

## RESEARCH COMMUNICATION

# Inhibition of 1,2-Dimethylhydrazine-Induced Mucin-Depleted Foci and *O*<sup>6</sup>-Methylguanine DNA Adducts in the Rat Colorectum by Boiled Garlic Powder

Takeshi Chihara<sup>1</sup>, Kan Shimpo<sup>1\*</sup>, Takaaki Kaneko<sup>1</sup>, Hidehiko Beppu<sup>1</sup>, Kenmei Mizutani<sup>1</sup>, Takashi Higashiguchi<sup>2</sup>, Shigeru Sonoda<sup>1</sup>

### Abstract

The scavenging capacity of reactive oxygen species, such as hydroxyl radicals, is reported not to decrease in boiled garlic (an odorless garlic preparation). We therefore examined the modifying effect of boiled garlic powder (BGP) on 1,2-dimethylhydrazine-induced mucin-depleted foci (MDF) and aberrant crypt foci (ACF), preneoplastic lesions, in the rat colorectum. Male F344 rats (5 weeks old) were fed a basal diet, or experimental diets containing 5% or 1% BGP for 5 weeks. One week later, all rats were injected s.c. with DMH (40 mg/kg, once weekly for 2 weeks). At 10 weeks of age, all the rats were sacrificed, and the colorectum was evaluated for MDF and ACF. In rats given DMH and the 5% or 1% BGP diets (Groups 2 and 3), the numbers of MDF decreased significantly in a dose-dependent manner, compared with the DMH and basal diet value (Group 1) ( $p < 0.01$ ). The numbers of ACF in Group 2, but not Group 3, showed a non-significant tendency to decrease. Next, the effects of BGP on the formation of DMH-induced *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) DNA adducts in rats were studied. Male F344 rats (5 weeks old) were fed the basal diet, or 10% BGP diet for 5 weeks. All rats were injected i.p. once with 40 mg/kg DMH at the end of week 5. The animals were sacrificed 6 hours after DMH injection to analyze the *O*<sup>6</sup>-MeG DNA adducts in the colorectal mucosa. Dietary administration of BGP significantly inhibited the *O*<sup>6</sup>-MeG DNA adduct levels in the colorectal mucosa, compared with the controls ( $p < 0.01$ ). These results suggested that BGP may exert chemopreventive effects against colon carcinogenesis at least in the initiation stage.

**Keywords:** Boiled garlic powder (BGP) - mucin-depleted foci (MDF) - *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) DNA adducts

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### Introduction

Several experimental and epidemiological studies have indicated the anticarcinogenic potential of garlic and its active constituents (see reviews; Fleischauer and Arab, 2001; Das, 2002; Khanum et al., 2004; Shukla and Kalra, 2007).

However, raw, crushed garlic has a strong odor which is favored by garlic lovers, but rejected by many people. Furthermore, people's breath smells after eating raw garlic, but it is known that the odor of heat-treated garlic is not as strong as that of raw garlic odor. Tamaki et al., (1999; 2008) measured the odor after *in vitro* or *in vivo* ingestion of raw or heated garlic, using an electronic nose, gas chromatography and sensory analysis, and demonstrated that the strength of garlic odor was stronger for raw garlic than for heat-treated garlic both in *in vivo* and *in vitro* studies.

We previously prepared heated garlic powder (=boiled garlic powder; BGP) and found that post-initiation-

stage feeding of BGP inhibited both the development of *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG)-induced duodenal and jejunal tumors and an increase in erythrocyte polyamine levels in C57BL/6 mice (Shimpo et al., 2002).

Recently, Pedraza-Chaverri et al., (2006) demonstrated that heating before or after garlic cutting was unable to eliminate the capacity of the extracts to scavenge superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, and therefore suggested that (a) the compound(s) involved in the scavenging capacity of garlic extracts are essentially heat stable and (b) the scavenging capacity of the garlic extracts is not related to alliinase activity. Prasad et al., (1996) also previously showed that garlic extract is a powerful scavenger of hydroxyl radicals and that heating reduces its activity slightly. Furthermore, alliin, the main component in extracts from boiled garlic, microwave-treated garlic and pickled garlic, scavenges hydroxyl radicals (Kourounakis and Rekka, 1991; Chung, 2006).

It is known that colon carcinogenesis induced by

<sup>1</sup>Fujita Memorial Nanakuri Institute, Fujita Health University, <sup>2</sup>Department of Surgery & Palliative Medicine, Fujita Health University School of Medicine. \*For correspondence : shimpo@fujita-hu.ac.jp

azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH) is mediated by reactive oxygen species, mainly hydroxyl radicals (Shamsuddin et al., 1988; Gamberini and Leite, 1997).

In the present study, we examined the modifying effect of the initiation-stage feeding of BGP on the formation of DMH-induced aberrant crypt foci (ACF) and mucin-depleted foci (MDF), putative premalignant lesions, in the rat colorectum.

## Materials and Methods

### Materials and Animals

1,2-Dimethylhydrazine (DMH) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and a 1 mM EDTA solution (pH 6.5) was prepared. Alcian blue was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade and were commercially available. BGP was prepared as follows. Fresh garlic bulbs were obtained from a local market. Unpeeled garlic bulbs were heated in boiling water for 15 min to inactivate alliinase. The boiled garlic bulbs were peeled, and the cloves were crushed and homogenized for 1 min using a Polytron. The homogenate was freeze-dried. The analysis of the boiled garlic powder used in this study gave the following results (calculated as dry weight); alliin (27.0 mg/g) and alliin-producing potential (none). The powder was finely pulverized with an Oster Powder Blender (Osaka Chemical Co., Ltd., Osaka, Japan) and added to the basal diet at 5% or 1% (Experiment 1), or 10% (Experiment 2), and thoroughly mixed using a ball mill. Male F344 rats (4 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 on a basal diet, the Oriental MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan), in an animal facility controlled at a temperature of  $23\pm 5^{\circ}\text{C}$ ,  $60\pm 5\%$  humidity, and with 12-h light/dark cycle. The care and use for the animals was according to the 'Guidelines for the Management of Laboratory Animals in Fujita Health University' and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

### Analysis of mucin-depleted foci and aberrant crypt foci

As in Experiment 1, after acclimatization for 1 week, the animals were divided into 3 groups and basal diet (Group 1), 5% BGP in the diet (Group 2), or 1% BGP diet (Group 3) were administered to each group for 8 weeks. On days 0 and 7 all rats were given s.c. injections of 40 mg/kg DMH. At 8 weeks after the first DMH treatment, all animals were anaesthetized with diethyl ether, killed by exsanguination, and carefully autopsied. The procedure of identification and quantification of MDF and ACF was performed as described by Yoshimi et al., (2004). Briefly, the colon was removed, flushed with saline, cut open along the longitudinal axis, attached flat on a paper filter, and fixed in 10% buffered formalin. After fixation, specimens 2 cm were cut. Each piece was rinsed in 3% acetic acid and stained with a 1% solution of Alcian blue (pH 2.5). Each piece was placed on a slide glass with the mucosal surface

facing upward and the numbers of MDF and ACF were counted under a microscope at a magnification of  $\times 40$ .

### DNA adduct formation

This experiment (Experiment 2) was performed as described by Herron and Shank (1981) with some modifications (Shimpo et al., 2003). After acclimatization for 1 week, the rats were fed the basal diet or 10% BGP in the diet for 5 weeks. At the end of week 5, all rats were given one i.p. injection 40 mg/kg DMH. Six hours after injection, they were anesthetized with diethylether, sacrificed by exsanguination, and carefully autopsied. The colorectum was immediately removed and rinsed in ice-cold phosphate buffered saline (PBS). It was then cut open longitudinally and washed with PBS to remove the colorectal contents. Next, it was placed on a glass plate and the mucosa was scraped off with a glass slide. These samples were kept at  $-80^{\circ}\text{C}$  until analysis of DNA adducts.

### Analysis of DNA adducts

Tissue DNA from the colorectal mucosa was isolated by phenol extraction and precipitated by ethanol. The pellet was suspended in 0.1 M HCl (5 mg/ml), and hydrolyzed at  $70^{\circ}\text{C}$  for 30 min to release the purines as free bases. The DNA hydrolysates were analyzed by the method of Herron and Shank (1981) with some modifications. The procedure was performed by high-performance liquid chromatography (HPLC) with a Gulliver series system (JASCO, Tokyo) equipped with a pump (PU-980), a degasser (DG-980-50) and an 821-FP intelligent spectrofluorometer at 366 nm emission with excitation at 286 nm. An analytic column, Whatman Partsil-10 SCX,  $250\times 4.5$  mm (GL Sciences Inc., Tokyo), with the same type of guard column was used. The  $O^6$ -MeG was eluted with a 0.05 M ammonium phosphate solution at pH 2.5 and a flow rate of 1.0 ml/min. Results are expressed as the ratio of  $O^6$ -MeG to guanine (G) in nmol/ $\mu\text{mol}$ .

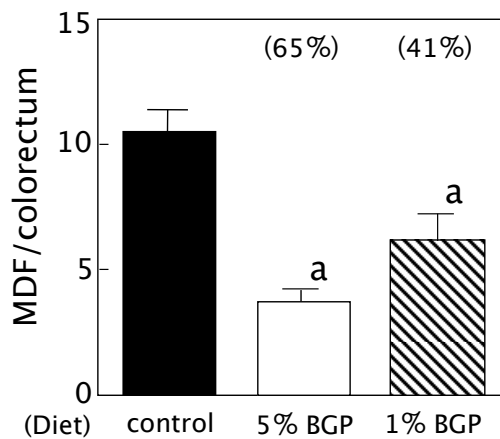
### Statistical analysis

The values are expressed as mean $\pm$ SE. Statistical analysis of the MDF and ACF formation were compared by Dunnett's multiple comparisons test. The DNA adducts were compared by unpaired *t*-test. These procedures were performed with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

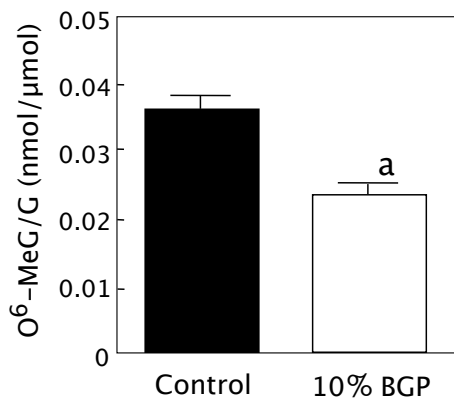
## Results

No rats died during the experimental period. The body weight and food consumption were not different among the 3 groups (data not shown).

To examine the chemopreventive effects against colorectal carcinogenesis in rats by BGP, 2 putative preneoplastic endpoints were used: the ACF discovered by Bird (1987) and the MDF firstly described by Caderni et al., (2003). As shown in Figure 1, total numbers of MDF/colorectum in Group 2 rats (treated with DMH and 5% BGP diet;  $3.7 \pm 0.6$ ) and Group 3 rats (treated with DMH and 1% BGP diet;  $6.2 \pm 1.0$ ) decreased significantly in a dose-dependent manner, compared with the values in Group 1 rats (treated with DMH and basal diet;  $10.5 \pm$



**Figure 1. Effect of BGP on DMH-induced MDF formation.** Each bar represents the mean  $\pm$  SE; Data in parentheses indicates percentage inhibition of MDF; \*Significantly different from control ( $p < 0.01$ ; Dunnett's multiple comparisons test)



**Figure 2. Effects of BGP on the Formation of DMH-Induced O<sup>6</sup>-MeG DNA Adducts in the Rat Colorectal Mucosa.** Each bar represents the mean  $\pm$  SE; \*Significantly different from control ( $p < 0.01$ ; unpaired *t*-test)

1.0) (percentage inhibition: 65% and 41%, respectively; both  $p < 0.01$ ). On the other hand, total numbers of ACF/colorectum in Group 2 rats ( $127.7 \pm 10.5$ ) tended to decrease compared with the values in Group 1 rats ( $155.8 \pm 19.6$ ; percentage inhibition: 18%), although this was not significant. However, there were no significant differences between Group 3 rats ( $152.5 \pm 9.5$ ) and Group 1 rats.

As a second step (Experiment 2), we examined the effect of BGP on the formation of DMH-induced DNA adducts (O<sup>6</sup>-MeG) in the rat colorectum, to clarify the possible mechanisms involved in its inhibition of MDF formation. The results shown in Figure 2 indicate that the O<sup>6</sup>-MeG levels in the colorectum of rats fed 10% BGP decreased by 41.7% compared with those in the colorectum of rats fed the basal diet ( $p < 0.01$ ).

## Discussion

To test whether boiled garlic powder (BGP) affects colorectal carcinogenesis induced in rats by DMH, 2 putative preneoplastic endpoints were used: the ACF discovered by Bird (1987) and MDF the firstly described by Caderni et al., (2003). The results demonstrated that

dietary administration of BGP significantly inhibited the formation of DMH-induced MDF (not ACF) in the rat colorectum. Since MDF are known to be more specific precancerous lesions of colorectal carcinogenesis than ACF (Femia et al., 2004; Yoshimi et al., 2004), our results suggest that BGP may inhibit DMH-induced colorectal carcinogenesis in rats, at least in the initiation stage.

We next examined whether BGP inhibited the formation of DMH-induced O<sup>6</sup>-MeG DNA adducts in the rat colorectum, because DNA adducts are believed to be an initial step in carcinogenesis. The results showed that BGP significantly inhibited O<sup>6</sup>-MeG DNA adduct levels in the colorectal mucosa compared with the controls. In addition, we further examined whether post-initiation administration of dietary BGP inhibited DMH-induced MDF formation in the rat colorectum, and the results showed that the total numbers of MDF/colorectum in 2% BGP diet rats ( $7.3 \pm 0.7$ ; 43.8%) decreased compared with the values in basal diet rats ( $13.0 \pm 2.9$ ; although this was not significant because of the small numbers of in the two groups) (Chihara et al., unpublished data). Therefore, the present results suggest that BGP has a chemopreventive effect against colorectal carcinogenesis, although long-term carcinogenesis experiments to test the inhibitory effects of BGP on the development of colorectal cancer in rodents are required.

The mechanisms by which BGP inhibited formation of DMH-induced MDF (preneoplastic lesions) and O<sup>6</sup>-MeG DNA adducts in the rat colorectum have not been clearly elucidated. Recently, we demonstrated that high temperature- and pressure-treated garlic (HTPG) inhibited DMH-induced MDF and O<sup>6</sup>-MeG DNA formation in the rat colorectum and reduced the activity of cytochrome P450 (CYP) 2E1 (a phase 1 enzyme) and increased the activities of phase 2 enzymes, quinone reductase (QR) and glutathione *S*-transferase (GST) in the rat liver (Chihara et al., 2009). However, we previously found that BGP feeding (10% in diet for 5 weeks) did not change the activities of CYP2E1, QR or GST in the livers of mice and rats (Chihara et al., 2005). In the present study, different mechanisms may therefore have been involved in the inhibitory actions of DMH-induced MDF and O<sup>6</sup>-MeG DNA adducts in the rat colorectum.

It seems that colon carcinogenesis induced by AOM or DMH is mediated by reactive oxygen species, mainly the hydroxyl radicals (Shamsuddin et al., 1988; Gamberini and Leite, 1997). Prasad et al. (1996) showed that garlic extract is a powerful scavenger of hydroxyl radicals and that heating reduces its activity slightly. Pedraza-Chaverri et al. (2006) also examined the reactive oxygen species scavenging capacity of different cooked garlic preparations, and the results indicated that heating before or after garlic cutting was unable to eliminate the capacity of the extracts to scavenge hydrogen peroxide, superoxide anion and hydroxyl radicals. From these data, the authors suggested that (a) the compound(s) involved in the scavenging capacity of garlic extracts are essentially heat stable and (b) the scavenging capacity of the garlic extracts is not related to alliinase activity (Pedraza-Chaverri et al., 2006). Furthermore, alliin, the main component in extracts from boiled garlic, microwave-treated garlic and pickled

garlic, scavenges hydroxyl radicals (Kourounakis and Rekka, 1991; Chung, 2006). Therefore, the mechanisms by which dietary BGP (mainly alliin) suppress DMH-induced MDF (pre-malignant lesions) and *O*<sup>6</sup>-MeG DNA adduct formation in the rat colorectum may be responsible, at least partly, for the hydroxyl radical scavenging action.

On the other hand, garlic contains abundant carbohydrates, and the main components of the carbohydrates are inulin-type fructans (beta (2,1) fructans) (Lawson, 1996). Interestingly, there is a lot of experimental evidence that inulin-type fructans modulated colon cancer risks in human colon cells, in animals, and in a human intervention trial (Pool-Zobel, 2005; Pool-Zobel and Sauer, 2007). Thus, in the present study inulin-type fructans as major nonsulfur compounds of BGP, together with alliin (a major sulfur compound of BGP), may exert inhibitory effects on MDF and *O*<sup>6</sup>-MeG formation in the rat colorectum.

In conclusion, the results of this study demonstrated that initiation-stage feeding of BGP inhibited DMH-induced MDF and *O*<sup>6</sup>-MeG DNA adduct formation in the rat colorectum. These results indicate that BGP may have anticarcinogenic activity in the colorectum.

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