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 mucosa 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). Methylation was 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.

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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 (Mergusunguiculatus) (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 Campyo (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 harvested 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, Tozu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for Helicobacter treatment were inoculated with ~105 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; CGL, 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/6J, 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 degree 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 Histovet 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 Vector 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 gland images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric gland images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan).

**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 peripheral blood cells (14) were purchased and 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 animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

**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 PCR. Gerbils in the NaCl group showed very high Ki-67 labeling indices. 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, SC3, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

**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.
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.

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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Infiltration of mononuclear cells</th>
<th>Infiltration of polymorphonuclear cells</th>
<th>Intestinal metaplasia</th>
<th>Heterotopic proliferative glands</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>2.8 ± 0.5*</td>
<td>2.3 ± 0.7*</td>
<td>0.9 ± 0.6*</td>
<td>1.4 ± 0.9*</td>
</tr>
<tr>
<td>SS1</td>
<td>1.6 ± 0.5*</td>
<td>1.1 ± 0.7*</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>HF</td>
<td>1.6 ± 0.8*</td>
<td>0.7 ± 0.5*</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.8</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.0 ± 0.0</td>
<td>0.9 ± 0.3*</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>MNU</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

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.
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.
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).
respective) 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 by dextran sulfate sodium-induced colitis (Katsurano et al., submitted for publication) can trigger aberrant methylation induction with cell proliferation. Expression of T-cell activation is required only for the initiation or maintenance of 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 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, Il6, 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 groups, which were positive strains. 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.

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.
Specific inflammation relates to methylation induction


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