

Black Garlic (*Allium sativum*) Extracts Enhance the Immune System

Danan Wang¹ • Yonghui Feng¹ • Jun Liu¹ • Jianzhong Yan¹ •
Meiru Wang¹ • Jin-ichi Sasaki^{2*} • Changlong Lu^{1**}

¹ Institute of Immunology China Medical University, 92 North Second Road, Heping District, Shenyang 110001, China

² Department of Clinical Immunology Hirosaki University School of Health Sciences, 66-1 Honcho, Hirosaki 036-8565, Japan

Corresponding authors: * jnsasaki@tk2.so-net.ne.jp ** clu@mail.cmu.edu.cn

ABSTRACT

Black garlic (*Allium sativum*) was created from ordinary fresh garlic by processing (aging) it in a temperature (65-80°C)- and humidity (70-80%)-controlled room for a month. The heat-extracts of black garlic were rich in S-allyl-L-cysteine (SAC) and enforced anti-tumor activity with a 50% cure rate of BALB/c mouse fibrosarcoma; however, its mechanism still remains unsolved until now. Experiments were carried out to clarify anti-tumor mechanisms using spleen cells culture system obtained from black garlic extracts-treated mice. The black garlic extracts enhanced the cellular immunity by raising the activity of NK (natural killer) cells which was thought to play a critical role in eradication of tumor cells *in vivo*. Further cytokines of NO (nitric oxide), IFN- γ (interferon- γ), IL-2 (interleukin-2), and TNF- α (tumor necrosis factor- α) were preferentially generated from the extracts-treated mouse spleen cells; however, the amount of IL-4 (interleukin-4), which is considered to be associated with the humoral immunity (antibody production such as IgG and IgE), decreased in the culture supernatants of spleen cells.

Keywords: anti-tumor potency, cytokine generation, enforcement of immune system, IgE, NK cells activity, S-allyl-L-cysteine

INTRODUCTION

Garlic has been used world-wide as a traditional medicine for over 4000 years to treat several disorders such as arthritis, diabetes and infectious diseases (common cold, malaria, and tuberculosis) (Bratman 2000; Espirito Santo *et al.* 2007). Further, the microbiologist Louis Pasteur demonstrated the bactericidal properties of garlic; later it was called "Russian penicillin" in the Second World War II because, after running out of antibiotics, the Russian government turned to this ancient treatment for its soldiers.

There is an increased concern about the trustworthiness of different sources of information about functional foods to improve and strengthen health by blending their inherent functions into daily food life (Mazza 1998; Bratman 2000; Sasaki 2006). Under these social requirements, we initiated studies to find novel bio-functions in foods and consequently provided individuals with beneficial information on squid fish (Takaya *et al.* 1994; Sasaki *et al.* 1997; Naraoka *et al.* 2000), garlic (Sasaki *et al.* 1999, 2003), mushroom (Sasaki *et al.* 2000), and sweet corn (Sasaki *et al.* 2003). The past reports on the functions of garlic were to reduce blood pressure, lower high cholesterol, prevent heart attacks and cancer, and inhibit microbe growth (Bratman 2000; Espirito Santo *et al.* 2007). We have additionally demonstrated novel bio-actions of garlic such as antibacterial potency against *Bacillus anthracis* (used as a bio-weapon in 2001 in the USA) and enterohemorrhagic *Escherichia coli* O157 (which caused the historical vast food poisoning in 1996, in Japan), which were never described before and anticoagulation, antioxidant and detoxification activity against organophosphate compounds causing poisoning (Sasaki 2006). Recently, a new type of garlic, black garlic, was developed in Japan by processing (aging) ordinary fresh garlic in a temperature- and humidity-controlled room without using any artificial additives. The final products developed in this manner were black in color with less or non-stimulating smell, a fruit-like sweetness, and are



Fig. 1 Processed black garlic clove. Fresh white garlic (left) changed its color to black (right) by processing in a temperature- and humidity-controlled room for a month.

readily edible just by peeling.

In a previous report (Sasaki *et al.* 2007), we described a strong anti-tumor activity of the black garlic (extracts) in the Meth A fibrosarcoma model of BALB/c mice, which showed a 50% cure rate with no direct cytotoxicity of the extracts against tumor cells in a test of mixture cultivation of extracts and tumor cells. These results suggested the probable association of the enhanced immune system due to bio-functional potency of black garlic (extracts) to eradicate transplanted tumor cells (Sasaki *et al.* 2007). In this paper, we present the immune enhancement potency of black garlic (extracts), which is possibly associated with the eradication of tumor cells in this mouse model.

MATERIALS AND METHODS

Processed (aged) black garlic

Black garlic was created by maintaining fresh garlic (cloves) in a temperature (65-80°C)- and humidity (70-80%)-controlled room for 30-40 days without any additional treatments and additives. Fresh white garlic changed its color from white to brown and eventually became black a month later, caused by the Maillard and Browning Reaction (Fig. 1). Black garlic has a soft fruity taste

with a non-irritating odor.

Animals and experimental design

Six- to 8-week-old female C57BL/6 mice were purchased from Academia Sinica Shanghai Experimental Animal Center (Shanghai, China) and housed in pathogen-free conditions. Five mice weighing 20 g formed one cage, and eight cages were prepared for the following experimental analyses. Animals were kept in a temperature- and humidity-controlled room throughout experiment in The Animal Experimental House, China Medical University. Each mouse in the experimental groups received an intra-peritoneal injection of the black garlic extracts with a dosage of 100 µg/0.1 mL/day for 5 days. Animal treatment with extracts ceased from day 6 to the end of experiment. Five mice in an individual group were sacrificed daily to isolate spleen (cells) and serum for immunological analyses. The isolated spleens and sera were preserved at -80°C until initiation of analyses, as described next. The above described animal experiments were approved by the Animal Ethics Committee of Hirotsuki University, Japan and China Medical University, China.

Black garlic extraction

Black garlic with 6 cloves (initial weight; around 30 g) were chopped and mashed for heat extraction in water (30 g/100 mL) at 90-100°C, 1-2 h to obtain water-soluble compounds. The extracted solution was centrifuged at 3000 rpm for 20 min to isolate the supernatants, frozen at -80°C then freeze-drying (lyophilized) (Freeze Dryer, BFD-2, Nihon Freezer Co. Ltd., Japan) for the immunological activity tests.

Chemical analyses

The extracts were used to measure the amount of S-allyl-L-cysteine (SAC) (an important bio-functional compound) and γ-glutamyl-S-allyl-L-cysteine (GSAC) (precursor of SAC) by liquid chromatography (Alliance 2696, Co. Ltd., Japan) under the following analytical conditions: column size (2 mm×50 mm), column temp (40°C), flow speed (0.2 ml/min), injection volume (5 µL), mobile phase [water (85): 10 mM ammonium formate (10): methanol (5)].

Antiradical activity assay

Antioxidant potency of the black garlic extracts was measured by the DPPH (1,1-diphenyl-2-picrylhydrazyl) (Wako Co. Ltd., Japan) method and expressed by mg consumed to reduce 50% of 1,1-diphenyl-2-picrylhydrazyl (RS50%). Extracts were mixed with DPPH (0.1 mM) in ethanol. After 20 min incubation at room temperature, absorbance at 217 nm was read.

NK (natural killer) cell activity assay in spleen cells prepared from the black garlic extracts-treated mice

Cell number of the Yac-1 as target cells for the test of NK cells activity (Cell Bank of the Chinese Academy of Sciences; No. 13917329935) was adjusted to 1.0×10^5 /mL in 10% FBS (inactivated fetal bovine serum) RPMI 1640 (Gibco Co. Ltd., USA) and used for the test of NK cells activity. The number of spleen cells as effector cells was adjusted to 1.0×10^6 /100 µL/micro-plate well

(Costa Co. Ltd., USA) and that of target cells to 1.0×10^4 /100 µL/well. They were co-cultivated in a 96-well micro-plate for 2 h under 5.0% CO₂-air at 37°C. Then 100 µL of the cultured supernatants was transferred into a new well for additional 10 min cultivation under 5.0% CO₂-air at 37°C. LDH (lactate dehydrogenase) substrate (Amersco Co. Ltd., USA) at 100 µL/well was kept for 10 min in the dark. The enzymatic reaction was stopped by adding citric acid at 30.0 µL (1.0 mol/L)/well. Triplicate experiments were performed for statistical evaluation.

NO (nitric oxide) induction in spleen cells prepared from the black garlic extracts-treated mice

For analysis of NO production spleen cells at 5.0×10^6 /mL were suspended in 10% FBS RPMI 1640 medium and incubated under 5.0% CO₂-air at 37°C for 48 h and culture supernatants were collected to measure the amount produced (Cao *et al.* 1998). Briefly, 0.1 mL of the culture supernatants and 0.1 mL of Griess solution (Wako Co. Ltd., Japan) were mixed in a well of the micro-plate and kept at room temperature for 10 min to measure the OD value at 550 nm.

Cytokine production in spleen cells prepared from the black garlic extracts-treated mice

Spleen cells at 5.0×10^6 /mL suspended in 10% FBS RPMI 1640 medium were incubated under 5.0% CO₂-air at 37°C for 48 h and culture supernatants were provided for cytokine measurement using the following ELISA Kit System: IL-2 (R&D System, Inc. DY 402, USA), TNF-α (R&D System, Inc. DY 410, USA), IL-4 (R&D System, Inc. DY 404, USA), and IFN-γ (eBioscience, Inc. 88-7314-88, USA). Triplicate measurements were carried out for statistical evaluation.

Statistic evaluation

The student's *t*-test (Windows 16.0, SPSS) was employed to evaluate the statistic significance between experimental and control groups (* *P* < 0.05, ***P* < 0.001).

RESULTS AND DISCUSSION

Anti-tumor potency of the black garlic extracts

Anti-tumor potency in newly developed black garlic (extracts) reached 50% in the 1.0 mg treatment after three injections on day 2, 4, 6 after tumor transplantation (Sasaki *et al.* 2007). The average tumor size in non-cured mice of the black garlic-treated mice was half that of the non-treated control group. By contrast, the fresh garlic extracts used as a reference failed to eradicate the transplanted tumor, even though the average tumor size in non-cured animal was less than that of non-treated control mice. These results indicated the presence of strong anti-tumor potency in the black garlic (extracts).

Chemical composition of the black garlic extracts

Carbohydrate content, which is probably associated with sweetness in black garlic, increased from 28.7% in fresh garlic to 47.0% in black garlic (Sasaki *et al.* 2007). Other

Table 1 Effect of the black garlic extracts on the cytokines production from the extracts-treated mouse spleen cells (**P* < 0.05, ***P* < 0.001).

Days	IL-2 (pg/ml)	IL-4 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	NO (µM)
Control	20.1 ± 3.9	20.0 ± 2.1	27.3 ± 8.2	17.4 ± 1.5	11.6 ± 0.9
6	15.9 ± 2.3	16.6 ± 2.8	47.0 ± 5.9*↑	24.5 ± 1.7*↑	11.2 ± 2.3
7	15.5 ± 1.8*↓	17.7 ± 1.7	60.7 ± 3.9*↑	34.7 ± 4.3**↑	10.4 ± 1.2*↑
8	18.2 ± 4.0	15.5 ± 1.0*↓	68.7 ± 0.6**↑	105.0 ± 55.7*↑	26.9 ± 1.0**↑
9	18.0 ± 2.6	15.9 ± 1.3*↓	56.5 ± 3.4**↑	30.0 ± 4.9*↑	26.1 ± 1.1**↑
10	16.2 ± 1.8	15.9 ± 1.6*↓	51.0 ± 8.7*↑	18.1 ± 0.7	26.3 ± 1.8**↑
11	28.0 ± 3.6*↑	16.5 ± 1.0*↓	44.0 ± 5.4*↑	32.2 ± 4.3*↑	28.8 ± 2.9**↑
12	18.6 ± 3.0	17.2 ± 1.2*↓	29.0 ± 5.4	19.7 ± 1.4	26.0 ± 2.5*↑

*Mice were treated with the black garlic extracts for 5 days. From day 6 to 12 (non treatment period) cytokine amounts were measured in supernatant of spleen cells culture.

compounds such as protein and lipid did not show any quantitative differences compared with those of fresh garlic. However, individual amino acid cysteine, phenylalanine, tyrosine, leucine, valine, alanine, glycine, glutamic acid, and aspartic acid increased considerably following processing (Sasaki *et al.* 2007).

The important substance in the black garlic is considered to be water-soluble SAC (Jones *et al.* 2007). After aging garlic, SAC increased in the processed black garlic, reaching 194 $\mu\text{g/g}$ after 40 days aging (24 $\mu\text{g/g}$ before aging began). γ -Glutamyl-S-allyl-L-cysteine (GSAC), considered to be a precursor of SAC, decreased from 748 to 247 $\mu\text{g/g}$ after aging (Sasaki *et al.* 2007), suggesting that GSAC was converted into SAC in the processing period. The creation of black garlic in this manner is not a microbe-associated fermentation, but a Maillard and Browning reaction, because the processing temperature of garlic was 70°C, which did not allow bacterial growth to elicit fermentation. Actually we could not detect *Lactobacillus* (species) growth in the black garlic incubation (Sasaki *et al.* 2007).

Antiradical activity of black garlic

One of the beneficial activities of black garlic is its antioxidant potency, which is much more efficient to fight oxidant-related DNA damage (Siess *et al.* 2007). In a study to assess the antigenotoxic activity of the organo-sulfur compounds in the human hepatoma cell line Hep G2 model, the garlic compounds protect it from DNA damage (Belloire *et al.* 2006). In our experiments on processed black garlic extracts, the antioxidant potency increased considerably (25-fold) more than fresh garlic (Sasaki *et al.* 2007). Increased activity implies a potential protection effect against DNA damage due to active oxygen, which is one of the major causative agents of cancer outbreak.

Enhancement of immune system *in vivo* by black garlic extracts

No cytotoxicity was observed in the black garlic extracts against Meth A tumor cells in a mixture cultivation assay (Sasaki *et al.* 2007), indicating the presence of another anti-tumor mechanism(s) such as the association of the immune system for eradication of tumor cells. To obtain direct evidence of the participation of the immune system for tumor eradication, we designed immunological analyses using spleen cells obtained from black garlic extracts-treated mice. Our concern was first directed to NK cells activity in the spleen, because NK cells play a crucial role in eliminating harmful cells such as tumor cells, virus infected cells, among others (Charles *et al.* 2001). The activity of NK cells increased and reached a maximum 10 days after the experiment began (5 days after ceasing the black garlic extracts treatment) ($P < 0.05$) (Fig. 2). Further, cytokines IFN- γ , TNF- α and IL-2, which were generated from Th1 (T lymphocytes helper 1), and NO generated from macrophages reached a maximum 8 days later (3 days after ceasing the extracts treatment), and that of IL-2 6 days later (Table 1). There was a time lag in reaching maximum between cytokine production and activity of NK cells. This indicates that the black garlic extracts worked first on both T lymphocytes and macrophages to activate them, and cytokines released from these activated cells enhanced the activity of NK cells to attack abnormal cells like tumor cells. The extracts-activated macrophages additionally supported NK cells activity by producing cytokine NO to collaborate with NK cells to fight tumor cells (Ishikawa *et al.* 2006).

Unlike the increase of Th1 cytokines IFN- γ , TNF- α , and NO, Th2 (T lymphocytes helper 2) cytokine IL-4 decreased in the culture supernatants of spleen cells (Table 1). IL-4 is a cytokine that potentially activates B lymphocytes that regulate generation of the allergic-associating antibody IgE *in vivo* (Charles *et al.* 2001). We were recently informed by the allergic patients who regularly take black garlic mitigate clinical symptoms of rhinitis and epiphora. These important

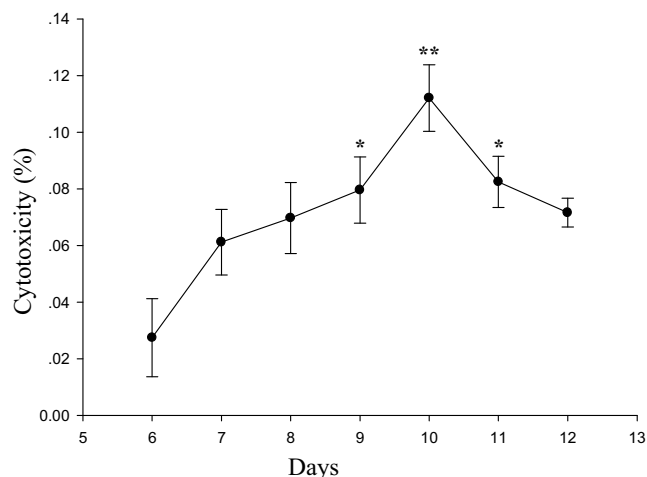


Fig. 2 NK (natural killer) cells activity in the spleen cells prepared from the black garlic extracts-treated mice. Mice were treated with the black garlic extracts for 5 days, then, treatment was ceased. The cytotoxic activity (mean \pm S.D) of NK cells gradually increased and it reached maximum on day 10 (5 days later from last injection) (* $P < 0.05$, ** $P < 0.001$).

findings correspond well to our result of decreasing IL-4 in our animal experiment. Less IL-4 *in vivo* implies less activity of B lymphocytes that eventually lead to lower production of the antibody IgE. We should confirm accuracy of this hypothetical consideration in more precise experiments.

Enhancement of the immune system by black garlic might be due to water-soluble SAC (Jones *et al.* 2007). In fact, there was a report in rat experiments in which aged garlic extracts rich in SAC significantly reduced the outbreak of colon tumor and aberrant crypt foci, indicating the presence of a chemo-preventive effect on carcinogenesis through suppression of tumor cells proliferation (Katsuki *et al.* 2006). The aged garlic used in their experiments was different from ours in the manner of processing. Their extracts were prepared by keeping fresh garlic soaked in alcoholic-water solution for 1-2 years. The mainly active compound in both extracts might be SAC, although a definite study will be required to define the principal effective substance to fight cancer and other diseases.

The black garlic used in this experiment has been just recently developed by a Japanese rice shop owner by repeating experiments to establish an appropriate processing method. Therefore, we lack sufficient knowledge on the nature of black garlic, including its bio-functional compounds. Our colleague has recently created a new type of garlic called "amber garlic" that is light yellow-brown in color containing more SAC than black garlic (Yamazaki 2005). Analytical studies are constantly in progress to find additional novel functions on the newly developed "amber garlic" together with black garlic.

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