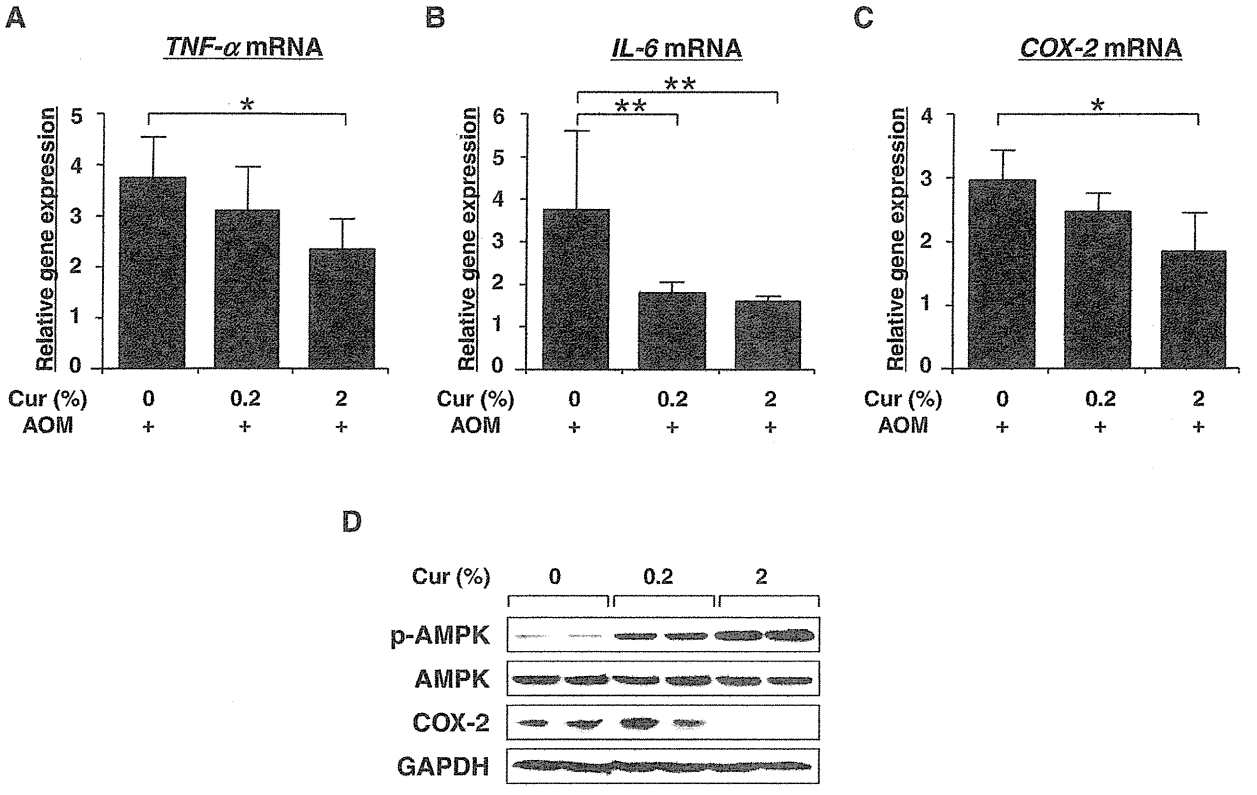

FIG. 1. Effect of curcumin (Cur) on the levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), and cyclooxygenase-2 (COX-2) (C) mRNAs and on the activation of AMP-activated kinase (AMPK) and expression of COX-2 proteins (D) in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. cDNA was synthesized from the colonic mucosa and real-time RT-PCR was performed using TNF-α (A), IL-6 (B), and COX-2 (C) specific primers. The expression levels of these genes were normalized to the level of the GAPDH gene. Each experiment was done in triplicate and the average was subsequently calculated. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. Total proteins were extracted from the scraped colonic mucosa and equivalent amounts of proteins were examined by a Western blot analysis using phosphorylated AMPK (p-AMPK), AMPK, and COX-2 specific antibodies (D). Two lanes represent protein samples from 2 different mice in each group (Groups 3 to 5). An antibody to GAPDH served as a loading control.

(activation) of AMPK protein in the colonic mucosa of AOM-treated mice. In addition, there was an apparent decrease in the expression levels of COX-2 proteins in the colonic mucosa of mice after feeding the diet containing 2% curcumin (Fig. 1D).

Effects of Curcumin on the NF-κB Activity in the Colonic Mucosa of AOM-Injected *db/db* Mice

Curcumin inhibits the activation of NF-κB and this is one of the critical mechanisms for the antiinflammatory effects of this agent (12,13). Therefore, the effects of curcumin on NF-κB activity were examined in the colonic mucosa of AOM-injected mice. As shown in Fig. 2, the indices of phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitium of curcumin-supplemented mice were significantly smaller than those of the curcumin-untreated mice ($P < 0.01$, for each comparison), thus indicating that curcumin supplementation significantly inhibits the NF-κB activity in the colonic mucosa, mainly crypt and inflammatory cells, of the AOM-treated *db/db* mice.

Effects of Curcumin on the Serum Levels of Adiponectin and Leptin and on the Relative Weights of White Adipose Tissue in *db/db* Mice

The serum concentrations of adiponectin in the 2% curcumin-treated groups were significantly higher than those of the groups that did not receive curcumin ($P < 0.01$ and $P < 0.05$), regardless of AOM-injection (Fig. 3A). In AOM-injected groups, administration of both doses of curcumin showed a significant decrease in the serum levels of leptin ($P < 0.05$ for each comparison) when compared to mice that did not receive curcumin (Fig. 3B). In addition, administration of curcumin also significantly reduced the relative weights of white adipose tissue (periorchis and retroperitoneum) compared with the untreated groups ($P < 0.05$ and $P < 0.01$), regardless of AOM-treatment (Fig. 3C).

DISCUSSION

Obesity-related physiological alterations, such as a state of chronic inflammation, are known to influence the risk of CRC development (1,2). This is the first study to report evidence

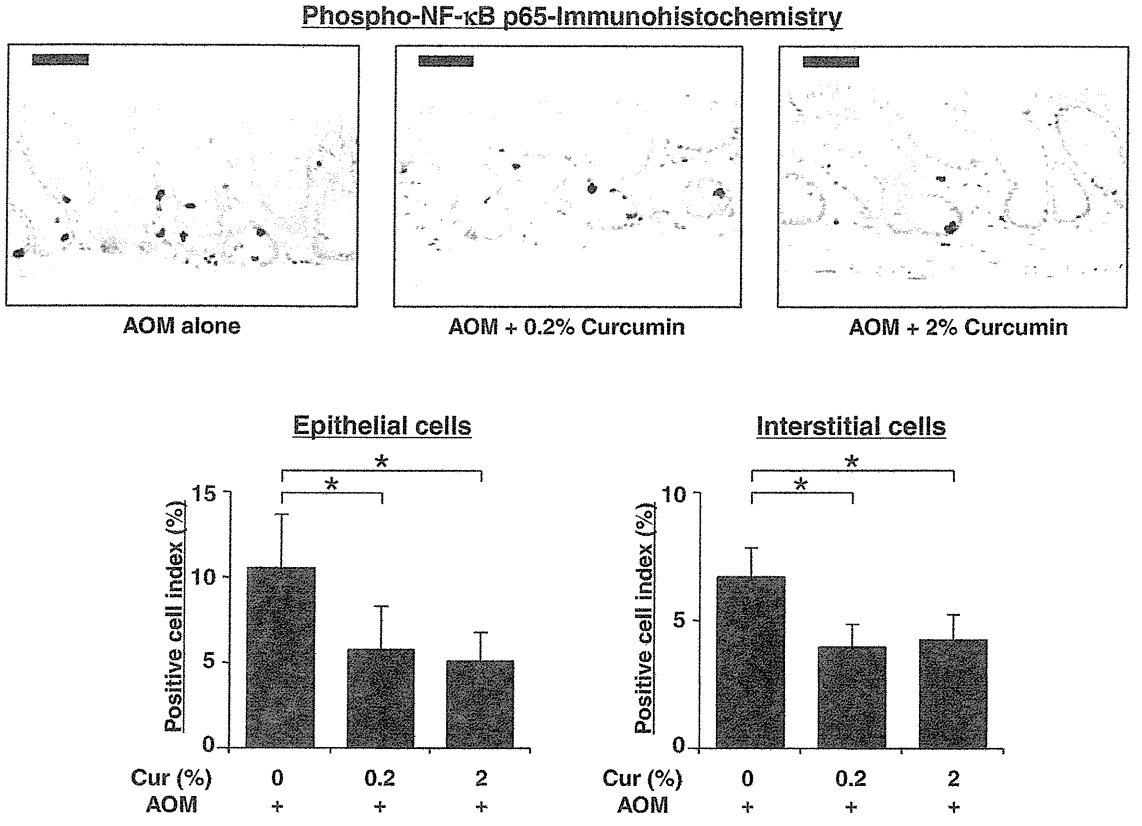

FIG. 2. Effects of curcumin (Cur) on the nuclear factor (NF)-κB activity in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. Sections of the colon were stained with antiphospho-NF-κB p65 antibody. Representative photographs from each group are shown in the upper panels. The positive cell indices, which were determined by counting the phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitial inflammatory cells, are shown in the lower panels. Bars: 50 μm. $^*P < 0.01$ compared to mice not fed with curcumin.

of curcumin effectively inhibiting the development of putative precursor lesions (ACF and BCAC) of colonic adenocarcinoma in the obese and diabetic *db/db* mice (Table 1). These suppressive effects are most likely associated with the decrease in expression of proinflammatory cytokines TNF-α and IL-6 in the colonic mucosa of AOM-treated *db/db* mice (Figs. 1A and 1B), although other anticancer mechanisms, such as modulation of cell proliferation and apoptosis, could be considered (12–14). The expression levels of COX-2, which represents an early response to proinflammatory mediators and a critical target for CRC chemoprevention (17), are also inhibited in the colonic mucosa of mice fed with curcumin (Figs. 1C and 1D). In addition, curcumin supplementation inhibited NF-κB activity, which is involved in regulation of COX-2 expression (26), in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2). These findings are consistent with those of a previous study indicating that curcumin inhibits the induction of COX-2 by TNF-α stimulus via the inhibition of NF-κB activation in human colon epithelial cells (27). Curcumin suppresses the growth of CRC-derived cells by inhibiting the expression of COX-2 (16). Curcumin also inhibits the secretion of TNF-α and IL-6 from monocytes cultured under high-glucose conditions, and lowers the serum levels of TNF-α and IL-6 in diabetic rats (21). Therefore, overexpression of inflammatory mediators, especially TNF-α, IL-6, and COX-2, which are relevant to excessive storage of lipid, might represent critical targets of curcumin to prevent the development of obesity-related CRC.

The present study also demonstrated the first evidence that administration of curcumin markedly enhances AMPK activation in the colonic mucosa of obese and diabetic mice (Fig. 1D). This finding is significant because AMPK, which functions as a major metabolic switch for maintenance of energy homeostasis, controls processes relevant to tumor development and therefore may be a promising target for cancer chemoprevention (28). For instance, the antidiabetic drug metformin, a pharmacological AMPK activator, exerts growth inhibitory effects on colon cancer xenografts as well as on human CRC cells (29,30). Pitavastatin, which is used to treat hyperlipidemia, inhibits development of AOM-induced BCAC in *db/db* mice by activating AMPK and decreasing the expression levels of TNF-α, IL-6, and COX-2 mRNAs in the colonic mucosa (8). Curcumin also inhibits proliferation and induces apoptosis in human CRC cells

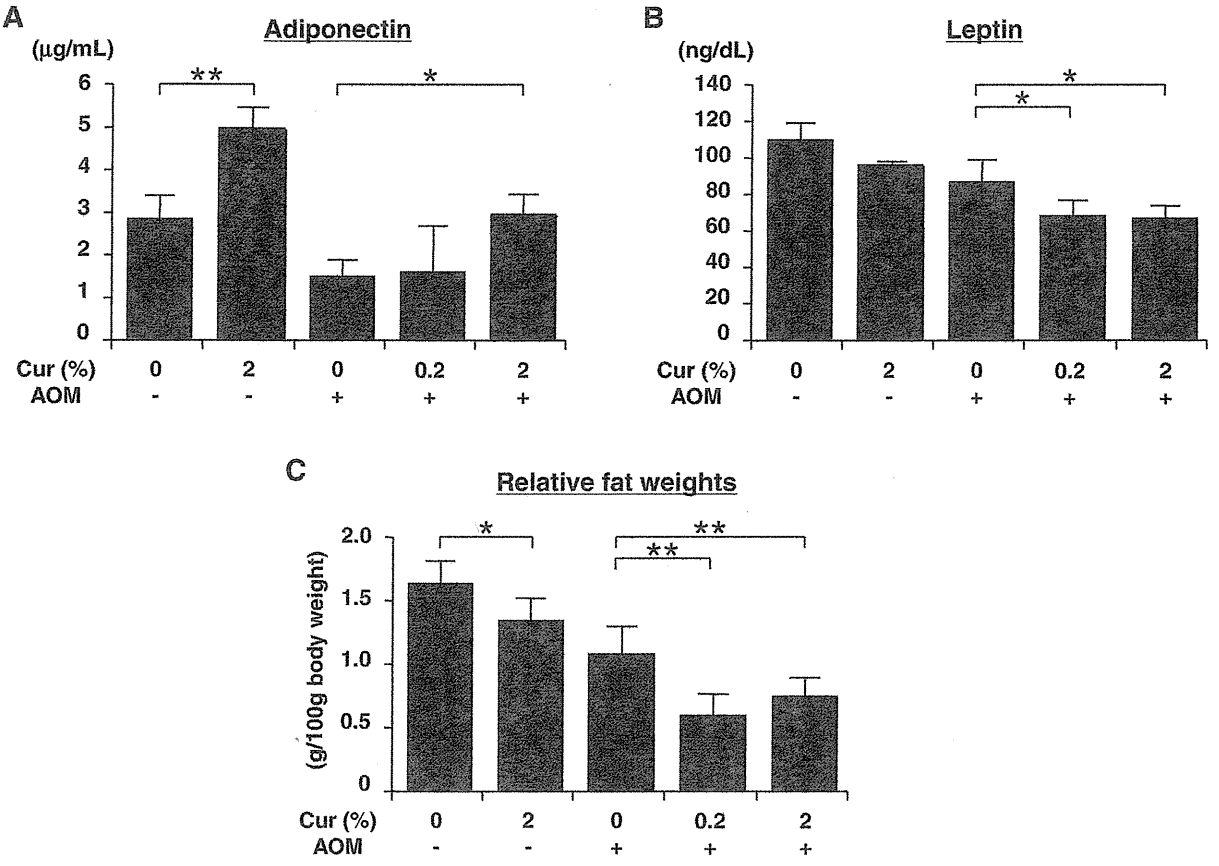

FIG. 3. Effect of curcumin (Cur) on the serum levels of adiponectin and leptin and on the relative weights of fat in experimental mice. The serum concentrations of adiponectin (A) and leptin (B) were measured by using an enzyme immunoassay. At sacrifice, white adipose tissue of the periorchis and retroperitoneum was excised and the relative weights (g/100 g body weight) were calculated (C). Values are expressed as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. AOM indicates azoxymethane.

by activating AMPK and decreasing COX-2 expression (15). These reports (8,15,29,30), together with our present findings (Table 1 and Fig. 1D), suggest that curcumin may prevent the development of obesity-related colorectal carcinogenesis, at least in part, through the activation of AMPK.

Dysregulation of adipocytokines is also involved in the link between obesity and colorectal carcinogenesis (1,2). In particular, a low level of serum adiponectin, which raises the risk of colorectal tumorigenesis (3,4), is associated with obesity-based chronic inflammation because adiponectin exerts antiinflammatory effects by downregulating the production of TNF-α and IL-6 (31). Adiponectin inhibits the growth of CRC cells through the activation of AMPK and suppresses colonic epithelial proliferation in mice that are fed a high-fat diet (32,33). On the other hand, a higher level of serum leptin, which is proportional to the amount of body fat stored, plays a role in enhancing the development of obesity- and inflammation-related CRC (5–9). Leptin also increases cell growth and promotes cell mobility and invasion in CRC-derived cells (34,35). These reports suggest that targeting the imbalance of adiponectin and leptin might be an effective strategy for preventing obesity-related CRC. Indeed, improvement of adipocytokine imbalance by certain agents may contribute to the inhibition of obesity-related tumorigenesis in the colorectum (6–8). Therefore, our findings that dietary curcumin significantly increased the levels of adiponectin as well as decreased the levels of leptin in the serum of *db/db* mice, which might be caused by the reduction in white adipose tissue (Fig. 3), is important to explain the chemopreventive effects of this agent on obesity-related colorectal carcinogenesis. Similarly, it was also reported that curcumin administration ameliorates diabetes and increases the expression of adiponectin both in serum and adipose tissue of leptin-deficient *ob/ob* mice (20). Therefore, in addition to the improvement of metabolic abnormalities, curcumin has the potential to suppress obesity-related colorectal carcinogenesis by affecting the serum levels of adiponectin and leptin.

The limitation of present study is that the dose of curcumin in Group 5 (2%) is relatively high when considering the use of this

agent in clinical practice. However, our histopathologic examination revealed no alterations in the vital organs of the mice that received the diet containing 2% curcumin. Our ongoing study on the safety and chemopreventive ability of different doses of curcumin may show dose-response efficacy in inhibiting colorectal tumorigenesis of mice, as suggested for ACF-inhibition in this study (see Group 4 in Table 1). Moreover, a recent clinical trial has shown a significant result indicating that a daily oral dose of 3.6 g curcumin, which is well tolerated at patients with advanced CRC (36), achieves pharmacologically efficacious levels in the colorectum with negligible distribution of this agent outside the gut (37). This finding suggests that regions of the gastrointestinal tract, such as the colorectum, may be more appropriate targets for curcumin to exert its cancer chemopreventive effects because such organs are directly exposed to high concentrations of this agent.

It should be emphasized again that obesity and obesity-related metabolic abnormalities represent serious global health care problems, and CRC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis, which includes chronic inflammation and adipocytokine imbalance, might be a promising strategy for treating obese individuals who are at an increased risk of developing CRC. Curcumin, which has been shown to exert various chemopreventive and anticancer properties (12–14), appears to be a potentially effective candidate for this purpose because this phytochemical can attenuate inflammation while improving the imbalance of adipocytokines and can, furthermore, prevent the development of colonic precancerous lesions in obese and diabetic *db/db* mice.

ACKNOWLEDGMENTS

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Regular article

Modulatory Effects of Capsaicin on *N*-diethylnitrosamine (DEN)-induced Mutagenesis in *Salmonella typhimurium* YG7108 and DEN-induced Hepatocarcinogenesis in *gpt* Delta Transgenic Rats

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Capsaicin from the red chili pepper is a prospective chemopreventive agent. To explore the possible antigenotoxic effects of capsaicin on *N*-diethylnitrosamine (DEN)-induced mutagenesis *in vitro*, we conducted bacterial mutation assays with *Salmonella typhimurium* YG7108, a sensitive strain to mutagenic alkylating agents. Capsaicin was not mutagenic either with or without S9 activation. Unexpectedly,it enhanced the mutagenicity of DEN in the presence of S9 activation significantly. Capsaicin also enhanced the mutagenicity of 2-aminoanthracene and benzo[*a*]pyrene in the presence of S9 activation and benzo[*a*]pyrene diolepoxide in the absence of S9 activation. However, it reduced the mutagenicity of ethylnitrosourea in the absence of S9 activation. To examine whether capsaicin modulates DEN-induced mutagenesis and hepatocarcinogenesis *in vivo*, we took advantage of *gpt* delta rats, transgenic rodents that carry reporter genes for mutations. Female *gpt* delta rats were given drinking water containing 40 ppm DEN for five weeks. They were fed diets containing capsaicin at doses of 0, 100 or 500 ppm for seven weeks, starting one week before the DEN treatment. Samples were collected at weeks 7 and 32, respectively, for mutagenicity and carcinogenicity assays. DEN enhanced *gpt* mutant frequency more than 200 fold in the liver. However, capsaicin displayed no modulating effects on the mutagenesis. Rather, it reduced the number of liver neoplasms, especially liver cell adenomas, in a dose-dependent manner although the reduction in hepatocellular carcinoma was statistically insignificant. These results suggest that chemopreventive effect of capsaicin against DEN-induced hepatocarcinogenesis is slight and that the effect is not due to antimutagenesis. The results also caution that chemopreventive effects of chemicals should be examined not only *in vitro* but also *in vivo* with multiple indexes, e.g., *in vitro* and *in vivo* mutations and pathological examinations.

Key words: capsaicin, chemoprevention, *N*-diethylnitrosamine, hepatocarcinogenicity, *gpt* delta transgenic rats

Introduction

Capsaicin is the principal pungent constituent of hot red chili peppers, which are the most frequently consumed spices in the world (1). In addition to a spicy dietary ingredient, capsaicine is known to exhibit various biological activities, such as inhibition of CYP-depenent xenobiotic metabolism, inhibition of cellular signal transduction and induction of apoptosis (1–6). Thus, it is expected that capsaicin can be chemopreventive against tumors via antigenotoxic mechanisms. In fact, it is reproted that dietary exposure to capsaicin suppresses azoxymethane-induced colon tumors in rats (7). Capsaicin also inhibits DNA binding of aflatoxin B1 in the presence of *in vitro* metabolic activation by S9 enzymes (8). Several reports suggest, however, that capsaicin itself is mutagenic in Ames bacterial mutation assays (9–11), V79 mammalian gene mutation assays (12) and micronucleus assays *in vivo* (13), and one report suspects the carcinogenicity (14). It remains elusive, there-

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fore, whether capsaicin is chempreventive and if so whether antigenotoxic mechanisms are involved in the chemopreventive action.

To examine the possible chemopreventive effects of capsaicin against *N*-diethylnitrosamine (DEN), we conducted *in vitro* and *in vivo* mutation assays. We chose DEN as a target carcinogen because it induces hepatocarcinoma effectively in a genotoxic manner (15). In addition, liver cancer is one of the most prevalent cancer diseases world wide (16). We employed *Salmonella typhimurium* YG7108 (17,18), a sensitive strain to mutagenicity of various alkylating agents, for *in vitro* mutation assays and Fischer 344 *gpt* delta transgenic rats for *in vivo* assays (19). The strain *S. typhimurium* YG7108 is sensitive to mutagenicity of alkylating agents because it lacks DNA repair enzymes of O^6-alkylguanine alkyltransferases encoded by the ada_{ST} and ogt_{ST} genes (17,18). *gpt* delta transgenic rats carry reporter genes for *in vivo* mutations (20). Point mutations and deletions can be identified in any organs or tissues of F344 rats and the mutations are analyzable at the sequence level (19). Chemopreventive effects of capsaicin against tumor induction was histopathologically evaluated in the liver of DEN-treated F344 *gpt* delta rats. Glutathione *S*-transferase placenta form (GST-P) positive foci are frequently used as an indicator of pre-neoplastic lesions of liver of rats because this bioassay shows good correlations with long-term carcinogenicity results (21). The results suggest that capcaisin suppresses DEN-induced heptatocarcinogenesis slightly. However, the chemopreventive effect is not due to antigenotoxic mechanims because capsaicin displayed no antimutgenic activity against DEN-induced mutations *in vivo*. Capsaicin is not mutagenic *in vitro* and *in vivo*. Because of the complex properties, chemopreventive effects of capsaicin should be further evaluated via multiple indexes such as mutations and proliferating lesions (preneoplasms and neoplasms) induced by other genotoxic carcinogens.

Materials and Methods

Materials: Capsaicin (synthetic, *N*-vanillylnonanamide, CAS: 2444-46-4) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The purity was >96%. DEN (CAS No.: 55-18-5) and ethylnitrosourea (ENU; CAS: 759-73-9) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-Aminoanthracen (2-AA; CAS No.: 103404-81-5), benzo[*a*]pyrene (BP; CAS No.: 50-32-8), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1; CAS No.: 67730-11-4), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Benzo[*a*]pyrene-dihydrodiol epoxide (BPDE; CAS No.: 60268-85-1) was purchased from Midwest Research Institute Global (Missouri, MO, USA). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Bacterial reverse mutation test (Ames test): Modulating effects of capsaicin against DEN-induced mutagenesis were assayed in a bacterial reverse mutation assay using *S. typhimurium* tester strains YG7108 (17,18), as TA1535 but is $\Delta ada_{ST}\Delta ogt_{ST}$, in the presence of S9 enzymes. The test was conducted by the pre-incubation method with modifications (22). Briefly, capsaicin dissolved in DMSO was mixed with S9 mix for 5 min on ice. Then, DEN dissolved in distilled water was added, followed by addition of overnight culture of *S. typhimurium* YG7108. The mixture was incubated for 20 min at 37°C and poured onto agar plates with soft agar. The plates were incubated for 2 days at 37°C. Assays were performed on triplicate. When the antimutagenic effects of capsaicin on other chemicals were examined, the test conditions, i.e., *S. typhimurium* strains and requirements for S9 mix, were as follows: 2-AA and Glu-P-1, TA98, +S9 mix; BP, TA100, +S9 mix; BPDE, TA100, −S9 mix; ENU, YG7108, −S9 mix.

Animals, diet and housing conditions: Female six-week-old F344 *gpt* delta transgenic rats (19) were obtained from Japan SLC and housed three or four animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23±2°C; relative humidity, 60±5%; with a 12:12-h light-dark cycle and free access to CRF-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water.

Treatments of animals: The protocol for this study was approved by the Animal Care and Utilization Committee of Kanazawa Medical University. Fifty-four rats were randomly divided into five groups (Fig. 1). Groups

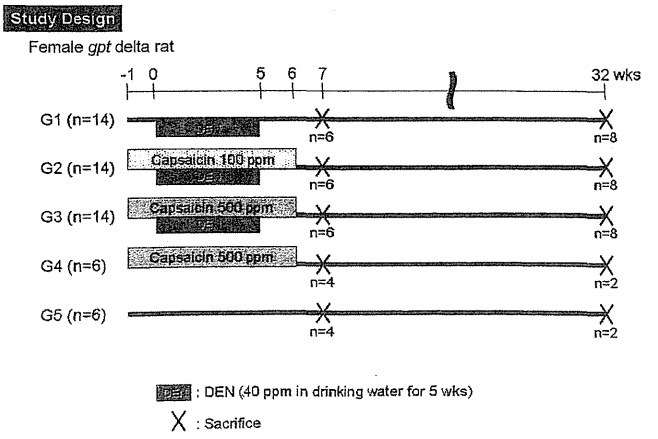

Fig. 1. Experimental protocol to examine *in vivo* modulating effects of capsaicin against DEN-induced mutagenesis and hepatocarcinogenesis using F344 *gpt* delta rats.

1 through 3 were treated with 40 ppm DEN in drinking water for five weeks. Group 2 was fed diets containing 100 ppm capsaicin, and Groups 3 and 4 were fed diets containing 500 ppm capsaicin for seven weeks, starting one week before DEN administration. Group 5 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly, and experimental diet consumption was recorded. The experiment was terminated at 32 weeks after the start of DEN administration. During the study, animals were killed at week 7 to determine the effects of capsaicin on the mutation frequency. At autopsy, liver, kidneys and spleen were macroscopically examined for the presence of pathologic lesions, and then isolated. Tissues were fixed in 10% buffered formalin and processed to hematoxylin and eosin (HE) stained sections. Neoplastic lesions of liver were histopathologically classified into adenomas and hepatocellular carcinomas (HCC). Left lobes of the livers from rats sacrificed at week 7 were excised and frozen in liquid nitrogen for mutation assay. Then, remaining livers were fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained by HE and histopathologically examined.

DNA isolation, *in vitro* packaging and *gpt* mutation assay: High-molecular-weight genomic DNA was extracted from the liver using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA, USA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* assay was conducted according to previously published methods (23). The mutant frequencies of the *gpt* gene (*gpt* MFs) in the liver were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of rescued plasmids.

Immunohistochemical procedures: Liver sections of 3 μm thickness from short period groups were treated with rabbit anti-rat GST-P antibody (1:1,000; Medical & Biological Laboratories, Nagoya, Japan). Immunohistochemical staining was done by the avidin-biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Inc., Burlingame, CA, USA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:200. Sections were lightly counterstained with hematoxylin for microscopic examination. Areas and numbers of GST-P-positive foci larger than 0.1 mm in diameter of the liver sections were quantitatively measured with an image processor for analytical pathology (IPAP-WIN; Sumika Technos Company, Osaka, Japan).

Statistical analysis: The statistical significance of the difference in the value of MFs between the treated groups and negative controls was analyzed by Student's *t*-test. A p value less than 0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight and pathological data were examined by Dunnett and Tukey multiple comparison post tests using GraghPad InStat (GraphPad Software, Inc., La Jolla, CA, USA) to compare the differences. The tumor incidence was examined by Yates $m \times n$ χ^2 test.

Results

Capsaicin enhanced mutagenicity of DEN in *S. typhimurium* YG7108: To examine modulating effects of capsaicin on DEN-induced mutagenicity, bacterial mutation assays with *S. typhimurium* YG7108 were performed (Fig. 2). Capsaicin itself was not mutagenic with or without S9 activation in *S. typhimurium* TA98, TA100 and YG7108. DEN itself at a dose of 200 μg/plate induced about 500 His^+ revertants per plate in the presence of S9 activation. When capsaicin was added in the reaction mixture, it enhanced the mutagenicity of DEN in a dose-dependent manner, and the number of His^+ revertants per plate reached about 4,000 at a dose of 50 μg capsaicin/plate. We also examined the modulating effects with 2-AA, BP and Glu-P-1 in the presence of S9 activation, and with ENU and BPDE in the absence of S9 activation. We used *S. typhimurium* strains TA98 or TA100 when the test chemicals were not alkylating agents. Capsaicin substantially enhanced mutations induced by 2-AA and BP in a dose-dependent manner as in the case of DEN. It enhanced mutations induced by BPDE dose-dependently at doses less than 100 μg of capsaicin per plate. Capsaicin slightly enhanced the mutagenicity of Glu-P-1 at lower doses and then reduced it at higher doses. Capsaicin reduced the mutagenicity of ENU.

General observation of *in vivo* study: To reveal modulating effects of capsaicin *in vivo*, genotoxicity assay and carcinogenesis study were conducted with *gpt* delta rats. Consumptions of capsaicin-mixed diets were 25% lower than those of normal diet at the first experimental week (Groups 2 to 4, data not shown). It recovered, however, to the level similar to the control group at the second experimental week. During drinking administration of 40 ppm DEN, growth of body weight of DEN-treated animals was slightly reduced (Groups 1 to 3, data not shown). At week 7, their body weight except for Group 3, which received DEN plus 500 ppm capsaicin (Suppl. Table 1, available at http://www.j-ems.org/journal/) did not differ from that of the control group. This difference was, however, not observed at week 32 (Suppl. Table 2, available at http://www.j-ems.org/journal/). Organ weight did not show any differences among the groups (Suppl. Table 1 and Suppl. Table 2).

Capsaicin did not affect formation of preneoplastic hepatocellular lesions: DEN induced small GST-P positive foci in liver at week 7 (Group 1, Table 1).

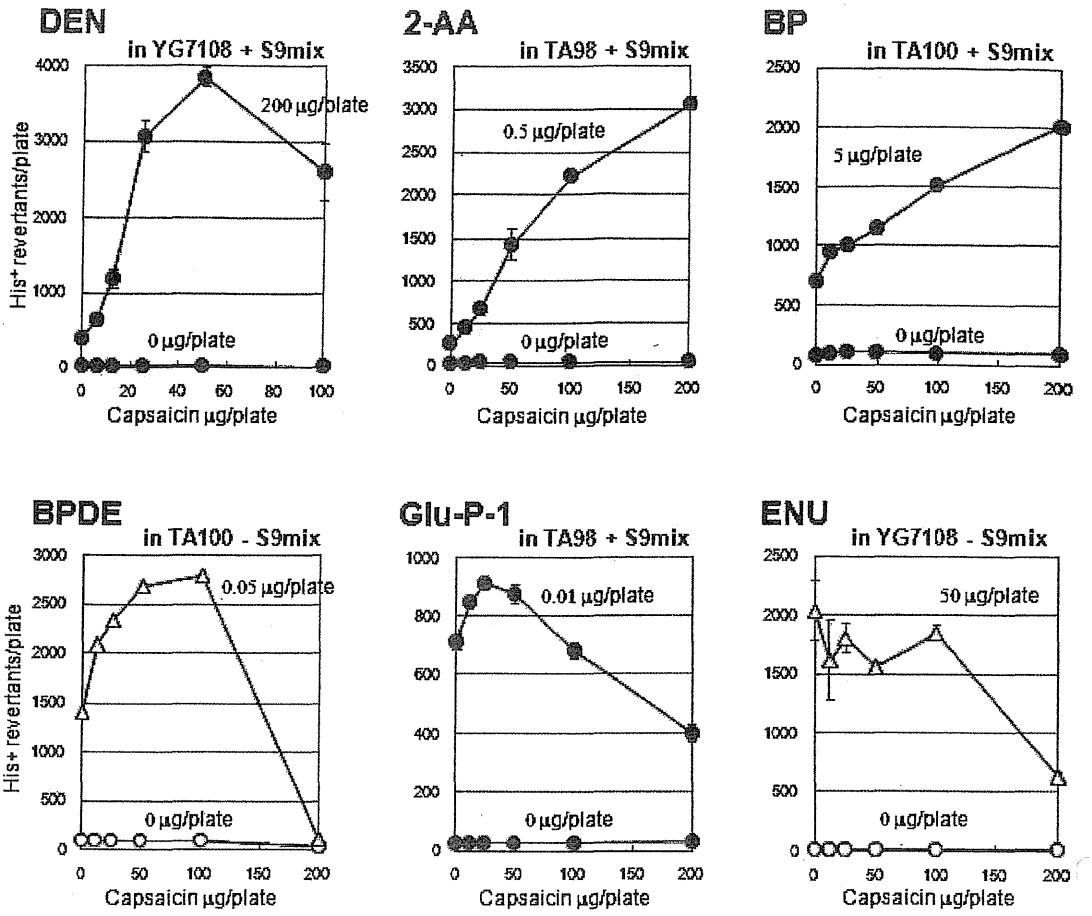

Fig. 2. Modulating effects of capsaicin on *in vitro* mutagenesis induced by DEN, 2-AA, BP, BPDE, Glu-P-1 and ENU. Doses of each chemical, 0.01–200 μg/plate, in the condition with chemical were indicated on the graph and doses of capsaicin were indicated on the X axis. Strains and S9 mix conditions are indicated on each panel.

Table 1. Quantification of GST-P positive foci at week 7

Group	Treatment		No. of rats	No. of foci [No./cm²]	Area of foci [mm²/cm²]
	DEN	Test chemical			
1	+	—	6	15.425 ± 7.233*	0.445 ± 0.280
2	+	Capsaicin 100 ppm	6	16.553 ± 10.543	0.538 ± 0.429
3	+	Capsaicin 500 ppm	6	20.405 ± 14.939	0.718 ± 0.816
4	−	Capsaicin 500 ppm	4	0.000 ± 0.000	0.000 ± 0.000
5	−	—	4	0.000 ± 0.000	0.000 ± 0.000

*Mean ± SD. Experimental period was 7 weeks. Values were compared with Group 1 by Student's *t*-test.

Quantitative analysis (number and area) of the lesions did not show any difference among the DEN-treated groups, i.e., Groups 1, 2 and 3 (Table 1). These preneoplastic lesions did not develop in the liver of rats treated without DEN (Groups 4 and 5).

Capsaicin did not affect gene mutations *in vivo*: DEN treatments enhanced *gpt* MF in the liver 200 times over the control levels (Table 2 and Suppl. Table 3, available at http://www.j-ems.org/journal/). Capsaicin was non-genotoxic (Group 4). Unlike the *in vitro* results, capsaicin treatments did not show any substantial effects on DEN-induced mutagenesis *in vivo*. The *gpt* MFs of Groups 2 and 3, which received DEN plus 100 and 500 ppm capsaicin, respectively, were not different from that of Group 1 that received DEN alone (Table 2).

Capsaicin slightly suppressed hepatocarcinogenesis: The incidence and multiplicity of tumors at week 32 were slightly reduced in the capsaicin treated groups (Groups 2 and 3, Table 3). The treatment with capsaicin

decreased the incidence and multiplicity of liver tumors by 20–40% and 47–64%, respectively. The number of adenomas per rat was significantly decreased by the capsaicin treatment ($p<0.05$), although the reduction of HCCs was statistically insignificant.

Discussion

In this study, we examined the modulating effects of capsaicin on DEN-induced mutagenesis *in vitro* and *in vivo*. We could not confirm previously reported mutagenicity of capsaicin in *S. typhimurium* TA98 and TA100 in the presence of S9 activation (9,11,13). Capsaicin was not mutagenic not only *in vitro* (Fig. 2) but also *in vivo* (Table 2). Purity of the samples may account for the different findings (24). Instead, we found that capsaicin effectively enhanced mutagenicity of DEN, 2-AA and BP in the presence of S9 activation *in vitro* (Fig. 2). At first, we assumed that capsaicin might modulate the activities of CYP enzymes involved in metabolic activation of the xenobiotics, thereby enhancing the mutagenesis. In fact, it is suggested that metabolites of capsaicin bind microsomal proteins, such as CYP enzymes (25–27). However, in this study capsaicin also enhanced mutations induced by BPDE without S9 activation. Therefore, we suggested that capsaicin modulated not only metabolic activation but also mutagenesis and/or DNA repair. Interestingly, capsaicin displayed opposite modulating effects on mutagenesis, i.e., reduction of mutagenesis by Glu-P-1 in the presence of S9 enzymes and by ENU in the absence of S9 (Fig. 2). These results suggest that capsaicin can suppress metabolic activation by S9 enzymes and mutagenesis/DNA repair in some cases. It is puzzling, however, why capsaicin enhanced mutations induced by DEN but reduced those induced by ENU, although both DEN and ENU induce mutagenic O^6-ethylguanine in DNA. One possible explanation for the complex modulating effects is that capsaicin might enhance membrane permeability of bacteria to chemical carcinogens, thereby displaying various modulating effects on the mutagenicity of chemicals. Complex modulating effects of capsaicin *in vitro* have been described by Huynh and Teel (28). They have reported that capsaicin at doses of 0.25 μmol (76.3 μg) and 0.5 μmol (152.7 μg) per plate reduced mutations in *S. typhimurium* TA98 induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and Glu-P-1, but enhanced those by Trp-P-2 in the presence of S9 enzymes (28). Collectively, our results illustrated in Fig. 2 along with other reports (2,29,30) suggest that capsaicin has ability to modulate multiple steps leading to mutations at least *in vitro*.

In contrast, capsaicin neither affected development of DEN-induced preneoplastic lesions, GST-P positive foci (Table 1) nor mutagenesis in the liver of rats (Table 2). These results were unexpected because capsaicin substantially enhanced the mutagenicity of DEN *in vitro* (Fig. 2) and it is reported that capsaicin inhibits metabolism of carcinogens, including dimethylnitrosamine (DMN), which are preferentially activated by CYP2E1 (26,27). In the report, capsaicin at 0.25 μmol (76.3 μg) per plate reduces the mutagenicity of DMN in

Table 2. Mutant frequency in Liver at week 7

Group	Treatment		No. of rats	Mutant frequency ($\times 10^{-6}$)
	DEN	Test chemical		
1	+	—	6	225.31±52.51*
2	+	Capsaicin 100 ppm	6	254.76±83.47
3	+	Capsaicin 500 ppm	6	245.10±114.52
4	−	Capsaicin 500 ppm	4	1.10±1.09
5	−	—	4	1.49±1.90

*Mean±SD. Values were examined by Student's *t*-test. Significant differences among groups 1, 2 and 3 were not observed.

Table 3. Pathological findings in liver at week 32

Group	Treatment		No. of rats	Incidence			Multiplicity		
				No. of rats with tumors			No. of tumors/ rats		
	DEN	Test chemical		Total	AD	HCC	Total	AD	HCC
1	+	—	8	8 (100%)	7 (88%)	5 (63%)	2.75±2.12*	1.75±1.28	1.00±1.07
2	+	Capsaicin 100 ppm	8	6 (75%)	5 (63%)	4 (50%)	1.38±1.30	0.75±0.71	0.63±0.74
3	+	Capsaicin 500 ppm	8	5 (63%)	5 (63%)	3 (38%)	1.13±1.13	0.63±0.52†	0.50±0.76
4	−	Capsaicin 500 ppm	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00
5	−	—	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00

AD, adenoma; HCC, hepatocellular carcinoma. *Mean±SD. †Significantly different from group 1 by Dunnett multiple comparison test ($p<0.05$).

S. typhimurium TA100 by more than 50% (26). A possible reason for the discrepancy between *in vitro* and *in vivo* findings is that, unlike *in vitro* where capsaicin can interact with S9 enzymes at high concentrations, the local concentration (tissue distribution) in the liver of rats might not be high enough to modulate the activity of CYP enzymes. In fact, there is a report suggesting that capsaicin did not inhibit any of CYP enzyme activities at concentrations occurring after ingestion of chili peppers (31). Direct inhibition may be observed at much higher concentrations. Although exact concentrations in the liver are unavailable in this study, the negative modulating effects of capsaicin in the liver of rats caution that chemopreventive effects of chemicals should be evaluated not only *in vitro* but also *in vivo*.

Interestingly, capsaicin slightly suppressed the incidence and multiplicity of hepatocellular tumors at week 32. Although the reduction in the multiplicity of liver cell carcinomas did not reach the statistical significance, the value of liver cell adenomas was significantly reduced by feeding with capsaicin at a dose level of 500 ppm ($p<0.05$, Table 3). Capsaicin was previously demonstrated to be chemopreventive against azoxymethane-induced colon carcinogenesis (7). Capsaicin has multiple biological activities, such as block of signal transduction pathways leading to carcinogenesis, induction of apoptosis, cell-cycle delay and anti-inflammation (1,3,5,6,32). It is unclear which biological activities are involved in the slight reduction of DEN-induced hapatocarcinogenesis. However, we suggest that antimutagenesis does not play roles in the weak chemopreventive ability because of the negative modulating effects on DEN-induced mutagenesis *in vivo*.

In summary, we revealed that capsaicin was slightly chemopreventive against liver cell tumors induced by DEN in rats through mechanisms other than antigenotoxicity. Our study highlights the importance of employment of multiple biological parameters, such as mutations and pathological biomarkers, to investigate the mechanisms underlying the chemopreventive effects of chemicals. In this regard, F344 *gpt* delta rats (19) are quite useful, because mutations *in vivo* as well as pathological alterations (incidences and multiplicities of tumors and preneoplasms) can be analyzed at the same time in target tissues of the same rats that received carcinogens and/or test agents.

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Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation

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Chronic inflammation is deeply involved in induction of aberrant DNA methylation, but it is unclear whether any type of persistent inflammation can induce methylation and how induction of cell proliferation is involved. In this study, Mongolian gerbils were treated with five kinds of inflammation inducers [*Helicobacter pylori* with cytotoxin-associated gene A (CagA), *H.pylori* without CagA, *Helicobacter felis*, 50% ethanol (EtOH) and saturated sodium chloride (NaCl) solution]. Two control groups were treated with a mutagenic carcinogen that induces little inflammation (20 p.p.m. of *N*-methyl-*N*-nitrosourea) and without any treatment. After 20 weeks, chronic inflammation with lymphocyte and macrophage infiltration was prominent in the three *Helicobacter* groups, whereas neutrophil infiltration was mainly observed in the EtOH and NaCl groups. Methylation levels of eight CpG islands significantly increased only in the three *Helicobacter* groups. By Ki-67 staining, cell proliferation was most strongly induced in the NaCl group, demonstrating that induction of cell proliferation is not sufficient for methylation induction. Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* showed increased expression specifically in the three *Helicobacter* groups. In human gastric mucosae infected by *H.pylori*, *NOS2* and *TNF* were also increased. These data showed that inflammation due to infection of the three *Helicobacter* strains has a strong potential to induce methylation, regardless of their CagA statuses, and increased cell proliferation was not sufficient for methylation induction. It was suggested that specific types of inflammation characterized by expression of specific inflammation-related genes, along with increased cell proliferation, are necessary for methylation induction.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is deeply involved in human carcinogenesis (1,2). As inducers of aberrant DNA methylation, aging and chronic inflammation have been suggested because methylation was present in colonic tissues of the aged (3) and patients with long-standing ulcerative colitis (4–6), in the liver with chronic hepatitis (7) and in gastric tissues with *Helicobacter pylori* (*H.pylori*)-induced gastritis (8,9). Especially in the stomach, accumulation levels of aberrant methylation correlate with risk of gastric cancers (8,10–12). Chronic inflammation is characterized by transition of inflammatory cell types from polymorphonuclear cells (mainly neutrophils) to mononuclear cells (lymphocytes and macrophages) and persistent cell proliferation (13). However, it is still unclear whether chronic inflammation with infiltration of mononuclear cells and expression of specific genes or simply persistent inflammation is important for methylation induction and how cell proliferation is involved in it.

As an animal model for methylation induction, we recently demonstrated that inflammation triggered by *H.pylori* infection induces aberrant methylation in the stomach of Mongolian gerbils (*Meriones unguiculatus*) (14). In the gerbil stomach, *H.pylori* with a bacterial virulence factor, cytotoxin-associated gene A (CagA), which is associated with a high risk of human gastric cancers (15), can induce more severe inflammation than that without (16). *Helicobacter felis*, which does not possess CagA (17), can induce chronic gastritis without direct damage of epithelial cells (18,19). High concentrations of ethanol (EtOH) and sodium chloride (NaCl) can induce gastric erosion associated with inflammation (20–22). Their repeated administration can induce persistent inflammation with cell proliferation without transition of inflammatory cell types. In contrast, little inflammation is induced by *N*-methyl-*N*-nitrosourea (MNU), a mutagenic gastric carcinogen (23).

Regarding inflammation-related genes, high expression of *IFNG*, *IL1B*, *TNF*, *NOS2* and *COX2* has been reported in human gastritis induced by *H.pylori* infection (24,25). Also in gerbils, high expression of *Ifng*, *Il1b*, *Cox2* and *Nos2* has been observed (26,27). Our previous time-course study after *H.pylori* infection and eradication in gerbils showed that expression levels of *Cxcl2*, *Il1b*, *Nos2* and *Tnf* were correlated with methylation levels in gastric epithelial cells (GECs) (14). In humans, a polymorphism of *IL1B* is associated with gastric cancer risk (28) and with methylation of multiple genes in gastric cancers (29).

In this study, using five inducers of inflammation (*H.pylori* with CagA, *H.pylori* without CagA, *H.felis*, EtOH and NaCl) and a carcinogen control (MNU), we aimed to clarify the roles of transition of inflammatory cell types, induction of cell proliferation and specific inflammation-related genes in methylation induction.

Materials and methods

Preparation of Helicobacter strains

Helicobacter pylori with CagA (ATCC 43504, also known as NCTC 11637) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Helicobacter pylori* without CagA, SS1, was kindly provided by Professor Takashi Joh at Nagoya City University (30). *Helicobacter felis* (ATCC 49179) was also obtained from ATCC. Each strain was inoculated in Brucella broth (Becton Dickson, Cockeysville, MD) with 7% vol/vol heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an AnaeroPack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 h. For the culture of *H.felis*, 0.1% wt/vol of BactoAgar (Becton Dickson) was supplemented. Before harvesting bacteria, their mobility and shape were confirmed under phase contrast microscopy.

Animal experiments and sample preparation

Five-week-old male Mongolian gerbils (MGS/Sea; Kyudo, Tosu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for *Helicobacter* treatment were inoculated with $\sim 10^8$ CFU/gerbil of *H.pylori* ATCC 43504 (ATCC group), *H.pylori* SS1 (SS1 group) or *H.felis* (HF group) and were kept without further treatment. Gerbils in groups of EtOH and NaCl treatment were administered with 5 ml/kg body wt of 50% EtOH group and saturated NaCl group, respectively, by gavage twice a week from 5 to 25 weeks of age. Gerbils in the group of MNU treatment (MNU group) were administered with 20 p.p.m. of MNU (Sigma–Aldrich, St Louis, MO) in drinking water from 5 to 25 weeks of age. A control group was kept without any treatment.

Abbreviations: CagA, cytotoxin-associated gene A; CGI, CpG island; Dnmt, DNA methyltransferase; EtOH, ethanol; GEC, gastric epithelial cell; MNU, *N*-methyl-*N*-nitrosourea; NaCl, sodium chloride; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcriptase–polymerase chain reaction.

At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

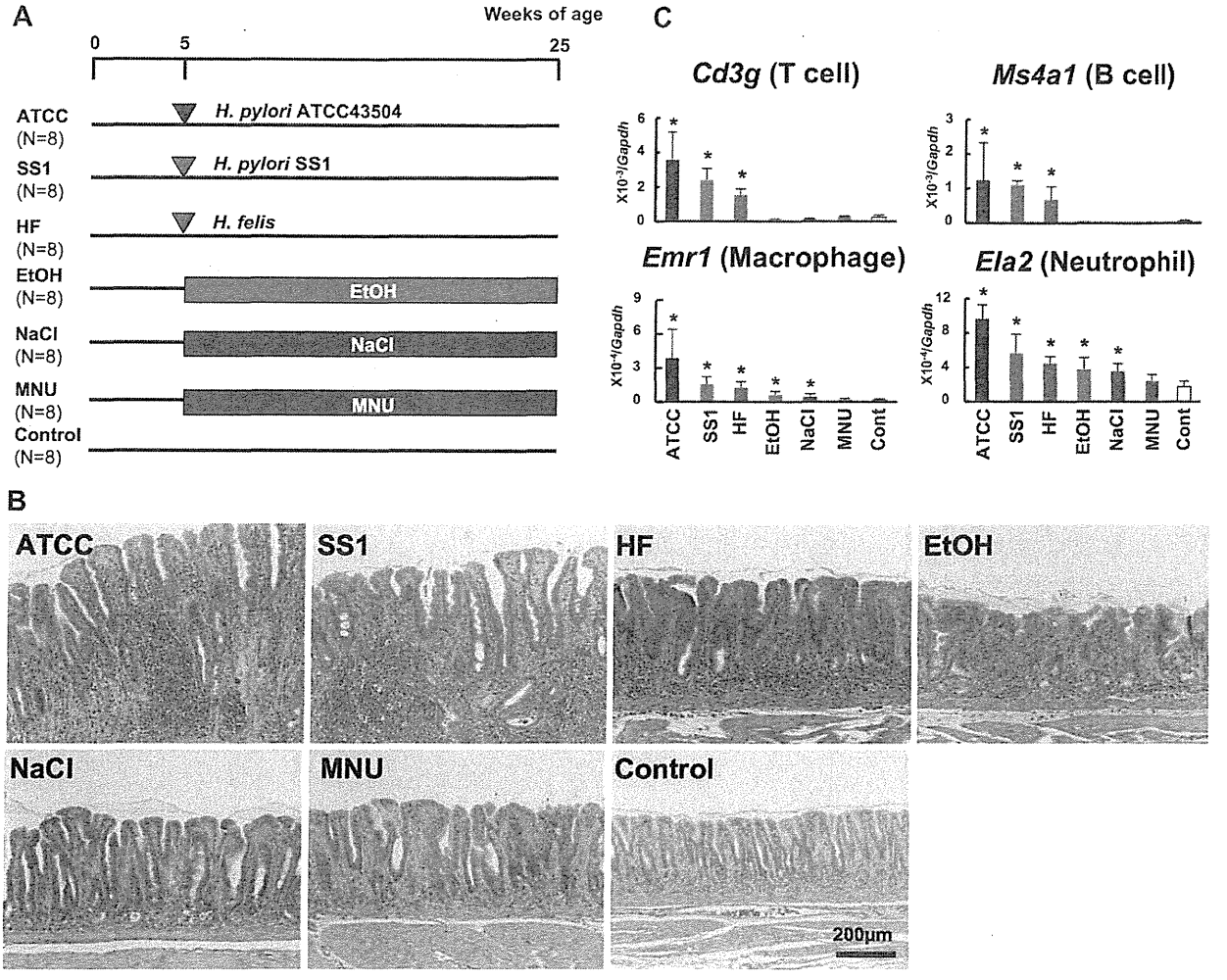

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^{*}P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
*$P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.

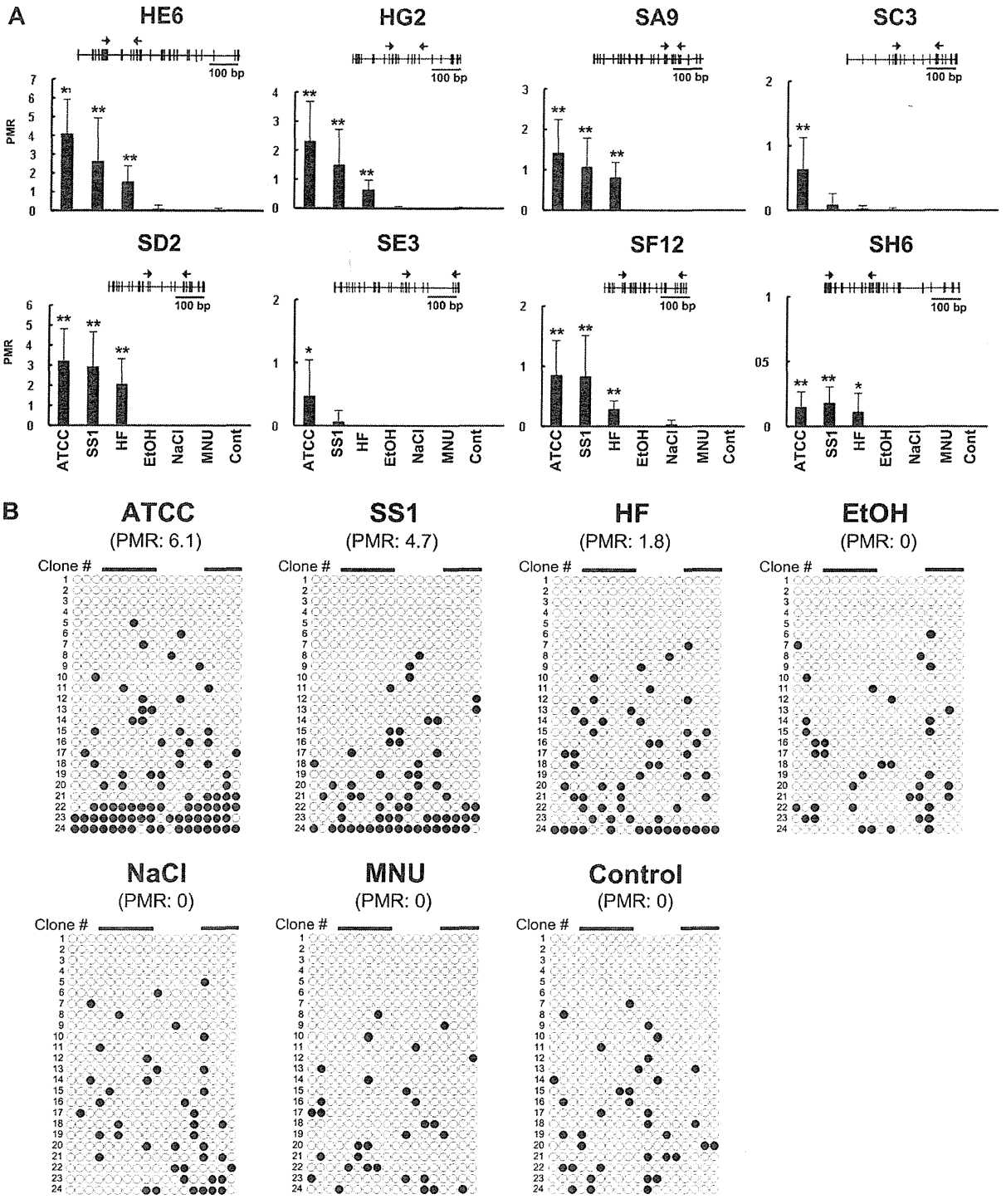

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

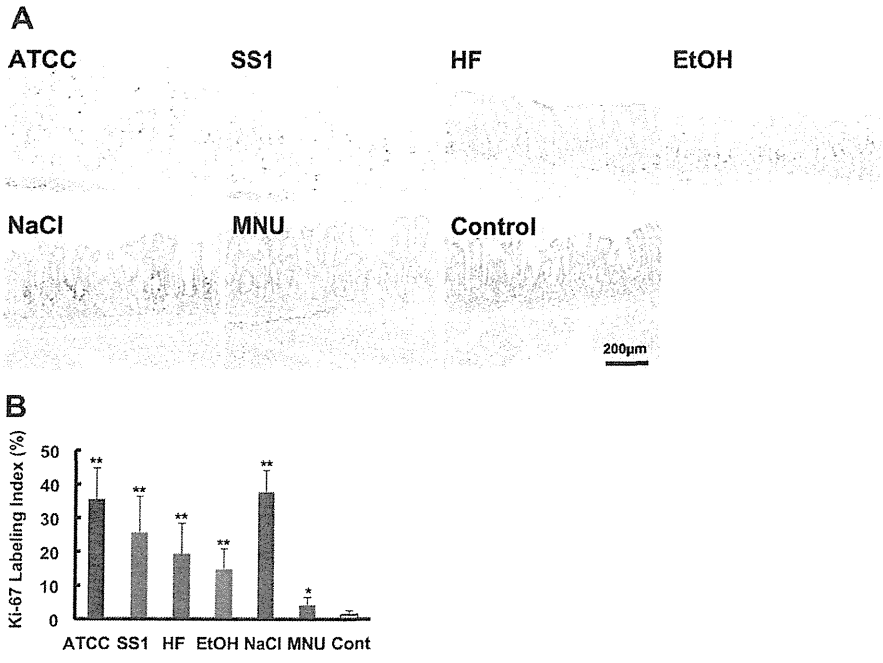

Fig. 3. Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase of cell proliferation.

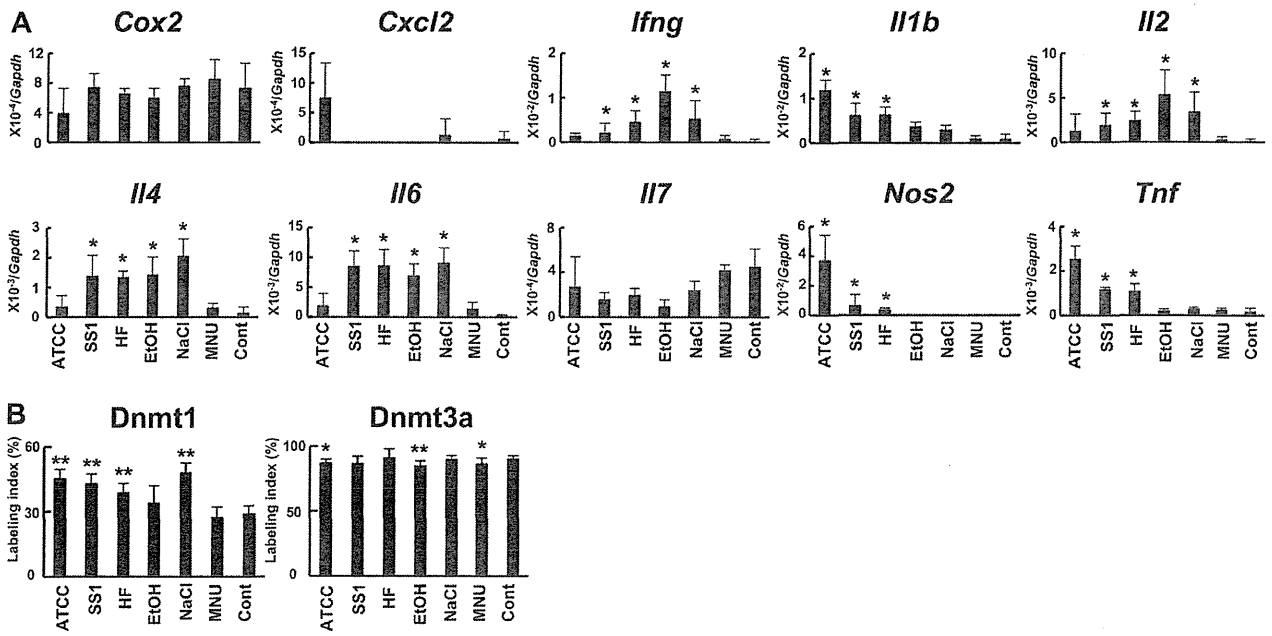

Fig. 4. Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* groups. (**B**) The fractions of GECs expressing Dnmt proteins in gastric glands by immunohistochemistry. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

Human relevance of inflammation-related gene expression

To address whether upregulation of specific inflammation-related genes are common in the human stomach, we conducted qRT–PCR of *COX2*, *IFNG*, *IL1B*, *IL6*, *NOS2* and *TNF* using human gastric mucosa samples with and without *H.pylori* infection. Expression levels of *NOS2* and *TNF* were markedly upregulated (27- and 3-fold,

respectively) also in human gastric mucosae (Figure 5). However, *IL1B* expression tended to be lower in gastric mucosae of *H.pylori*-infected individuals.

Discussion

Among the five groups with inflammation, aberrant methylation was induced only in the three *Helicobacter* groups, which showed inflammation with infiltration of mononuclear cells, increased expression of *Il1b*, *Nos2* and *Tnf* and increased cell proliferation. In the EtOH and NaCl groups, these agents were administered repeatedly for 20 weeks, and increased cell proliferation was present at the end of the experiment. The increased proliferation was considered to have persisted for this period because thickening of lamina propria was observed in these two groups. Nevertheless, aberrant methylation was not induced, at least in the CGIs analyzed here. This showed that cell proliferation alone is not sufficient for methylation induction and suggested that both specific types of inflammation and increased cell proliferation are necessary for induction of aberrant methylation.

The inflammation induced in the *Helicobacter* groups was characterized by infiltration of mononuclear cells (lymphocytes and macrophages). In our previous study, suppression of T-cell activation by cyclosporin A remarkably repressed inflammatory response and methylation induction triggered by *H.pylori* infection (14), showing that T-cell activation is involved in methylation induction in this system. However, our recent study in mouse colon demonstrated that aberrant methylation can be induced even in severe combined immunodeficiency mice, which lack functional T and B cells, by dextran sulfate sodium-induced colitis (Katsurano *et al.*, submitted for publication). It is known that, even in severe combined immunodeficiency mice, colitis with macrophage infiltration can be induced (36). If a common mechanism for methylation induction is present in *H.pylori*-infected gastric mucosae and dextran sulfate sodium-treated colonic mucosae, infiltration of macrophages is a candidate for the proximate effector that transmits signal for methylation induction to epithelial cells. It can be considered that, in *H.pylori*-infected gastric mucosae, activation of T cells is required only for the initiation or maintenance of inflammation capable of inducing aberrant DNA methylation.

Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* were specifically upregulated in the three *Helicobacter* groups. These three genes are reported to be overexpressed also in human chronic inflammation associated with cancers, such as ulcerative colitis and hepatitis (37–40). *IL1B* promoter polymorphism is associated with risk of human gastric cancers (28) and aberrant methylation of multiple genes in gastric cancers (29). The lack of its upregulation in human gastric mucosae infected with *H.pylori* could be because most of them had superficial gastritis and had already increased *IL1B* expression. *NOS2*, which encodes nitric oxide synthase, was upregulated *in vitro* by administration of IL1B and nitric oxide donors induced methylation of *FMR1* and *HPRT* (41). These suggest that IL1B and NOS2 might be involved in methylation induction. On the other hand, *Ifng*, *Il2*, *Il4* and *Il6* were upregulated mainly in the EtOH and NaCl groups, in which no methylation was induced, and also in the SS1 and HF groups, in which methylation induction levels were lower than in the ATCC group. This suggested a possibility that some (one) of the genes could suppress methylation induction.

SS1 and *H.felis*, which lack CagA, were capable of inducing aberrant methylation although the capacity was weaker than the CagA-positive strain (*H.pylori* ATCC 43504). CagA-positive *H.pylori* strains are known to induce severe gastritis in Mongolian gerbils (16) as confirmed in this study, and this explains their stronger capacity to induce methylation. The three inflammation-related genes associated with methylation induction (*Il1b*, *Nos2* and *Tnf*) had the highest expression in the ATCC group among the three *Helicobacter* groups. CagA-positive *H.pylori* seems to promote methylation induction by maximizing expression of such genes and minimizing expression of genes that suppress methylation induction.

Dnmts are the final effectors to methylate DNA, and their overexpression was observed in various human cancers (35). Immunohistochemical analyses here revealed that Dnmt1 was upregulated in gastric mucosae of gerbils in the three *Helicobacter*-infected groups and the NaCl-treated group. However, the highest expression was observed in the NaCl group, where methylation was not induced. This result indicated that expression of Dnmt1 was not associated with methylation induction but with cell proliferation. Expression of Dnmt3a was significantly but slightly decreased in the ATCC group and this also suggested that the expression itself is not involved in aberrant methylation induction. However, due to the lack of an appropriate antibody, we were not able to exclude the possibility that upregulation of Dnmt3b is involved in methylation induction. Therefore, disturbance in the local balance between Dnmts and factors that protect DNA from aberrant methylation, such as the presence of RNA

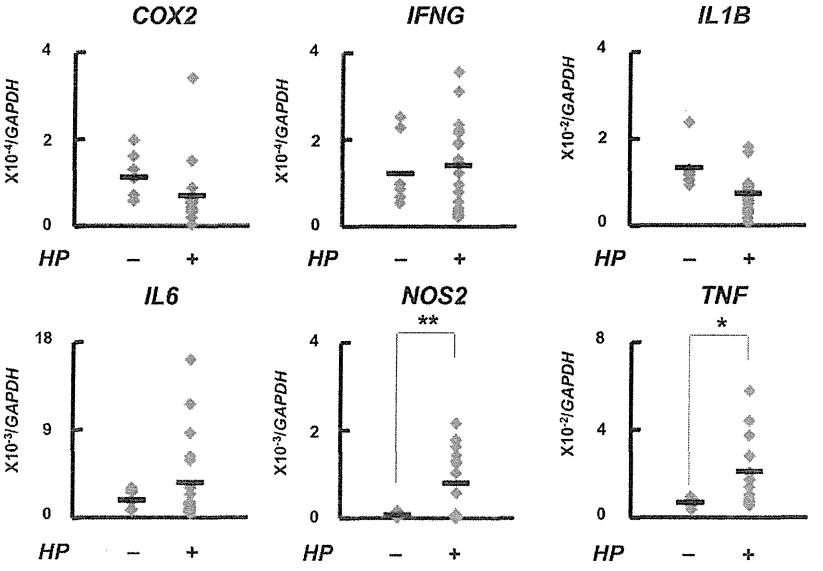

Fig. 5. Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

polymerase II (42) and/or possible overexpression of Dnmt3b might be involved in methylation induction.

In conclusion, inflammation due to infection of *Helicobacter* strains had a high capacity to induce methylation in GECs, regardless of their CagA status. Increased cell proliferation was not sufficient for methylation induction. Therefore, specific types of inflammation, characterized by infiltration of mononuclear cells and expression of specific inflammation-related genes, along with increased cell proliferation were considered to be necessary for methylation induction.

Supplementary material

Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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RESEARCH COMMUNICATION

Long-term High-dose Proton Pump Inhibitor Administration to *Helicobacter pylori*-infected Mongolian gerbils Enhances Neuroendocrine tumor Development in the Glandular Stomach

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Abstract

Proton pump inhibitors (PPIs) are routinely used for control of upper gastrointestinal disorders, often with long-term application. However, there has been some concern about the long-term safety and the possibility of cancer induction and development of neuroendocrine tumors (NET) in the stomach. We therefore analyzed the influence of PPI use on tumor development histologically, immunohistochemically, and serologically in the glandular stomachs of Helicobacter pylori (Hp)-infected and uninfected Mongolian gerbils (MGs). 53 MGs were divided into 6 groups: Hp+25PPI, Hp+5PPI, Hp, 25PPI, 5PPI, and controls. The high-dose Hp+25PPI and 25PPI groups received the PPI (lansoprazole) at 25mg/kg/day, and the low-dose Hp+5PPI and 5PPI groups were given 5mg/kg/day. After 50 or 100 weeks, animals were sacrificed humanely, and the glandular stomach samples were evaluated histologically and phenotypically, using antibodies against chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP). Serum gastrin levels were also examined. NETs occurred in the Hp+25PPI, Hp+5PPI, Hp, and 25PPI groups, but there was no synergistic effect between Hp-infection and high-dose PPI administration. Serum gastrin was increased statistically by Hp infection and high-dose PPI administration, but not influenced by the low-dose. The NETs featured expression of CgA, but not gastrin or GIP. In conclusions, PPI at low dose had no influence on development of carcinomas and NETs in the Hp-infected and uninfected glandular MG stomach, suggesting clinical safety. However, PPI at high dose increased NET development and serum gastrin in the MG model.

Keywords: Neuroendocrine tumors - proton pump inhibitor - *Helicobacter pylori* - Mongolian gerbil

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Introduction

Proton pump inhibitors (PPIs) are routinely used to control upper gastrointestinal disorders such as peptic ulcers and gastro-esophageal reflux disease (GERD), often for long periods of time. However, there has been some concern about their long-term safety (Poulsen et al., 2009). Most PPI users have moderate hypergastrinemia due to the inhibition of gastric acid secretion (Lamberts et al., 1988; Klinkenberg-Knol et al., 1994), which may increase the development of neuroendocrine tumors (NETs) (Bardram et al., 1986; GillenMcColl, 2001) and carcinomas (Laine et al., 2000; Waldum et al., 2005; Kuipers, 2006) in the stomach. It has been reported that the long-term PPI use is associated with an increased incidence of atrophic gastritis (Kuipers et al., 1996), a precursor of stomach cancer (Kuipers et al., 1996; YeNyren, 2003) in patients with the *Helicobacter pylori* (Hp), but concrete conclusions have yet to be drawn.

The Mongolian gerbil (MG) model is useful for examining the link between Hp infection and human stomach disorders, as the lesions induced by Hp in this experimental animal resemble those apparent in man (Hirayama et al., 1996). The Hp-infected and chemical carcinogen-treated MG has proved very useful for the analysis of stomach carcinogenesis (Tatematsu et al., 2005). Recently, several reports have shown development of NETs in Hp-infected MGs (Kagawa et al., 2002; Cao et al., 2008). Regarding the histogenesis of cancers and NETs in the gastrointestinal tract, we have previously

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