
RESEARCH COMMUNICATION

Inhibitory Effects of Heated Garlic on *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine-induced Carcinogenesis in the Duodenum and Jejunum of C57BL/6 Mice

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Abstract

We examined the modifying effects of heated garlic (*Allium sativum* L.) on *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)-induced duodenal and jejunal carcinogenesis in mice. Heated garlic powder used in this study was prepared as follows: unpeeled garlic bulbs were blanched in boiling water for 6 min, and then peeled, the cloves being crushed, homogenized, and finally freeze-dried. The garlic powder had almost undetectable alliinase activity and was rich in alliin (the main sulfur compound of heated garlic; 22.1 mg/g dry weight). Male C57BL/6 mice were given ENNG (100 mg/l) in drinking water for the first 4 weeks, and then basal diet (Group 1), or 10% (Group 2), 3% (Group 3) or 1% (Group 4) heated garlic in the diet for 30 weeks. At the termination of the experiment, the incidences of duodenal tumors in Groups 1-3 were significantly lower than those in Group 1, and the multiplicities in Group 2 were significantly lower than those in Group 1. Additionally, the incidences and/or multiplicities of the jejunal tumors in Groups 2 and 4 were also significantly lower than those in Group 1. In this study, we also examined changes in erythrocyte polyamine levels. Values for Group 1 were significantly greater than those in the control group, and this elevation in Group 1 were significantly inhibited by dietary heated garlic (10% in the diet; Group 2). These results indicated that the post-initiation-stage feeding of heated garlic, especially at 10% in the diet, inhibits ENNG-induced duodenal and jejunal carcinogenesis in mice.

Key Words: Heated garlic (*Allium sativum* L.) - anti-tumor promotion - mouse duodenal and jejunal carcinogenesis - *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) - erythrocyte polyamines

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Introduction

Garlic (*Allium sativum* L.) has been used worldwide not only as a flavoring but also as a traditional medicine since ancient times. During the past several decades, many researchers have reported on various pharmacological effects of garlic and certain constituents of garlic or their transformation products (see reviews and books; Mochizuki et al., 1995; Agarwal, 1996; Koch and Lawson, 1996; WHO, 1999; Ariga and Seki, 2000; Saito, 2000). The most important chemical constituents reported from garlic are the

organosulfur compounds and the sulfur chemical profiles of garlic products are reflected by the processing procedure (Mochizuki et al., 1995; Koch and Lawson, 1996; WHO, 1999; Ariga and Seki, 2000; Saito, 2000). Garlic has a typical odor which is favored by garlic lovers but rejected by people who dislike it, and the latter are the majority, at least in Western society (except in some Mediterranean countries) and also in Japan. Thus, attempts to hide or mask the typical gastric odor in medicinal products have been numerous, especially in the patent literature (Pentz and Siegers, 1996; Koch, 1996).

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Recently, we reported that post-initiation-phase feeding of freeze-dried *Aloe arborescens* whole leaf powder (Aloe) inhibited the development of *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG)-induced duodenal tumors in mice (Chihara et al., 2000a). In addition, we also found that the inhibition of the ENNG-induced tumorigenesis by Aloe alone was identical to the inhibition by a mixture of Aloe and heated garlic (Shimpo et al., 2000). The heated garlic powder was alliinase-inactivated, alliin-enriched, and therefore odor-minimized (Shimpo et al., 2000).

The present study was designed to examine the effect of heated garlic alone on ENNG-induced duodenal and jejunal carcinogenesis in mice.

Materials and Methods

Materials

Male C57BL/6 mice (7 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in groups of four or five in plastic cages on woodchip bedding and fed on 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 for the animals was according to the 'Guideline fo the Care and Use of Laboratory Animals' of Fujita Health University. 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. Freeze-dried powder of heated garlic was provided by Yurika Co. Ltd. (Hisai, Japan). The garlic powder was prepared as follows. Fresh garlic bulbs were obtained from a local market. Unpeeled garlic bulbs were blanched in boiling water for 6 min to inactivate alliinase. The blanched 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 blanched garlic powder used in this study gave the following results (calculated as dry weight); alliin (22.1 mg/g) and alliin-producing potential (0.03 mg/g). The powder was finely pulverized with an Oster Power Blender (Osaka Chemical Co., Ltd., Osaka, Japan) and added to the basal diet at 10%, 3%, or 1%, and throughly mixed using a ball mill. All other chemicals were of the highest grade available and were obtained commercially.

Experimental protocol

After acclimatization for 1 week, the animals were divided into 7 groups as shown in Figure 1. Mice in Groups 1, 2, 3 and 4 were given basal diet and ENNG (100 mg/l) in drinking water *ad libitum* for the first 4 weeks. Then, these groups were shifted to tap water, and Groups 1, 2, 3 and 4 were given basal diet, or 10%, 3% or 1% heated garlic powder in basal diet *ad libitum* for 30 weeks, respectively.

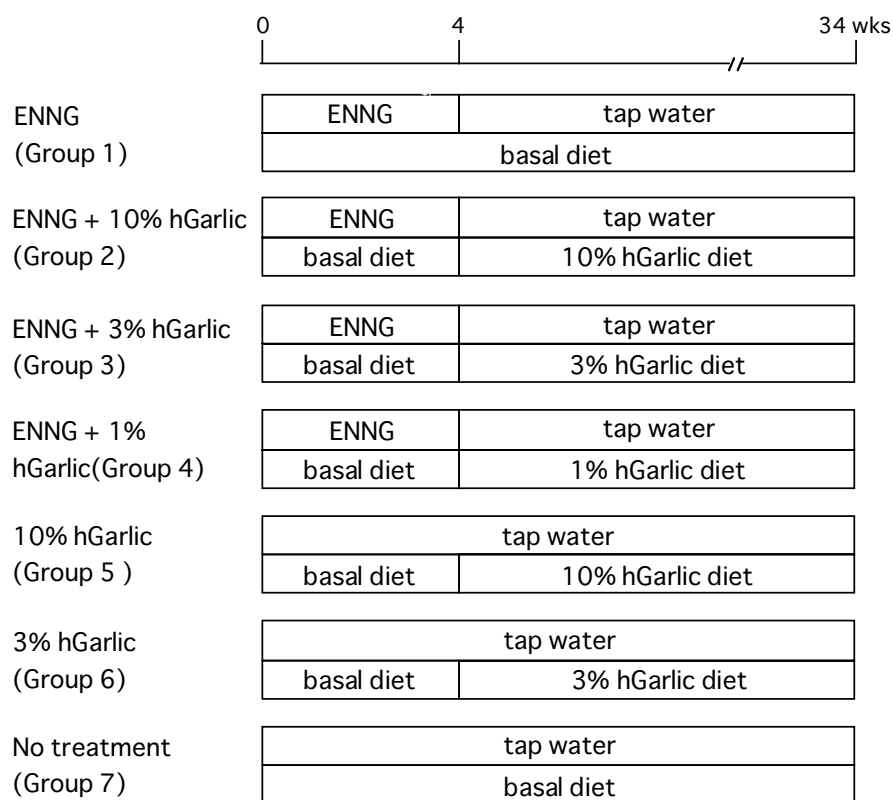


Figure 1. Experimental Schedule

Abbreviations: ENNG, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine (100 mg/l); hGarlic, heated garlic powder

Mice in Groups 5, 6 and 7 were given basal diet for the first 4 weeks, and then given 10% or 3% heated garlic powder in the basal diet, or the basal diet *ad libitum* for 30 weeks, respectively. Mice in these 3 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 into heparin-coated syringes. 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 any unusual mass lesions 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.

Determination of erythrocyte polyamine levels

Erythrocyte polyamines were determined as described previously (Chihara et al., 2000a). Briefly, heparinized blood samples were centrifuged at 600 x g for 10 min at 4 °C. After removal of plasma and the buffy coat layer, packed erythrocytes were hemolyzed with water, and extracted into 10% perchloric acid. The polyamines in the cell extracts were derivatized with dansyl chloride at 70°C for 10 min and extracted with cyclohexane. The cyclohexane phase was dried. The residues were redissolved in 70% acetonitrile and separated on a Puresil C column (5 μ m, 4.6 x 250 mm; Nihon Waters K.K., Tokyo¹⁸, Japan), with an acetonitrile-water gradient as the mobile phase. Eluted dansyl polyamines were detected with a fluorescence detector.

Statistical analysis

Statistical analysis of the tumor incidence (percentage of tumor-bearing mice) was compared by the Fisher's exact

test. The tumor multiplicity (average number of tumors per mouse) and erythrocyte polyamine levels were compared by the Kruskal-Wallis test (nonparametric ANOVA) followed by the Dunn's multiple comparisons test. These procedures were performed with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

General observations

There was no significant difference in the final body weight (Table 1), the weight of organs and the average food consumption (data not shown) among the seven groups. Hematological parameters were also not affected by treatments with ENNG and/or heated garlic (data not shown). Five mice died before completion of the study, two from Group 1 at 28 and 32 weeks, one from Group 2 at 28 weeks and two from Group 3 at 28 weeks. These mice were included in the tumor analysis, because all the deaths might be due to gastrointestinal tumors induced by ENNG.

ENNG-induced carcinogenesis

The effects of heated garlic on ENNG-induced mouse duodenal and jejunal tumorigenesis are summarized in Table 1-A. The incidences of adenomas, adenocarcinomas and total tumors (adenomas + adenocarcinomas) in the duodenum in Group 1 were 24%, 80% and 88%, respectively. The incidences of adenocarcinomas in Groups 2 and 3 were significantly lower than those in Group 1 (p<0.01 or p<0.05). The incidences of total tumors in Groups 2, 3 and 4 were also significantly lower than those in Group 1 (p<0.01 or p<0.05). The multiplicities of adenomas, adenocarcinomas and total tumors in Group 1 were 0.36±0.14, 1.44±0.24 and 1.80±0.27 (mean±SE), respectively. The multiplicities of adenocarcinomas and total tumors in Group 2 were significantly lower than those in Group 1 (p<0.05), but those

Table 1. Effects of Heated Garlic on ENNG-induced Carcinogenesis in the Duodenum and Jejunum of C57BL/6 Mice

Group/Treatment	No. of mice	Body wt ^a (g)	Tumor incidence (%) ^b			Tumor multiplicity ^c		
			AD	ADC	Total	AD	ADC	Total
A. Duodenum								
1/ ENNG alone	25	45.5 ± 0.9	24	80	88	0.36 ± 0.14	1.44 ± 0.24	1.80 ± 0.27
2/ ENNG → 10% hGarlic	25	45.8 ± 0.9	16	40 ^d	48 ^d	0.16 ± 0.07	0.76 ± 0.23 ^f	0.92 ± 0.28 ^f
3/ ENNG → 3% hGarlic	25	43.5 ± 1.4	24	48 ^e	60 ^e	0.32 ± 0.14	1.28 ± 0.38	1.60 ± 0.38
4/ ENNG → 1% hGarlic	25	46.4 ± 0.9	16	60	64 ^e	0.16 ± 0.07	1.12 ± 0.27	1.28 ± 0.30
B. Jejunum								
1/ ENNG alone	25	45.5 ± 0.9	0	24	24	0	0.36 ± 0.15	0.36 ± 0.15
2/ ENNG → 10% hGarlic	25	45.8 ± 0.9	0	4 ^e	4 ^e	0	0.04 ± 0.04 ^f	0.04 ± 0.04
3/ ENNG → 3% hGarlic	25	43.5 ± 1.4	4	8	12	0.04 ± 0.04	0.08 ± 0.06	0.12 ± 0.07
4/ ENNG → 1% hGarlic	25	46.4 ± 0.9	0	0 ^e	0 ^e	0	0 ^g	0 ^f

Abbreviations: AD, adenoma; ADC, adenocarcinoma; ^aBody weight at experimental termination (mean ± SE); ^bPercentage of tumor-bearing mice; ^cAverage number of tumors per mouse (means ± SE); ^{d,e}Significantly different from Group 1 by the Fisher's exact test (^dP<0.01; ^eP<0.05); ^{f,g}Significantly different from group 1 by the Kruskal-Wallis test with the Dunn's test (^fP<0.05; ^gP<0.01)

in Groups 3 and 4 did not significantly differ from those in Group 1. There were no significant differences in the incidences and multiplicities of adenomas in all the ENNG-treated groups.

As shown in Table 1-B, there were a few tumors in the mouse jejunum in the present study. The incidences of adenocarcinomas and total tumors in Group 1 were 24% and 24%, respectively. The incidences of adenocarcinomas and total tumors in Groups 2 and 4 were significantly lower than those in Group 1 (all $p < 0.05$). The multiplicities of adenocarcinomas and total tumors in Group 1 were 0.36 ± 0.15 and 0.36 ± 0.15 , respectively. The multiplicities of adenocarcinomas in Groups 2 and 4 were significantly lower than those in Group 1 ($p < 0.05$ or $p < 0.01$). The multiplicities of total tumors in Group 4 were significantly lower than those in Group 1 ($p < 0.05$). There was only one adenoma in the jejunum of Group 3 in all the ENNG-treated groups. No duodenal and jejunal tumors were found in the animals that did not receive ENNG.

In addition, the multiplicities of adenomas of glandular stomach were 0.24 ± 0.09 , 0.20 ± 0.10 , 0.08 ± 0.06 and 0.16 ± 0.08 in Groups 1 - 4, respectively. The multiplicities of adenocarcinomas of glandular stomach were 0.08 ± 0.06 , 0.08 ± 0.06 , 0.08 ± 0.06 and 0 in Groups 1 - 4, respectively. There were no significant differences in the incidences (data not shown) and multiplicities among Groups 1 - 4. There were a few dysplasias in the esophagus and forestomach of the mice in Groups 1 - 4, but the differences were not significant (data not shown). No tumors were found in other organs of the animals in all groups.

Erythrocyte polyamine levels

Since it has been suggested that the erythrocyte polyamine levels may be one of the biomarkers in chemopreventive studies and may parallel the morphological evaluation, we also examined the changes in the erythrocyte polyamine levels in ENNG-induced mouse duodenal and jejunal tumorigenesis by dietary heated garlic in this study. Table 2 shows the erythrocyte polyamine levels of mice in this experiment. The erythrocyte spermidine and spermine levels in Group 1 were significantly greater than those in the control group (Groups 5 + 6 + 7) ($p < 0.05$ or $p < 0.01$).

The erythrocyte spermidine levels in Group 2 were significantly lower than those in Group 1 ($p < 0.05$), and the erythrocyte spermine levels in Group 2 tended to be lower than those in Group 1. However, these polyamine levels in Groups 3 and 4 were not significantly lower than those in Group 1.

Discussion

The ENNG-induced mouse duodenal tumorigenesis model was developed by Matsuyama et al. (1975). This model was used to examine the inhibitory effect on tumor promotion of some phytochemicals in the upper gastrointestinal tract (Fujita et al., 1989; Huang et al., 1994). Previously, we also used this model to examine the effects of *Aloe arborescens*. In the experiment, the tumor incidence of the ENNG-alone group was 44%. Our results were similar to those (45%) reported by Huang et al. (1994). However, the data were lower than the tumor incidence (63%) of Fujita et al. (1989). In the present study, thus, we prolonged the tumor promotion (post-initiation) stage from 16 weeks to 30 weeks. As a result, the duodenal tumor incidence and multiplicity of the ENNG-alone group rose to 88% and 1.80 ± 0.27 (mean \pm SE), respectively. In addition, 80% of the tumors were histologically adenocarcinomas. Neoplastic lesions were also observed in the jejunum, although the tumor incidence and multiplicities were much lower compared with those of the duodenum. Additionally, glandular stomach tumors (adenomas and adenocarcinomas) and dysplasias of the esophagus and forestomach were seen in a small number of mice.

In this experiment, we examined the effects of heated garlic (alliin content, 22.1 mg/g dry weight; almost undetectable alliinase activity; 10%, 3% or 1% in the diet) on duodenal tumorigenesis in mice. Feeding with 10%, 3% and 1% heated garlic significantly decreased the tumor incidences of adenocarcinomas and/or total tumors in the duodenum. Feeding with 10% heated garlic, but not 3% or 1% heated garlic, also significantly decreased the multiplicities of the adenocarcinomas and total tumors in the duodenum. Dietary heated garlic also significantly reduced the incidences and/or multiplicities of jejunal tumors

Table 2. Erythrocyte Polyamine Levels

Group/Treatment	No.	Spermidine	Spermine
1/ ENNG alone	23	208.9 ± 64.2^a	25.0 ± 4.6^a
2/ ENNG → 10% hGarlic	24	100.6 ± 27.6^b	16.1 ± 3.9
3/ ENNG → 3% hGarlic	23	218.4 ± 63.9	31.0 ± 8.3
4/ ENNG → 1% hGarlic	25	175.6 ± 65.2	24.7 ± 7.8
5/ 10% hGarlic	5	39.2 ± 4.9	6.4 ± 0.8
6/ 3% hGarlic	5	36.4 ± 4.9	5.2 ± 0.3
7/ No treatment	5	43.4 ± 2.8	6.3 ± 0.4
5+6+7/ Controls	15	39.7 ± 2.4^b	6.0 ± 0.3^c

^aMean \pm SE (nmol/ml packed erythrocytes); ^{b,c}Significantly different from Group 1 by the Kruskal-Wallis test with the Dunn's test ($bp < 0.05$, $cp < 0.01$)

to an almost similar extent as in the duodenum.

In the present study, we measured the erythrocyte polyamine level as a marker of cell proliferation, and we found that feeding with heated garlic (10% in diet) significantly suppressed the increase in the erythrocyte polyamine level in ENNG-treated mice. We previously demonstrated the significance of erythrocyte polyamine determination in various carcinogenic models (Hibino et al., 1990; Shimpo et al., 1996; Chihara et al., 2000a, 2000b). Mouloux et al. (1991) reported that circulating polyamines, mainly erythrocyte polyamines, are markers of cellular proliferation, factors involved in maintaining the state of malignant proliferation, and have a potential role in the homeostatic control of cellular growth. They, therefore, have characteristics which are similar to those observed for intracellular polyamines. Tanaka et al. (1998) also measured the blood polyamine level as an intermediate biomarker in chemopreventive studies, and they showed that total polyamine level in blood was well correlated with that in the target organ (colonic mucosa). Thus, the results of the present study on erythrocyte polyamines also support the potential of heated garlic in reducing the development of tumors.

Numerous reports, including several epidemiological studies, indicate that garlic and its organosulfur compounds inhibit chemically-induced and transplanted tumors in experimental animals (Agarwal, 1996; Reuter et al., 1996; Milner, 2001; Fleischauer and Arab, 2001; Yang et al., 2001; Fukushima et al., 2001). However, few studies have reported on the effect of heated garlic and its major organosulfur compound alliin on chemical carcinogenesis. Therefore, it is believed that our present study is the first report on cancer chemoprevention using heated garlic.

The mechanisms of the inhibitory effects of dietary heated garlic on ENNG-induced duodenal and jejunal tumorigenesis in mice are unknown. Alliin, a major sulfur compound of heated garlic, was reported to have no antioxidative activity in a linoleic acid oxidation system in vitro (Hirata and Matsushima, 1996). However, Kourounakis and Rekkas (1991) reported that alliin was a very good hydroxyl radical scavenger. Prasad et al. (1996) also indicated that garlic extract was a powerful scavenger of hydroxyl radical and that heating reduces its activity slightly. In addition, Sheela and Augusti (1995) reported that when cholesterol-fed rats were fed a very large dose of alliin (200 mg/kg body weight) for 2 months, those animals resulted in significantly decreased tissue peroxides and increased tissue glutathione, SOD activity, and catalase activity. Our preliminary findings in the present study also indicated that feeding of heated garlic significantly suppressed the lipid peroxide levels in plasma of ENNG-treated mice (data not shown). On the other hand, Salman et al. (1999) showed that alliin in vitro exerts an immunomodulatory effect on certain functions of the peripheral blood cells. Kasuga et al. (2001) also showed that treatment with heated garlic extract significantly enhanced killer cell activity in splenic cells prepared from Sarcoma 180-bearing mice, whereas it

increased natural killer (NK) cell activity slightly. Thus, the antitumor action mechanisms of heated garlic may be due to free radical scavenging activity and immune system modulation; however, further experiments are necessary to clarify these points.

In conclusion, we found that post-initiation-stage feeding of heated garlic powder inhibited both the development of ENNG-induced duodenal and jejunal tumors and the increase in erythrocyte polyamine levels in C57BL/6 mice.

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