Aged black garlic extract induces inhibition of gastric cancer cell growth *in vitro* and *in vivo*

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Abstract. There is mounting evidence that garlic extracts possess significant anticancer actions. However, no studies have been reported on the effects of aged black garlic extracts (ABGE) on gastric cancer *in vitro* or *in vivo*. To examine the potential action of ABGE against gastric cancer, the present study evaluated its effect on the inhibition of cell proliferation and induction of apoptosis in SGC-7901 human gastric cancer cells. Additionally, we performed an *in vivo* study by inoculating the murine foregastric carcinoma cell line in Kunming mice and treating them with various doses of ABGE (0, 200, 400 and 800 mg/kg, intraperitoneally) for 2 weeks. Dose-dependent apoptosis was detected in ABGE-treated cells in *in vitro* studies. In tumor-bearing mice, significant antitumor effects of ABGE were observed, such as growth inhibition of inoculated tumors. Further investigation of serum superoxide dismutases, glutathione peroxidase, interleukin-2 and the increased indices of spleen and thymus indicated that the anticancer action of ABGE may be partly due to its antioxidant and immunomodulative effects.

Introduction

Gastric cancer is the fourth most common cancer worldwide, with ~700,000 confirmed deaths annually (1). Epidemiological studies indicate that gastric cancer is associated with a low intake of fruits and vegetables (2). Surgery, chemotherapy, and radiotherapy are still the major conventional cancer therapies for this malignancy (3). However, these therapies have numerous limitations due to low response and poor outcome, indicating that there is an urgent need for the development of novel therapeutic strategies.

Garlic (*Allium sativum*) has been used for disease prevention and treatment for over 4,000 years. Numerous favorable biological and pharmacological effects of consumption of garlic preparations have been reported in culture cells *in vitro* and in animal models *in vivo* (4). Of the beneficial actions of garlic, such as antitumorigenesis, antiatherosclerosis, blood sugar modulation and antibiosis, inhibition of the growth of cancer is perhaps the most notable (5). Preclinical studies and population-based case control studies provide the most compelling evidence linking garlic and related foods with activity against cancer, confirming an inverse correlation between dietary intake of *Allium* vegetables and the risk of various cancer types (6).

Among the numerous garlic preparations available, aged black garlic extract (ABGE) is used as a major component of non-prescription tonics and dietary supplements (7). Compared to raw garlic, it has been shown to be more potent in many of the therapeutic properties of garlic, such as immunomodulative and antioxidative effects (8). In addition, ABGE, which is produced by a long-term extraction from garlic in aqueous ethanol, does not cause adverse events and has been confirmed to be safe in preclinical trials (9).

Although a number of epidemiological studies present promising evidence regarding the role of garlic in gastric cancer etiology, the pharmacological mechanism by which ABGE may inhibit gastric cancer remains unclear. In the present study, the immunomodulative and antioxidant effects of ABGE were investigated in SMG-7901 cells *in vitro* and in a transplantable mouse model *in vivo* to investigate its potential use as an anti-cancer agent for gastric cancer. To the best of our knowledge, this is the first study to explore the effect of ABGE in gastric cancer.

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Materials and methods

Preparation of aged black garlic extract. Aged black garlic was purchased from Huagu Garlic Co., Ltd. (Dalian, China). ABGE was prepared by the following steps: briefly, 100 g of peeled aged black garlic was crushed with 100 ml of distilled water in a mortar. The crushed material was carefully decanted by pressing and 100 ml of aqueous extract was collected and designated as 1 g/ml of ABGE. The ABGE solution was kept at -80˚C before use.

Cell culture and treatment. SGC-7901 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37˚C in a humidified incubator supplemented with 5% CO_2.

The cell subgroups of the *in vitro* study were designed as follows: group A, SGC-7901 cells without treatment as a negative control; group B, SGC-7901 cells treated with a low dose of ABGE (10 mg/ml); group C, SGC-7901 cells treated with a middle dose of ABGE (50 mg/ml); group D, SGC-7901 cells treated with a high dose of ABGE (100 mg/ml); group E, SGC-7901 cells treated with cisplatin (20 μg/ml) as a positive control. SGC-7901 cells were seeded at a density of $5x10^4$ cells per well in 6-well plates and incubated for 24 h. ABGE was added to the culture media at various concentrations in the 0-100 mg/ml range, and the cells were then incubated for 48 h. All cells were plated in culture medium containing serum into 6-well plates at the same time. Each concentration was examined in duplicate, whereas the whole experiment was performed 3 times. After 48 h, the cells in the 5 groups were collected, washed twice with PBS, and fixed in 70% ethanol overnight at -20˚C. The ethanol was then removed by centrifugation. Cell density was adjusted to $5x10^5$ cell/ml with PBS buffer, and 500 μl of mixed staining solution was added to the cells for 30 min in the dark. Cells were analyzed by flow cytometry for detection of apoptosis. The mixed staining solution contained 0.125 g sodium citrate, 0.75 ml Triton X-100, 0.03 g propidium iodide (PI), and 0.01 g RNase in a final volume of 250 ml (Sigma, St. Louis, MO, USA).

Animal experimentation. Male white mice of the Kunming strain, were acquired from the Laboratory Animal Center of Shandong Luye Biology Co. Ltd. [License No. (Lu): SCXK No. 021501], weighing 20±2 g. The animals were housed in an environmentally controlled room at 21-22˚C with a 12-h light/dark cycle 08:00-20:00. All procedures involving animals were approved by the institutional Animal Care and Use Committee and followed the guidelines set by the Medicine Animal Research Policies Committee of Shandong Province.

The mice were randomly divided into 5 groups and received different treatments. Briefly, mice of different groups were subcutaneously inoculated with $8x10^5$ murine forestomach cells (MFC) under the right axilla. The tumor-bearing mice models were successfully established 1 week after inoculation and the experimental animal groups were set up as follows: group A, tumor-bearing control mice with daily intraperitoneal (i.p.) injection of 100 mg/kg saline water as a negative control; group B, tumor-bearing mice injected with 200 mg/kg (low dose) ABGE; group C, tumor-bearing mice injected with 400 mg/kg (medium dose) ABGE; group D, tumor-bearing mice injected with 800 mg/kg (high dose) ABGE; and group E, tumor-bearing mice injected with 60 mg/kg cyclophosphamide (CTX) as a positive control. The number of animals in each group at the beginning of the experiment was 12. ABGE was administered to the assigned groups at 17:00 every day for 2 weeks via i.p. injection, after which the tumor tissues were excised and weighed. During the experiment, the long and short diameters of the tumors were measured at specified time points (days 1, 4, 7, 11 and 14), and the tumor volume (V) was calculated using the following formula: $V=4/39\pi \times L/2 \times (W/2)^2$, where L and W are the long and short axes, respectively (10). Mice were sacrificed by cervical dislocation on day 14. Spleen, thymus and tumor tissues were removed and weighed. The index of the spleen (or thymus) was calculated from the formula: Spleen (or thymus) index = weight of spleen (or thymus)/ body weight x 100%. The inhibition rate of tumor growth=(average tumor weight of the control group - average tumor weight of the test group)/average tumor weight of the control group x 100% (11). Meanwhile, the whole blood from each animal was collected in a 14-ml Falcon tube. The remaining blood was centrifuged at 1,600 g for 15 min, and separated serum was preserved at -80˚C until further analysis.

Activity detection of serum superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in tumor-bearing mice. SOD activity was determined with the xanthine oxidase-cytochrome c method according to a previous study (12). The cytochrome c reduction by superoxide anions generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm. Activity values were expressed in U/ml; 1 unit of SOD was defined as the amount of sample producing 50% inhibition under the assay conditions.

Measurement of GSH-Px activity was based on the method described by Kaynar *et al* (13). The whole activity of GSH-Px was evaluated using the oxidation of glutathione (GSH) reacted with cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The reduction in the absorbance of NADPH at 340 nm was measured. One unit of GSH-Px activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of reduced glutathione (GSH) by cumene hydroperoxide to oxidized GSH per min at pH 7.0 at 37˚C.

Concentration detection of serum interleukin-2 (IL-2) in tumor-bearing mice. The enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of IL-2 from blood serum in treated mice as previously described (14). The serum samples (50 μl) were added to each well of the reaction plate. The reaction plate was stored at 37˚C for 1 h, and was subsequently washed 4 times and blotted on filter paper. The enzyme-labeled antibody (50 μl) was placed in each well and was stored at 37˚C for 1.5 h; the reaction plate was then washed as described above. The substrate A (50 μl) and B (50 μl) was placed into each well and allowed to react for 15 min in the dark at 37˚C. Lastly, one drop of stop buffer was added to each

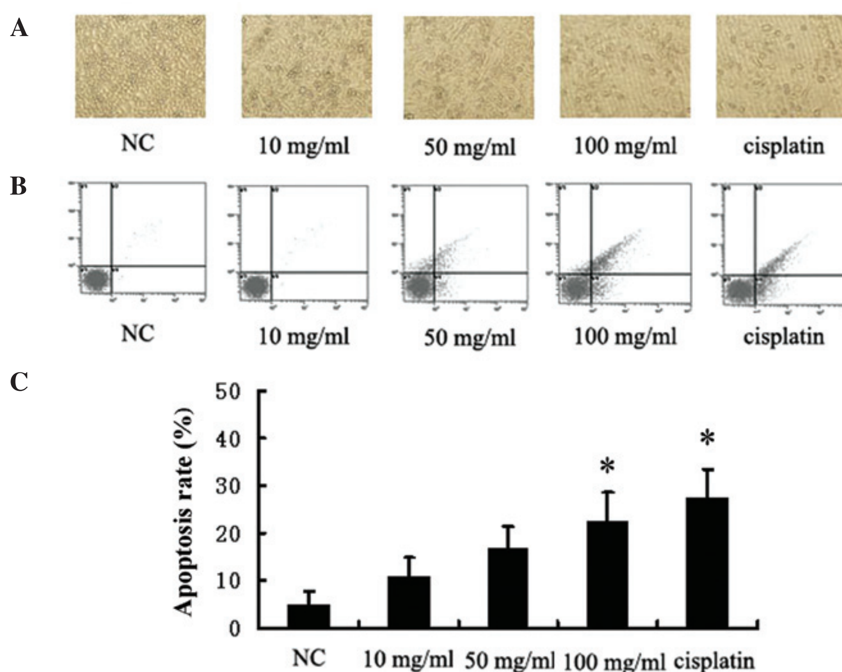

Figure 1. Effect of ABGE on the proliferation and apoptosis of SGC-7901 cells *in vitro*. SGC-7901 cells were treated with various concentrations of ABGE for 48 h; SGC-7901 cells without treatment were used as a negative control and SGC-7901 cells treated with cisplatin (20 μg/ml) as a positive control, repectively. The cells were then harvested, stained with PI, and analyzed by flow cytometry. (A) Morphological analysis of SGC-7901 cells treated with various concentrations of ABGE using phase-contrast microscopy. (B) Representative results of apoptosis assessment by Annexin-V/PI double-staining assay. (C) ABGE induced a statistically significant increase of apoptosis in SGC-7901 cells. Results are represented as the mean ± SD from three independent experiments. The data were analyzed using the Student's t-test. *Significant difference from negative control (p<0.05). ABGE, aged black garlic extract; NC, negative control.

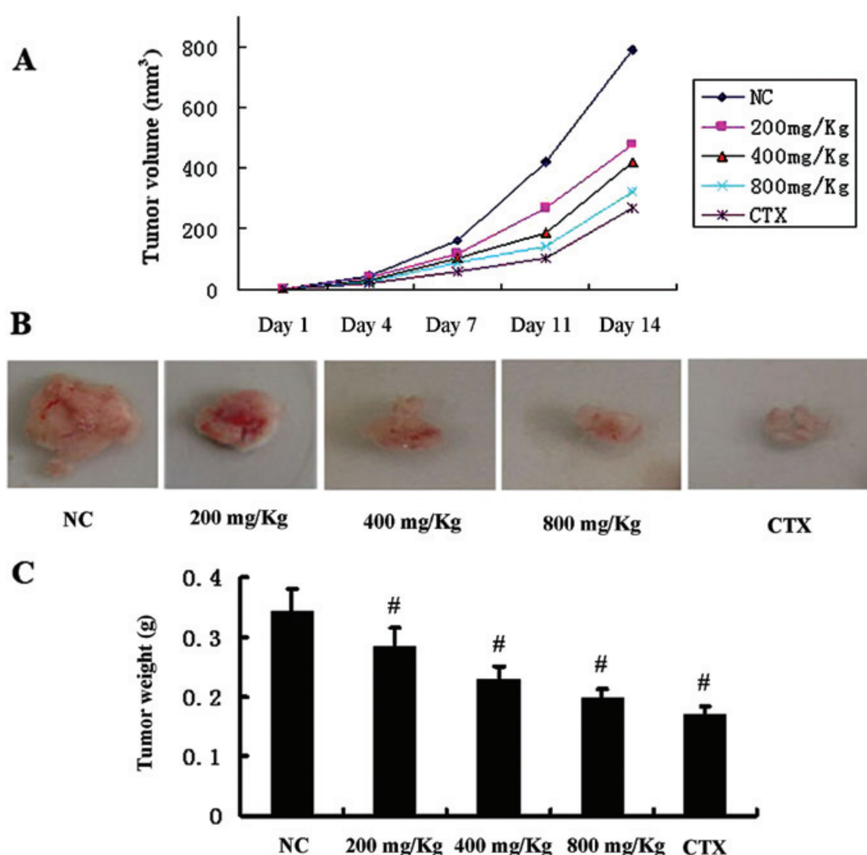

Figure 2. Anti-tumor effects of ABGE *in vivo*. (A) Comparison of tumor volume between tumor-bearing ABGE-treated mice and negative control. The tumor volumes in different groups were measured on days 1, 4, 7, 11 and 14. The tumor volumes were calculated as described in Materials and methods. (B) Gross observation of tumor in MFC-inoculating mice. Representative tumors from each group are shown. (C) Average tumor weight of sacrificed animals at day 14. Results are represented as the mean ± SD. All data were compared with the negative control and were analyzed using the Student's t-test. #Significant differences from the negative control (p<0.01). ABGE, aged black garlic extract; NC, negative control; CTX, group treated with cyclophosphamide as a positive control.

Table I. Effects of ABGE on tumor growth and index of spleen and thymus in tumor-bearing mice.

Group (n=12)	Body weight (g)	Tumor weight (g)	Inhibitory rate (IR, %)	Spleen index (mg/g)	Thymus index (mg/g)
Negative control	22.87±0.98	0.342±0.0373	-	63.85±9.68	28.55±5.75
ABGE (200 mg/kg)	22.15±1.78	0.284±0.031[b]	17.0	81.37±14.65[b]	37.15±6.02[b]
ABGE (400 mg/kg)	23.02±1.37	0.228±0.023[b]	33.3	88.32±18.41[b]	41.38±5.63[b]
ABGE (800 mg/kg)	22.38±1.65	0.198±0.016[b]	42.1	96.13±16.83[b]	43.55±7.38[b]
CTX	19.35±1.14[b]	0.169±0.0139[b]	50.6	48.94±13.78[a]	18.13±6.67[b]

The inhibitory rate of tumor and the index of spleen and thymus were calculated as described in Materials and methods. All values are presented as mean ± SD of 12 mice in relevant groups. [a]$p<0.05$; [b]$p<0.01$ vs. negative control; CTX, cyclophosphamide positive control.

well and mixed, from which absorption was then detected at 450 nm. The levels of IL-2 were determined according to the standard curve of absorption-concentration.

Statistical Analysis. All values were expressed as mean ± standard deviation (SD). Differences were evaluated using the Statistical Package for Social Science 13.0. Statistical analysis was performed using two-sided Student's t-test. Differences were considered statistically significant at $p<0.05$.

Results

Effect of ABGE on the apoptosis of SGC-7901 cells. First, SGC-7901 cells were used as an *in vitro* model to evaluate their apoptotic status following treatment with three dosages of ABGE. It was observed that the morphology of ABGE-treated cells changed from a more elongated fibroblast-like morphology to a round, smaller and packed appearance of epithelial cells 24 h after exposure to ABGE, indicating that SGC-7901 cells began to exhibit morphological features of apoptosis (Fig. 1A). Flow cytometry (FCM) was performed to further confirm the effects of ABGE on the induction of apoptosis. FCM with PI staining revealed that the apoptosis rates of SGC-7901 cell line treated with 10, 50 or 100 mg/ml ABGE for 48 h were 10.9±3.9%, 16.7±4.8% and 22.7±5.8%, respectively, and were significantly higher than that in the negative control (Fig. 1B and C). The results of FCM indicated that the effect of ABGE on apoptosis of SGC-7901 cells occurred in a dose-dependent manner.

Effect of ABGE on inoculated tumors in mice. To determine whether ABGE inhibits tumor growth *in vivo*, an equal number of MFC cells ($8x10^5$) were injected into the right axilla of Kunming mice to produce a tumor-bearing model. During the experiment, we measured the tumor volumes of different groups at 1, 4, 7, 11 and 14 days, respectively. As shown in Fig. 2A, the tumor volumes from low, medium and high doses of ABGE-treated tumor-bearing mice were significantly reduced. On the final day (day 14), the mice were sacrificed. Tissues of spleen, thymus and tumor were then removed and weighed. As shown in Fig. 2B and C the tumor weights of tumor-bearing mice in the ABGE-treated and positive groups markedly decreased compared with the negative control ($p<0.01$). According to the afore-mentioned formula, the inhibitory rates of tumor growth in the 3 ABGE groups and the positive control were 17.0, 33.3, 42.1 and 50.6%, respectively. Similarly, the index of spleen (or thymus) gradually increased with the increased dosage of ABGE (Table I). Moreover, it should be noted that no toxicity judged by parallel monitoring of body weight was observed in ABGE-treated mice (Table I). These results indicate that the tumor growth rate was notably inhibited in mice following treatment with ABGE as indicated by monitoring of the proliferation of spleen (or thymus).

Activity of serum SOD and GSH-Px in tumor-bearing mice. To further understand the molecular mechanisms of action of ABGE against the MFC cell growth, we investigated the effects of ABGE on activities of antioxidant enzymes in tumor-bearing mice. Fig. 3A and B show the effect of ABGE on activities of SOD and GSH-Px in the serum. The analysis indicated that, compared with the negative control, ABGE significantly increased the activity of SOD and GSH-Px in a dose-dependent manner ($p<0.01$).

Treatment of ABGE increased concentration of serum IL-2 in tumor-bearing mice. To determine whether treatment of ABGE has an immunomodulative action, concentrations of serum IL-2 in MFC-bearing mice were assessed. As shown in Fig. 3C, ELISA analysis indicated that, compared with the negative control, the activity of IL-2 increased slightly with the low-dosage ABGE treatment ($p>0.05$). In contrast, significant increase of IL-2 was observed in the medium- and high-dosage groups compared with negative control group ($p<0.01$).

Discussion

Gastric cancer is one of the most common human malignant tumors. Epidemiological investigations have provided evidence that gastric carcinogenesis is a complex, multistep and multifactorial event (15). Considering that reactive oxygen species (ROS) are capable of changing a number of cellular events involved in the cancer process by which cancer cells escape the immune system, the status of oxidative stress and immunity response may be a crucial factor in the development of gastric cancer. Despite continuous progress in the development of conventional therapies such as surgery, chemotherapy, and radiotherapy, as well as novel target-protein-based cancer therapy, gastric cancer is still the second highest cause of

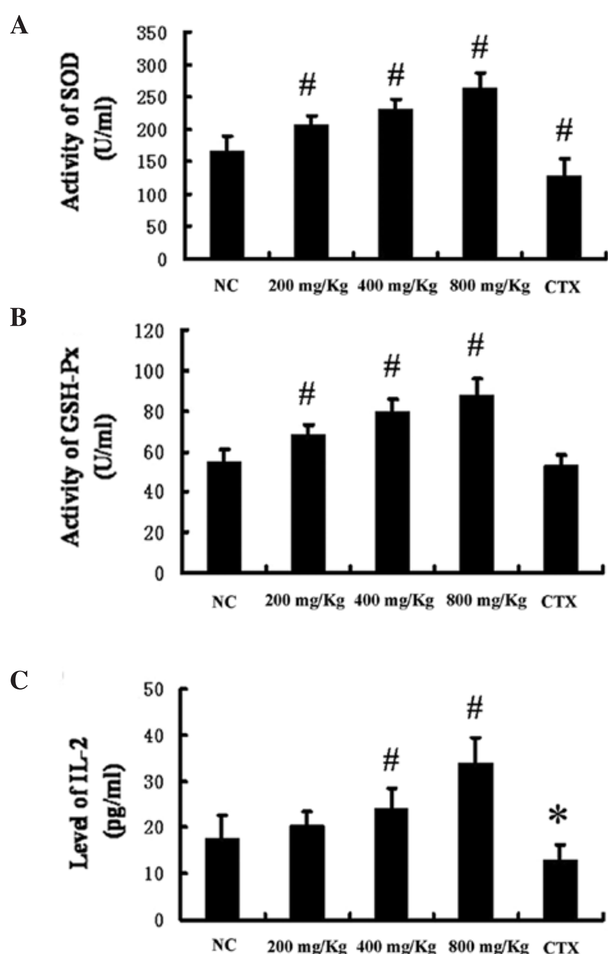

Figure 3. Levels of SOD, GSH-Px and IL-2 in serum of MFC-inoculating mice. (A) Activities of serum SOD in tumor-bearing mice. (B) Activities of serum GSH-Px in tumor-bearing mice. (C) Concentrations of serum IL-2 in tumor-bearing mice. Values are presented as mean ± SD of 12 mice in relevant groups. Data were compared with the negative control and were analyzed using the Student's t-test. Significant differences from the negative control are indicated by $^{*}p<0.05$ or $^{\#}p<0.01$. NC, negative control; CTX, group treated with cyclophosphamide as a positive control.

cancer-related mortality, indicating that the value of these therapies in the management of tumors may have reached a plateau (16). Nutritional support is a recent advancement in the domain of diet-based therapies (17). As a formidable prophylactic and therapeutic medicinal food, garlic and its extracts are significant components of strategies to prevent and cure various malignancies (18,19). To investigate this potential connection, the antioxidant and immunopotentiating effects of ABGE on gastric cancer were examined in SGC-7901 cells *in vitro* and in a tumor-bearing mouse model *in vivo*, respectively.

In the last few years, a number of reports have demonstrated the antiproliferative effects of several compounds derived from garlic. The effects displayed by garlic derivatives include induction of apoptosis, regulation of cell cycle progression and modification of pathways of signal transduction, all of which are cancer-reducing events (20,21). In the present study, the apoptosis of SGC-7901 cells increased significantly in a dose-dependent manner following treatment with ABGE. These data are in agreement with those of previous studies. Organosulfur compounds (OSC), components of garlic extraction, can suppress growth of cancer cells of different anatomical locations and modulate a number of key elements in cellular signal transduction pathways related to the apoptotic process (22). In the tumor-bearing mouse model, administration of ABGE effectively suppressed the development of inoculated tumor cells, indicating that ABGE also plays an anticancer role *in vivo*. Several early studies focused on the analysis of the effects of treatment with garlic or its components on the tumorigenic capabilities of the inoculated tumor cells in test animals. In these studies, reduced tumor growth and increased survival were also observed (23,24).

Formation of ROS is a normal consequence of essential biochemical processes. Low levels of ROS are harmless and necessary in several processes such as intracellular messaging, immunity and defence against microorganisms. In contrast, growing evidence indicates that ROS may lead to a variety of biological responses, including the induction of apoptosis, cell cycle arrest and formation of DNA mutations, which are associated with the pathogenesis of several diseases, including cancer (25-27). Therefore, the balance between ROS production and antioxidant defenses appears to one of the most significant physiological factors for cell transformation.

There are numerous mechanisms by which cells defend themselves against oxidants, forming a defense system mainly comprising antioxidant enzymes. SODs are generally regarded as the first line of antioxidant enzymes that protect cells against ROS, and may contribute to protection against carcinogenesis or tumor progression (28). Another antioxidant enzyme is GSH-Px, which is also a significant H_2O_2-scavenging enzyme (29). Although a number of reports have studied the level of SOD and GSH-Px in humans and animal cancer types, the data are conflicting and controversial (30,31). The plausible explanations for this include: i) the fact that treatment strategies that are effective in one type of cancer may be ineffective in another type, and ii) the differences between the cancerous tissues studied.

ROS are also involved in the pathogenesis of gastric malignancies (32). To date, the correlation between administration of ABGE and its effect on the status of SOD and GSH-Px have yet to be fully investigated. In this study, the activities of SOD and GSH-Px were significantly increased by the administration of ABGE in an animal model. Considering that ROS are inversely correlated to the induction of apoptosis (as mentioned above), these results offer a plausible explanation of the antioxidative and antiproliferative effects of ABGE. In the context of the previous literature, administration of ABGE may exert its antioxidative role directly and indirectly. During the aging process of ABGE, compounds in fresh garlic are converted into stable and water-soluble OSCs such as S-allyl cysteine (SAC) and S-allylmercapto-L-cysteine (SAMC). It has been reported that SAC and SAMC possess high radical scavenging activity, which directly removes ROS (33,34). In addition to this direct effect, ABGE also increases the activity of antioxidant enzymes, so that excessive ROS is indirectly cleared (35). Consistent with our study, enhancement of the circulating levels of SOD and GSH-Px were observed following administration of garlic extract in the circulation of tumor-bearing animals.

In addition to ROS, the host immune system plays another major role in tumorigenesis. A number of studies have shown

that the mechanism by which cancer cells escape detection by the body's immune system plays a key role in the occurrence and development of malignant tumors (36). As a significant immunomodulator, IL-2 is an immune factor secreted by helper T-lymphocytes that serves as a crucial growth and activation factor for cytotoxic lymphocytes, macrophages, natural killer cells and B lymphocytes (37). To date, studies on the effects of garlic to enhance immunocompetence in cancer cells have focused mainly on bladder cancer, since superficial bladder tumors have been shown to be sensitive to several biological response modulators and to immunomodulators in particular. The results demonstrated that administration of ABGE stimulates proliferation of lymphocytes and macrophage phagocytosis, induces splenic hypertrophy and the infiltration of macrophages and lymphocytes, and stimulates release of IL-2 in transplanted tumors (38). It has been reported that the effective dose of ABGE to enhance immune responses ranged from 1.8 g/day to 10 g/day, indicating that it is more practical for future complementary and alternative therapy (39). In the present study, the level of IL-2 increased gradually in tumor-bearing mice depending on the dosage of ABGE. Moreover, the index of spleen and thymus also increased significantly following administration of ABGE, indicating the existence of hypertrophy of the spleen and thymus and the effective stimulation of the immune response. Recent studies using purified OSCs from garlic have provided a new insight into its immunomodulatory effects. Studies on the effect of diallyl sulfide (DAS) on the serum levels of IL-2 revealed that administration of DAS significantly enhanced serum IL-2 levels in angiogenesis-induced animals compared to untreated control animals (40). In studies of human T cells, SAC was found to inhibit activation of the nuclear protein of the Rel oncogene family (nuclear factor-κB). This protein can regulate immune function with the induction of TNF-α or H_2O_2 (41). Taking into account that most OSCs in aged garlic are water-soluble, it must be one of the active substances in garlic preparations and account for at least a portion of garlic's immunomodulatory activities.

In summary, the present study indicates that ABGE treatment inhibits growth of SGC-7901 cells by inducing apoptosis *in vitro*. More importantly, we present the first evidence that ABGE is an effective anticancer garlic preparation in a mouse model. The beneficial effects of ABGE may be partly due to its antioxidative and immunomodulatory activities. Due to its broad range of beneficial effects, serious assessment of ABGE in clinical trials is warranted for the prevention and treatment of gastric cancer.

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