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[**Original Article**]

SYNTHETIC OLIGODEOXYNUCLEOTIDES INDUCE GASTRITIS IN MICE

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Abstract: To investigate whether DNA directly induces gastritis and/or peptic ulcer, we injected synthetic DNA including CpG motif (CpG-DNA) to mouse stomach. BALB/c mice were injected with either saline, acetic acid (AA), CpG-DNA, or Control-DNA. Mice were sacrificed, and sections of the stomachs were stained with hematoxylin and eosin. The lesions were histopathologically scored from 0 to 4 based on the extent of the inflammation. Populations of neutrophils and mononuclear cells infiltrated to the lesion were calculated. IFN-γ mRNA expression at the injection site was analyzed by RT-PCR. The number of CpG motifs included in the complete genomes of *H. pylori* HP26695 and J99, *Escherichia coli* O157, and *Salmonella Typhi* was determined by genomic analysis of these bacteria. Intragastric injection with CpG-DNA induced gastritis, and statistical analysis of histological scores revealed a significant difference between saline vs CpG-DNA ($p=0.037$). The population of mononuclear cells infiltrated to the lesions was significantly higher in mice injected with CpG-DNA than that injected with AA ($p=0.0061$). IFN-γ mRNA expression was detected in the CpG-DNA group. While *H. pylori* includes multiple CpG motifs in its genome, it has fewer than the other pathogenic gram-negative bacilli. We conclude that synthetic DNA including CpG motif directly causes gastritis in mice and induces IFN-γ production in the stomach. Bacterial DNA including CpG motif is known to stimulate innate immunity and to cause inflammation. Thus, *H. pylori* genomic DNA may be one of the virulent factors involved in *H. pylori* infection.

Key words: gastritis, CpG motif, *Helicobacter pylori*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is widely recognized as a pathogen that can induce chronic active gastritis, and infection with the bacterium is a risk factor for the development of peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. Ammonia[1] and toxin such as CagA[2,3] and vacuolating cytotoxin (VacA)[4] have been identified as virulent factors. However, further research is required to identify additional *H. pylori* virulent factors, as previous studies have not provided clear answers to the following questions; why do most patients with gastric colonization by *H. pylori* remain asymptomatic, and why is *H. pylori* infection associated with the development of neoplasms?

In our previous study[5], we observed gastritis in mice received intragastric injection with plasmid DNA including CpG motif. CpG motif is composed of the sequence 5′-purine-purine-CG-pyrimidine-pyrimidine-3′ and has been shown to function as ligand for toll like receptor (TLR) 9. In the present study, we investigated whether direct intragastric injection of synthetic DNA containing CpG motif induces gastritis and/or peptic ulcer in mice to assess whether *H. pylori* genomic DNA is a potential virulent factor for inducing gastritis.

MATERIALS AND METHODS

Animals

This study was performed under the guidelines on Animal Experiments in Fukushima Medical University and Japanese Government Animal Protection and Management Law (No. 105). Female BALB/c mice 8 to 12 weeks old were purchased from Charles River Japan (Yokohama, Japan) and used in all experiments.

Reagents

Five percent acetic acid (AA), synthesized oligodeoxynucleotides (ODNs) containing CpG motif (CpG-DNA), and Control-DNA (Bex, Tokyo, Japan) dissolved in saline were used for injection. The ODNs were composed of the following sequences;

CpG-DNA: 5′-TGACTGTGAACGTTCGAGATGA-3′,
Control-DNA: 5′-TGACTGTGAACCTTAGAGATGA-3′.

Intragastric injection

Sixteen mice were divided into 4 groups of 4 each. The groups received one of the following intragastric injections: saline, 5% acetic acid (AA), 20 μg of CpG-DNA (CpG-DNA), and 20 μg of Control-DNA. Intragastric injection was performed as previously described[5,6]. In brief, mice were anesthetized peritoneally with 1.25 mg of pentobarbital sodium (Dainabot, Osaka, Japan), and a small incision was

made with a scalpel in the upper middle portion of the abdomen. The stomach was exposed, and 25 μl of each solution was injected into the submucosal layer of the glandular portion of the anterior wall with a microinjection capillary (Eppendorf, Tokyo, Japan). The incision was immediately closed after injection.

Histological analysis

Mice were sacrificed 4 days after the injection for histological analysis. Extirpated stomachs were fixed with 20% buffered formalin, embedded in paraffin, and serial sections of the specimens were stained with hematoxylin and eosin. After dehydration and mounting, the sections were observed by an individual blinded to the treatments and were scored referring to the previous study[7]: 0=no lesion, 1=mucosal edema, 2=focal neutrophil infiltration, 3=diffuse neutrophil infiltration in all layers, 4=ulceration. Data were analyzed for statistical significance by Mann-Whitney's U test using StatView J-5.0 computer software (SAS Institute Inc., NC, USA) and P values <0.05 were considered significant.

A total of 100 infiltrated cells in each lesion were counted, and neutrophil and mononuclear cell populations were calculated in mice injected with AA or CpG-DNA. Data were analyzed for statistical significance by unpaired t-test using StatView J-5.0 (SAS Institute Inc., NC, USA). P values <0.05 were considered significant.

RT-PCR

To analyze interferon (IFN)-γ mRNA expression, total cellular RNA was extracted from stomach tissue located at the injection site, and mRNA was used to synthesize single-stranded cDNA as previously described[8]. PCR was performed using HotStarTag Master Mix Kit (QIAGEN, Germany) and primers (Greiner, Japan) with the following PCR conditions: 94°C for 30 sec, 60°C for 15 sec, and 72°C for 30 sec, for 35 cycles. The following primer sequences were used:

IFN-γ sense: 5′-TACTGCCAGGGCACAGTCATTGAA-3′
IFN-γ anti sense: 5′-TACTGCCAGGGCACAGTCATTGAA-3′
βactine sense: 5′-GACATGGAGAAGATCTGGCACCACA-3′
βactine anti sense: 5′-ATCTCCTGCTCGAAGTCTAGAGCAA-3′

PCR products were visualized by electrophoresis on 2% agarose gels after staining with ethidium bromide. The intensity of the β actine band was used as an internal standard.

Genomic analysis

The number of CpG motifs (5′-purine-purine-CG-pyrimidine-pyrimidine-3′) included in the complete genomes of *H. pylori* HP26695, *H. pylori* J99, *Escherichia coli* (*E. coli*) O157: H7 EDL933, and *Salmonella enterica subsp. enterica serovar Typhi* (*Salmonella Typhi*) was analyzed using GENETYX−SV/RC Version 6.1.0, and the population of CpG motifs to total DNA was calculated.

RESULTS

CpG-DNA induced gastritis in mice

Histological examination revealed that intragastric injection with saline did not induce ulcers (Fig. 1A a and b). In contrast, intragastric injection with AA induced ulcer accompanied by massive infiltration of neutrophils and mononuclear cells (Fig. 1B a and b). CpG-DNA induced mild infiltration of neutrophils and mononuclear cells and hyperplasia of the glandular epithelium (Fig. 1C a and b), while Control-DNA induced mild gastritis (data not shown). Statistical analysis of histological scores revealed a significant difference between the saline vs AA groups ($p=0.013$) and between the saline vs CpG-DNA groups ($p=0.037$) by Mann-Whitney's U test (Fig. 2). The histological score of CpG-DNA was higher than that of Control DNA ; however, the difference was not significant. The difference in histological scores between saline vs Control-DNA was not significant either.

The populations of neutrophils and mononuclear cells that infiltrated the lesions by intragastric injection of AA were $97.5\pm0.3\%$ and $2.5\pm0.3\%$, respectively, whereas those associated with injection of CpG-DNA were $74.0\pm5.7\%$ and $26.0\pm5.7\%$, respectively (Table 1). The population of neutrophils was significantly higher in mice injected with AA than that injected with CpG-DNA ($p=0.0061$) by unpaired t-test. The population of mononuclear cells was higher in mice injected with CpG-DNA than that injected with AA ($p=0.0061$).

On day 7, ulcers were healed in the AA group, while focal cell infiltration with hyperplasia was observed in the CpG-DNA group (data not shown).

Table 1. Populations of neutrophil and mononuclear cell infiltrated into stomachs injected with acetic acid or CpG-DNA.

Material	Neutrophil (%)	Mononuclear cell (%)
Acetic acid	97.5 ± 0.3	2.5 ± 0.3
CpG-DNA	$74.0\pm5.7^{*}$	$26.0\pm5.7^{\dagger}$

Data represent the mean±SE. *,†, $p=0.0061$

CpG-DNA induced IFN-γ mRNA expression in the stomach

IFN-γ mRNA expression was detected in the CpG-DNA and Control-DNA groups on day 3 ; CpG-DNA induced stronger expression than Control-DNA (Fig. 3). On day 7, IFN-γ mRNA expression was no longer detected in either group. Results are representative of 3 similar and independent experiments.

H. pylori includes CpG motifs in their genomic DNA

H. pylori HP26695 strain includes 9,075 CpG motifs in its genome (1,667,867 nucleotides in length ; 0.552%), while the J99 strain has 9,081 motifs within its

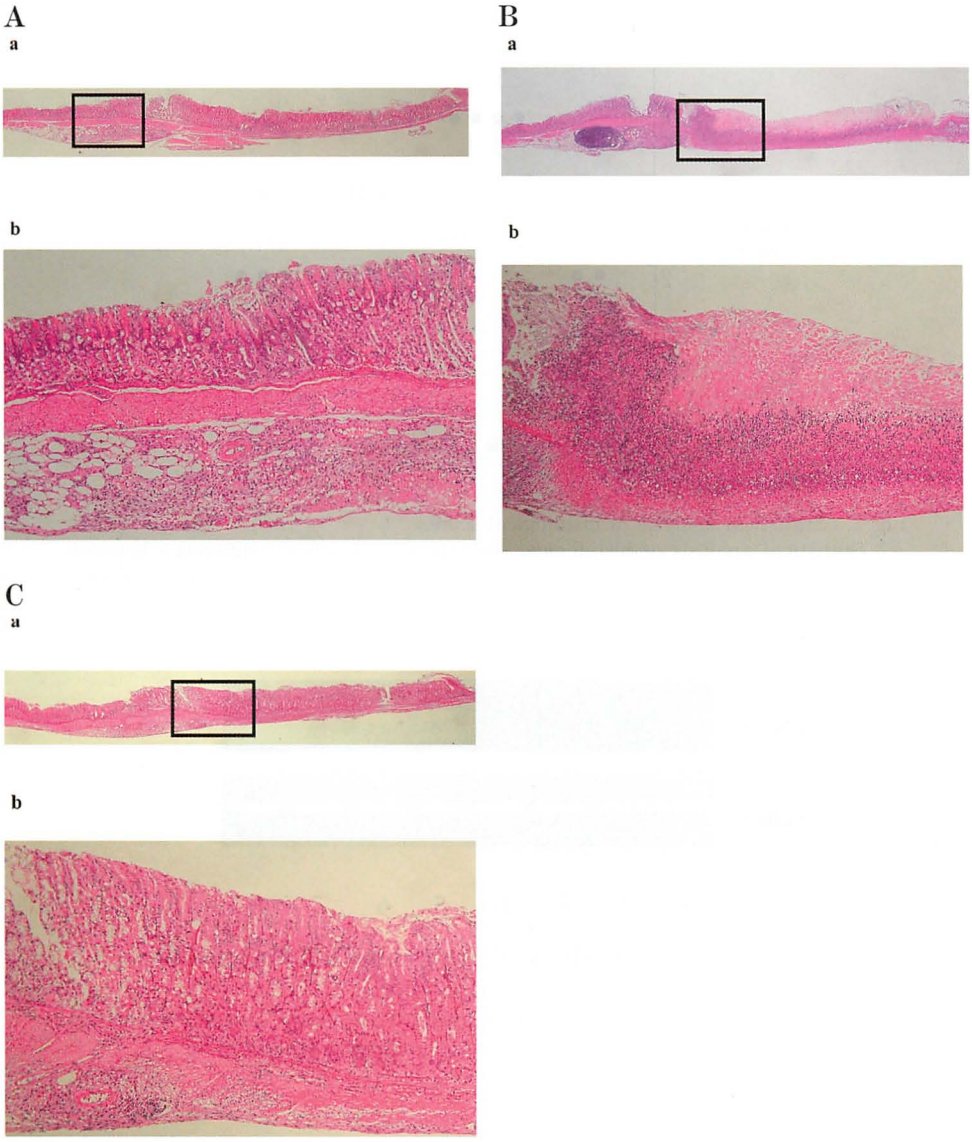

Fig. 1. Histological findings of stomach 4 days after injection (hematoxylin and eosin).
(A) Gastric wall injected with saline shows neutrophil infiltration and fibrinous exudates in serosa but no ulcer. (B) Gastric wall injected with acetic acid shows acute gastric ulcer extending to the serosa, with necrosis and massive infiltration of neutrophils and mononuclear cells. (C) Gastric wall injected with CpG-DNA shows no ulcer and hyperplasia of glands. Mild infiltration of neutrophils and mononuclear cells is observed in the lamina propria, submucosa, and serosa. Panel a, 40×; panel b, 200×. Photomicrographs shown in panel b represent areas delimited by the rectangles in panel a.

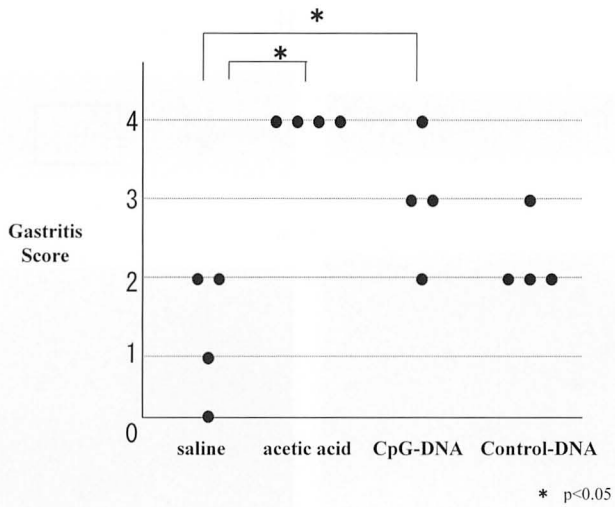

Fig. 2. Statistical analysis of histological score.
Statistical analysis of histological score reveals significant difference between saline vs acetic acid ($p=0.013$) and saline vs CpG-DNA ($p=0.037$) by Mann-Whitney's U test.

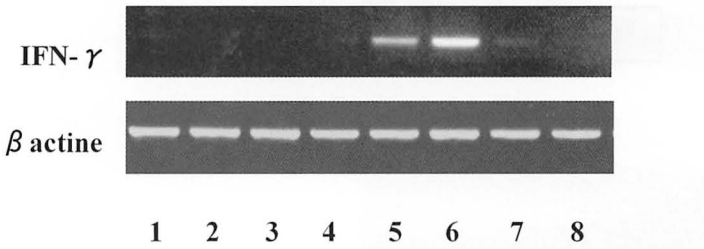

Fig. 3. IFN-γ mRNA expression by RT-PCR.
IFN-γ mRNA expression is detected in the CpG-DNA and Control-DNA groups on day 3 of the experiment, and stronger expression is noticed in the CpG-DNA group.

Table 2. Number of CpG motifs in bacterial genomic DNA.

Bacteria	Number of CpG motifs	Total genome size (nt)	CpG DNA/total DNA (%)
H. pylori J99	9,081	1,667,867	0.544
H. pylori 2669	9,075	1,643,831	0.552
Escherichia coli O157 : H7 EDL933	28,674	4,639,221	0.618
Salmonella enterica subsp. enterica serovar Typhi CT18	33,038	4,809,037	0.687

nt, nucleotides

164,381-nucleotide genome (0.544%). The number of CpG motifs and the population of CpG motifs to total DNA in these two strains are lower than that of the other pathogenic gram negative bacilli, such as *Eschericia coli* O157 : H7 EDL933 (28,674 motifs within 4,639,221 nucleotides ; 0.618%) and *Salmonella Typhi* (33,038 motifs within 4,809,037 nucleotides ; 0.687%) (Table 2).

DISCUSSION

In this study, we demonstrated that intragastric injection of synthetic ODN containing CpG motif induced gastritis in mice. Based on the AA ulcer models used in peptic ulcer research[6], we directly injected AA into the gastric wall of mice. Histological analysis confirmed the induction of gastric ulcer and gastritis accompanied by neutrophil infiltration following intragastric injection with AA, yielding a positive control of this study. While injection of CpG-DNA did not cause ulcer, our results indicate that CpG-DNA can induce gastritis.

The DNA used in this study included a CpG motif within 20 bases of synthetic ODNs. CpG motif is composed of an unmethylated CpG dinucleotide core within a particular base context[9,10], and they have been shown to function as ligand for TLR9[11]. While these sequences are found at predictable frequencies within microbial genomes, mammalian DNA contains fewer CpG motifs, with the cytosines usually being methylated[12]. DNA derivatives that include CpG motifs have been shown to activate innate immunity[13]. For example, a single injection of CpG-DNA induces a detectable increase in IFN-γ and interleukin-12 levels in mouse serum[8]. Furthermore, plasmid DNA or synthetic ODN including CpG motifs were shown to exacerbate encephalitis[14], arthritis[15], nephritis[16], and inflammatory bowel disease[17] in animal models accompanied by the upregulation of inflammatory cytokines such as IFN-γ and tumor necrosis factor-α in lymph nodes or inflammatory tissues. However, no previous studies have reported that CpG-DNA directly induces gastritis.

Gastritis caused by *H. pylori* infection is characterized by chronic inflammation accompanied by infiltration of neutrophils and plasma cells. In this study, the gastritis caused by CpG-DNA was observed shortly after injection (i.e., it was acute), and the population of mononuclear cells that infiltrated the lesion was larger in mice received CpG-DNA than in mice received AA. Furthermore, administration of CpG-DNA induced IFN-γ mRNA expression at the injection lesion in the stomach. IFN-γ is known to play an important role in the induction of gastric inflammation caused by *H. pylori* infection[18]. IFN-γ increases the bacterial attachment as well as the induction of apoptosis in gastric epithelial cells[19]. In humans, the percentage of IFN-γ producing cells in the gastric mucosa diminishes significantly after *H. pylori* eradication[20]. Our findings indicate that in addition to ammonia and toxins, DNA induces gastritis, which partly depends on IFN-γ produced in the stomach. This is the first report to demonstrate DNA directly induces gastritis.

Although this study did not confirm the hypothesis that *H. pylori* genomic DNA directly causes gastritis, the results indicate that microbial DNA is a potential virulent factor for inducing gastritis, considering the fact *H. pylori* is the only bacterium that survives for prolonged periods in the stomach. While *H. pylori* includes CpG motifs in its genomic DNA, the number of motifs is relatively low compared to the other pathogenic bacteria, such as *E. coli* O157 or *Salmonella Typhi.* It is possible that the relatively smaller number of CpG motifs are able to induce chronic inflammation in the stomach because they are continuously provided via long-term *H. pylori* infection.

Recently, TLR4, TLR5, and TLR9 were found to be expressed in human gastric epithelium[21]. Of note, previous reports suggested that gastric epithelial cells that present MHC class II and accessory molecules such as B7-1 and B7-2 may play an important role as antigen-presenting cells[22-24]. We suggest that gastric epithelial cells and circulating antigen-presenting cells recognize CpG-DNA via TLR9, thereby inducing IFN-γ expression.

In conclusion, we propose that microbial DNA has a potential to induce gastritis, as we have found that synthetic ODNs including CpG motif directly cause gastritis in mice, characterized by a predominantly mononuclear cell infiltration and IFN-γ production at the injection lesion. Considering the fact that *H. pylori* is the only bacterium that survives in the stomach, its genomic DNA cannot be ignored as a potential virulent factors for gastritis. Further investigations, such as in vivo analysis of *H. pylori* genomic DNA is required to demonstrate whether *H. pylori* DNA is one of the virulent factors that induces diseases associated with *H. pylori* infection.

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