The mean PH-VEGFR-1 mRNA level in HCC patients was 9.1285 × 10^{-3} (range: 1.2774 × 10^{-3}-174.4928 × 10^{-3}). HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptor; PH: Peripheral blood; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

29.5885 × 10^{-3}). The mean BM-VEGFR-1 mRNA level of all HCC patients was higher than that of the normal controls (Figure 1). The HCC patients were then divided into two groups ("High" and "Low") according to their BM-VEGFR-1 mRNA level; the cut-off value was 1.5664 × 10^{-3}, which was the 25th percentile value of the BM-VEGFR-1 mRNA levels in the HCC cohort. The number of patients in the High group was 81 (71.1%) and 33 (28.9%) were assigned to the Low group.

Expression of VEGFR-1 mRNA in peripheral blood

The mean VEGFR-1 mRNA/GAPDH ratio in the peripheral blood (PH-VEGFR-1 mRNA) of the normal controls was 2.4944 × 10^{-3} (range: 1.0730 × 10^{-3} to 4.6958 × 10^{-3}). The mean PH-VEGFR-1 mRNA level in the HCC patients was 9.1285 × 10^{-3} (range: 1.2774 × 10^{-3} to 174.4928 × 10^{-3}). The PH-VEGFR-1 mRNA level of almost all HCC patients was higher than that of the normal controls (Figure 2). The HCC patients were divided into high and low groups according to their PH-VEGFR-1 mRNA level. The cut-off value was 4.0238 × 10^{-3}, which was in the 25th percentile of the PH-VEGFR-1 mRNA level of HCC patients. The number of patients in the high group was 78 (75.0%) with 26 (25.0%) placed in the Low group. Peripheral blood samples were available for 104 patients only.

Clinical significance of the BM- and PH-VEGFR-1, and BM- and PH-AFP mRNA levels

The status of the BM-AFP mRNA levels was correlated with microscopically detectable portal invasion, whereas that of PH-AFP mRNA was found to correlate with the serum AFP and AFP-L3 levels, the number of tumors, microscopic portal invasion, and microscopic intrahepatic metastasis (Table 1). The number of tumors, serum albumin level, and a noncancerous liver were significantly correlated with the BM-VEGFR-1 mRNA level (Table 2).

Patient outcomes

Mortality: By the end of our study, 42 of the HCC patients under analysis had died; 35 from HCC, three from liver failure and four from another malignant disease. The 1-, 2- and 3-year patient survival rates for this cohort were determined to be 92.1%, 85.9% and 78.7%, respectively.

HCC recurrence: HCC recurred in 80 patients (70.2%); in the remnant liver in 48 cases (60%), in the remnant liver and remote tissue in 20 (25%), and in the remote tissue alone in 12 (15%). The 1-, 2- and 3-year DFS rates were 67.5%, 49.8% and 34.4%, respectively. We found a significant tendency for patients who were positive for BM-AFP mRNA to experience recurrence within 1 year of their surgery compared with patients who were negative for this transcript (Table 3).

HCC classification according to VEGFR-1 and AFP mRNA status

Patients were classified into four groups according to the level/status of their BM-VEGFR-1 and BM-AFP mRNA as follows: group A (n = 23), BM-VEGFR-1/BM-AFP mRNA = low/negative; group B (n = 57) high/negative; group C (n = 10) low/positive; group D (n = 24), high/positive. This classification was correlated with disease recurrence within or more than 1 year after surgery. Significantly, in the groups in which patients were negative for BM-AFP mRNA, only three patients (13.0%) experienced recurrence in group A, whereas 17 (29.0%) in group B experienced recurrence within 1 year of surgery (Table 3). Classification of the HCC cases in the current study cohort by their PH-VEGFR-1 and BM-AFP (P = 0.1024), BM-VEGFR-1 and PH-AFP (P = 0.2100), and

Table 1 Characteristics of hepatocellular carcinoma patients according to their bone marrow-α-fetoprotein and peripheral blood-α-fetoprotein mRNA profile

		BM-AFP mRNA		*P* value	PH-AFP mRNA		*P* value
		Positive (n = 34)	Negative (n = 80)		Positive (n = 6)	Negative (n = 105)	
Sex	Male	27	67	0.5774	5	86	0.9294
	Female	7	13		1	19	
Age (yr)	≤ 60	19	34	0.1900	5	47	0.0655
	> 60	15	46		1	58	
HBsAg	+	19	30	0.0697	3	45	0.7312
	-	15	50		3	60	
HCV	+	12	34	0.4731	2	43	0.7116
	-	22	46		4	62	
Albumin	≤ 4.0 mg/dL	13	33	0.7641	3	41	0.5937
	> 4.0 mg/dL	21	47		3	64	
Total bilirubin	≤ 0.7 mg/dL	21	45	0.5853	4	60	0.6461
	≥ 0.8 mg/dL	13	35		2	45	
ICGR15	≤ 15%	22	39	0.1181	3	56	0.8736
	> 15%	12	41		3	49	
Anatomical resection	Yes	25	54	0.5231	4	73	0.8826
	No	9	26		2	32	
AFP	≤ 200 ng/mL	21	57	0.3189	1	76	0.0040
	> 200 ng/nL	13	23		5	29	
AFPL3	≤ 15%	21	58	0.2556	2	76	0.0418
	> 15%	13	22		4	29	
PIVKA-Ⅱ	≤ 40 mAU/mL	8	30	0.1477	1	36	0.3732
	> 40 mAU/mL	26	50		5	69	
Tumor number	Solitary	25	57	0.8804	2	78	0.0259
	Multiple	9	22		4	26	
Tumor size	≤ 2 cm	2	10	0.2922	0	12	0.3806
	> 2 cm	32	70		6	93	
Differentiation	Well	0	7	0.0737	0	7	0.5859
	Moderately	26	49		3	70	
	Poorly	7	24		3	27	
vp	Positive	14	18	0.0423	5	25	0.0014
	Negative	20	62		1	80	
vv	Positive	3	4	0.4366	1	5	0.2098
	Negative	31	76		5	100	
im	Positive	12	18	0.1558	4	25	0.0201
	Negative	22	62		2	80	
Noncancerous liver Liver cirrhosis		11	28	0.6833	1	36	0.3501
Non liver cirrhosis		23	49		5	66	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; HCV: Anti-hepatitis C virus antibody; AR: Patients who underwent anatomical resection; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

PH-VEGFR-1 and PH-AFP (P = 0.2138) mRNA status showed no such correlation. The DFS curve of group A was significantly better than that of group B, C or D (P = 0.0437, P = 0.0325, P = 0.0225, respectively; Figure 3).

Univariate analysis further revealed that age, hepatitis B surface antigen (HBsAg), albumin, AFP, AFPL3, PIVKA-Ⅱ, the number of tumors, tumor size, portal vein invasion, hepatic vein invasion, intrahepatic metastasis, BM-AFP mRNA and classification by BM-VEGFR-1/BM-AFP mRNA are important risk factors for HCC early recurrence (Table 3). Multivariate analysis revealed that albumin ≤ 4.0 mg/dL and positive portal vein invasion were independent risk factors for recurrence within 1 year of surgery. Although BM-AFP mRNA positivity was not a significant factor by multivariate analysis, it was still found to be an important factor in predicting an early recurrence in HCC cases (P = 0.0761, Table 4).

DISCUSSION

In our current study, we found a significant tendency for HCC patients who were positive for BM-AFP mRNA to experience disease recurrence within 1 year of surgery. Patients with low BM-VEGFR-1 mRNA and who were negative for BM-AFP mRNA experienced early recurrence in 3/23 cases, whereas in 57 cases with high BM-VEGFR-1 and BM-AFP mRNA, 17 recurrences were observed. Hence, BM-AFP mRNA positivity is an important predictor of early HCC recurrence after curative hepatectomy due to hematogenic spread. BM-VEGFR-1 mRNA was also found to be associated with early HCC recurrence.

The time between hepatectomy and recurrence of metachronous *de novo* tumors is longer than that of intrahepatic metastases[15], therefore, early recurrence of these

Table 2 Characteristics of hepatocellular carcinoma patients according to their bone marrow-vascular endothelial growth factor receptor-1 and peripheral blood-vascular endothelial growth factor receptor-1 mRNA profile

		BM-VEGFR1		*P* value	PH-VEGFR1		*P* value
		High (*n* = 81)	Low (*n* = 33)		High (*n* = 78)	Low (*n* = 26)	
Sex	Male	67	27	0.9090	64	22	0.7647
	Female	14	6		14	4	
Age (yr)	≤ 60	40	13	0.3322	37	12	0.9097
	> 60	41	20		41	14	
HBsAg	+	35	14	0.9387	34	12	0.8197
	-	46	19		44	14	
HCV	+	36	10	0.1628	32	9	0.5624
	-	45	23		46	17	
Albumin	≤ 4.0 mg/dL	38	8	0.0252	27	12	0.2926
	> 4.0 mg/dL	43	25		51	14	
Total bilirubin	≤ 0.7 mg/dL	48	18	0.6439	51	11	0.0378
	≥ 0.8 mg/dL	33	15		27	15	
ICGR15	≤ 15%	41	20	0.3322	44	13	0.5695
	> 15%	40	13		34	13	
Anatomical resection	Yes	55	24	0.6124	56	18	0.8026
	No	26	9		22	8	
AFP	≤ 200 ng/mL	54	24	0.5278	57	16	0.2653
	> 200 ng/nL	27	9		21	10	
AFPL3	≤ 15%	54	25	0.3399	59	15	0.0802
	> 15%	27	8		19	11	
PIVKA-II	≤ 40 mAU/mL	24	14	0.1888	25	10	0.5491
	> 40 mAU/mL	57	19		53	16	
Tumor number	Solitary	53	29	0.0068	55	18	0.8867
	Multiple	28	3		23	7	
Tumor size	≤ 2 cm	9	3	0.7499	10	2	0.4784
	> 2 cm	72	30		68	24	
Differentiation	Well	4	3	0.1151	6	1	0.1614
	Moderately	51	25		56	11	
	Poorly	26	5		16	14	
vp	Positive	26	6	0.1337	21	7	...
	Negative	55	27		57	19	
vv	Positive	6	1	0.3773	4	1	0.7913
	Negative	75	32		74	25	
im	Positive	23	7	0.4296	24	5	0.2559
	Negative	58	26		54	21	
Noncancerous liver cirrhosis		34	5	0.0061	26	9	0.9962
Non liver cirrhosis		45	27		49	17	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; VEGFR: Vascular endothelial growth factor receptor; HCV: Anti-hepatitis C virus antibody; AR: Patients who underwent anatomical resection; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

lesions (within 1 year) is thought to be dependent on hematogenic spread. By real-time quantitative RT-PCR, we found in our current analyses that, although the AFP/GAPDH mRNA ratios in the liver tissues were generally constant among normal control subjects, they were markedly different among HCC patients. This indicated highly variable AFP synthesis activity among individual HCC cells. It has been shown that high AFP mRNA levels reflect the presence of HCC cells[13]. In our present study, the 1-year survival and DFS rates of HCC patients who were positive for AFP mRNA were 86.5% and 54.5%, respectively. Hence, we analyzed the relationship between early recurrence and the preoperative status of the BM- and PH-AFP, and the BM- and PH-VEGFR-1 mRNA.

Although we found in our present experiments that the BM-AFP mRNA status significantly correlates with early HCC recurrence, the BM-VEGFR-1, PH-VEGFR-1 and PH-AFP mRNA levels did not correlate with this outcome. However, classifying the HCC cases in our cohort using the BM-VEGFR-1/BM-AFP mRNA levels showed a correlation with early recurrence ($P = 0.0228$). Based on these findings, we speculate that the preoperative presence of cancer cells in the bone marrow is an important and essential driver of early HCC recurrence due to hematogenic spread, although we did not detect any changes in the AFP or VEGFR1 mRNA levels in the bone marrow and peripheral blood after surgical intervention in recurrent cases. The importance of the coexistence of disseminated cancer cells and VEGFR-1-positive hematopoietic bone marrow progenitor cells was further supported by the improved DFS curve of HCC patients that were negative for BM-AFP mRNA, and that showed low BM-VEGFR-1 transcript levels as compared with the other three patient groups. On the other hand, we surmised that the relationship between BM-VEGFR-1 mRNA and hematogenic spread in HCC

Table 3 Clinical factors related to early hepatocellular carcinoma recurrence after curative hepatectomy

		Recurrence over 1 year (77)	Recurrence within 1 year (37)	*P* value
Sex	Male	64	30	0.7980
	Female	13	7	
Age (yr)	≤ 60	30	23	0.0200
	> 60	47	14	
HBsAg	+	24	25	0.0002
	-	53	12	
HCV	+	34	12	0.2322
	-	43	25	
Albumin	≤ 4.0 mg/dL	24	22	0.0039
	> 4.0 mg/dL	53	15	
Total bilirubin	≤ 0.7 mg/dL	47	19	0.3266
	≥ 0.8 mg/dL	30	18	
ICGR15	≤ 15%	43	18	0.4708
	> 15%	34	19	
Anatomical resection	Yes	55	24	0.4769
	No	22	13	
AFP	≤ 200 ng/mL	59	19	0.0066
	> 200 ng/nL	18	18	
AFPL3	≤ 15%	58	21	0.0442
	> 15%	19	16	
PIVKA-II	≤ 40 mAU/mL	32	6	0.0072
	> 40 mAU/mL	45	31	
Tumor number	Solitary	62	20	0.0021
	Multiple	14	17	
Tumor size	≤ 2 cm	36	6	0.0016
	> 2 cm	41	31	
Differentiation	Well	7	0	0.1631
	Moderately	52	13	
	Poorly	18	24	
vp	Positive	11	16	< 0.0001
	Negative	66	21	
vv	Positive	2	5	0.0230
	Negative	75	32	
im	Positive	13	17	0.0010
	Negative	64	20	
BM VEGFR1	Low	24	9	0.4506
	High	53	28	
PH VEGFR1	Low	18	8	
	High	54	24	
BM AFP mRNA	Positive	17	17	0.0091
	Negative	60	20	
PH AFP mRNA	Positive	2	4	0.0569
	Negative	74	31	
BM-AFP mRNA/ BM-VEGFR1	Negative/low	20	3	0.0228
	Negative/high	40	17	
	Positive/low	4	6	
	Positive/high	13	11	

BM: Bone marrow; PH: Peripheral blood; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; VEGFR: Vascular endothelial growth factor receptor; HCV: Anti-hepatitis C virus antibody; ICGR15: Indocyanine green retention rate at 15 min; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

during hematogenic recurrence was not stronger than that in gastric cancer, because it has been reported in a clinically relevant and widely used preclinical study model that blockade of VEGFR-1 activity does not affect the formation of spontaneous metastases[16].

In our present study, the BM-VEGFR-1 mRNA level

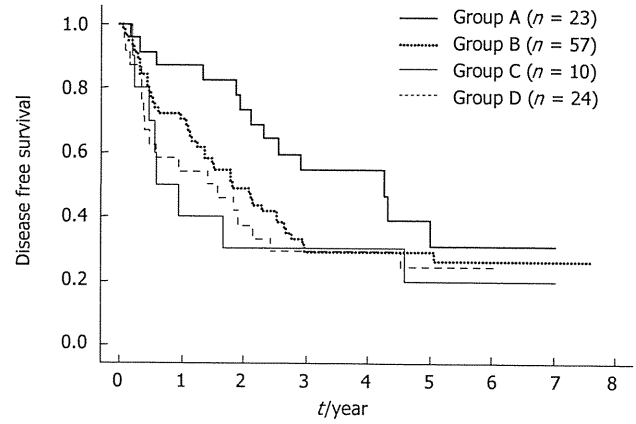

Figure 3 Patients were classified into four groups according to the level/status of their bone marrow-vascular endothelial growth factor receptor-1 and bone marrow-α-fetoprotein mRNA as follows: group A (*n* = 23), bone marrow-vascular endothelial growth factor receptor-1/bone marrow-α-fetoprotein mRNA = low/negative; group B (*n* = 57) high/negative; group C (*n* = 10) low/positive; group D (*n* = 24), high/positive. The disease-free survival (DFS) curve of group A was significantly better than that of groups B, C or D (*P* = 0.0437, *P* = 0.0325, *P* = 0.0225).

of all HCC patients was higher than that in the normal controls, and the PH-VEGFR-1 mRNA levels of almost all of these patients were also higher than in the normal controls. Direct evidence for the role of the chemokine stromal-cell derived factor-1 [SDF-1, also known as chemokine CXC ligand (CXCL)12] in regulating the mobilization of proangiogenic bone marrow cells *in vivo* has been demonstrated by plasma elevation of SDF-1, which stimulates the mobilization of chemokine CXC receptor (CXCR) 4^+ bone marrow cells, including hematopoietic stem cells and endothelial progenitor cells[17,18]. SDF-1 not only promotes revascularization by engaging with CXCR4 expressed on vascular cells but also supports the mobilization of proangiogenic $CXCR4^+$ $VEGFR1^+$ hematopoietic cells[19]. In contrast, Li *et al*[20] have reported a much higher expression level of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhotic, adenocarcinoma or normal liver tissues. Hence, we speculate that VEGFR-1-positive hematopoietic bone marrow progenitor cells might be regulated and recruited by a mechanism similar to the SDF-1-CXCR4 pathway in most HCC patients. On the basis of our current data and the results of these earlier reports, we further predict that, in almost all patients with HCC, a pre-metastatic niche might have already been initiated by VEGFR-1-positive hematopoietic bone marrow progenitor cells. The levels of BM- and PH-VEGFR-1 mRNA were not found to correlate with early recurrence in each of the HCC patients, although BM-AFP mRNA positivity was significantly associated with early recurrence. These findings thus indicate that the initiation of a pre-metastatic niche be recognized as a first but essential step in the development of metastasis that requires the presence of disseminated cancer cells. This hypothesis is supported by our finding that patients negative for BM-AFP mRNA and with low levels of BM-VEGFR-1 mRNA show the lowest rate of recurrence among all of the groups analyzed.

Table 4 Multivariate analyses of variables that are predictive of early hepatocellular carcinoma recurrence after curative hepatectomy

	P value	Risk ratio	95% CI
Age ≤ 60 yr	0.0899	3.147	0.836-11.838
HBsAg +	0.3601	1.821	0.504-6.571
Albumin ≤ 4.0 mg/dL	0.0038	6.536	1.832-23.256
AFP > 200 ng/nL	0.2571	2.330	0.539-10.067
AFPL3 ≤ 15%	0.4379	1.869	0.385-9.090
PIVKA-II > 40 mAU/mL	0.1494	2.959	0.677-12.987
Tumor number solitary	0.9127	1.088	0.240-4.938
Tumor size > 3 cm	0.1177	3.026	0.756-12.114
vp positive	0.0069	6.639	1.681-26.219
vv positive	0.2221	0.234	0.023-2.408
im positive	0.2307	2.508	0.557-11.289
BM AFP mRNA: positive	0.0761	2.704	0.901-8.113

CI: Confidence interval; BM: Bone marrow; AFP: α-fetoprotein; HBsAg: Hepatitis B surface antigen; vp: Microscopic tumor thrombus in the portal vein; vv: Microscopic tumor thrombus in the hepatic vein; im: Microscopic intrahepatic metastasis.

It has been shown in several previous studies that the detection of micrometastases from solid tumors in bone marrow samples can be an important prognostic indicator with high specificity[3-7,11]. The release of carcinoma cells from the bone marrow into the peripheral blood can be induced by cytokine treatment[21]. Hence, the bone marrow might function as an important reservoir and a source of disseminated cancer cells that can subsequently spread into other organs. Moreover, the bone marrow itself may become altered in response to chemokines produced by the primary tumor and thereby enhance the metastatic capabilities of tumor cells that reside within it[22]. It has been reported that VEGFR-1-positive cells promote tumor adherence and growth[11]. VEGFR signaling is a crucial inducer of angiogenesis, enables primary tumor growth, and probably releases micrometastases from dormancy[23]. In our current study, only the classification by BM-VEGFR-1 and BM-AFP mRNA was correlated with early recurrence. Hence, the coexistence of bone-marrow-derived hematopoietic progenitor cells that express VEGFR-1 in the bone marrow, and not in the peripheral blood, might be advantageous for various cancer cells in the bone marrow in terms of metastasis.

In conclusion, the evaluation of BM-AFP mRNA and BM-VEGFR-1 mRNA in patients with HCC shows great promise as a predictor of recurrence in curatively resected HCC.

ACKNOWLEDGMENTS

The authors wish to thank the staff of the Department of General Surgery, Graduate School of Medicine, Hokkaido University, for their kind cooperation.

COMMENTS

Background

α-fetoprotein (AFP) mRNA, which represents disseminate cancer cells, is related to recurrence of hepatocellular carcinoma (HCC). Bone-marrow-derived hematopoietic progenitor cells that express vascular endothelial growth factor receptor (VEGFR)-1 home to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of tumor cells.

Research frontiers

It has been reported that simultaneous presence of isolated tumor cells and VEGFR-1 expression at pre-metastatic sites is clinically significant for disease progression in gastric cancer. With regard to HCC, there has been no study about the association between the presence of isolated cancer cells and the expression of VEGFR-1. In the present study, we tried to determine whether expression of AFP mRNA and VEGFR-1 in bone marrow and peripheral blood detected by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) could predict early recurrence in consecutive patients after curative hepatic resection.

Innovations and breakthroughs

There was a significant tendency for patients who were positive for AFP mRNA in bone marrow to experience recurrence within 1 year after surgery compared to those negative for AFP mRNA in bone marrow. The VEGFR-1 mRNA level in bone marrow in all HCC patients was higher than that of normal controls. It was supposed that this initiation of pre-metastatic niche represented by the high level of VEGFR-1mRNA might be recognized as only the first step and as a necessary condition for development of metastasis, and required the subsequent presence of disseminated cancer cells represented by AFP mRNA.

Applications

The evaluation of AFP mRNA and VEGFR-1 mRNA in bone marrow in patients with HCC could be very important for the prediction of recurrence of curatively resected HCC.

Peer review

This study found that the expression of AFP and VEGFR-1 mRNA in bone marrow detected by real-time quantitative RT-PCR predicted early recurrence in consecutive patients after curative hepatic resection. This finding is very important to elucidate the mechanism of metastasis and recurrence by hematogenic spread of HCC cells.

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S- Editor Lv S **L- Editor** Kerr C **E- Editor** Zhang DN

Transplant Immunology 26 (2012) 42–49

Contents lists available at SciVerse ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

NK026680 inhibits T-cell function in an IL-2-dependent manner and prolongs cardiac allograft survival in rats

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ARTICLE INFO

Article history:
Received 16 June 2011
Received in revised form 7 October 2011
Accepted 8 October 2011

Keywords:
NK026680
Triazolopyrimidine
Rat
Heart transplantation
T cells

ABSTRACT

NK026680 is a triazolopyrimidine derivative that has been shown to inhibit dendritic cell maturation and activation. Here, we examined the immunosuppressive properties of NK026680 on T-cell function and assessed its immunosuppressive efficacy in an ACI ($RT1^{av1}$ haplotype) to Lewis ($RT1^{l}$) rat heart transplantation model. The effects of NK026680 on T-cell proliferation, activation, and cytokine production were investigated *in vitro*. Heart transplant recipient rats were administered NK026680 daily for 14 days post-transplantation. In addition to graft survival time, alloimmune responses and graft histology at 4–10 days post-transplantation were assessed. NK026680 was found to inhibit proliferation, CD25 upregulation, IL-2 production, and cell cycle progression in αCD3/αCD28-stimulated murine T cells. These effects were likely due to suppression of the p38 mitogen-activated protein kinase pathway and the subsequent inhibition of p65, c-Fos, and to a lesser extent, c-Jun. Daily NK026680 treatment suppressed alloimmune responses, prevented cellular infiltration into allografts, and prolonged graft survival. The anti-rejection effects of NK026680 were enhanced by tacrolimus. In conclusion, NK026680 inhibits the activation of T cells and prolongs cardiac allograft survival in rats. These features make it a potential candidate immunosuppressant for the treatment of organ transplant patients in the future.

1. Introduction

The long-term administration of calcineurin inhibitors (CNIs) leads to an increased risk of the development of unfavorable side effects [1,2]. Recently, regimens that avoid the use of CNIs have been successfully adopted by transplant centers. There have been reports that these regimens can lead to reductions in drug-related adverse events along with equivalent outcomes in allograft survival following transplantation [3–7]. Despite this, CNIs are still key immunosuppressants in practice, and safer alternatives are limited. Thus, the development of new agents that have less drug-related side effects is essential.

Triazolopyrimidines are synthetic heterocycles with valuable bioactivity. Trapidil, a platelet-derived growth factor antagonist, is one of the major triazolopyrimidines and has a broad spectrum of biological activities. Previous studies have shown its protective effects in ischemia–reperfusion injury in some experimental models [8–12] and its clinical benefits in controlling angina pectoris and reducing angiographic in-stent restenosis [13,14]. These effects are due to trapidil's myriad of pharmacological properties that include nitroglycerine-like vasodilating action, inhibition of platelet aggregation, facilitation of the biosynthesis of prostacyclin, inhibition of thromboxane A2, and reduction of lipid peroxidation. Trapidil has also been reported to suppress IL-6, IL-12, and TNF-α production by blocking CD40 expression on monocytes and macrophages in humans [15,16].

NK026680 is a novel triazolopyrimidine derivative compound that also has very potent bioactivity. It has been reported to ameliorate mortality in acute lethal graft-versus-host disease in both MHC classes I and II disparate mice [17], prevent glomerulonephritis and perinuclear antineutrophil cytoplasmic antibody production in SCG/Kj mice [18], and prolong liver allograft survival in rats [19]. The findings in these reports suggested that such effects may be the result of impaired dendritic cell (DC) function. Here, we studied the effects of

Abbreviations: AP-1, activator protein-1; CNI, calcineurin inhibitor; DC, dendritic cell; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MLR, mixed lymphocyte reaction; MST, median survival time; NF-κB, nuclear factor-kappa B; NFAT, nuclear factor of activated T cells; SAPK, stress-activated protein kinase.

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doi:10.1016/j.trim.2011.10.002