Glutathione-Dependent Cell Cycle G1 Arrest and Apoptosis Induction in Human Lung Cancer A549 Cells Caused by Methylseleninic Acid: Comparison with Sodium Selenite

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The aim of the present study was to clarify the mechanism underlying the inhibition of cell proliferation in human lung cancer A549 cells by selenium (Se) compounds. Methylseleninic acid (CH₃SeO₂H, abbreviated as MSA), a synthetic Se compound, is a direct precursor of active methylselenol (CH₃SeH) and is considered to be one of beneficial agents for cancer prevention and therapy. Sodium selenite (Na₂SeO₃), an inorganic Se form, is utilized in clinical Se supplementation. MSA markedly inhibited the growth of A549 cells at a concentration of 2.5 × 10⁻³ mol/L for 1 d. On Day 1, Na₂SeO₃ also inhibited A549 cell growth at the concentration of 7.5 × 10⁻⁴ mol/L. These compounds induced cell cycle arrest at the G₁ phase and apoptosis under the inhibitory condition. Reduced glutathione (GSH) is critical to MSA or Na₂SeO₃ metabolism. The depletion of intracellular GSH suppressed Na₂SeO₃-induced G₁ arrest, but promoted Na₂SeO₃-induced apoptosis. Therefore, Na₂SeO₃ appears to have directly induced apoptosis. In contrast, the MSA-induced G₁ arrest was alleviated by a marked decrease in GSH content. Additionally, the depletion of GSH slightly suppressed MSA-induced apoptosis. The difference in inhibitory effects between MSA and Na₂SeO₃ may be due to this variation in GSH-related metabolism. After exposure of A549 cells to MSA, the GSH content was significantly decreased. These results indicate that because MSA-induced G₁ arrest and apoptosis induction are enhanced by GSH, the maintenance of GSH is essential for the effective anticancer action of MSA in A549 cells.

Key words methylseleninic acid; cell cycle arrest; apoptosis; reduced glutathione; human lung cancer A549 cell

Cancer is a major public health problem in many parts of the world. Lung cancer is a common cause of cancer death; in 2008, lung cancer accounted for approximately 12.7% of all new cancers and 18.2% of all cancer mortality, or approximately 1.4 million deaths worldwide. Decreasing the number of deaths due to lung cancer is an important issue.

Pneumocytes are exposed not only to exogenous oxidants, but also to endogenous active oxygen species (ROS) generated by aerobic metabolism. Oxidative stress generally occurs when the balance between ROS and antioxidants is disrupted in cells. Several mechanisms have been proposed for the involvement of oxidative stress in the development of lung cancer.

Cells are protected against oxidative damage by maintaining the activities of several antioxidant enzymes and redox-regulating proteins. Selenium (Se) is an essential trace element in mammals and is also a well-known antioxidant. Several epidemiological studies have suggested inverse associations between serum Se levels and a number of different cancers. Prospective studies have also provided scientific evidence for the beneficial effects of Se in reducing the risk of lung cancer. Many in vitro and preclinical studies showed the antitumor effect by MSA. The function and underlying mechanism of Se in the prevention and treatment of cancer remain unclear.

Approximately 25 selenoproteins have been identified in the human genome to date. The glutathione peroxidase and thioredoxin reductase families are the most important selenoproteins functioning as antioxidant enzymes. In addition to these selenoproteins, monomethylated Se compounds have been shown to act as protein redox modulators. Methylselenol (CH₃SeH) has been hypothesized to be a critical Se metabolite for anticancer activity. The routes of the metabolism of Se compounds differ according to their chemical forms (Chart 1). The inorganic Se compound, selenite (SeO₃⁻), was shown to converge in order to produce CH₃SeH via hydrogen selenide (H₂Se). Methylseleninic acid (MSA), a synthetic Se compound, was reduced to CH₃SeH by reduced glutathione (GSH) through non-enzymatic reactions.

The aim of the present study was to determine whether the GSH-related metabolism of MSA was involved in cell cycle arrest and apoptosis induction in lung cancer A549 cells. We first compared the inhibitory effects of MSA and Na₂SeO₃ on cell growth. MSA and Na₂SeO₃ inhibited cell growth, arrested the cell cycle at the G₁ phase and induced apoptosis. We showed that unlike Na₂SeO₃, MSA depressed both G₁ arrest and apoptosis by a depletion of GSH. Our results suggest that the GSH content in A549 cells is important in anticancer effect by MSA.

MATERIALS AND METHODS

Chemicals MSA, Na₂SeO₃, and buthionine sulfoximine (BSO) were obtained from Sigma-Aldrich Co., LLC (St. Louis, MO, U.S.A.). Ham’s F-12K medium and fetal bovine serum (FBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and BioWest SAS (Nuaillé, France). SBD-F was purchased from Dojindo (Kumamoto, Japan). All other reagents were of the highest grade commercially available.

Cell Culture and Treatment The human lung tumor-derived cell line, A549, was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). These cells were cultured in Ham’s F-12K medium supplemented...
with 10% FBS in an atmosphere of 5% CO₂ at 37°C in a humidified incubator. The culture medium contained the antibiotics penicillin (100 units/mL) and streptomycin (100 units/mL). Cell cultures were used 2–3 d after plating at 70–80% confluence. Stock solutions of MSA, Na₂SeO₃, and BSO were prepared in MilliQ water and filter-sterilized using 0.22 μm filters before being stored at 4°C. In experiments involving BSO, the cells were pretreated with 2.5 × 10⁻⁵ mol/L of BSO for 20 h.

**Determination of the Cell Number** Cells were seeded in 96-well plates (Nunc A/S, Roskilde, Denmark) at initial concentrations of 7.5 × 10³ cells/well in 10% FBS-supplemented Ham’s F-12K medium. Different concentrations of Se compounds were added 1 d after seeding. After 0–3 d of cultivation, the total amount of DNA in the cells grown was determined using the ethidium bromide (EtBr) fluorescence method.

**Determination of the Intracellular GSH Content** The content of GSH was determined by HPLC using SBD-F as a fluorescent reagent according to a previously described method. Standard curves was liner over the range of 2 to 1000 nmol/mL. Protein contents were quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) according to the manufacturer’s procedure.

**Cell Cycle Analysis** The cell cycle was analyzed using flow cytometry with propidium iodide (PI) staining. A549 cells were seeded in a 60-mm tissue culture dish (BD Falcon, Durham, NC, U.S.A.) at an initial concentration of 9.1 × 10⁵ cells/dish. To achieve synchronization, cells were starved in serum-free medium for 2 d. When returned to regular growth medium, cells were exposed to 2.5 × 10⁻⁶ mol/L MSA or 7.5 × 10⁻⁶ mol/L Na₂SeO₃. After being treated for 1 d, cells were trypsinized, washed in phosphate buffered saline (PBS), and fixed overnight in 1 mL of 70% ethanol at −20°C. The ethanol solution was subsequently removed after centrifugation, and cells were re-suspended in 1 mL PBS containing PI (50 μg/mL) and RNase (100 μg/mL). After being incubated at 37°C for 30 min, cells were subjected to DNA content analysis using flow cytometry (CytoACE, JASCO Co., Tokyo, Japan). Data were analyzed with the software contained in CytoACE.

**Detection of Apoptosis** Cells were seeded in 96-well plates at initial concentrations of 7.5 × 10³ cells/well in 10% FBS-supplemented Ham’s F-12K medium. One day after seeding, cells were exposed to 2.5 × 10⁻⁶ mol/L MSA or 7.5 × 10⁻⁶ mol/L Na₂SeO₃. After 1-d cultures, the stained cells were analyzed using the ArrayScan VTI HCS Reader (Thermo Fisher Scientific Inc.) Images were acquired for each fluorescence channel, Hoechst 33342, Annexin V, and PI, using suitable filters with a 10× objective. Automatic focusing was performed in the nuclear channel to ensure focusing regardless of the staining intensities in the other channels. Not less than 500 cells were analyzed in each well, and the proportion of apoptotic cells stained by Annexin V to the total number of cells stained by Hoechst 33342 was then calculated.

**RNA Isolation and Polymerase Chain Reaction (PCR)** Cells were seeded in a 60-mm tissue culture dish at an initial concentration of 9.1 × 10⁵ cells/dish in 10% FBS-supplemented Ham’s F-12K medium. Cells were exposed to 7.5 × 10⁻⁶ mol/L Na₂SeO₃ or 2.5 × 10⁻⁶ mol/L MSA for 1 d. Total RNA was iso-
lated from cells using Sepasol reagent (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer’s protocols and was then quantified spectrophotometrically. Total RNA (1 µg) in each sample was reverse-transcribed using the High Capacity cDNA transcription kit. To measure cell cycle-related gene expression, PCR was performed using the human cell cycle RT² Profiler™ PCR Array System (SuperArray Biosciences Co., Frederick, MD, U.S.A.) according to the manufacturer’s procedure.

Statistical Analysis Statistical significance between two groups was examined using unpaired Student’s t-test. A probability of 0.05 or less was regarded as significant.

RESULTS
MSA Inhibited the Proliferation of A549 Cells To assess the effectiveness of MSA as therapeutic agents in lung cancer, we investigated the effects of MSA and Na₂SeO₃ on the proliferation of A549 cells. After 3 d, MSA and Na₂SeO₃ at each concentration of greater than 2.5×10⁻⁶ mol/L significantly inhibited the cell growth (Fig. 1). Interestingly, dose-dependent inhibitory effects were observed when cells were treated with a concentration greater than 2.5×10⁻⁶ mol/L MSA for 1 d, as shown in Fig. 1A. Meanwhile, a marked reduction in viable cell counts was only observed in cells exposed to Na₂SeO₃ for 1 d after the treatment with a concentration of 7.5×10⁻⁶ mol/L (Fig. 1B).

Fig. 1. Dose- and Time-Dependent Effects of MSA (A) and Na₂SeO₃ (B) on the Proliferation of A549 Cells
Cells were exposed to MSA or Na₂SeO₃ at a concentration of 2.5×10⁻⁶ mol/L or 7.5×10⁻⁶ mol/L for 1 d, respectively. The values are the mean±S.D. (n=5). **p<0.01 vs. control.

Fig. 2. Cell Cycle Analysis of A549 Cells Exposed to MSA and Na₂SeO₃
Cells were synchronized by serum starvation for 2 d. Cells were treated with BSO (2.5×10⁻⁵ mol/L) 20 h before MSA or Na₂SeO₃. When returned to regular growth medium, cells were exposed to MSA (2.5×10⁻⁶ mol/L) or Na₂SeO₃ (7.5×10⁻⁶ mol/L) for 1 d.

A. Control
G₀/G₁ : 53.05%
S : 24.47%
G₂/M : 22.48%

B. MSA
G₀/G₁ : 67.37%
S : 5.92%
G₂/M : 26.71%

C. Na₂SeO₃
G₀/G₁ : 65.68%
S : 16.11%
G₂/M : 18.21%

D. BSO
G₀/G₁ : 52.57%
S : 24.14%
G₂/M : 23.29%

E. BSO+MSA
G₀/G₁ : 53.22%
S : 21.40%
G₂/M : 25.38%

F. BSO+Na₂SeO₃
G₀/G₁ : 52.33%
S : 24.23%
G₂/M : 22.44%
MSA Inhibited Cell Progression in A549 Cells  To determine whether the inhibition in growth by MSA in A549 cells was associated with the arrest of cells in a particular phase, we analyzed cell cycle distribution profiles for MSA- or Na2SeO3-exposed cells. A549 cells were deprived of FBS for cell cycle synchronization. After being starved for 2 d, 67.1% of cells were in the G0/G1 phase, 19.4% in the S phase, and 13.5% in the G2/M phase. As shown in Fig. 2A, re-feeding with 10% FBS for 1 d resulted in the enrichment of the S (24.5%) and G2/M (22.5%) phases in these cells. Although MSA was administered concurrently with 10% FBS, the cell cycle distribution pattern remained unchanged after 1 d (Fig. 2B). Over 67% of cells were consecutively inhibited at the G0/G1 phase. Similar results were observed with the co-administration of Na2SeO3 and 10% FBS (Fig. 2C). These results suggested that MSA and Na2SeO3 may inhibit cell cycle progression from the G1 to the S phase in A549 cells.

The expression of 84 genes related to regulation of the cell cycle was measured using the Human Cell Cycle RT2 Profiler™ PCR Array (SuperArray Biosciences Co., Frederick, MD, U.S.A.). In A549 cells treated with MSA, as shown in Table 1, the gene expression level of p27kip1 was increased to over 200% that of untreated control cells, while those of CDK2 and cyclin E1 were decreased to approximately 80 and 60% that of control cells, respectively. These results suggested that MSA may cause cell cycle G1 arrest by down-regulating cyclin E1 and up-regulating p27kip1.

MSA Induced Apoptosis in A549 Cells  We determined whether the inhibition of growth in A549 cells by MSA was associated with the induction of apoptosis. On 1 d, 3.1±1.1% of the untreated A549 cells were apoptotic (Fig. 3A). MSA and Na2SeO3 increased the percentage of apoptotic cells to 22.5±6.1% and 14.7±4.2%, respectively (Figs. 3B, C). These results clearly indicated that the MSA and Na2SeO3 treatments clearly induced apoptosis in A549 cells.

Intracellular GSH Enhanced MSA-Induced G1 Arrest and Apoptosis  To confirm whether GSH-related metabolism of MSA contributed to G1 arrest and apoptosis, we further examined cell cycle distribution profiles and apoptosis induction after BSO-pretreated cells were exposed to MSA or Na2SeO3. Our results revealed that the GSH content in A549 cells was markedly decreased by the MSA treatment for 1 d (Fig. 4). The pretreatment of A549 cells with BSO, a GSH synthesis inhibitor, was decreased the GSH content to below the detectable limits of our assay. After starvation and re-feeding with 10% FBS, the cell cycle distribution pattern in BSO-pretreated cells was similar to that in the control cells (Fig. 2D). When MSA or Na2SeO3 was given concurrently with 10% FBS, the cycle was measured using the Human Cell Cycle RT2 Profiler™ PCR Array (SuperArray Biosciences Co., Frederick, MD, U.S.A.). In A549 cells treated with MSA, as shown in Table 1, the gene expression level of p27kip1 was increased to over 200% that of untreated control cells, while those of CDK2 and cyclin E1 were decreased to approximately 80 and 60% that of control cells, respectively. These results suggested that MSA may cause cell cycle G1 arrest by down-regulating cyclin E1 and up-regulating p27kip1.

Table 1. Effect of MSA on Gene Expression Levels in A549 Cells

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Relative expression (β-actin)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>MSA</td>
</tr>
<tr>
<td>p27kip1</td>
<td>4.94×10^-7 (100%) 1.10×10^-5 (2227%)</td>
</tr>
<tr>
<td>CDK2</td>
<td>2.84×10^-4 (100%) 2.37×10^-4 (83.5%)</td>
</tr>
<tr>
<td>Cyclin E1</td>
<td>1.15×10^-3 (100%) 7.35×10^-4 (63.9%)</td>
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RT2 Profiler™ RCR array analysis of 24 h treatment of A549 cells with 2.5×10^-6 mol/L MSA. The values are presented as the relative expression levels compared with β-actin.

**Fig. 3. Detection of MSA- or Na2SeO3-Induced Apoptotic Cells in A549 Cells**

Cells were treated with BSO (2.5×10^-5 mol/L) 20h before the administration of Na2SeO3 or MSA. Cells were then exposed to MSA (2.5×10^-6 mol/L) or Na2SeO3 (7.5×10^-6 mol/L) for 1 d. The values are the mean±S.D. (n=3–4). Blue: Hoechst 33342 stain; Green: Annexin V stain (apoptotic cells); Red: Propidium iodide stain (dead cells).
BSO-pretreated cells were passaged and released into the cell cycle (Figs. 2E, F). These results suggested that the GSH-related metabolism of MSA or Na₂SeO₃ enhances the G₁ arrest of A549 cells.

The percentage of apoptotic cells was slightly higher in BSO-pretreated cells than in control cells (Fig. 3D). When BSO-pretreated cells were exposed to MSA for 1 d, the percentage of apoptotic cells was reduced (Figs. 3B, E). In contrast, when BSO-pretreated cells were exposed to Na₂SeO₃ for 1 d, the percentage of apoptotic cells was approximately 2-fold higher than that in BSO-untreated cells (Figs. 3C, F). These results suggested that the intracellular GSH plays an important role in the difference in inhibitory effects between MSA and Na₂SeO₃.

**DISCUSSION**

In the present study, we investigated growth inhibition as an index of the anticancer effects of MSA in human lung cancer A549 cells, and attempted to explore the underlying mechanisms. The inhibition of cell growth by the selenoamino acid selenomethionine was negligible until a concentration of 1×10⁻⁵ mol/L for 1 d (data not shown). MSA and Na₂SeO₃ significantly inhibited the cell growth at concentrations higher than 2.5×10⁻⁵ mol/L and 7.5×10⁻⁶ mol/L, respectively (Fig. 1). Although the inhibitory concentration of Na₂SeO₃ may be slightly higher than human blood levels, MSA or Na₂SeO₃ may prove useful as anticancer agents for lung cancer.

MSA and Na₂SeO₃ arrested cell progression at G₁ phase under each inhibitory condition (Figs. 2B, C). Concurrently, an increase was observed in the ratio of apoptotic cells in A549 cells treated with MSA or Na₂SeO₃ (Figs. 3B, C). Our results for Na₂SeO₃ were consistent with previous findings in which 6×10⁻⁶ mol/L Na₂SeO₃ for 1 d suppressed the growth of A549 cells that was attributed to the increase in the sub-G₁ phase and the induction of apoptosis. Previous studies examined the cell cycle arrest and induction of apoptosis by MSA in other cancer cell lines, but not the A549 cell line. Sinha et al. reported that MSA at a low concentration (5×10⁻⁶ mol/L) inhibited the growth of the mouse mammary epithelial tumor cell line, TM6 at the G₁ phase, whereas a high concentration (5×10⁻⁵ mol/L) could induce apoptosis. Another study showed that 5×10⁻⁴ mol/L MSA inhibited the growth of MCF10AT1 and MCF10AT3B premalignant human breast cells, and a high concentration could induce apoptosis. Our results indicated that a concentration of 2.5×10⁻⁴ mol/L MSA may be sufficient for the G₁ arrest and induction of apoptosis in A549 cells. Homma et al. suggested that NF-E2-related factor 2 (Nrf2), key transcription regulator for antioxidant-related enzymes such as glutathione synthase and glutathione reductase, was abundantly expressed in A549 cells. Therefore, intracellular GSH content in A549 cells is probably abundant. In A549 cells, the cell cycle progression and/or apoptosis induction may be highly influenced by MSA and Na₂SeO₃ because these Se compounds are quickly reduced to their respective intermediate metabolites by GSH.

The gene expression level of p27Kip1, a cyclin-dependent kinase inhibitor protein, was significantly higher in A549 cells treated with MSA than in control cells, whereas the gene expression levels of CDK2 and cyclin E1 were lower. The p27Kip1 protein was previously shown to bind with cyclin E1/CDK2 complexes and inhibit the kinase activity of CDK2. These effects inhibited the hyperphosphorylation of retinoblastoma (Rb) and increased the amount of the Rb-bound E2F1 transcription factor, thereby delaying the expression of gene products required for G₁/S transition in cells.

It remains clear whether the anticancer effects of MSA or Na₂SeO₃ are enhanced or suppressed by their metabolism, which was the focus of the present study. The anticancer mechanisms ascribed to these Se compounds may be dependent on their chemical forms and metabolic transformations. A previous study reported that the active metabolite of many Se compounds is CH₃SeH, which has been associated with the superior anticancer effects of Se. CH₃SeH was previously shown to induce G₁ cell cycle arrest and apoptosis via several cancer signaling genes.

Intracellular GSH is critical to metabolism of MSA and Na₂SeO₃, in addition to being a protective antioxidant which scavenges ROS and maintains the stability of intracellular redox status. In fact, the content of GSH in A549 cells was 2.58±0.48 nmol/µg protein (Fig. 4). When cells were treated with a concentration of MSA or Na₂SeO₃ that induced apoptosis, GSH contents tended to decrease compared to control group (Fig. 4). MSA and Na₂SeO₃ are non-enzymatically metabolized by GSH, which is ubiquitously present in cells (Chart 1). H₂Se produced by GSH from Na₂SeO₃ is further transformed to selenoprotein for utilization and/or to selenosugar for excretion. H₃Se is also involved in the generation of ROS such as the superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). On the other hand, as for MSA, Lui et al. inferred that the mechanism of ROS generation is through the selenide route: MSA catalyzes by GSH to CH₃SeH, and the CH₃SeH is demethylated to H₂Se by demethylase, which is critical in generation of ROS. CH₃SeH may directly react with oxygen to produce the free radical CH₃Se⁻ and ROS. MSA may be taken up into cells easily and then quickly catalyzed by GSH to CH₃SeH. We propose that the difference in GSH depletion between MSA and Na₂SeO₃ is attributed to the metabolic rate or intermediary metabolites of Se. We hypothesized that the intracellular GSH may play a key role in the cell cycle arrest and apoptosis induced by MSA or Na₂SeO₃. The GSH content decreased to undetectable levels in cells pretreated with BSO. Hence, the BSO pretreatment was presumed to inhibit the GSH-related metabolism in cells. Our results showed that G₁ arrest caused by MSA or Na₂SeO₃ was
suppressed when their GSH-related metabolism was inhibited by the BSO pretreatment (Fig. 2). We also confirmed that the gene expression level of p27Kip1 was not increased when BSO-pretreated cells were exposed to MSA (data not shown).

The percentage of apoptotic cells was slightly lower in cells treated simultaneously with BSO and MSA than in those treated with MSA alone (Figs. 3B, E). In contrast, our results indicated apoptosis was more severe in cells treated with both BSO and Na2SeO3 than in those treated with Na2SeO3 alone (Figs. 3C, F). These results demonstrated that MSA-induced apoptosis was enhanced by GSH-related metabolism. Unlike MSA, Na2SeO3 per se appeared to directly induce apoptosis. In the presence of GSH, MSA may be more effective at inducing apoptosis than Na2SeO3 (Figs. 3B, C). In A549 cells, however, the MSA treatment in particular induced a more rapid decline in GSH than the Na2SeO3 treatment (Fig. 4). From these results, the maintaining of intracellular GSH content is important in the more effective anticancer action via apoptosis induction by MSA in A549 cells. H2Se reacted with molecular oxygen to produce ROS; the formation of which induced DNA single-strand breaks leading to cell death by apoptosis.20) ROS are also known to be mediators of p53-initiated apoptosis.34–36)

Intracellular ROS levels were slightly higher following the treatment with both BSO and Na2SeO3 than the treatment with Na2SeO3 (data not shown). This ROS generation would appear to mediate Na2SeO3 per se-induced apoptosis; however, the mechanism responsible remains unknown. MSA also may be induce generation of ROS. However, some studies have reported that MSA-induced caspase-mediated apoptosis does not require p53.37,38) Jiang et al. reported that MSA induced apoptosis in DU-145 human prostate cancer cell through mitochondrial cytochrome c release and caspase activation.39)

In conclusion, we here demonstrated that micromolar concentrations of MSA and Na2SeO3 markedly inhibited the growth of A549 cells. The GSH-related metabolism of MSA and Na2SeO3 were closely related to the G1 arrest. It is assumed that MSA induces G1 arrest by down-regulating cyclin E1 and up-regulating p27Kip1. MSA was simultaneously shown to enhance the apoptosis induction in the presence of intracellular GSH. In contrast, Na2SeO3 per se slightly enhanced apoptosis. The difference in inhibitory effects between MSA and Na2SeO3 may be due to the GSH-dependent apoptosis. Further studies are needed in order to determine the ultimate chemical forms relevant to the induction of G1 arrest and apoptosis in A549 cells.

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