Induction of apoptosis by *S*-allylmercapto-L-cysteine, a biotransformed garlic derivative, on a human gastric cancer cell line

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Received September 25, 2007; Accepted January 24, 2008

Abstract. Epidemiological and experimental carcinogenesis studies provide evidence that certain components of garlic have anti-cancer activity. Although the biotransformed garlic derivative *S*-allylmercapto-L-cysteine (SAMC) has been reported to show an inhibitory effect on tumorigenesis, the mechanisms are poorly understood. The present study investigated the effect of SAMC on the growth of human gastric cancer SNU-1 cells. Upon treatment with SAMC, a concentration-dependent inhibition of cell proliferation was observed and cells developed many of the hallmark features of apoptosis, including DNA fragmentation and an increase in the sub-diploid population. The anti-proliferative and apoptotic effect of SAMC was associated with the induction of Bax, p53, and caspase-9, rather than the induction of Bcl-2 and p21. Mitochondrial cytochrome *c* activation and an *in vitro* caspase-3 activity assay demonstrated that the activation of caspases accompanies the apoptotic effect of SAMC, which mediates cell death. These results suggest that the apoptotic effect of SAMC on gastric cancer SNU-1 cells may be connected with caspase-3 activation through the induction of Bax and p53, rather then Bcl-2 and p21.

Introduction

The stereotypical features of a cell undergoing apoptosis include cell shrinkage, blebbing, phosphatidylserine externalization, DNA fragmentation, and nuclear condensation (1). These events are preceded by the activation of several cascades, which then induce apoptosis via the cleavage of key substrates (2,3). Early in the apoptotic process, mitochondria release cytochrome *c*, which enters the cytosol and forms a complex with Apaf-1 and a pro-form of caspase-9 (4). These complexes induce the activation of other caspases, specifically caspase-3, -6 and -7 (5). Caspase activity is responsible, either directly or indirectly, for the cleavage of several intracellular proteins, which are characteristically lysed during apoptosis (6). Additionally, the p53 protein functions, in part, by responding to DNA damage and inducing apoptosis, which is likely to be a crucial aspect of its function as a tumor suppressor (7,8). Wild-type p53 protein arrests DNA-damaged cells in the G1 phase by inducing the cyclin-dependent kinase (CDK) inhibitor p21WAF1/CIPI (9), and unrepaired cells might be eliminated during apoptosis by inducing Bax and repressing Bcl-2 factors (10,11). The pro-apoptotic protein, Bax, induces the release of cytochrome *c* and the activation of caspase, resulting in apoptosis; this process is blocked by anti-apoptotic proteins such as Bcl-2 and Bcl-xL (12). In many cancer therapies, the induction of apoptosis in tumor cells has been shown to be the general rule for anti-cancer mechanism conjuncts.

Garlic, a plant of the *Allium* genus, has been used for disease prevention and treatment in many different cultures, especially for diseases of the gastrointestinal tract. Epidemiological investigations in China, Italy, and the US have provided evidence that the regular consumption of garlic and related garlic products decreases the risk of stomach and colon cancers (13). Carcinogenesis studies showed that allyl sulfides such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), typical components of garlic, inhibited tumor cell proliferation in culture and in chemically induced tumors in experimental animals, for cancers such as colon (14), breast (15) and lung (16). These allyl sulfides (mono, di and tri) are oily compounds and decomposition products formed from allicine (13). Most of these allyl sulfides, which are absorbed in the gastrointestinal tract, were also reported to biotransform to the corresponding allylmercapto glutathione *S*-conjugate after reacting with endogenous antioxidants, such as cysteine and reduced glutathione (GSH) (13,17,18). In as much as the intracellular concentration of GSH is in the millimolar range (19), interactions of this type significantly alter the biological fate of the original garlic components (i.e. allyl sulfides) and affect redox balance within cells. The allylmercapto glutathione *S*-conjugate, *S*-allylmercapto-L-cysteine (SAMC), which is biotransformed from allyl sulfides and from the naturally occurring water-soluble garlic derivatives (Fig. 1), also

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Key words: apoptosis, *S*-allylmercapto-L-cysteine, caspase protein, cell death

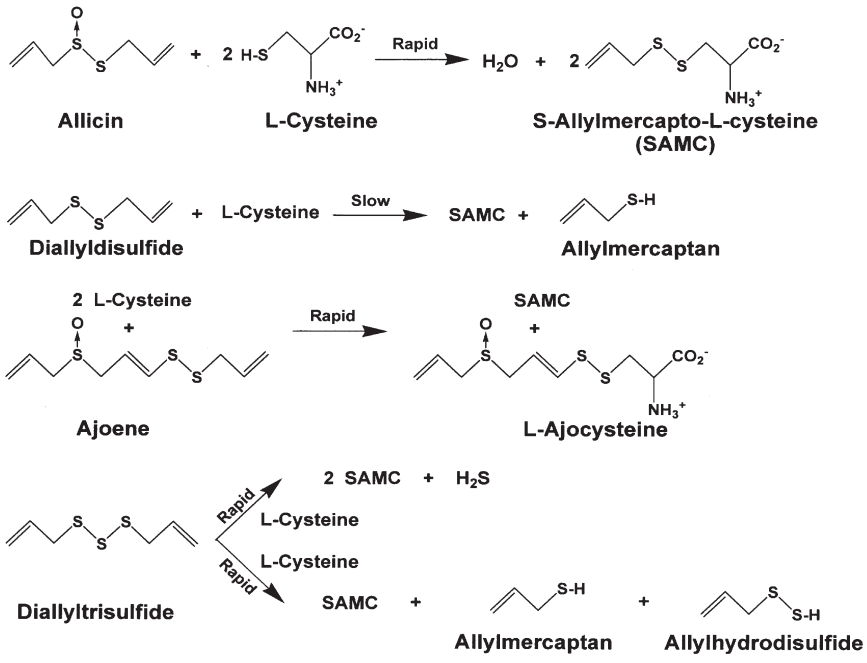

Figure 1. Formation of *S*-allylmercapto-L-cysteine (SAMC) from *allium*-derived polysulfides. Due to the high concentrations of glutathione (GSH) in most cells, it is also possible for GSH to replace L-cysteine in the reactions shown, generating the corresponding allylmercapto glutathione *S*-conjugate. Adapted from (13).

inhibited tumorigenesis (13). In addition, SAMC reportedly inhibits the growth of, and induces apoptosis in, SW480 and HT-29 human colon cancer cells (20). However, any inhibitory effects of SAMC on stomach cancer cell proliferation have not yet been reported.

In the present study, the effects of SAMC on the growth of the human gastric cancer cell line, SNU-1, were investigated. The findings suggest that SAMC-mediated apoptosis is not associated with changes in the level of p21 or Bcl-2 expression. However, the levels of p53 and Bax expression increased dramatically in a concentration-dependent manner. The release of cytochrome *c* and the activation of caspase-3 and caspase-9 were also observed.

Materials and methods

Cell culture. The human gastric cancer SNU-1 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in minimal RPMI-1640 medium (Gibco, BRL, Rockville, MD) that contained 10% fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO) and 1% antibiotic-antimycotic (Gibco), in an atmosphere of 5% CO_2 and 95% air at 37˚C.

Allium derivative. SAMC (*S*-allylmercapto-L-cysteine) was generously supplied by Wakunaga of America Co., Ltd. (Misson Viejo, CA, USA). A stock solution of SAMC was prepared fresh in PBS.

MTT assay. This assay is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan by mitochondrial enzymes as previously described (21). SNU-1 cells were seeded at a density of $5x10^4$ cells per well in 24-well plates and incubated for 24 h. SAMC was dissolved in PBS and added to the culture media at various concentrations in the 0-400 μM range, and the cells were then incubated for 48 h. Stock MTT solution (120 μl) was added into each well under dark conditions, and the plates were incubated at 37˚C for 4 h. After centrifugation, 1 ml of DMSO diluted with ethylalcohol (1:1) was added in order to dissolve the formazan. After agitation for 10 min at room temperature, 100 μl of each solution was transferred to 96-well plates, and the absorbance value of each well was read at 540 nm using an ELISA reader (Model 550 Microplate Reader, Bio-Rad, USA).

Quantitative analysis of fragmented DNA. SNU-1 cells were incubated in growth medium for 4 h with 1 μCi/ml [^{3}H]-thymidine (Amersham Pharmacia Biotech., UK) (22). The cells were then washed twice with PBS and incubated for 48 h after treatment with SAMC. The cells were washed and lysed with lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% Triton X-100) (23). Low and high molecular weight DNA was separated by centrifugation and the amount of [^{3}H]-thymidine of each supernatant was determined with a liquid scintillation counter (Beckmann, USA). The percent change of DNA fragments was calculated as follows: % fragments = [cpm of small DNA/(cpm of small DNA + cpm of large DNA) x100].

RNA extraction and reverse transcription-PCR (RT-PCR). Following SAMC treatment, total RNA was isolated with TRI Reagent (Gibco). cDNA was synthesized from 5 μg of total RNA with 200 units of MMLV-RT (Promega Co., Madison, WI) and 500 ng of oligo-dT primer. For amplification of cDNAs, oligonucleotide primers and cycle parameters for PCR were designed as follows: Bax (sense, 5'-ATG GAC GGG TCC GGG GAG-3'; anti-sense, 5'-TGG AAG AAG

ATG GGC TGA-3'), Bcl-2 (sense, 5'-CAG CTG CAC CTG ACG-3'; anti-sense, 5'-GCT GGG TAG GTG GTG CAT-3'), p53 (sense, 5'-GCT CTG ACT GTA CCA CCA TCC-3'; anti-sense, 5'-CTC TCG GAA CAT CTC GAA GCG-3'), p21 (sense, 5'-CTC AGA GGA GGC GCC ATG-3'; anti-sense, 5'-GGG CGG ATT AGG GCT TCC-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3'; anti-sense, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'). PCR products were electrophoresed on agarose gel, and photographed under UV light, after staining with ethidium bromide. The band intensity of the PCR product was analysed after normalization with a ß-actin band using the image analysing software, TINA 2.0 (Fuji Inc., Japan).

Preparation of cytosolic extracts and immunoblotting. After SAMC treatment, the cells were collected and re-suspended in 500 μl of extraction buffer (50 mM Pipes-KOH, 220 mM mannitol, 68 mM sucrose, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors). After 30 min of incubation on ice, the cells were homogenized using a glass dounce and a tight pestle (50 strokes). Cell homogenates were centrifuged and 10 μl of protein was loaded onto 15% SDS-polyacrylamide gels (12). Mitochondrial cytochrome *c* was detected with anti-cytochrome *c* monoclonal antibody (PharMingen).

Caspase-3 assay. After SAMC treatment, SNU-1 cells were harvested, washed twice with ice-cold PBS, and re-suspended in lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml leupeptin). The remaining protocol followed the manufacturer's instruction (Bio-Rad Lab., Hercules, CA). The fluorescence was measured in a microplate reader (Bio-Tek Instruments, Winooski, VT) using 360 nm excitation and 530 nm emission. Data were expressed as fold-induction of caspase-3 activity compared to that of control cells.

Immunoblot analysis. SNU-1 cells were treated with SAMC for 48 h and lysed with lysis buffer (40 mM Tris-HCl 7.4, 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors). Total protein (50 mg) was electrophoresed using 15% SDS-polyacrylamide gels, and used for immunoblot analysis with anti-Bcl-2, anti-actin, anti-caspase-3 polyclonal antibodies and anti-p21 monoclonal antibody (Santa Cruz Inc., Santa Cruz, CA). Monoclonal anti-p53 and polyclonal anti-Bax antibodies were purchased from Calbiochem (Cambridge, MA).

Flow cytometry analysis. After treatment with SAMC, the cells were washed with cold PBS and re-suspended in PBS. The DNA content of the cells was measured using a DNA staining kit (CycleTest Plus DNA Reagent Kit, Becton Dickinson, Heidelberg, Germany). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Data were acquired using CellQuest Software with a FACScalibur (Becton Dickinson) flow cytometry system using 20,000 cells per analysis. Cell cycle distributions were calculated using ModFit LT v2.0 software (Verity Software House, Topsham, ME).

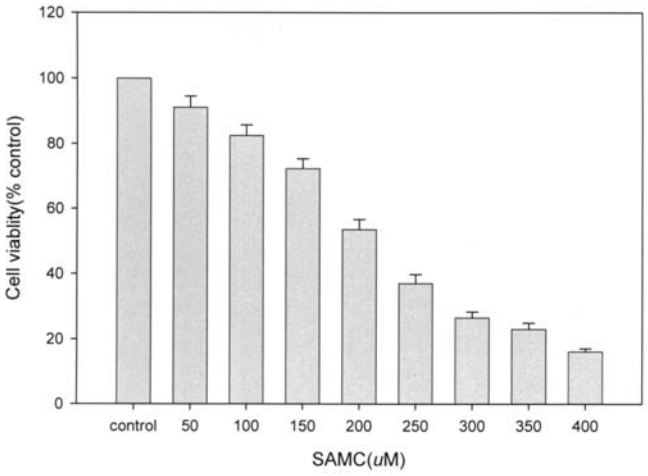

Figure 2. Decreased cell viability by *S*-allylmercapto-L-cysteine (SAMC) in SNU-1 cells. After treatment with SAMC for 48 h, the cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition (medium without SAMC) in which the cells were grown. Data represent the mean values of four replicates, with bars indicating SEM.

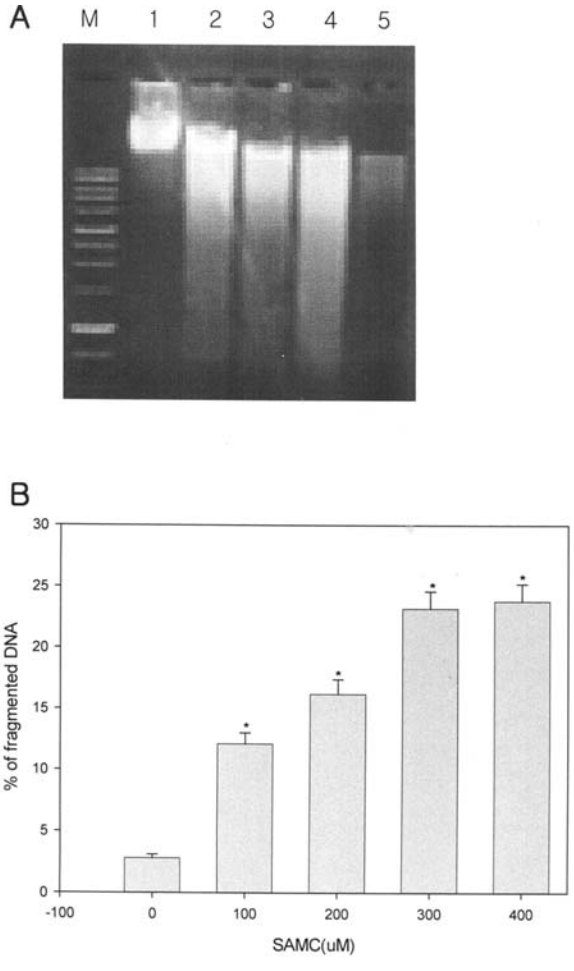

Figure 3. Effect of *S*-allylmercapto-L-cysteine (SAMC) on DNA fragmentation of SNU-1 cells. (A) SNU-1 cells were treated with increasing concentrations of SAMC for 48 h. Lane M, DNA marker; lane 1, control; lane 2, 100 μM; lane 3, 200 μM; lane 4, 300 μM; lane 5, 400 μM of SAMC. (B) The fragmented DNA was quantitated using [^{3}H]-thymidine incorporation. Data were presented as the percentage of cpm of fragmented DNA compared to total cpm. Data represents the mean values of four replicates with bars indicating SEM. $^*p<0.05$ compared to control.

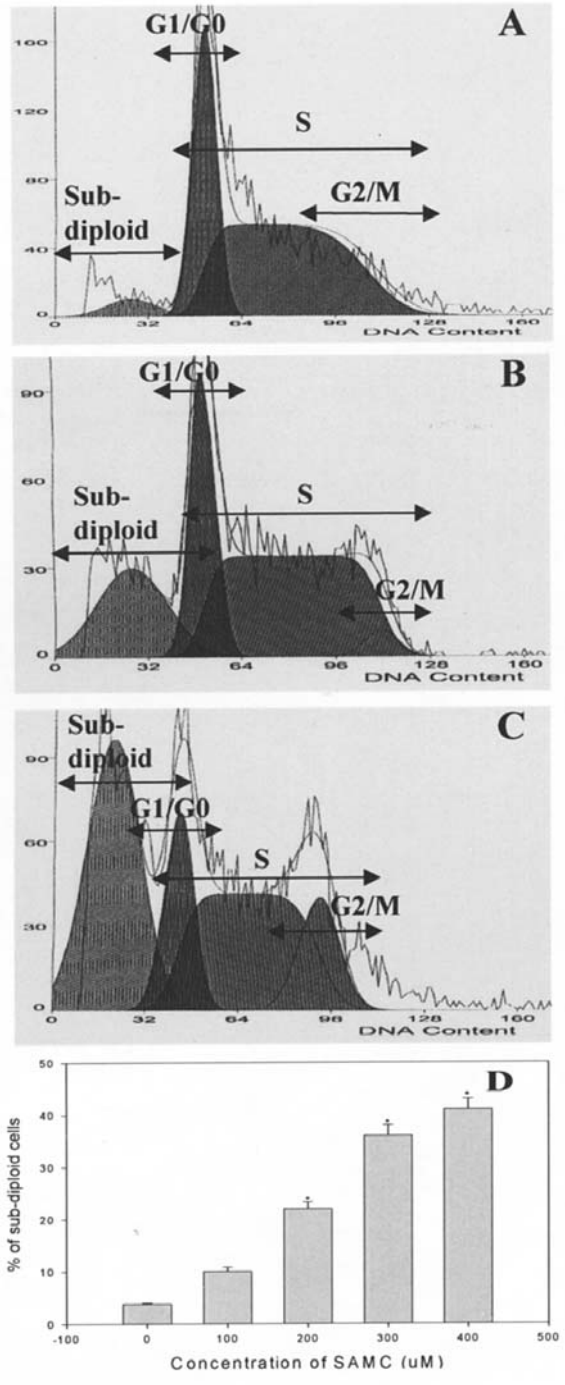

Figure 4. Increase of sub-diploid cells after SAMC treatment. (A) Control, (B) SNU-1 cells treated for 48 h with 200 μM and (C) 400 μM of SAMC. (D) The percentage of sub-diploid cells in each concentration of SAMC. Cells were stained with PI, and the number of sub-diploid cells was counted using FACScan flow cytometry. Cells with a sub-diploid DNA content (>5% of G_0 content) were considered to be apoptotic. Cell cycle distribution was analysed with ModFit LT v2.0. Data represent the mean values of three replicates, with bars indicating SEM. *p<0.05 compared to control.

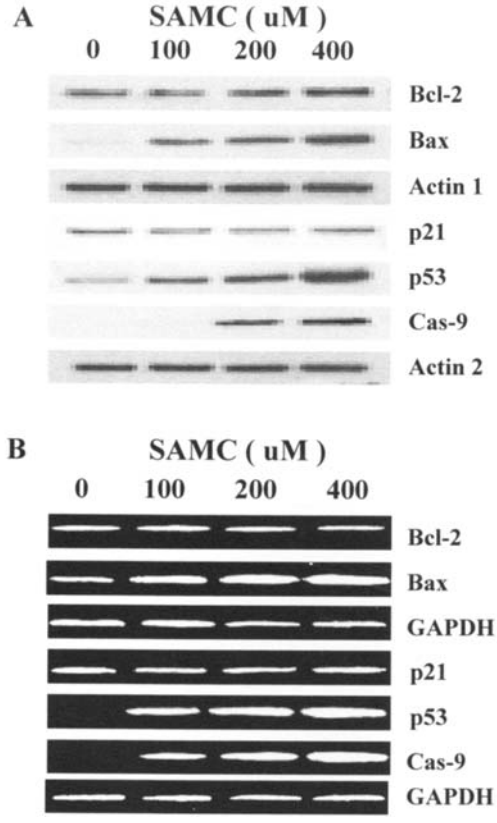

Figure 5. Effect of SAMC on the levels of Bcl-2, Bax, p53, p21 and caspase-9 expression on human gastric cancer SNU-1 cells after 48 h of incubation. (A) Immunoblot analysis. Lysed and cellular proteins were separated onto 10 or 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the corresponding antibodies. (B) DNA analysis by RT-PCR amplification. Total RNA was isolated and RT-PCR was performed using the indicated primers.

Results

Growth inhibition by SAMC. SAMC showed a dose-dependent inhibitory effect on SNU-1 cell proliferation. Viability was inhibited >48% and >80% in SNU-1 cells exposed to 200 and 400 μM of SAMC respectively, as compared to untreated cells. With increasing concentrations (0-300) μM of SAMC, the viability of SNU-1 cells was inhibited in a concentration dependent manner (Fig. 2).

Induction of apoptosis by SAMC. Genomic DNA fragmentation, one of the hallmarks of apoptosis, was induced with increasing concentrations (100-400 μM) of SAMC (Fig. 3A). The percentage of DNA fragmentation showed the same results in the quantitative analysis of fragmented DNA using [^{3}H]-thymidine incorporation (Fig. 3B). Those results were also confirmed by the quantitation of apoptotic sub-diploid cells. As shown in Fig. 4, the proportion of sub-diploid cells shown by flow cytometry analysis increased from 10 to 41% with increasing concentrations of 100-400 μM of SAMC. These results suggest that, following SAMC treatment, SNU-1 cells show apoptosis and that there is a good correspondence between the extent of apoptosis and growth inhibition.

Induction of Bax by SAMC. Cellular proteins and total RNAs were isolated after 48 h of incubation with SAMC, and immunoblotting and RT-PCR were performed against Bcl-2 and Bax in order to examine the expression of the Bcl-2 family in SAMC-induced apoptosis. As shown in Fig. 5, SAMC did not affect the levels of Bcl-2 mRNA or protein,

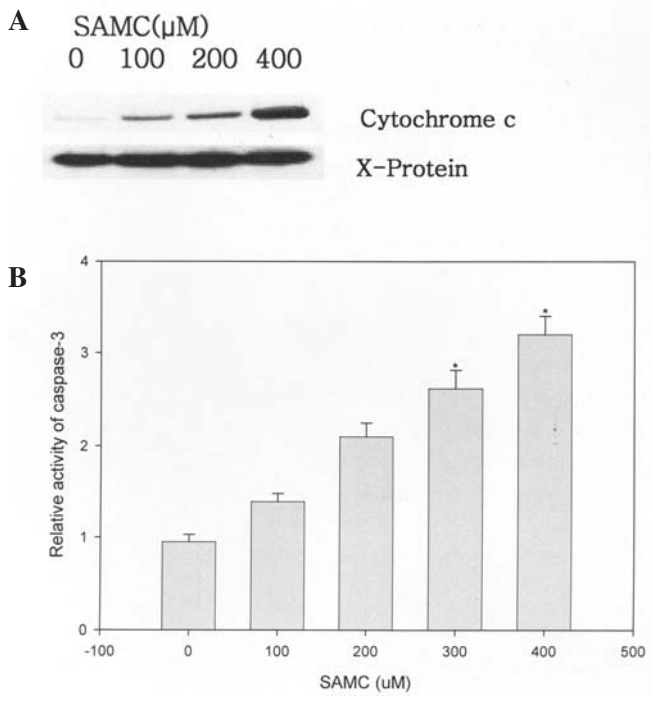

Figure 6. Induction of cytochrome *c* release and caspase-3 activity by SAMC. SNU-1 cells were treated with each concentration of SAMC for 48 h. (A) Mitochondrial cytochrome *c* was detected by anti-cytochrome *c* monoclonal antibody. The aggregated cytochrome *c*, X-protein bands were used to normalize the protein loading. (B) caspase-3 activity was measured by reading samples in a fluorescence microplate reader. Data represents the relative activity of caspase-3 after normalization with protein amounts. Data represents the mean values of three replicates, with bars indicating SEM. *p<0.05 compared to control.

whereas the levels of Bax mRNA and protein were significantly increased by SAMC treatment in a concentration-dependent manner. The expression of tumor suppressor p53, CDK inhibitor p21, and caspase-9 in SNU-1 cells treated with SAMC was also examined. The levels of the protein and mRNA of p21 remained unchanged, but the levels of both the protein and mRNA of p53 were significantly increased by treatment with SAMC. We then examined the activation of caspase-9, which cleaves and activates downstream caspases. The protein and mRNA levels of caspase-9 increased significantly in a concentration-dependent manner. These results suggest that the apoptotic effects of SAMC on SNU-1 cells are caused by the induction of the proteins and mRNAs of Bax, p53, and caspase-9, rather than the induction of the proteins and mRNAs of Bcl-2 and p21.

Cytochrome c release and caspase-3 activation. The activation of caspases is regulated by the release of cytochrome *c* from mitochondria to the cytosol (24,25). The present study showed that cytochrome *c* release was induced and markedly increased by treatment with SAMC for 48 h (Fig. 6). We confirmed these results using a caspase-3 activity assay. As shown in Fig. 6, caspase-3 activities were increased 2.1-, 2.6-, and 3.1-fold by treatment with SAMC (200, 300, and 400 μM, respectively). These results suggest that SAMC induces apoptosis through the release of mitochondrial cytochrome *c*; then a complex form with Apaf-1 and a pro-form of caspase-9 may subsequently activate caspase-3.

Discussion

The present results clearly demonstrate that SAMC induces apoptosis in human gastric cancer SNU-1 cells in culture, which explains its anti-proliferation activity. These results also suggest that the induction of apoptotic cell death by SAMC occurred via the mitochondrial pathway that activates caspases.

Apoptosis is a systematically regulated process that involves the expression of many gene products. Of the major genes that regulate apoptosis, the anti-apoptotic Bcl-2 gene and the pro-apoptotic Bax gene are of particular interest. Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope, and may register damage to these compartments and affect their behavior, perhaps by modifying the flux of small molecules or proteins (26). Bcl-2 may be activated by Ser70 phosphorylation, but may be inactivated or otherwise altered by the phosphorylation of several loop sites, such as Jun kinase (JNK) (27). Bcl-2 protects against various cytotoxic insults, such as UV-irradiation, cytokine withdrawal, and cytotoxic drugs (28). However, pro-apoptotic Bax protein translocates to mitochondria upon exposure to the stimuli of apoptosis (29), and induces the release of cytochrome *c* and the activation of caspase *in vitro* (11). 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 this study, no change was observed for Bcl-2 levels in SNU-1 cells, but the level of the pro-apoptotic gene, Bax, increased significantly in a concentration-dependent manner, resulting in a decrease in the ratio of Bcl-2 to Bax (Fig. 5). Additionally, the expression levels of p21 mRNA and protein were not changed by SAMC treatment, but the expression levels of p53 mRNA and protein were increased in a concentration-dependent manner. These results suggest that the apoptotic effect of SAMC is associated with an increased expression level of the pro-apoptotic Bax gene and p53 without an alteration in the expression of Bcl-2 or p21.

Thus, the pro-survival Bcl-2 subfamily and pro-apoptotic Bax subfamily can oppositely regulate apoptosis through the control of cytochrome *c* release from mitochondria, resulting in caspase activation (11). Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode *C. elegans*, is related to mammalian interleukin-1 ß-converting enzyme (30,31). Caspases share similarities in their amino acid sequences, structures, and substrate specificities (32). This study demonstrated that SAMC treatment of SNU-1 cells caused a concentration-dependent activation of caspase-3, one of the main executers of the apoptotic process (33,34). This study also showed that activation of caspase-3 is regulated by the release of cytochrome *c* from mitochondria to the cytosol (Fig. 6). Caspase-9 was also activated in a concentration-dependent manner (Fig. 5). These results suggest that released cytochrome *c* enters the cytosol and might activate caspase-9 after forming a complex with Apaf-1 and a pro-form of caspase-9 (4). Therefore, these data indicate that the pathway for apoptosis by SAMC exists, in part, due to the increased expression of Bax, and Bax induces

cytochrome *c* release and caspase activation, resulting in apoptosis.

Taken together, these findings suggest that SAMC exhibits apoptotic effects through a down-regulation of Bcl-2/Bax and an activation of caspase-3. Further studies are needed to identify the active compounds that confer the anti-cancer activity of SAMC.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-521-C00299). We thank Dr John T. Pinto (Burke Research Institute, Cornell Medical College) for helpful suggestions.

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