Anticancer Activity of *S*-Allylmercapto-L-cysteine on Implanted Tumor of Human Gastric Cancer Cell

Yongkyu LEE,^{*,a} Hejin KIM,^a Jinhwa LEE,^b and Kyongtai KIM^{c,d}

^aDepartment of Food and Biotechnology, Dongseo University; ^bDepartment of Clinical Laboratory Science, Dongseo University; Jurae-Dong, Sasang-gu, San 69-1, Busan 617-716, Korea; and ^cDepartment of Life Science, Division of Molecular and Life Science, Pohang University of Science and Technology; and ^dDivision of Integrative Bioscience & Biotechnology, Pohang University of Science and Technology; 790-784 San 31, Hyoja-dong, Nam-gu, Pohang, Gyungbuk 790-784, Republic of Korea. Received November 16, 2010; accepted February 9, 2011

Allylmercapto glutathione *S*-conjugate, *S*-allylmercapto-L-cysteine (SAMC), which is biotransformed from allyl sulfides and from naturally occurring water-soluble garlic derivatives, has been known to inhibit tumorigenesis. We found that SAMC was able to induce apoptosis in gastric cancer cells *in vitro*. We report that SAMC inhibited tumor growth rate by 31.36% and 37.78% at doses of 100 and 300 mg/kg, respectively. Apoptosis in the implanted tumor cells was manifested by apoptotic characteristics, including morphological changes of chromatin crescent, cell shrinkage and membrane blebbing. The apoptosis index of 100 mg/kg and 300 mg/kg of SAMC was $20.74 \pm 2.50\%$ and $30.61 \pm 2.42\%$, respectively, by terminal deoxy-nucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining. The positive rate of B-cell lymphoma 2 (bcl-2) protein expression of control, 100 mg/kg SAMC and 300 mg/kg SAMC was $15.20 \pm 1.67\%$, $10.94 \pm 1.57\%$, and $8.24 \pm 1.07\%$, respectively, by immunohistochemical staining. The positive rate of bax protein expression of control, 100 mg/kg SAMC and 300 mg/kg SAMC was $15.30 \pm 1.90\%$, $23.18 \pm 1.81\%$, and $25.26 \pm 3.03\%$, respectively. We also observed decreases in bcl-2 mRNA and increases in bax mRNA by SAMC in a dose-dependent manner by reverse transcription-polymerase chain reaction (RT-PCR). These results suggest that SAMC may regulate bcl-2 and bax to induce apoptosis in transplanted tumor cells.

Key words apoptosis; *S*-allylmercapto-L-cysteine; tumor cell; bax protein; B-cell lymphoma 2 protein

Apoptosis is a complex process that involves many regulatory proteins such as B-cell lymphoma 2 (bcl-2) family proteins and other anti- or pro-apoptotic proteins. Of the major genes that regulate apoptosis, the anti-apoptotic bcl-2 gene and the pro-apoptotic bax gene are of particular interest. They have homologous amino acid sequences.^{1–4} Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope, and may damage these compartments and affect their behavior, perhaps by modifying the flux of small molecules or proteins.⁵ Bcl-2 protects against various cytotoxic insults, such as UV-irradiation, cytokine withdrawal, and cytotoxic drugs.⁶ However, pro-apoptotic bax protein translocates to mitochondria upon exposure to apoptotic stimuli,⁷ and induces cytochrome *c* release and caspase activation *in vitro*.⁸ However, this process is blocked by anti-apoptotic proteins such as bcl-2 and bcl-xL.^{9,10} Therefore, anti-apoptotic bcl-2 subfamily proteins and pro-apoptotic bax subfamily proteins can oppositely regulate apoptosis through the control of cytochrome *c* release, resulting in caspase activation.⁸ Overexpression of bax could promote cell death.^{11–15} Conversely, overexpression of anti-apoptotic proteins such as bcl-2 could repress the function of bax.^{16–20} Thus, it has been suggested that the ratio of the level of pro-apoptotic bax to that of anti-apoptotic bcl-2 determines whether a cell responds to an apoptotic signal.²¹ In many cancer therapeutic studies, induction of apoptosis in tumor cells has been shown to be the generalized rule for anti-cancer mechanism conjoints.^{22–24}

Garlic, a plant within the *Allium* genus, has been used in many different cultures for disease prevention and treatment, especially for diseases of the gastrointestinal tract. Carcinogenesis studies have shown that allyl sulfides such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide

(DATS), typical components of garlic, inhibit tumor cell proliferation in culture and chemically induced tumors in experimental animals, such as colon cancer,²⁵ breast cancer,²⁶ and lung cancer.²⁷ Most of these allyl sulfides, which are absorbed in the gastrointestinal tract, are also reported to biotransform to the corresponding allylmercapto glutathione *S*-conjugate after reacting with endogenous antioxidants, such as cysteine and reduced glutathione (GSH).^{28,29} This allylmercapto glutathione *S*-conjugate, *S*-allylmercapto-L-cysteine (SAMC), which is biotransformed from allyl sulfides and from the naturally occurring water-soluble garlic derivatives, also inhibits tumorigenesis.³⁰ The fact that the chemical instability of these allyl sulfides does not limit their efficacy suggests that SAMC is the main bioactive metabolite.³¹ Although not present in raw garlic, SAMC can be found as a stable orally bioavailable organosulfur compound in aged garlic. In addition, SAMC reportedly inhibits growth and induces apoptosis in SW480 and HT 29 human colon cancer cells³² and prostate cancer cells.³³ Our group also reported that SAMC induces apoptosis in primary gastric cancer cells.³⁴ SAMC exhibits apoptotic effects through down-regulation of bcl-2/bax proteins and activation of caspase-3 in the gastric cancer cell line.

Despite the abundant *in vitro* evidence of the effects of garlic-related compounds, the *in vivo* anticancer effect of SAMC has rarely been reported. This study was designed to investigate the apoptotic effect of SAMC on implanted tumors of human gastric cancer cells in nude mice. Understanding the relationship between apoptosis by SAMC and expression of bcl-2/bax *in vivo* may allow for its clinical application.

* To whom correspondence should be addressed. e-mail: lyk@dongseo.ac.kr

MATERIALS AND METHODS

Chemicals *S*-Allylmercapto-L-cysteine (SAMC) was a generous gift from Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan). It was fed to mice as an acidified (pH 4.5) suspension of 30 mg/ml in 10% (w/v) dextrose, 1% (w/v) gum Arabic (Sigma-Aldrich, St. Louis, U.S.A.). Adriamycin was used as a positive control. *In situ* cell detection kit, anti-bcl-2 and anti-bax monoclonal antibodies were purchased from IMGENEX Co. (San Diego, CA, U.S.A.). Male BALB/c nude mice (6 week old, 19–23 g) were obtained from Central Lab. Animal Inc. in Korea.

Cell Culture The previously described technique was used.³⁴⁾ Human stomach cancer cells, MKN-45, from KCLB (Korean Cell Line Bank) were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS).

Tumor Implantation 5×10^6 cells/0.2 ml/mice of human gastric cancer cells (KMN-45) were injected into subcutaneous tissues of nude mice. The animals commenced treatment on day 2 of control (vehicle, $n=7$), 100 mg/kg (low dose) *S*-allylmercapto-L-cysteine ($n=7$), and 300 mg/kg (high dose) *S*-allylmercapto-L-cystein ($n=7$) by daily orogastric feeding and adriamycin (2 mg/kg), a positive control, were daily injected intraperitoneally. Then, changes of tumor volume were measured with Calipus (Mitutoyo Corp., Japan) twice a week, and tumor inhibition rate of each group was calculated according to the following formula.

$$\text{inhibitory rate (IR) of tumor growth} = \frac{C(V_1 - V_0) - T(V_1 - V_0)}{C(V_1 - V_0)}$$

Where C is control group, T is treated group, V_1 is the volume before treatment (mm^3), V_0 is the volume after treated (mm^3).

This experiment was approved by the ethics committee for animal experiments at Dongseo University. When experimental animals are used, we follow the NIH guidelines for laboratory animals³⁵⁾ and the Korean revised law for animal protection, 8852. All precautions were taken to care for the animals and to minimize any pain or discomfort.

Tumor Morphology After 24 d of treatment, the mice were euthanized by cervical dislocation and meticulously examined at postmortem. Paraffin-embedded tumor samples were cut into $1 \mu\text{m}$ sections and attached on glass slides. After deparaffination and dehydration, samples are hematoxylin and eosin (H&E) stained. H&E stained ultrathin sections were observed with a light microscope (BX41TF, Olympus, Japan) with magnification $\times 1000$.

Tunel Assay Paraffin-embedded tumor samples were cut into $5 \mu\text{m}$ sections and attached on silane coated glass slides (MUTO, Japan). Apoptosis in the tumor was measured with *In Situ* Cell Death Detection kit, POD (Roche, Germany). The tunel assay was carried out following the procedures directions. Tissue sections were deparaffinize in xylene, and hydrated through graded ethanol and then treated with proteinase K (20 $\mu\text{g}/\text{ml}$, Sigma, U.S.A.) for 20 min at RT. And endogenous peroxidase was blocked by 0.3% H_2O_2 in methanol for 15 min at RT. After the reaction, tissue sections were treated with Tris-HCl with 3% bovine serum albumin for 30 min and then incubated with tunel reaction mixture [containing terminal deoxynucleotidyl transferase from calf thymus (enzyme solution) and nucleotide mixture (label so-

lution)] for 1 h at 37°C in a humidified chamber. Tissue samples were then combined with Converter-POD (containing anti-fluorescein antibody Fab fragments from sheep, conjugated with horse-radish peroxidase), followed by washing and DAB (Sigma, U.S.A.) color reaction. During the tunel procedure samples were washed in phosphate buffered saline (PBS). Tissue sections were counterstained by hematoxylin, dehydrated through graded ethanol, cleared in xylene, and mounted. Slides were observed with a light microscope (BX41TF, Olympus, Japan) for histological analysis. As a negative control, a section was incubated with label solution only instead of using the tunel reaction mixture. One thousand tumor cells were counted in five different sites of each tissue section at a magnification of $200\times$. The apoptotic index (AI) was calculated as follows:

$$\text{AI (\%)} = (\text{number of apoptotic cells} / \text{total number of cells}) \times 100$$

Immunohistochemical Staining After deparaffination, the tissue sections were heated at 100°C for 20 min in 10 mM sodium citrate buffer with 0.05% Tween 20 (pH 6.0) for antigen retrieval. Then the sections were incubated with anti-Bax or anti-Bcl-2 antibodies (IMGENEX) at a 1 : 200 dilution at 4°C overnight. After washed with PBS, the secondary antibody, Alexa Fluor[®] 488 conjugated anti-rabbit immunoglobulin G (IgG) (Invitrogen), was added and cells were incubated at room temperature for 1 h. Then cells were counterstained with 2 $\mu\text{g}/\text{ml}$ Hoechst dye for visualizing the nuclei. Finally, cells were observed with fluorescence microscopy and anti-Bax or anti-Bcl-2 positive cells were detected. The number of whole cells and positive cells were counted using image analysis software (ImageJTM) (NIH, Bethesda, MD, U.S.A.).

RT-PCR Tumor samples were cryopreserved in liquid nitrogen and total RNA was extracted as previously described technique.³⁴⁾ Concentration of RNA was determined by the absorption at 260 nm. The primers for bcl-2, bax and β -actin were as follows: β -actin (500 bp) 5'-GTGGGGCGC-CCCAGGCACCA-3' (sense); 5'-CTCCTTAATGTCACG-CACGATTTC-3' (antisense); bcl-2 (716 bp) 5'-GGAAATA-TGGCGCAGCT-3' (sense); 5'-TCACTTGTGGCCAGAT-3' (anti-sense); bax (508 bp) 5'-CCAGCTCTGAGCAGAT-CAT-3' (sense), 5'-TATCAGCCCATCTTCTTCC-3' (anti-sense). Polymerase chain reactions were performed in a 50 μl reaction volume. PCR products were electrophoresed on agarose gel, and photographed under UV light, after staining with ethidium bromide.

Data Analyses Data were analyzed by one-way ANOVA, Student's *t*-test or Tukey's HSD test.

RESULTS

Tumor Inhibition Rate and Morphological Changes by SAMC An inhibitory effect was observed in all therapeutic groups and the inhibition rate by SAMC at the dose of 100 mg/kg and 300 mg/kg was 31.36% and 37.78%. This showed significance at $p < 0.05$ and $p < 0.01$ vs. control group, respectively. The inhibition rate by the positive control, Adriamycin, at 2 mg/kg was 49.18% ($p < 0.01$) (Table 1). The cells in the control group had normal morphological structures, but some cells in the therapeutic groups (SAMC, 100, 300 mg/kg) had apoptotic characteristics including chro-

Table 1. Inhibitory Effect of SAMC on Tumor Volume of Implanted Tumors in Nude Mice (Mean±S.D.)

Group	Number of animals		Volume of tumors (mm ³)		Inhibition rate %
	Beginning	Ending	Beginning	Ending	
Control group					
10% (w/v) dextrose + 1% (w/v) gum arabic	7	7	40.36±12.33	2075.04±330.72	
SAMC					
100 mg/kg	7	7	42.01±14.12	1438.28±406.09*	31.36
300 mg/kg	7	7	46.26±13.20	1311.97±109.97**	37.78
Positive control adriamycin (2 mg/kg)	7	7	51.36±11.36	1085.23±396.49**	49.18

* $p < 0.05$, ** $p < 0.01$ vs. control group by student's *t*-test.

Fig. 1. Microscopic Structures of Transplanted Tumor Cells and Apoptotic Transplanted Tumor Cells Induced by SAMC (Original Magnification: ×1000) of H&E Staining

Animal ID: 5 (A: control), 10 (B: SAMC, 100 mg/kg), 20 (C: SAMC, 300 mg/kg) and 26 (D: adriamycin, 2 mg/kg).

matin crescent, cell shrinkage and membrane blebbing (Fig. 1). Positive control (Adriamycin, 2 mg/kg) induced more drastic apoptotic characteristics.

Apoptotic Tumor Cells by SAMC Positive staining was clear in nuclei (Fig. 2). The apoptosis index of control, 100 mg/kg SAMC and 300 mg/kg SAMC was 4.42 ± 0.63 , 20.74 ± 2.50 and 30.61 ± 2.42 , respectively. These showed significance at $p < 0.01$ vs. control group. The AI value of the positive control, Adriamycin (2 mg/kg) was 42.01 ± 3.01 ($p < 0.01$) (Table 2).

Expression of Bcl-2 and Bax Proteins Positive staining was located in the cytoplasm. The positive rate of bcl-2 protein expression in control, 100 mg/kg SAMC and 300 mg/kg SAMC was $15.20 \pm 1.67\%$, $10.94 \pm 1.57\%$ and $8.24 \pm 1.07\%$ ($p < 0.05$), respectively. The positive rate of the positive control, Adriamycin (2 mg/kg), was $5.30 \pm 1.18\%$ ($p < 0.01$). The positive rate of bax protein expression in control, 100 mg/kg SAMC and 300 mg/kg SAMC was $15.30 \pm 1.90\%$, $23.18 \pm 1.81\%$, and $25.26 \pm 3.03\%$ ($p < 0.05$), respectively, by immunohistochemical staining. The positive rate of the positive control, 2 mg/kg Adriamycin, was $26.60 \pm 1.82\%$ ($p < 0.05$). 300 mg/kg SAMC and 2 mg/kg Adriamycin showed signifi-

Fig. 2. Apoptotic Tumor Cells/Human Stomach Cancer MKN-45 Cell Line Xenograft (Original Magnification: ×400)

TUNEL positive cells are observed in the tumor mass (arrows). Animal ID: 5 (A: control), 10 (B: SAMC, 100 mg/kg), 20 (C: SAMC, 300 mg/kg) and 26 (D: adriamycin, 2 mg/kg).

Table 2. Apoptotic Index (AI) of Implanted Tumors in Nude Mice (Mean±S.D.)

	Control	SAMC 100 mg/kg	SAMC 300 mg/kg	Adriamycin 2 mg/kg
AI (%)	4.42 ± 0.63	$20.74 \pm 2.50^{**}$	$30.61 \pm 2.42^{**}$	$42.01 \pm 3.01^{**}$

** $p < 0.01$ vs. control group by student's *t*-test.

Table 3. Positive Rate of Bcl-2 and Bax Proteins of Implanted Tumors in Nude Mice

	Bcl-2 (%) Mean±S.E.	Bax (%) Mean±S.E.
Control	15.20 ± 1.67	15.30 ± 1.90
SAMC 100 mg/kg	10.94 ± 1.57	23.18 ± 1.81
SAMC 300 mg/kg	$8.24 \pm 1.07^{*}$	$25.26 \pm 3.03^{*}$
Adriamycin 2 mg/kg	$5.30 \pm 1.18^{**}$	$26.60 \pm 1.82^{*}$

* $p < 0.05$, ** $p < 0.01$ vs. control group by Turkey HSD test.

cance at the 5% level (Table 3).

Expression of Bcl-2 and Bax mRNA The density of bax mRNA in control, 100 mg/kg SAMC, 300 mg/kg SAMC and Adriamycin was increased progressively, and the density of bcl-2 mRNA in control, 100 mg/kg SAMC, 300 mg/kg SAMC and Adriamycin decreased progressively according to RT-PCR (Fig. 3).

Fig. 3. Expression of Bcl-2 mRNA and Bax mRNA in Apoptotic Transplanted Tumor Cells Induced by SAMC

Total RNA was extracted, and of bcl-2, bax and β -actin mRNA levels were determined by RT-PCR. β -Actin was used as an internal control.

DISCUSSION

Uncovering new anti-tumor drugs with less toxicity is an ongoing challenge in cancer research. The original source of SAMC, garlic, a plant within the *Allium* genus, has been used in many different cultures for disease prevention and treatment, especially for diseases of the gastrointestinal tract. Epidemiological investigations in China, Italy, and America have provided evidence that regular consumption of garlic and related garlic products decreases the risks of stomach and colon cancers.³⁰⁾ Although not present in raw garlic, SAMC is a major *in vivo* metabolic product of the garlic compounds allidine and diallyl disulfides,³⁶⁾ both of which are well known to show anticancer properties.^{37–40)} This allylmercapto glutathione *S*-conjugate, SAMC, which is bio-transformed from allyl sulfides and from the naturally occurring water-soluble garlic derivatives, also inhibits tumorigenesis.³⁰⁾ SAMC also reportedly inhibits growth and induces apoptosis in SW480 and HT 29 human colon cancer cells,³²⁾ and prostate cancer cells.³³⁾ This anticancer activity of SAMC *in vitro*^{30–34)} might be related to the induction of tumor cell apoptosis.

In a previous study, we showed that SAMC was able to induce apoptosis in gastric cancer cells. The apoptosis accompanied down-regulation of the anti-apoptotic gene, bcl-2, at the same time as up-regulation of the pro-apoptotic gene, bax. In this study, we evaluated the apoptotic effect of SAMC on an implanted tumor of gastric cancer cells, and investigated the molecular mechanisms further to provide a theoretical basis for the therapeutic application of SAMC. We observed the inhibitory effect of SAMC in two therapeutic groups. Tumor cells in the control group had normal structures, but some tumor cells in the therapeutic groups had apoptotic characteristics. The apoptosis index of SAMC at doses of 100 and 300 mg/kg was increased. The protein level of bcl-2 was decreased with SAMC at doses of 100 and 300 mg/kg, but that of bax was increased. The density of bcl-2 mRNA induced by SAMC at doses of 100 and 300 mg/kg decreased progressively and the density of bax mRNA increased progressively. We hypothesized that the lowered ratio of bcl-2/bax by the altered transcriptional regulation by SAMC triggered the apoptosis of implanted tumor cells. Our results demonstrate that SAMC was able to induce the apoptosis of implanted tumors of gastric cancer cells in nude mice. SAMC may be involved in down-regulating the expression of the apoptosis-regulated gene bcl-2 and up-regulating

the expression of the pro-apoptotic gene bax. These data suggest that SAMC may be used as a chemotherapeutic drug in anti-tumor chemotherapy.

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