Anticancer Activity of S-Allylmercaptal-cysteine on Implanted Tumor of Human Gastric Cancer Cell

Yongkyu Lee, Hejin Kim, Jinhwa Lee, and Kyongtai Kim

*Department of Food and Biotechnology, Dongseo University; †Department of Clinical Laboratory Science, Dongseo University; ‡Department of Life Science, Division of Molecular and Life Science, Pohang University of Science and Technology; ‡Department of Life Science, Division 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

S-Allylmercaptal-glutathione S-conjugate, S-allylmercaptal-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±2.50% and 30.61±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±1.67%, 10.94±1.57%, and 8.24±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±1.90%, 23.18±1.81%, and 25.26±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-allylmercaptal-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. 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. Bcl-2 protects against various cytotoxic insults, such as UV-irradiation, cytokine withdrawal, and cytotoxic drugs. However, pro-apoptotic bax protein translocates to mitochondria upon exposure to apoptotic stimuli, and induces cytochrome c release and caspase activation in vitro. However, this process is blocked by anti-apoptotic proteins such as bcl-2 and bcl-xL. 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. Overexpression of bax could promote cell death. Conversely, overexpression of anti-apoptotic proteins such as bcl-2 could repress the function of bax. 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. In many cancer therapeutic studies, induction of apoptosis in tumor cells has been shown to be the generalized rule for anti-cancer mechanism conjoints.

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 dialllyl 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, breast cancer, and lung cancer. Most of these allyl sulfides, which are absorbed in the gastrointestinal tract, are also reported to biotransform to the corresponding allylmercaptal-glutathione S-conjugate after reacting with endogenous antioxidants, such as cysteine and reduced glutathione (GSH). This allylmercaptal-glutathione S-conjugate is biotransformed from allyl sulfides and from the naturally occurring water-soluble garlic derivatives, which inhibits tumorogenesis. The fact that the chemical instability of these allyl sulfides does not limit their efficacy suggests that SAMC is the main bioactive metabolite. 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 cells and prostate cancer cells. Our group also reported that SAMC induces apoptosis in primary gastric cancer cells. 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.
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 acidic solution (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 death 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 (containing terminal deoxynucleotidyl transferase from calf thymus (enzyme solution) and nucleotide mixture (label solution)) 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×. The apoptotic index (AI) was calculated as follows:

\[ AI(\%) = \frac{\text{number of apoptotic cells}}{\text{total number of cells}} \times 100 \]

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 μg/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 (ImageJ™) (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: bcl-2 (508 bp) 5'-GTGGGGGCAGCCCCAGGACCA-3' (sense); 5'-CTCCTTAATGTACGGCATGATTCT-3' (antisense); bax (508 bp) 5'-GAAAATAGTGCGCAAGCTG3' (sense); 5'-TACCTGTTGGGCCAGATCAT-3' (anti-sense); β-actin (500 bp) 5'-CCAGCATCCTGACACACG-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-
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% (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±1.67%, 10.94±1.57% and 8.24±1.07% (\( p<0.05 \)), respectively. The positive rate of the positive control, Adriamycin (2 mg/kg), was 5.30±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±1.90%, 23.18±1.81%, and 25.26±3.03% (\( p<0.05 \)), respectively; by immunohistochemical staining. The positive rate of the positive control, 2 mg/kg Adriamycin, was 26.60±1.82% (\( p<0.05 \)). 300 mg/kg SAMC and 2 mg/kg Adriamycin showed significance 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).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Volume of tumors (mm³)</th>
<th>Inhibition rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Ending</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>7</td>
<td>7</td>
<td>40.36±12.33</td>
</tr>
<tr>
<td>10% (w/v) dextrose+1% (w/v) gum arabic</td>
<td>7</td>
<td>7</td>
<td>42.01±14.12</td>
</tr>
<tr>
<td>SAMC 100 mg/kg</td>
<td>7</td>
<td>7</td>
<td>46.26±13.20</td>
</tr>
<tr>
<td>SAMC 300 mg/kg</td>
<td>7</td>
<td>7</td>
<td>51.36±11.36</td>
</tr>
<tr>
<td>Positive control adriamycin (2 mg/kg)</td>
<td>7</td>
<td>7</td>
<td>42.01±3.01</td>
</tr>
</tbody>
</table>

\* \( p<0.05 \), ** \( p<0.01 \) vs. control group by student’s \( t \)-test.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>AI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.42±0.63</td>
</tr>
<tr>
<td>SAMC 100 mg/kg</td>
<td>20.74±2.50**</td>
</tr>
<tr>
<td>SAMC 300 mg/kg</td>
<td>30.61±2.42**</td>
</tr>
<tr>
<td>Adriamycin 2 mg/kg</td>
<td>42.01±3.01**</td>
</tr>
</tbody>
</table>

**++ \( p<0.01 \) vs. control group by student’s \( t \)-test.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Bcl-2 (%) Mean±S.E.</th>
<th>Bax (%) Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.20±1.67</td>
<td>15.30±1.90</td>
</tr>
<tr>
<td>SAMC 100 mg/kg</td>
<td>10.94±1.57</td>
<td>23.18±1.81</td>
</tr>
<tr>
<td>SAMC 300 mg/kg</td>
<td>8.24±1.07**</td>
<td>25.26±3.03*</td>
</tr>
<tr>
<td>Adriamycin 2 mg/kg</td>
<td>5.30±1.18**</td>
<td>26.60±1.82*</td>
</tr>
</tbody>
</table>

\* \( p<0.05 \), ** \( p<0.01 \) vs. control group by Turkey HSD test.
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. Although not present in raw garlic, SAMC is a major in vivo metabolite with the capacity to induce apoptosis in SW480 and HT 29 human colon cancer cells, and prostate cancer cells. This anticancer activity of SAMC in vitro 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, and up-regulation of bax 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, but some tumor cells in the therapeutic groups had served the inhibitory effect of SAMC in two therapeutic groups.

REFERENCES

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