Oral Intake of N-acetyl-D-glucosamine Suppresses the Growth of Helicobacter Pylori in Gastric Mucosa of Mongolian Gerbils by Increasing Gland Mucus

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Abstract—Oral intake of N-acetyl-D-glucosamine (GlcNAc) for 24 weeks suppressed the growth of Helicobacter pylori in the gastric mucosa of Mongolian gerbils. Inhibition was confirmed by quantitative real-time polymerase chain reaction (PCR). Histochemical analysis revealed that, as compared with intake of normal chow, intake of GlcNAc led to a significant increase in gland mucus, which contains gland mucus cell mucin, in the stomach of gerbils infected with H. pylori. Gland mucus cell mucin has O-glycans that contain terminal α1,4-linked GlcNAc residues, which are known to suppress H. pylori growth. We conclude that oral GlcNAc intake led to an increase in terminal α1,4-linked GlcNAc residues in gland mucus cell mucin and this increase in terminal GlcNAc residues inhibited the growth of H. pylori in the gerbil’s stomach.

Index Terms—N-acetyl-D-glucosamine, Helicobacter pylori, mucin, gland mucus cell mucin, bacterial infection, antibiotics

I. INTRODUCTION

Recently, there has been growing interest in bacterial infections. Helicobacter pylori is one of the most well-known Gram-negative bacteria, and it infects the stomach in around half of the world's population [1]. Marshall and Warren first succeeded in culturing the bacterium in 1982 [2], [3]. Although most individuals infected by H. pylori show no symptoms, the bacterium has been regarded as a leading cause of gastric malignancies [4]. In general, antibiotics based on proton pump inhibitors have been used to treat H. pylori infection [5]; however, increasing numbers of patients with H. pylori infection have resistance to such antibiotics [5]. As a result, these therapies do not guarantee universal success [6]. Therefore, it’s imperative to identify new antimicrobial agents for the treatment of H. pylori infection.

Kawakubo et al. have demonstrated previously that the terminal α1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues on O-glycans in gastric mucins have antibiotic function against H. pylori [7]. Gastric mucins are histochemically classified into two types: surface mucous cell mucin, which is secreted by surface mucous cells; and gland mucus cell mucin, which is secreted by gland mucous cells including mucous neck cells, cardiac gland cells, and pyloric gland cells [8]. H. pylori rarely colonizes regions expressing gland mucous cell mucin because of the presence of terminal α1,4-linked GlcNAc residues on O-glycans in the mucin [9]. The terminal α1,4-linked GlcNAc residues inhibit cholesterol α-glucosyltransferase in H. pylori. This enzyme is responsible for the synthesis of cholesteryl α-D-glucosides [7], which are major components of the cell wall of the bacterium. Inhibition of cholesterol α-glucosyltransferase therefore leads to a decrease in components, and consequently inhibits the growth of H. pylori [7].

Collectively, these findings suggest that increasing the gland mucous cell mucin, which contains O-glycans with antibiotic functional GlcNAc, may lead to efficient inhibition of the growth of H. pylori. Furthermore, if oral intake of GlcNAc increases the amount of the gland mucous cell mucin, GlcNAc may acts as an efficient antibiotic by controlling the ratio and relative amount of the two types of mucins. To test this hypothesis, here we evaluated the effects of oral GlcNAc intake on inhibition of H. pylori growth in the stomach of Mongolian gerbils.

II. MATERIALS AND METHODS

GlcNAc was prepared by acid and enzymatic hydrolysis of chitin [10]. Pathogen-free and H. pylori ATCC 43504-infected male Mongolian gerbils, aged 5 weeks, were purchased from Kyudo Co. Ltd. (Saga, Japan). The Mongolian gerbils were housed on a 12-h light/dark cycle. Body weights were measured weekly, beginning a week after infection with H. pylori. Pathogen-free and H. pylori-infected gerbils that had increased IgG values in sera, aged 17 weeks, were randomly assigned to 2 groups (groups 1 and 2, and groups 3 and 4, respectively). Group 1 comprised three pathogen-free gerbils that were allowed free access to normal rodent chow (CE-2, CREA Japan Inc., Tokyo, Japan) and water. Group 2 comprised three pathogen-free gerbils that were allowed free access to normal rodent chow (CE-2, CREA Japan Inc., Tokyo, Japan) and water.

Manuscript received July 24, 2014; revised December 1, 2014.
chow and 5% GlcNAc solution. Group 3 comprised six *H. pylori*-infected gerbils that were allowed free access to normal rodent chow and water. Group 4 comprised four *H. pylori*-infected gerbils that were allowed free access to normal rodent chow and 5% GlcNAc solution. At 24 weeks after infection, all gerbils (aged 29 weeks) were sacrificed under ether anesthesia after fasting for 40 hours and their stomachs were excised. Each stomach was washed with 0.1 M phosphate-buffered saline and the wet weight was determined (Fig. 1).

### Figure 1. Experimental schedules

- Standard diet
- 5% N-acetylglucosamine
- Inoculation of *H. pylori*
- Dissection of Mongolian gerbil

#### A. Measurements of Anti-*H. pylori* IgG Titers.

Values of anti-*H. pylori* IgG in gerbil sera were determined by an ELISA kit (E-Plate Eiken *H. pylori* antibody, Eiken Chemical Co., Ltd., Tokyo, Japan). Serum samples were centrifuged; the supernatants were then diluted 1,000 fold with phosphate buffer and analyzed according to the manufacturer’s instructions except that peroxidase-conjugated rabbit anti-mouse IgG (Dako Japan Co., Japan) was used as the secondary antibody. A value greater than 10 U/mL (absorbance at 490 nm >0.1) was considered to be positive for *H. pylori* infection.

#### B. Real-Time Polymerase Chain Reaction and Relative Quantitative Analysis.

An approximately 25-mg sample was taken from the gastric mucosa of each gerbil. The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA according to the manufacturer’s instructions. For *H. pylori* quantification, we employed relative quantitative real-time polymerase chain reaction (PCR) to quantify the relative dosages of the *H. pylori*-specific urease A gene using the gerbil-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal standard. Urease A gene levels were expressed as percentages relative to the *H. pylori*-inoculated control group (group 3), which was set at 100%. The PCR primers were: Urease A, sense 5’-TGTCTCGGACAGCCGGTCAAACT-3’ and antisense 5’-GCTGTCGCCCTGCAATGTCATAAGC-3’ GAPDH, sense 5’-AAGGCGACATCAAGGTCGAGAAGC-3’ and antisense 5’-CAACATCTCGGACGCGCATCG-3’ PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) and the QuantiTect SYBR Green PCR Kit (Qiagen). The cycling conditions included an initial denaturation for 15 min at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

#### C. Histochemical Analysis.

The washed stomachs were fixed in Carnoy’s fixative for 2 h and then cut along the long axis, dehydrated through ethanol (99.5% ethanol overnight, anhydrous ethanol for 1 h), cleared in xylene (30 min, 3 times), and embedded in paraffin. Paraffin sections, 3–m thick, were stained with galactose oxidase-cold thionin Schiff reaction/paradoxical concanavalin A staining (GOTS-PCS) to identify surface mucous cell mucin and gland mucous cell mucin [11], [12]. The samples were observed and analyzed with an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan), Axioshot 2 microscope (Carl Zeiss, Jena, Germany), and image analyzer software (WinRoof, Mitani Co., Ltd, Fukui, Japan).

#### D. Data Analysis

Data are expressed as the mean ± S.E. Values were compared between groups by using the unpaired two-tailed t-test. P<0.05 was taken to indicate a statistically significant difference.

### III. RESULTS

#### A. Anti-*H. pylori* IgG titers

The anti-*H. pylori* IgG titer in each gerbil was measured at 6, 12, and 24 weeks after *H. pylori* infection. The titers in groups 1 and 2 remained below 0.065 throughout the study. Gerbils in groups 3 or 4 that had weak titers (<0.1) were considered to be negative for *H. pylori* infection and were excluded at 12 weeks. The mean titers of the remaining gerbils in groups 3 and 4 were 0.087±0.009 and 0.361±0.116 at 6 weeks, 0.466±0.059 and 0.856±0.241 at 24 weeks (Fig. 2), respectively. There was no significant difference between groups 3 and 4 over the experimental period.

![IgG antibody titers against *H. pylori* in gerbils at 24 weeks after *H. pylori* infection.](image2.png)
B. Body Weight

The average body weight in groups 1 and 2 comprising pathogen-free gerbils were consistently higher than those in H. pylori-infected groups 3 and 4 during the study (data not shown). At 24 weeks after H. pylori infection, the mean body weight in groups 1 and 2 was 107±5 g and 98±9 g, respectively. Compared with group 3 (75±4 g), the body weight of group 4 (89±4 g), which had 5% GlcNAc solution, showed a tendency toward recovery from the decrease caused by H. pylori infection, but the difference was not significant (p=0.054).

C. Wet Weight of Stomach

The wet weight of stomach as a percentage of body weight increased due to H. pylori infection in group 3 (1.64±0.11). As compared with group 3, by contrast, the mean stomach weight of group 4 (1.23±0.17) tended to be suppressed (p=0.066).

D. Relative Urease A Gene Levels

Relative urease A gene levels were significantly suppressed in group 4 (25.5±9.5%) as compared with group 3 (100%). In the pathogen-free gerbils (groups 1 and 2), the gene was not detected.

E. Gastric Mucus

The grand mucus in gastric mucosa in group 4 (3.93±0.18 x10^5 pixel) was significantly increased as compared with group 3 (2.36±0.25 x10^5 pixel). As a result, there was a significant difference between the two groups (group 3, 5.97±0.50 x10^5 pixels; and group 4, 8.53±0.86 x10^5 pixels) in total gastric mucus despite the lack of difference in surface mucus.

IV. DISCUSSION

In this study, we examined the effects of oral GlcNAc intake on H. pylori-infected Mongolian gerbils. The gerbils in group 4 that had 5% GlcNAc solution tended towards recovery from both the loss of body weight and the increase in wet weight of stomach caused by H. pylori infection.
infection (Fig. 3 and Fig. 4). However, there were no significant differences between the gerbils in group 4 and the H. pylori-infected gerbils in group 3, which did not consume GlcNAc.

We also determined the relative dosages of the H. pylori specific urease A gene in the gastric mucosa of each gerbil by quantitative PCR. This method has been known as a useful tool for specific detection and quantification of H. pylori gene expression in gastric mucosa [13], [14]. The relative levels of urease A gene were significantly suppressed in group 4 (Fig. 5), which indicated that the growth of H. pylori in gastric mucosa of the gerbils was inhibited.

Furthermore, we conducted histochemical analyses of the gastric mucus in stomach by the dual staining method GOTS-PCS, which enables differentiation between surface mucous cell mucin and gland mucous cell mucin in surface mucus and gland mucus, respectively [11], [12]. Compared with intake of normal chow, oral intake of GlcNAc increased gland mucous cell mucin, but not surface mucous cell mucin in H. pylori-infected gerbils (Fig. 6). GlcNAca1,4Galβ-R in gland mucous cell mucin is responsible for the reactivity of paradoxical concanaavalin A staining (PCS) [15]. Therefore, this finding indicates that oral intake of GlcNAc increased O-glycans containing terminal α1,4-linked GlcNAc residues in gland mucus, which are known to suppress the growth of H. pylori by inhibiting its enzyme activity [7] and also to suppress tumor-promoting inflammation [16]. Accordingly, we concluded that the increase in gland mucous cell mucin suppressed the growth of H. pylori in gerbils that took GlcNAc. In this way, we demonstrated that oral intake of GlcNAc has a unique inhibitory mechanism in vivo. Although oral intake of GlcNAc by itself did not lead to the complete elimination of H. pylori, GlcNAc may act an agent that supplements the effects of other drugs.

V. CONCLUSION

In this paper, we evaluated the effect of oral intake of GlcNAc solution on Mongolian gerbils infected with H. pylori. The results showed that GlcNAc increased the amount of gland mucus in the gastric mucosa of the gerbils and significantly suppressed the growth of H. pylori. Therefore, oral GlcNAc intake is efficient for inhibiting the growth of H. pylori in the stomach.

REFERENCES


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