RESEARCH COMMUNICATION

Inhibition of ENNG-Induced Pyloric Stomach and Small Intestinal Carcinogenesis in Mice by High Temperature- and Pressure-Treated Garlic

Takaaki Kaneko[1], Kan Shimpo[1]*, Takeshi Chihara[1], Hidehiko Beppu[1], Akiko Tomatsu[1], Masanori Shinzato[2], Takamasa Yanagida[2], Tsutomu Ieike[2], Shigeru Sonoda[1], Akihiko Futamura[3], Akihiro Ito[3], Takashi Higashiguchi[3]

Abstract

High temperature- and pressure-treated garlic (HTPG) has been shown to have enhanced antioxidative activity and polyphenol contents. Previously, we reported that HTPG inhibited 1,2-dimethylhydrazine-induced mucin depleted foci (premalignant lesions) and O^6-methylguanine DNA adduct formation in the rat colorectum. In the present study, we investigated the modifying effects of HTPG on *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)-induced pyloric stomach and small intestinal carcinogenesis in mice. Male C57BL/6 mice were given ENNG (100 mg/l) in drinking water for the first 4 weeks, then a basal diet or diet containing 2% or 5% HTPG for 30 weeks. The incidence and multiplicity of pyloric stomach and small intestinal (duodenal and jejunal) tumors in the 2% HTPG group (but not in the 5% HTPG group) were significantly lower than those in the control group. Cell proliferation of normal-appearing duodenal mucosa was assessed by MIB-5 immunohistochemistry and shown to be significantly lower with 2% HTPG (but again not 5% HTPG) than in controls. These results in dicate that HTPG, at 2% in the diet, inhibited ENNG-induced pyloric stomach and small intestinal (especially duodenal) tumorigenesis in mice, associated with suppression of cell proliferation.

Keywords: Intestinal neoplasia - mice - high temperature- and pressure-treated garlic - chemoprevention

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Introduction

Garlic (*Allium sativum* L.) contains various chemical components, and production of physiological active components markedly varies depending on cooking and processing methods and these components exhibit diverse physiological functions (see reviews; Ariga & Seki, 2000; Milner, 2001; Khanum et al., 2004; Shukla & Kalra, 2007; Iciek et al., 2009). Most physiological activities and pharmacological actions are exhibited by odor components of the sulfide-generating system, such as diallyl disulfide, and artificially generated components, such as *S*-allylcysteine (SAC) and ajoene (Lawson, 1996; Amagase, 2006). However, many people dislike garlic smells in Japan, for which various odorless change methods have been attempted. We have also investigated an odorless change method. We prepared heated garlic powder in which alliinase enzyme synthesizing an odor component, allicin, was inactivated by boiling treatment of raw garlic bulbs, and showed that this heated garlic powder inhibited duodenal and jejunal tumorigenesis induced by *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG) in mice (Shimpo et al., 2002). When the influence of boiled garlic powder on formation of mucin-depleted foci (MDF) in 1, 2-dimethylhydradine (DMH)-induced rat colorectal precancerous lesions was investigated, the powder significantly reduced the number of MDF (Chihara et al., 2010).

On the other hand, it has recently been reported that the antioxidative activity, total polyphenol, and total flavonoid levels were increased in garlic juice treated with high-temperature and pressure (130 °C, 2 hours), and exhibited an anti-cancer effect on cultured human cancer cells (Jeong et al., 2006; Kwon et al., 2006). We prepared high-temperature and pressure-treated garlic (HTPG) using a simple autoclave and attempted measurement of antioxidative activity and total polyphenol, and observed that antioxidative activity was enhanced and total polyphenol levels were 25 times higher (Tomatsu et al., 2007). This was not due to generation of sulfur compounds of the sulfide system by the garlic-specific alliin-alliinase system, but it may have been due to changes in sulfide and non-sulfide components (e.g., γ-glutamyl-*S*-allylcysteine and fructan) originally having weak physiological activity to an extract with a strong antioxidative activity by high-temperature and pressure (Ryu et al., 2001; Amagase,

*[1]Fujita Memorial Nanakuri Institute, [2]School of Health Sciences, [3]Department of Surgery & Palliative Medicine, School of Medicine, Fujita Health University *For correspondence: shimpo@fujita-hu.ac.jp*

2006; Ichikawa et al., 2006). Thus, we firstly investigated the influences of HTPG on MDF and ACF formation in DMH-induced colorectal precancerous lesions and DMH-induced O^6-methylguanine (O^6-MeG) DNA adduct formation in colorectal mucosae and livers in rats. HTPG significantly inhibited MDF and O^6-MeG DNA adduct formations, significantly reduced the activity of a phase 1 liver detoxification enzyme, cytochrome P450 2E1 (CYP2E1), and significantly enhanced activities of phase 2 liver detoxification enzymes: quinone reductase and glutathione *S*-transferase activities (Chihara et al., 2009). In addition, HTPG administered in the post-initiation stage also inhibited MDF formation (Chihara et al., 2011).

In this study, we investigated the modifying effect of HTPG on ENNG-induced carcinogenesis in the pyloric stomach and small intestine in mice. We also performed Ki-67(MIB-5) staining to investigate the cell proliferative ability in the duodenum of the ENNG treatment groups.

Materials and Methods

Animals

Male C57BL/6 mice (7 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in groups of two or three in plastic cages on woodchip bedding and fed a basal diet, Oriental MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan), in an animal facility controlled at a temperature of 23±5 ˚C, 60±5 % humidity, and with a 12-h light/dark cycle. The care and use of animals was in accordance with the 'Guidelines for the Management of Laboratory Animals in Fujita Health University, Fujita Memorial Nanakuri Institute', and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

Chemicals

ENNG (Nacalai Tesque Co, Ltd., Kyoto, Japan) was dissolved in distilled water at 100 mg/l. ENNG solution was freshly prepared three times a week and protected from light by storage in black bottles.

HTPG preparation

HTPG preparation was described previously by Kwon et al. (2006) and Tomatsu et al. (2007). Briefly, garlic slices were pulverized. Then, 100 g of the pulverized garlic was mixed with 250 ml of hot water to inactive alliinase. The mixture was heated at 130 ˚C and pressured at 0.18 MPa in an autoclave for 2.5 hours and then freeze-dried. The powder was finely pulverized using a blender. Analysis of the HTPG used in this study gave the following results (calculated as dry weight); SAC (2.47 mg/g), 5-hydroxymethyl-2-furfural (5-HMF; 2.09 mg/g), and total polyphenol content (17.00 mg/g).

Experimental protocol

After acclimation for 1 week, animals were divided into 5 groups as shown in Figure 1. Mice in Groups 1, 2, and 3 were given basal diet and ENNG (100 mg/l) in drinking water *ad libitum* for the first 4 weeks. These groups were then shifted to tap water, and Groups 1, 2, and 3 were given basal diet, or 2% and 5% HTPG powder in basal diet *ad libitum* for 30 weeks, respectively. Mice in Groups 4, 5 were given basal diet for the first 4 weeks, and then given 5% HTPG powder in the basal diet or the basal diet *ad libitum* for 30 weeks, respectively. Mice in these 2 groups were given tap water throughout the entire experiment. The experiment was terminated 34 weeks after the start of ENNG treatment. All mice were anesthetized with diethyl ether and exsanguinated through the heart. The tongue, esophagus, stomach, small intestine, and large intestine were removed together. The stomach was opened along the greater curve and pinned. The esophagus, duodenum (4 cm distal from the pyloric ring), jejunum and ileum, large intestine, and unusual mass lesion were dissected. After mucosal lesions were noted, each tissue was fixed in 10% formalin, embedded in paraffin wax, and stained with hematoxylin-eosin for histological examination.

Hematological analysis

The following hematological parameters in animals that did not receive ENNG (Group 4 and 5) were measured using an automated hematology analyzer (Model XT-1800i; Sysmex Corp., Kobe, Japan): red blood count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (HCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), and platelet count (PTL).

Immunohistochemistry

Epithelial proliferation of the duodenum was determined by a slight modification of the method of Katsuki et al. (2006) and Levan et al. (2003). Briefly, sections were retrieved in an autoclave in citrate buffer (pH 6.0) for 20 min at 120 ˚C. Endogenous peroxidase activity was blocked by incubation of slides in absolute methanol containing 3% H_2O_2 for 15 min at room temperature. They were then incubated with anti-Ki-67; MIB-5 antibody (rat anti-mouse Ki-67 antigen clone TEC-3, DakoCytomation, Denmark) at its working dilution of 1:100. After 30 min at room temperature, they were treated with Histofine Simple Stain Mouse MAX-PO (Rat) reagent (Nichirei Bioscience, Tokyo, Japan) for 30 min. They were washed three times with PBS after each incubation, and 3, 3'-diaminobenzidine was employed as a chromogen. Nuclei were lightly counterstained with Mayer's hematoxylin solution. For determination of immunostaining, epithelial cells of the duodenum were counted from the lowest point

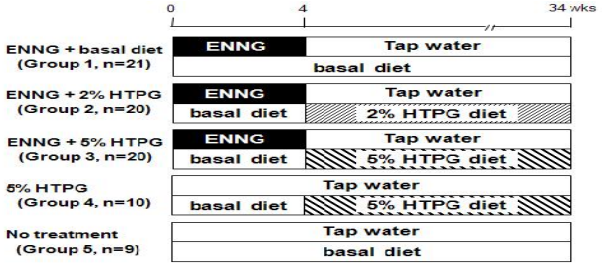

Figure 1. Experimental Schedule. Abbreviations: ENNG, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (100 mg/l); HTPG, high temperature- and pressure-treated garlic

of the crypt to the tip of the villus by light microscopy using 400 × magnification. The number of positively stained cells in each crypt column and villus was recorded. The results were defined as the ratio of the number of positive-stained cells to the total number of cells counted (at least 500), and then multiplied by 100.

Statistical analysis

Values are expressed as mean ±SE. Statistical analysis of the tumor incidence (percentage of tumor-bearing mice) was compared by the Fisher's exact test. Food consumption, body weight, organ weight, and tumor multiplicity (average number of tumors per mouse) was compared by the Kruskal-Wallis test (nonparametric ANOVA) followed by the Dunn's multiple comparisons test. MIB-5 labeling index was compared by ANOVA followed by the Dunnett Multiple Comparisons Test. These procedures were performed with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

General observations

As shown in Table 1, food consumption in Group 2 was significantly higher than that in Group 1. Food consumption in Group 3 was also increased, but not significantly so. There was no significant difference in the final body weight among the three groups (Table 1). Relative kidney weights in Group 2 were significantly higher than that in Group 1. Kidney weights in Group 3 were also increased, but not significantly so. There were no significant changes in absolute and relative organ weights expect relative to kidney weights among the three groups. There were no significant differences in food consumption, final body weight, and absolute and relative weight of the main organs such as the liver between Groups 4 and 5 (date not shown). In hematological findings, MCV (fl) in Group 4 (45.0±0.28) was significantly higher than that of Group 5 (43.9±0.39) ($p<0.05$), and MCH (pg) in Group 4 (14.5±0.06) was also significantly higher than that in Group 5 (14.2±0.08) ($p<0.01$). On the other hand, WBC ($10^3/\mu l$) in Group 4 (5.53±0.70) was significantly lower than that in Group 5 (8.66±0.82) ($p<0.01$).

Table 1. Food Consumption, Final Body Weight, and Organ Weight in ENNG-treated C57BL/6 Mice

Group	1	2	3
Diet	Basal diet	2% HTPG	5% HTPG
Number of animals	21	20	20
Food consumption (g/kg/day)	111.6±11.5	180.2±21.1[a]	149.1±14.6
Final body weight (g)	43.7±0.6	41.7±0.9	42.0±1.2
Absolute organ weight			
Liver (g)	1.84±0.11	1.77±0.12	1.70±0.12
Kidneys (g)	0.33±0.01	0.35±0.01	0.42±0.08
Relative organ weight (100 g body weight)			
Liver (g)	4.17±0.19	4.19±0.20	4.00±0.21
Kidneys (g)	0.74±0.01	0.84±0.02[a]	0.95±0.14

[a]Significantly different from Group 1 (P<0.01; Kruskal-Wallis test/Dunn's test)

ENNG-induced carcinogenesis

The effects of HTPG on ENNG-induced pyloric stomach and small intestinal (duodenal and jejunal) tumorigenesis in C57BL/6 mice are summarized in Table 2. In the small intestine, the incidences and multiplicities of adenomas in Group 2, but not those in Group 3, tended to be lower than those in Group 1, although not significantly so. In the pyloric stomach and small intestine, the incidences of adenomas and total tumors in Group 2 were significantly lower than those in Group 1 ($p<0.01$ or $p<0.05$). The multiplicities of adenomas and total tumors in Group 2 were also significantly lower than those in Group 1 (both $p<0.05$). However, there were no significant differences in the incidences and multiplicities of adenomas and total tumors between Groups 1 and 3.

MIB-5-labeling index

MIB-5-labeling indices were measured to assess cell proliferation in the duodenum. As shown in Figure 2, the MIB-5-labeling index of Group 2 (28.9 ± 1.4) was significantly lower than that in Group 1 (40.0 ± 2.1) ($p<0.01$), whereas the labeling index of Group 3 (38.3±1.8) was not significantly different from that in Group 1.

Table 2. Effects of HTPG on ENNG-induced Carcinogenesis in the Pyloric Stomach and Small Intestine of C57BL/6 Mice

Group	No. of mice	Tumor incidence (%)[a]			Tumor multiplicity[b]		
		AD	ADC	Total	AD	ADC	Total
A. Small intestine (duodenum and jejunum)							
1/ ENNG alone							
	21	19	4.8	24	0.3±0.2	0.1±0.1	0.4±0.2
2/ ENNG->2% HTPG							
	20	5	5	10	0.1±0.1	0.1±0.1	0.2±0.1
3/ ENNG->5% HTPG							
	20	20	5	25	0.3±0.1	0.2±0.2	0.5±0.2
B. Pyloric stomach and small intestine							
1/ ENNG alone							
	21	42.9	9.5	47.6	0.6±0.2	0.1±0.1	0.7±0.2
2/ ENNG-> 2% HTPG							
	20	5[c]	5	10[d]	0.1±0.1[c]	0.1±0.1	0.2±0.1[e]
3/ ENNG->5% HTPG							
	20	35	5	40	0.6±0.2	0.2±0.2	0.8±0.3

[a]Percentage of tumor-bearing mice; [b]Average number of tumors per mouse (means±SE); [c, d]Significantly different from Group 1 ([c]P<0.01;[d]P<0.05; Fisher's exact test); [e]Significantly different from Group 1 ([e]P<0.05; Kruskal-Wallis test/Dunn's test)

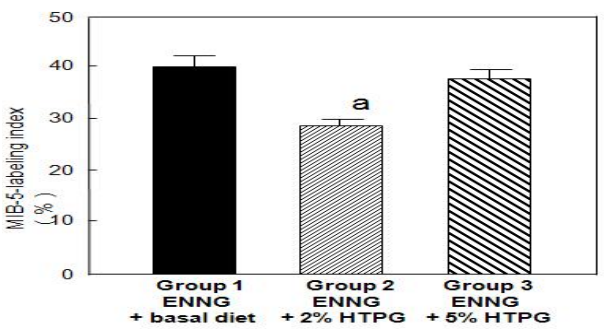

Figure 2. MIB-5-Labeling Index in Normal Duodenal Mucosa. [a]Significantly different from Group 1 (P<0.01; ANOVA/Dunnett multiple comparisons test)

Discussion

There have been many reports on anti-cancer and carcinogenesis-preventive effects of various garlic extracts (raw garlic extract, garlic oil, and aged garlic extract) and isolated garlic components (allyl methyl trisulfide, diallyl sulfide, and SAC) (Khanum et al., 2004; Shukla and Kalra, 2007). Black garlic, which has been recently sold widely in Japan (fermented and aged black garlic), has only a slight garlic smell and exhibits a marked antioxidative effect, attracting attention as a new health product (Sato et al., 2006a; Sasaki et al., 2007).

HTPG is a garlic material with a new possibility as a cancer-preventive food product. We investigated the influences of HTPG on ENNG-induced carcinogenesis in the pyloric stomach and small intestine in mice. When mice were fed the 2% HTPG-containing diet for 30 weeks in the post initiation stage (promotion stage) after ENNG administration, the incidence of tumors in the pyloric stomach and small intestine and the multiplicity of tumors in these mice were significantly lower than those in the basal diet group. In contrast, no significant differences were noted in the incidence or multiplicity of tumors in the 5% HTPG-containing diet group.

A cell proliferation marker, MIB-5, was stained to compare the positivity rate. The positivity rate was significantly lower in the 2% HTPG-containing diet group, but not in the 5% HTPG-containing diet group. This finding was consistent with tumor evaluation and may have been due to the influence of excess ingestion. Our collaborators (Yanagida et al., 2009) reported that no significant inhibitory effect on nitrosodimethylamine-induced renal cell carcinoma was obtained in mice fed a 5% HTPG-containing diet for 39 weeks (no 2% HTPG-containing diet group was set). On the other hand, we showed significant inhibition with a 3% HTPG-containing diet for 5 weeks and slight inhibition with a 1% HTPG-containing diet on DMH-induced MDF formation in the rat colorectum (Chihara et al., 2009). In addition, we observed significant inhibition by a 5-week feeding of a 10% HTPG-containing diet in DMH-induced DNA adduct formation experiment in rats (Chihara et al., 2009). Based on the series of carcinogenesis model experiments using HTPG with potent antioxidative activities, we assumed that inhibition of carcinogenesis was blocked by excess HTPG ingestion in animals fed the 5% HTPG-containing diet for 30-40 weeks in the post-initiation stage.

Similar cases have been reported. For example, Krestry et al. (2001) reported a chemopreventive effect of lyophilized black raspberries (LBRs)-containing diet on esophageal carcinogenesis in rats. Animals were fed 5 and 10% LBRs-containing diets throughout the experimental period or in the post-initiation stage, but no dose-dependent inhibition was noted: the longest post-initiation stage administration (35 weeks) of a 5% LBRs-containing diet significantly reduced the incidence and multiplicity of tumors, but no significant differences were noted in animals fed a 10% LBRs-containing diet. In contrast, dose-dependent inhibition was noted in the DNA adduct formation experiment in the initiation stage (5 or 10% LBRs-containing diet was given for 15 days). Based on these findings, they pointed out that the optimum dose of the chemopreventive component may vary depending on the age of animals, and long-term ingestion of high-dose LBRs may lead to interference with utilization of preventive components of LBRs by non-preventive components. A similar phenomenon may occur in our and collaborators' studies. We are planning to investigate these experimental conditions at a low dose.

The antioxidative effect may be a strong factor in the mechanism of cancer inhibition by HTPG. SAC is firstly considered as a component of HTPG. Katsuki et al. (2006) reported an inhibitory effect of SAC observed in a DMH-induced colorectal carcinogenesis experiment using mice. Garlic smell derived from sulfide components was reduced by autoclave treatment in HTPG, and the color of the powder became brown due to the Maillard reaction, suggesting that HTPG is similar to aged garlic extract (prepared by long-term aging of sliced raw garlic in alcoholic water (Amagase, 2006)) and fermented garlic (or black garlic) (Sato et al., 2006a; Kaneko et al., 2007; Sasaki et al., 2007; Wang et al., 2010), and contains *S*-allylmercaptocysteine, fructosyl arginine, and tetrahydro-β-carboline derivative, in addition to SAC (Imai et al., 1994; Ryu et al., 2001; Ichikawa et al., 2006; Sato et al., 2006b). A Korean research group which initially reported the HTPG production method identified an antioxidative substance, thiacremonone (Hwang et al., 2007), and showed that this substance alone exhibited an anticancer effect on human colon cancer cells and strengthened the growth inhibition effect of chemotherapeutic drugs (Ban et al., 2007; Ban et al., 2009).

It had been reported that HTPG also contains 5-HMF (Kwon et al., 2006), which was confirmed by our research team (Chihara et al., unpublished data). The pharmacological actions of 5-HMF on red blood cells and platelets have been reported, as well as the possibility to be metabolized to genotoxic and mutagenic 5-sulfooxymethylfulfural (Gato and Oka, 2009a; 2009b). However, according to the results of a long-term animal study performed by the U.S. National Toxicology Program (NTP), carcinogenicity of 5-HMF for rodents was noted only when the compound was administered at a high dose for 2 years, and it was only observed in females. (Natl Toxicol Program, 2010; Gato and Oka, 2009a; 2009b). Therefore, it was assumed that carcinogenesis was inhibited by HTPG through a complex modifying effect of the above various physiologically active components formed by autoclave treatment.

The incidence and multiplicity of tumors in the ENNG treatment groups (carcinogen-exposed groups) in this study were lower than those in our previous studies (Chihara et al., 2000; Shimpo et al., 2002), but there were many tumors in the pyloric stomach, not observed in the previous studies. It has been reported that the multiplicity of tumors in the pyloric stomach is increased with prolongation of the post-initiation stage after ENNG administration (Makita, 1991), suggesting that the post-initiation stage was too long.

Food intake in the ENNG treatment groups was higher in the 2 and 5% HTPG-containing diet groups than that

in the basal diet group, and it reached a 1.6 times higher intake in the 2% HTPG-containing diet group ($P<0.01$). In contrast, no significant difference was noted in food intake between the non-ENNG-treated basal and 5% HTPG-containing diet groups (no 2% HTPG-containing diet group was set). Based on these findings, mice initiated with ENNG had a higher palatability for HTPG (particularly for the 2% HTPG-containing diet), but, at present, it is unclear whether this was associated with carcinogenesis.

Regarding organ weights, the relative kidney weight was significantly higher in the 2% HTPG-containing diet group and slightly higher in the 5% HTPG-containing diet group than that in the basal diet group in the ENNG-treated groups. Similarly, in the non- ENNG-treated groups, it was slightly higher in the 5% HTPG-containing diet group. On hematological tests, the WBC count was significantly decreased in the non-ENNG-treated 5% HTPG-containing diet group (no hematological test was performed in the ENNG-treated groups). These findings on general condition showed an influence of excess ingestion in not only the 5%, but also partially in the 2% HTPG-containing diet group. We are planning to investigate the cancer preventive effect of HTPG at a low dose in consideration of palatability.

In conclusion, 30-week administration of the 2% HTPG-containing diet significantly inhibited ENNG-induced tumorigenesis in the pyloric stomach and small intestine in mice, and a reduction in intestinal cell proliferative ability by HTPG was suggested as its inhibitory mechanism.

Acknowledgements

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