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Inhibitory effect of *raphanobrassica* on *Helicobacter pylori*-induced gastritis in Mongolian gerbils

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ABSTRACT

Helicobacter pylori (*H. pylori*) infection is well known to be associated with chronic gastritis and also development of gastric cancer. *Raphanobrassica* (RB) is an intergeneric hybrid of the genera *Raphanus* (radish) and *Brassica* (cabbages) containing appreciable amounts of glucoraphanin (GR) and glucoraphenin (GRe), which are actively hydrolyzed by the enzyme myrosinase to sulforaphene and sulforaphene, respectively. Both of these metabolites exert antimicrobial and anti-inflammatory activity. The purpose of the present study was to investigate the effect of two freeze-dried products of RB (RB1 and RB2) on *H. pylori*-induced gastritis in Mongolian gerbils. Six-week-old male Mongolian gerbils were inoculated orally with *H. pylori* (ATCC 43504), and 2 weeks later were fed diets containing no additives or diets supplemented with 2% RB1 (containing both GR and GRe) or 2% RB2 (containing GR only) for 10 weeks. In the RB1, but not the RB2 group, mononuclear cell infiltration, mRNA expression of IL-6, and cell proliferation in the gastric mucosa were significantly suppressed. These results indicate that RB1 containing both GR and GRe exerted significant inhibitory effects on *H. pylori*-induced gastritis in Mongolian gerbils apparently mediated via suppression of IL-6 expression and chronic inflammation.

1. Introduction

Helicobacter pylori (*H. pylori*) infection is now recognized as a major causative factor for chronic gastritis and peptic ulcers, and there is compelling evidence indicating an association between *H. pylori*-induced chronic gastritis and development of stomach cancer (Uemura et al., 2001). Triple therapy with a proton pump inhibitor and two antimicrobials, amoxicillin and clarithromycin, is usually used for treatment of *H. pylori*-infected patients. However, considering the occurrence of antibiotic-resistance, the search for new preventive agents of *H. pylori* infection continues to be very important (Engstrand et al., 1997; Graham, 1998; Trust et al., 2001).

Raphanobrassica (RB), an intergeneric hybrid between the genera *Raphanus* (radish) and *Brassica* (cabbage), contains large amounts of glucoraphanin (GR) and glucoraphenin (GRe) (Schutze et al., 1999). GR, an inert glucosinolate precursor of sulforaphane (1-isothiocyanato-4-methylsulfinylbutane), is actively hydrolyzed by the enzyme myrosinase to sulforaphane (Fahey et al., 2001), which exerts antimicrobial, antioxidative, anti-inflammatory and antitumorigenic effects mediated largely via the transcription factor Nrf2 (Fahey et al., 1997, 2002; Fahey and Talalay, 1999; Ramos-Gomez et al., 2001). It has been shown that sulforaphane-rich broccoli sprouts can attenuate gastritis in *H. pylori*-infected mice via protecting cells from oxidative injury (Yanaka, 2009, 2011). GRe, an inert glucosinolate precursor of sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate), is actively hydrolyzed by the enzyme myrosinase to sulforaphene (Barillari et al., 2007; Song et al., 2013), which exerts antimicrobial, antiviral and antimutagenicity effects (Nakamura et al., 2001; Nastruzzi et al., 1996; Shishu and Kaur, 2009). Although RB is a breeding material for plant breeding in agriculture and is considered inedible as a vegetable, we have focused interest on whether it might be useful as a health supplement for chemoprevention of *H. pylori*-induced chronic gastritis, as it contains precursors of sulforaphane and sulforaphene.

The Mongolian gerbil is a useful animal model for the examination of *H. pylori*-induced chronic active gastritis (Hirayama et al., 1996). The purpose of the present study was to investigate the

Abbreviations: GR, glucoraphanin; GRe, glucoraphenin; *H. pylori*, *Helicobacter pylori*; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; IL, interleukin; Nrf2, NF-E2-related factor 2; NF-κB, nuclear factor-kappa B; RB, *raphanobrassica*; TNF-α, tumor necrosis factor-α; 8-OHdG, 8-hydroxydeoxyguanosine.

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effect of two kinds of freeze-dried products of RB, containing GR alone or together with GRe having a highly similar chemical structure (Fig. 1), on *H. pylori*-induced chronic active gastritis in Mongolian gerbils.

2. Materials and methods

2.1. Test chemicals and diets

Two kinds of the freeze-dried products of RB prepared by different freeze drying methods were kindly provided by the Vegetable and Ornamental Experiment Station, Nagano, Japan. RB1 contained GR and GRe, but RB2 only contained GR, possible due to loss of GRe during the freeze drying process. The contents of GR and GRe in RB1 and RB2 are shown in Table 1. Basal diet (powdered MF) and diets containing RB1 or RB2 were prepared by Oriental Yeast Co., Tokyo, Japan.

2.2. H. pylori culture

H. pylori was prepared by a established protocol as described previously (Shimizu et al., 1999; Toyoda et al., 2007, 2009). Briefly, *H. pylori* (ATCC 43504; American Type Culture Collection, Rockville, MD) was grown in Brucella broth (Becton Dickinson, Cockeysville, MD) containing 7% FBS at 37 °C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) at high humidity for 24 h. Broth cultures of *H. pylori* were checked under a phase contrast microscope for bacterial shape and motility.

2.3. Experimental design

The animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School. A total of 30 specific-pathogen-free 6-week-old male Mongolian gerbils (Meriones unguiculatus; MGS/Sea, Kyudo, Fukuoka, Japan) were housed in an air-conditioned biohazard room with a 12-h light/12-h dark cycle and allowed free access to food and water throughout the study. The gerbils were divided into 3 groups of 10 animals each: *H. pylori*-infection alone (*H. pylori* group); *H. pylori* infection → 2% RB1 (RB1 group), and *H. pylori* infection → 2% RB2 (RB2 group). The concentration of 2% was the highest doses that could be prepared for this experiment due to the limited amounts of RB1 and RB2 available. This dose is lower than 5% that is the maximum concentration in diet recommended for toxicity assay of food additive (International Life Sciences Institute, 2010). All animals were inoculated with *H. pylori* by a single oral gavage treatment with 1.0 ml of broth culture containing 1×10^8 CFU *H. pylori* at the beginning of the experiment. This is a well-established inoculation protocol in Mongolian gerbils, and successful inoculation has been confirmed by typical macroscopic and microscopic changes in the gastric mucosa (Shimizu et al., 1999; Toyoda et al., 2007, 2009) as shown in Figs. 2 and 3. From weeks 2 to 12, the gerbils received basal diet, basal diet supplemented with 2% RB1 (contains both GR and GRe), or basal diet supplemented with RB2 (contains GR only). At the end of experimental week 12, the animals were killed under deep anesthesia and the stomachs were excised. The stomachs of five animals in each group were used for histopathological analysis, and the stomachs of the other animals were used for measurement of 8-hydroxydeoxyguanosine (8-OHdG) formation and gene expression assays.

2.4. Histopathology and immunohistochemistry

The excised stomachs used for histopathology and immunohistochemistry were fixed in 10% neutral-buffered formalin for 24 h and sliced along the longitudinal axis into 4–8 strips of equal width and embedded in paraffin. Serial sections were prepared and stained with hematoxylin and eosin (H&E) for morphological examination. The antrum and corpus in the stomach were examined histopathologically for inflammation and epithelial changes. The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System (Dixon et al., 1996): infiltration of multinucleated neutrophils and mononuclear cells, development of heterotopic proliferative glands, and intestinal metaplasia were scored on a four-point scale (0–3; 0, normal; 1, mild; 2, moderate; 3, marked). Intestinal metaplasia was assessed by immunostaining with a rabbit polyclonal anti-Mucin 2 antibody (clone H-300, diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA). After immunostaining with a rabbit monoclonal anti-Ki-67 antibody (clone SP6, diluted 1:500, Epitomics, Burlingame, CA), cell proliferation was evaluated as the mean percentage of Ki-67-positive epithelial cells in the total cell population (>2000 cells) in glands selected randomly from both the antrum and corpus.

Table 1
Contents of GR and GRe in diets.

	GR (μg/100 mg dry weight)	GRe (μg/100 mg dry weight)	Total (GR + GRe) (μg/100 mg dry weight)
RB1	198	893	1091
RB2	136	0	136

GR: glucoraphanin; GRe: glucoraphenin.

2.5. RNA extraction and analysis of mRNA expression for inflammation-associated factor by relative quantitative real-time RT-PCR

To investigate the effects of RB1 and RB2 on cytokine expression in *H. pylori*-stimulated stomachs in gerbils, real-time RT-PCR analysis was performed. Approximately 40 mg of mucosa was homogenized and total RNA was isolated with TRIzol Reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol and stored at −80 °C until use. RNA concentrations were determined with a spectrophotometer (Nanodrop, ND-1000; LMS Co., Ltd., Tokyo, Japan). cDNA synthesis was performed using an Advantage RT-for-PCR Kit (Takara Bio Company, Shiga, Japan). Relative quantitative PCR for tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-6, IL-10, inducible nitric oxide synthase (iNOS), and IL-8 homologue (KC) was carried out using a 7300/7500 Real Time PCR system (Life Technologies Japan, Tokyo, Japan) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The PCR was performed as described earlier using the Quantifast SYBR Green PCR Kit (QIAGEN, Hilden, Germany) (Toyoda et al., 2009). The primer sequences for each gene are listed in Table 3. The real-time cycler conditions used were those recommended for the Quantifast kit. Relative quantification was performed using the internal control.

2.6. Measurement of 8-hydroxydeoxyguanosine (8-OHdG) formation in stomach tissue

DNA extraction and measurement of 8-OHdG were conducted by high-performance liquid chromatography (HPLC) as previously described (Nakae et al., 1995). Briefly, DNA was extracted from approximately 50 mg of frozen mucosa using a DNA Extraction WB kit (Wako pure Chemical Industries, Ltd., Osaka, Japan). Extracted DNA was digested to nucleosides by combined treatment with nuclease P1 and alkaline phosphatase. Finally, the samples were purified using an Ultrafree-MC Centrifugal Filter U and examined by HPLC. Peaks were assessed with electrochemical and UV detectors. The levels of 8-OHdG formation were determined by calibration against HPLC runs of standard samples which contained known amounts of authentic 8-OHdG and dG. The results are expressed as the number of 8-OHdG residues per 10^5 dGs.

2.7. Statistical analysis

Values are expressed as means ± standard deviations. Differences in histopathological scores for gastritis were analyzed by the Mann–Whitney test. Other data were examined using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. General observation

One animal in the *H. pylori*-infection alone group died in experimental week 2 for an unknown reason and was excluded from the

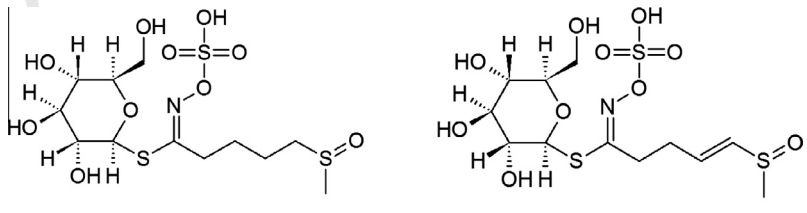

Fig. 1. Chemical structures of glucoraphanin (GR) and glucoraphenin (GRe).

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