Prevention of Helicobacter pylori–Induced Gastric Cancers in Gerbils by a DNA Demethylating Agent

Tohru Niwa1, Takeshi Toyoda2, Tetsuya Tsukamoto3, Akiko Mori1, Masae Tatematsu4, and Toshikazu Ushijima1

Abstract

Suppression of aberrant DNA methylation is a novel approach to cancer prevention, and so far, the efficacy of the strategy has not been evaluated in cancers associated with chronic inflammation. Gastric cancers induced by Helicobacter pylori infection are known to involve aberrant DNA methylation and associated with severe chronic inflammation in their early stages. Here, we aimed to clarify whether suppression of aberrant DNA methylation can prevent H. pylori–induced gastric cancers using a Mongolian gerbil model. Administration of a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), to gerbils (0.125 mg/kg for 50–55 weeks) decreased the incidence of gastric cancers induced by H. pylori infection and N-methyl-N-nitrosourea (MNU) treatment from 55.2% to 23.3% (P < 0.05). In gastric epithelial cells, DNA methylation levels of six CpG islands (HE6, HG2, SB1, SB5, SF12, and SH6) decreased to 46% to 68% (P < 0.05) of gerbils without 5-aza-dC treatment. Also, the global DNA methylation level decreased from 83.0% ± 4.5% to 80.3% ± 4.4% (mean ± SD) by 5-aza-dC treatment (P < 0.05). By 5-aza-dC treatment, Il1b and Nos2 were downregulated (42% and 58% of gerbils without, respectively) but Tnfα was upregulated (187%), suggesting that 5-aza-dC treatment induced dysregulation of inflammatory responses. No obvious adverse effect of 5-aza-dC treatment was observed, besides testicular atrophy. These results showed that 5-aza-dC treatment can prevent H. pylori–induced gastric cancers and suggested that removal of induced DNA methylation and/or suppression of DNA methylation induction can become a target for prevention of chronic inflammation–associated cancers. Cancer Prev Res; 6(4): 263–70. ©2013 AACR.

Introduction

DNA methylation is an epigenetic mechanism for gene regulation. Methylation of promoter CpG islands (CGIs) consistently suppresses expression of their downstream genes (1), and physiologic methylation of retrotransposons is involved in their transcriptional repression (2). In cancers, tumor suppressor genes are frequently inactivated by aberrant methylation of their promoter CGIs (3, 4). Such aberrant methylation is present not only in cancers but also in noncancerous tissues exposed to chronic inflammation, such as colonic mucosae with ulcerative colitis, liver tissues exposed to hepatitis, and gastric mucosae exposed to chronic gastritis (5–10). In the case of the stomach, Helicobacter pylori infection is known to induce severe chronic inflammation (11–13) and aberrant methylation in gastric epithelial cells (GEC; ref. 14). Accumulation levels of aberrant methylation in gastric mucosae correlate with risk of gastric cancers (8–10).

Suppression of aberrant methylation is considered as one of the novel targets for cancer chemoprevention (15, 16). Traditionally, chemoprevention has used substances based on 2 strategies: the anti-initiation and anti-promotion/progression strategies (17–19). In the former strategy, blockage of activity of carcinogens that induce genetic or epigenetic alterations and enhancement of repair systems have been targeted. In the latter strategy, suppression of proliferation of initiated cells and induction of their apoptosis have been targeted. However, neither of these strategies targeted removal of genetic or epigenetic alterations accumulated in the cells, which can be achieved by DNA demethylating agents, such as 5-aza-2′-deoxycytidine (5-aza-dC; refs. 20, 21).

The usefulness of DNA demethylating agents in cancer chemoprevention has been shown in several animal models, including intestinal tumors in Apcmin/+ mice (22, 23), prostate tumors in transgenic mice harboring probasin promoter-driven SV40 antigen (24), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone–induced mouse lung tumors (25), and 4-nitroquinoline 1-oxide–induced mouse liver tumors (26).
oral tumors (26). Genetic suppression of a maintenance DNA methyltransferase (Dnmt1) also suppressed tumor development in some of these models (22, 27, 28). However, so far, the efficacy of suppression of aberrant DNA methylation was not evaluated in chronic inflammation–associated cancers, in which aberrant DNA methylation is heavily involved (29, 30). From this aspect, gastric cancers induced by H. pylori infection of Mongolian gerbils (Meriones unguiculatus) have several advantages. In gerbils, H. pylori infection induces severe chronic inflammation, as in humans, and promotes gastric cancers initiated by N-methyl-N-nitrosourea (MNU; refs. 31, 32). Also, 10 CGIs have already been established as markers that can be methylated by H. pylori infection, and a critical role of inflammation triggered by H. pylori infection, not a direct effect of H. pylori, in methylation induction has been shown (14). In contrast, few markers for methylation induction have been isolated in H. pylori–or Helicobacter felis–infected mice, except Tff2 promoter (33).

In this study, using the gerbil model, we aimed to clarify whether 5-aza-dC treatment can prevent chronic inflammation–induced gastric cancers and evaluate its effects on methylation induction and inflammation triggered by H. pylori infection.

Materials and Methods

Animals and sample preparation

Male Mongolian gerbils (MGS/Sea) were purchased from Kyudo and divided into 10 groups (G1–6 in Fig. 1A and G7–10 in Fig. 2A). Gerbils were inoculated with H. pylori (~4 × 10⁸ colony-forming units (CFU)/gerbil, ATCC 43504; American Type Culture Collection) at 5 weeks of age (34). In a carcinogenicity experiment, 10 ppm of MNU (Sigma-Aldrich) was given in drinking water to gerbils. 5-Aza-dC (125 µg/kg body weight in sterilized PBS; Sigma-Aldrich) was administered to gerbils intraperitoneally twice per week. The dose was selected from the 3 doses (125, 250, and 500 µg/kg) tested in a preliminary experiment for lack of toxicity. Timing and duration of the treatments are shown in Figs. 1A and 2A.

The stomach was resected and cut along the greater curvature. In a carcinogenicity experiment (G1–6), the antral region was fixed in formalin for histologic analysis. From the body region, GECs were isolated by the gland isolation technique (35) and stored in 100% ethanol at −80°C until DNA extraction. The testes, small intestine, liver, and kidneys were resected, and half parts were fixed in formalin. The other halves were snap-frozen for DNA extraction. In an experiment to induce H. pylori–triggered gastritis (G7–10), the antral region was cut into 2 pieces—one was snap-frozen for RNA extraction and the other half was fixed in formalin.

In both experiments, samples (tissues or GECs) were digested with proteinase K, and gDNA was extracted by the standard phenol/chloroform method. RNA of gastric tissue was isolated using ISOGEN (Nippon Gene). Whole blood was obtained from the inferior vena cava, and gDNA was extracted by a QuickGene DNA Whole Blood Kit (Fujifilm).

All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

Histological analysis

Formalin-fixed tissues were sliced along the longitudinal axis into strips of 5 mm width and embedded in paraffin. Sections of 3 µm thickness were prepared and stained with hematoxylin and eosin. Neoplastic lesions in the stomach were diagnosed as previously described (36). The size of a gastric cancer was determined as the maximum diameter of the gastric cancer in the neighboring sections. The degree of infiltration of mononuclear and polymorphonuclear cells was graded on a 4-point scale (0–3; 0, no or faint; 1, mild; 2, moderate; 3, marked) as described (37).

Luminometric methylation assay

gDNA from whole blood was amplified by an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) and used as fully unmethylated DNA. The unmethylated DNA was methylated by SssI methylase (New England Biolabs) and used as fully methylated DNA. A series of standard DNA was prepared by serial mixing of the unmethylated DNA and the methylated DNA.

Luminometric methylation assay (LUMA) was conducted as described (38) with slight modifications. Briefly, 3 µg of DNA was digested with 2 pairs of restriction enzymes (HpaII and EcoRI or MspI and EcoRI) in independent tubes (all restriction enzymes were purchased from Toyobo). The DNA was purified with a DNA Clean & Concentrator Kit (Zymo Research) and eluted in 40 µL of an annealing buffer (2 mmol/L magnesium acetate and 20 mmol/L Tris-acetate, pH 7.6). Using the PSQ 96 Pyrosequencing System (Qiagen), 5′-CG overhang produced by HpaII (or MspI) and 5′-AATT overhang produced by EcoRI was sequenced, and an HpaII/EcoRI (or MspI/EcoRI) signal ratio was determined. An HpaII/MspI value was obtained as (HpaII/EcoRI)/(MspI/EcoRI) in each sample. The HpaII/MspI value was compared with those of the standard DNA series, and the global methylation level (GML), which is equivalent to the percentage of methylated DNA in the standard DNA, was determined.

Quantitative methylation-specific PCR

DNA digested with BanII was treated with sodium bisulfite as described (39) and used as a template for real-time PCR. With primer sets specific to methylated CGIs (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6; Supplementary Fig. S1) and a B2 repeat sequence, real-time PCR was carried out as described (14). On the basis of the copy number of sequences measured by real-time PCR, the methylation level was calculated as a percentage of the methylated reference (PMR), which was obtained as [(number of methylated fragments of a target CGI in sample)/(number of the B2 repeat in sample)]/[number of
methylated fragments of a target CGI in SssI-treated DNA)
(number of the B2 repeat in SssI-treated DNA) \times 100.

**Gene expression analyses**

The number of cDNA molecules was quantified by quantitative reverse transcriptase-PCR (qRT-PCR) using gene-specific primers (Il1b, Nos2, and Tnf) as described (14). The number of cDNA molecules of a target gene was normalized to that of Gapdh.

**Genomic PCR and sequencing**

A forward primer (5'-AGATTCCTTGATGCGCGGTCGTC-3') was designed in a region of the mouse Tnf promoter highly conserved with the human corresponding region. A reverse primer (5'-AGATTCCTTGATGCGCGGTCGTC-3') was designed on the gerbil Tnf mRNA sequence (AB177841). The gerbil Tnf promoter was amplified using these primers, and the PCR product was directly sequenced with the same primers. The obtained sequence was
registered in GenBank (AB762083.1). A CGI was searched by a EMBOSS CpG report program (40).

**Statistical analysis**

Statistical analyses were conducted by SPSS 13.0J (SPSS Japan Inc.). To evaluate significant difference between 2 independent groups of sample data, a Mann–Whitney U test was used. The difference of the proportion between 2 groups was evaluated by Fisher exact test.

**Results**

**Suppression of *H. pylori*/MNU-induced gastric carcinogenesis by 5-aza-dC**

To evaluate cancer prevention effects of 5-aza-dC, a carcinogenicity experiment was carried out (Fig. 1A). Among the gerbils with MNU treatment and *H. pylori* infection (G5 and G6), 5-aza-dC treatment decreased incidence of gastric cancers from 55.2% (G5) to 23.3% (G6, *P* < 0.05; Table 1). The incidence in G6 was similar to that in MNU-treated gerbils without *H. pylori* infection (G2, 20.7%). There were no significant differences in the tumor multiplicity and size among the groups. These results clearly showed that 5-aza-dC treatment suppressed *H. pylori*/MNU-induced gastric carcinogenesis in gerbils and suggested that it might have completely abrogated the cancer promotion effects of *H. pylori* infection.

Regardless of 5-aza-dC treatment, gerbils with MNU treatment (G2, G3, G5, and G6) showed low body weight than the gerbils without MNU treatment (G1 and G4), showing that the body weight loss was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S2). Survival rates started to decrease from 25 weeks of age, and the decrease was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S3). This showed that the dose of 5-aza-dC used in this study (125 mg/kg body weight) had no obvious effects on body weight and survival of gerbils.

**Reduction of DNA methylation levels in GECs by 5-aza-dC**

To confirm the demethylating effects of 5-aza-dC in vivo, methylation analyses were conducted in GECs. First, the global DNA methylation level was measured by LUMA, in which global CCGG methylation was measured by using

<table>
<thead>
<tr>
<th>Table 1. Suppression of gastric cancers by 5-aza-dC</th>
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<tr>
<td><strong>Adenocarcinoma</strong></td>
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<td><strong>Group</strong></td>
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<tr>
<td>G1, HP(−) + DW</td>
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<tr>
<td>G2, HP(−) + MNU + PBS</td>
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<td>G3, HP(−) + MNU + 5-aza-dC</td>
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<tr>
<td>G4, HP(+) + DW</td>
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<td>G5, HP(+) + MNU + PBS</td>
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<tr>
<td>G6, HP(+) + MNU + 5-aza-dC</td>
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NOTE: 5-Aza-dC treatment decreased incidence of gastric cancers (adenocarcinomas) from 55.2% (G5) to 23.3% (G6).

*P* < 0.05 compared with G3.

*P* < 0.05 compared with G5.
a combination of methylation-sensitive and -insensitive restriction enzymes and pyrosequencing. Among the gerbils with MNU treatment and H. pylori infection (G5 and G6), 5-aza-dC treatment decreased the global methylation level from 83.0% ± 4.5% (mean ± SD)% (G5) to 80.3% ± 4.4% (G6, P < 0.05; Fig. 1B). Among the MNU-treated gerbils without H. pylori infection (G2 and G3), the methylation level was decreased by 5-aza-dC treatment (from 84.4% ± 2.3% in G2 to 82.2% ± 2.4% in G3, P < 0.05). No significant influence of H. pylori infection or MNU treatment was observed. These results indicated that 5-aza-dC worked as a DNA demethylating agent in vivo and decreased global methylation levels in GECs.

Next, methylation of 10 CGIs, where H. pylori infection was previously shown to induce aberrant methylation (Supplementary Fig. S1; refs. 14, 41), was analyzed by quantitative methylation-specific PCR (qMSP). 5-Aza-dC treatment reduced methylation levels in G6 to 46% to 80% of those in G5 for 6 CGIs (HE6, HG2, SB1, SB5, SF12, and SH6; P < 0.05; Fig. 1C). These results showed that 5-aza-dC treatment suppressed methylation induction by H. pylori infection and MNU treatment in GECs. The methylation levels in G5 were higher than those in G4 whereas those in G2 were not elevated compared with those in G1, indicating that MNU treatment had an augmenting effect on H. pylori–induced aberrant methylation.

Dysregulation of inflammation-related genes by 5-aza-dC

Among 10 inflammation-related genes whose expression was examined in the stomach, expression of 3 genes (Il1b, Nos2, and Tnf) has been shown to be associated with induction of methylation in GECs (14, 41). Therefore, we examined whether 5-aza-dC treatment affected expression of these 3 genes in the stomach after H. pylori infection using H. pylori–infected and -uninfected gerbils without MNU treatment (Fig. 2A). In the H. pylori–infected gerbils with 5-aza-dC treatment (G10), expression levels of Il1b and Nos2 decreased to 42% and 58%, respectively (P < 0.01, respectively), of those in H. pylori–infected gerbils without 5-aza-dC treatment (G9; Fig. 2B). In contrast, Tnf was upregulated to 187% of G9 (P < 0.01). These results indicated that 5-aza-dC treatment caused up- and downregulation, namely dysregulation, of inflammation-related genes. As there was a possibility that upregulation of Tnf was due to demethylation of its promoter CGI, we sequenced its promoter region but found that there was no CGI (Supplementary Fig. S4). Because methylation of only promoters with CGIs can consistently silence their downstream genes (42), we considered that the upregulation of Tnf was unlikely to be due to demethylation by 5-aza-dC.

We also analyzed infiltration of inflammatory cells in the stomach. In gerbils with H. pylori infection (G9 and G10), 5-aza-dC treatment did not affect infiltration of mononuclear cells and polymorphonuclear cells (Fig. 2C). In gerbils without H. pylori infection (G7 and G8), there was little mononuclear cell and polymorphonuclear cell infiltration, and no effect of 5-aza-dC treatment was observed. These results showed that 5-aza-dC treatment did not affect infiltration of inflammatory cells.

Extra-gastric effects of 5-aza-dC treatment

To evaluate possible adverse effects of 5-aza-dC treatment, we first conducted macroscopic analysis. Although most organs did not show any abnormality, the testes were prominently atrophic in 5-aza-dC–treated gerbils [0.47 ± 0.05 (mean ± SD) g/2 testes in G3 and 0.50 ± 0.05 g/2 testes in G6] compared with gerbils in other groups (1.10 ± 0.11 g/2 testes in G2 and 1.09 ± 0.13 g/2 testes in G5).

We then conducted analysis of histopathologic abnormalities and global DNA methylation levels in the testes, small intestine, liver, and kidneys. In the testes of 5-aza-dC–treated gerbils, the numbers of spermatozoa and spermatids were markedly decreased regardless of H. pylori infection.
infection status (Fig. 3A). Despite the presence of hyperpermatogenesis, there was no significant decrease of the global methylation level (Fig. 3B). In contrast, in the small intestine, the global methylation level was reduced by 10.4% and 5.6% (G3 and G6, respectively Fig. 3B). However, no histologic changes compared with the untreated gerbils were observed (Fig. 3A). As for the liver and kidneys, there were no histologic abnormalities or reduction of global methylation levels in G3 and G6 (Supplementary Fig. S5).

Discussion

Our study using a gerbil model showed that 5-aza-dC treatment suppressed H. pylori/MNU-induced gastric cancers. This study showed for the first time that chemoprevention using a DNA demethylating agent is effective for chronic inflammation–associated cancers. As chronic inflammation contributes to about 25% of all cancer cases (43), and aberrant DNA methylation is frequently observed in tissues exposed to chronic inflammation (29), suppression of aberrant methylation might become an effective preventive approach for these types of cancers. This study also showed that induction of aberrant methylation is an important mechanism for gastric carcinogenesis by H. pylori infection.

As mechanisms of suppression of gastric cancers by 5-aza-dC, at least 2 modes of action were present. The first one was the DNA demethylating effect. 5-Aza-dC treatment decreased global methylation level in GECs and suppressed hypermethylation of CGIs. These results suggested that 5-aza-dC was capable of removing aberrant DNA methylation induced by H. pylori infection and thereby prevented cancer development. The second one was the effect on inflammation. It was previously shown that inflammation triggered by H. pylori infection is pivotal for aberrant methylation induction, and expression of inflammation-related genes (Il1b, Nos2, and Tnf) in the stomach is associated with the induction (14, 41). The present study showed that 5-aza-dC treatment dysregulated expression of these genes. Therefore, there is a possibility that altered balances among the related cytokines might have led to the reduced methylation induction.

Recently, in the stomach of hypergastrinemic INS-GAS mice, inhibition of H. pylori (H. felis)-induced global demethylation by folic acid supplementation was reported to suppress gastric dysplasia (44). The data are seemingly discordant with our data showing a cancer preventive effect by DNA demethylation. However, in the INS-GAS mice, global hypomethylation by H. felis was evident, suggesting that the demethylation plays important roles in the carcinogenesis. Hypermethylation by folic acid might exert the cancer preventive effect via suppression of global demethylation. In contrast, in our gerbil study, hypermethylation of CGIs by H. pylori infection, rather than global demethylation, was evident, suggesting that hypermethylation of CGIs rather than global demethylation was the major mechanism for the carcinogenesis. Thus, demethylation by 5-aza-dC was considered to have exerted the preventive effect via suppression of hypermethylation of CGIs.

No obvious adverse effect of 5-aza-dC treatment was observed besides hyperpermatogenesis in the testes. Hyperpermatogenesis due to 5-aza-dC treatment was reported in mice (24). As global hypomethylation was not detected in the testes of the 5-aza-dC-treated gerbils, the effect was speculated to be independent of its DNA demethylating activity. However, we cannot exclude the possibility that decrease of methylation was not detected due to immediate elimination of spermatozoa/spermatids with decreased DNA methylation. Regardless of the mechanism, the presence of this adverse effect precludes 5-aza-dC as a chemoprevention agent for the general population. However, chemoprevention by a DNA demethylating agent itself still might become a promising strategy if a DNA demethylating agent without such toxicity is developed.

Demethylating effects by 5-aza-dC were observed in the stomach and the small intestine, but not in the liver and the kidneys. Specific global demethylation in the stomach and intestine was also observed in the female ApcMmin/+ mice administered a demethylating agent, zebularine (23). Turnover of epithelial cells in these tissues is known to be very rapid, being 3 to 4 days in mice (45, 46). As demethylating effects of 5-aza-dC and zebularine are exerted after their incorporation into gDNA and DNA replication, rapid cell turnover in the stomach and intestine could explain the organ-specific global demethylation.

Individuals with a severe epigenetic field defect, in contrast with the general population, can be considered as a target population for cancer prevention using 5-aza-dC after careful balancing of its preventive and adverse effects. In the case of gastric cancers, eradication of H. pylori is the primary strategy for prevention (47), but the incidence of gastric cancers remains high, even after H. pylori eradication, especially in persons with intestinal metaplasia and gastric atrophy (48). Notably, aberrant methylation in gastric mucosa decreases by H. pylori eradication, but it does not disappear completely (14, 49). The level of the remaining methylation reflects the risk of gastric cancers (8, 9). As DNA demethylating agents are likely to remove such accumulated methylation and suppress gastric cancers development, these individuals with a severe epigenetic field defect may benefit from epigenetic chemoprevention.

In summary, treatment with 5-aza-dC effectively prevented gastric cancers induced by H. pylori infection in gerbils, suppressed DNA methylation in GECs, and induced dysregulation of inflammation. Chemoprevention with a DNA demethylating agent is expected to become an effective strategy for prevention of chronic inflammation–associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Authors' Contributions
Conception and design: T. Niwa, M. Tatematsu, T. Ushijima
Development of methodology: T. Niwa, T. Tsukamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Niwa, T. Toyota
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Niwa, T. Toyota
Writing, preparation, or revision of the manuscript: T. Niwa, T. Ushijima
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Mori
Study supervision: M. Tatematsu

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