Combination treatment with arsenic trioxide and sulindac augments their apoptotic potential in lung cancer cells through activation of caspase cascade and mitochondrial dysfunction

HAK-RYUL KIM[1], EUN-JUNG KIM[2], SEI-HOON YANG[1], EUN-TAIK JEONG[1], CHANNY PARK[2], SE-JIN KIM[2], MYUNG-JA YOUN[2], HONG-SEOB SO[2] and RAEKIL PARK[2]

Departments of [1]Internal Medicine and [2]Microbiology, Wonkwang University, School of Medicine, 344-2 Shinyong-dong Iksan, Jeonbuk 570-749, South Korea

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Abstract. Non-steroidal anti-inflammatory drugs (NSAIDs) are known to enhance the responsiveness of tumor cells toward chemotherapeutic drugs and radiation. However, the precise mechanism of synergistic enhancement in tumoricidal activity is not clearly known. Herein, we demonstrate that the combination treatment of arsenic trioxide (As_2O_3) and sulindac resulted in a synergistic augmentation of cytotoxicity toward NCI-H157 lung cancer cells, which was revealed as apoptosis accompanied by chromatin fragmentation and an increase in sub-G_0/G_1 fraction. In addition, combination treatment with As_2O_3 and sulindac increased the catalytic activity of caspase-3, -8, and -9 along with induction of Fas/FasL expression and cytosolic release of cytochrome c. Pharmacologic scavenging study of reactive oxygen species (ROS) revealed that synergistic augmentation of cytotoxicity was achieved by generation of ROS, which might modulate the expression of Bcl-2 family proteins, the activity of caspase-3, and mitochondrial membrane potential transition.

Introduction

Although many new therapeutic maneuvers have been developed in the treatment of cancers, mortality rates of lung cancer patients are currently high and increasing (1). The 5-year survival rate for lung cancer patients with stage IIa disease is 9-25% and with stage IIIb disease is only 3-7%. A number of new agents have become available for the treatment of lung cancer, but until now no chemotherapy regimens have offered a significant advantage over others (2). Therefore, new approaches for the treatment of lung cancer are needed. As a complementary therapy, gefitinib, an inhibitor of epidermal growth factor receptor tyrosine kinase, and Bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor, were clinically tried with chemotherapy regimens to treat lung cancer patients. However, these combination therapies did not have improved efficacy over chemotherapy regimens alone (3,4).

Arsenic trioxide has long been used as an anticancer agent in traditional Chinese medicine (5) and is currently used in the treatment of refractory and relapsed acute promyelocytic leukemia (APL) without severe marrow suppression (6). Some investigators have reported that As_2O_3 induces apoptosis in a variety of human solid tumor cell lines, including carcinomas of the esophagus, stomach, prostate, and ovary as well as neuroblastoma cells (7-10). However, the clinically relevant concentration is a major limiting factor in practical prescription for leukemia patients. The dose range of clinical use is 0.5-2 μM of As_2O_3 in the treatment of leukemia, whereas there should be over 5-10 μM for induction of apoptotic death in many solid tumor cells. As a consequence, the clinical use and efficacy of As_2O_3 have thus far been largely hampered in its universal application to various types of cancer patients, except in patients with APL.

Recently, NSAIDs have attracted much attention after the discovery that sulindac could induce the regression of colon adenomatous polyps in cancer therapy (11). Continuous NSAID administration results in a significant reduction of relative risk for colorectal cancer (12). Sulindac, a structural isoform of indomethacin, exerts antiproliferative and apoptotic effects which eventually lead to the regression of cancer cells (13,14). NSAIDs are known to enhance the cellular responsiveness of tumors toward chemotherapeutic drugs by apoptosis or anti-angiogenic effect and are also synergistic with radiotherapy by directly increasing cellular radiation sensitivity as well as inhibiting tumor neovascularization (15,16). In this study, we aimed to investigate whether combination treatment with As_2O_3 and sulindac augments their apoptotic potential in NCI-H157 human lung cancer cells.

Materials and methods

Materials. RPMI-1640, fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL Co. (Grand Island,

Correspondence to: Dr Raekil Park, Department of Microbiology, Wonkwang University, School of Medicine, 344-2 Shinyong-dong Iksan, Jeonbuk 570-749, South Korea
E-mail: rkpark@wonkwang.ac.kr

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NY). Arsenic trioxide, sulindac, methylthiazol-2-yl-2,5-diphynyl, tetrazolium bromide (MTT), propidium iodide (PI), bicinchoninic acid (BCA), dimethyl sulfoxide (DMSA), n-acetylcysteine (NAC), and glutathione (GSH) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against caspase-3, -8, -9, PARP, ICAD, FAS, FAS/L, Bax, Bid, and Bcl-x_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). JC-1 was obtained from Molecular Probes Co. (Eugene, OR), and antibody against anti-cytochrome c was obtained from Pharmigen Co. (San Diego, CA). Anti-rabbit IgG conjugated horseradish peroxidase antibody and enhanced chemiluminescent (ECL) kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and viability test. NCI-H157 human lung cancer cells were obtained from Korean Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS, and maintained in a humidified atmosphere of 5% CO_2 in air at 37˚C and maintained in log phase. Cell viability was determined by MTT assay. Cells ($5x10^4$/well) were seeded in 24-well plates, were added to MTT (0.5 mg/ml) and kept in a 37˚C, 5% CO_2 incubator for an additional 3 h. After three washes of cells with phosphate-buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in DMSO. Then, the optical density (OD) of each culture well was measured using Microplate reader (Titertek Multiskan, Flow Laboratories, North Ryde, Australia) at 590 nm. The OD of formazan formed in control cells was taken as 100% of viability and the positively-stained cells with MTT were expressed as the percentage (%) compared to control cells.

Hoechst 33342 staining. After treatment with reagents, cells were fixed in PBS with 3.7% paraformaldehyde for 10 min. Cells were washed twice with PBS and then incubated with 10 μM Hoechst 33342 (Sigma) in PBS at room temperature for 30 min. After incubation, cell morphology was observed under fluorescence microscope (Leica, Japan).

Flow cytometric analysis. The degree of apoptosis was determined by measuring the number of cells showing below the G_0/G_1 phase of DNA content from flow cytometric analysis after staining the cells with propidium-iodide (PI) as originally described by Crissman and Steinkamp (17). Cell cycle analysis was performed with FACScan equipped with Cell Quest software (Becton-Dickinson, San Jose, CA).

Preparation of genomic DNA and electrophoresis. The ladder pattern of DNA strand break was analyzed by agarose gel electrophoresis. Genomic DNA from H157 cells was isolated using a Wizard Genomic DNA purification kit (Promega). After ethanol precipitations, 5 μg of DNA was subjected to electrophoresis on 1.5% agarose for 3 h at 50 V. DNA was visualized by staining with ethidium bromide under UV light.

Caspase activity assay. To measure caspase activity, whole cell lysate was prepared in a lysis buffer as described previously and used to measure the catalytic activities of caspases (18). Cleavage of fluorogenic substrates, including 100 μM Ac-DEVD-AMC by caspase-3, Ac-IETD-AFC by caspase-8, and Ac-LEHD-AFC by caspase-9, was measured by spectrofluorometer (Jasco FR-777, Germany) at 380/460 and 405/505 nm, respectively.

Determination of H_2O_2 generation. The generation of intracellular H_2O_2 was determined as described by Root *et al* (19) by monitoring the decrease in fluorescence of scopoletin during its oxidation catalyzed by horseradish peroxidase (HRP). H157 cells were harvested and resuspended at $1x10^6$/ml in PBS without Ca^{++} and Mg^{++} in the presence of 5 μM scopoletin and 1 U/ml HRP in the dark. Then, cells were added to the wells of the microplate, which was prewarmd at 37˚C and contained 100 μl of reaction mixture with 10 μl of buffer. Data were analyzed by flow cytometry (Molecular Devices Co.) with an excitation and emission spectrum of 350 and 460 nm, respectively.

Fluorescent staining of mitochondrial membrane potential transition (MPT). H157 cells (10^7 cells/group) treated with sulindac or As_2O_3 were harvested, washed with PBS, and then incubated with 10 μg/ml JC-1 at 37˚C for 30 min. After incubation, cell morphology was observed at 530 nm under fluorescence microscope (MPS 60, Leica).

Western blotting. Cell extracts were separated by 10% SDS-PAGE under reduced conditions and transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS-T (25 mM Tris, pH 7.6, 138 mM NaCl and 0.05% Tween-20) for 1 h and probed with primary antibodies (1:1,000-1:5,000). After a series of washes, the membrane was further incubated with secondary antibody (1:2,000-1:10,000) conjugated with horseradish peroxidase (HRP). The immunoreactive signal was detected using an enhanced chemiluminescent (ECL) detection system (Amersham).

Preparation of cytosolic and mitochondrial fractions. Preparation of cytosolic and mitochondrial fractions was performed according to the previous report (20) with some modifications. In brief, H157 cells were harvested, washed with ice-cold PBS, and then incubated with 500 μM of buffer A (250 mM sucrose, 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin and pepstatin A) on ice for 30 min. Then, cells were disrupted by 20 passages through a 26-gauge needle. The disrupted cells were centrifuged at 750 x g for 10 min. The supernatant was centrifuged at 10000 x g for 25 min. After centrifugation, the cytosolic fraction was frozen at 70˚C. The pellet containing mitochondria was washed with ice-cold buffer A and then resuspended with cell lysis buffer. The resuspended pellet was incubated on ice for 30 min and then centrifuged at 10000 x g for 25 min. The supernatant was collected as cytosolic fraction of H157 cells.

Statistical analysis. Each experiment was performed at least three times, and all values are represented as means ± SD of triplicates. Student's t-test was used to analyze a statistical significance of the results. Values of $p<0.05$ were considered as statistically significant.

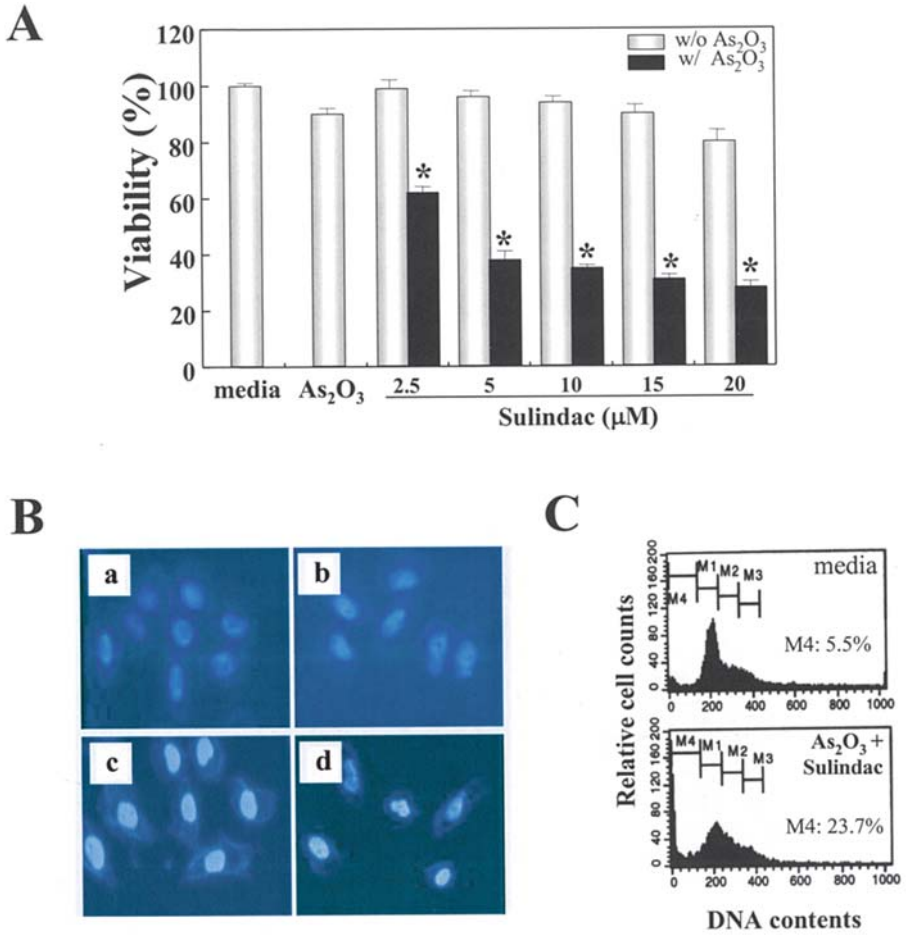

Figure 1. Combination of As_2O_3 with sulindac synergistically augmented apoptotic death of H157 cells. (A) Cells were treated with different concentrations of sulindac in the absence or presence of 2.5 μM As_2O_3 for 48 h and viability was determined by MTT assay. The data represent the mean ± SD of three independent experiments. *p<0.05 compared to control. (B) Cells were treated with As_2O_3 (2.5 μM), sulindac (5 μM), or combination of both for 48 h. Then, cells were stained with Hoechst 33342 dye and observed under fluorescent microscope. a, control cells; b, As_2O_3 only; c, sulindac only; d, As_2O_3 and sulindac. (C) Cellular DNA was stained with PI and cell cycle was analyzed by flow cytometry. The data represent one of three independent experiments.

Results

Combination of As_2O_3 with sulindac synergistically augmented apoptotic activity of NCI-H157 cells. To test the synergism in cytotoxicity, NCI-H157 cells were treated with As_2O_3 and sulindac and then viability was measured by MTT assay (Fig. 1A). Neither single exposure of H157 cells to 2.5 μM As_2O_3 alone nor various concentrations of sulindac alone affected the viability at 48 h. However, combination of 2.5 μM As_2O_3 with varying doses of sulindac from 2.5 to 20 μM resulted in a significant decrease of the viability of H157 cells in a dose-dependent fashion ($p<0.05$). Next, to provide the nature of synergistic cytotoxicity of As_2O_3 and sulindac, the phenotypic characteristics of apoptosis were examined. Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h, stained with Hoechst dye 33342, and visualized under fluorescent microscope (Fig. 1B). The nuclei of the control culture as well as of the single-treatment group with As_2O_3 and sulindac were an oval round shape with homogenous intensity whereas those of cells simultaneously treated with As_2O_3 and sulindac demonstrated a condensed and fragmented shape with irregularity in staining homogeneity. DNA fragmentation by combination treatment with As_2O_3 and sulindac was further analyzed as the sub-G_0/G_1 fraction of cell cycle analysis by flow cytometry in H157 cells stained with PI (Fig. 1C). In contrast to the control culture, combination treatment with As_2O_3 and sulindac markedly increased the accumulation of the sub-G_0/G_1 fraction from 5.5% to 23.7%, respectively. These data, collectively, indicate that combined treatment with As_2O_3 and sulindac may activate apoptotic signaling pathway in H157 cells.

Combination treatment with As_2O_3 and sulindac increased the catalytic activity of caspase-8. The role of death receptor is well known in apoptosis of lung cancer cells after treatment with various chemotherapeutic agents (21). Thus, we first examined whether combination treatment of As_2O_3 and sulindac affects the expression of Fas and FasL in H157 cells (Fig. 2A). Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for up to 48 h and lysate was used to carry out Western blotting for Fas and FasL. The date revealed that combination treatment with As_2O_3 and sulindac resulted in a marked increase in the expression of Fas and FasL proteins in a time-dependent fashion with the same kinetics. We next measured the catalytic

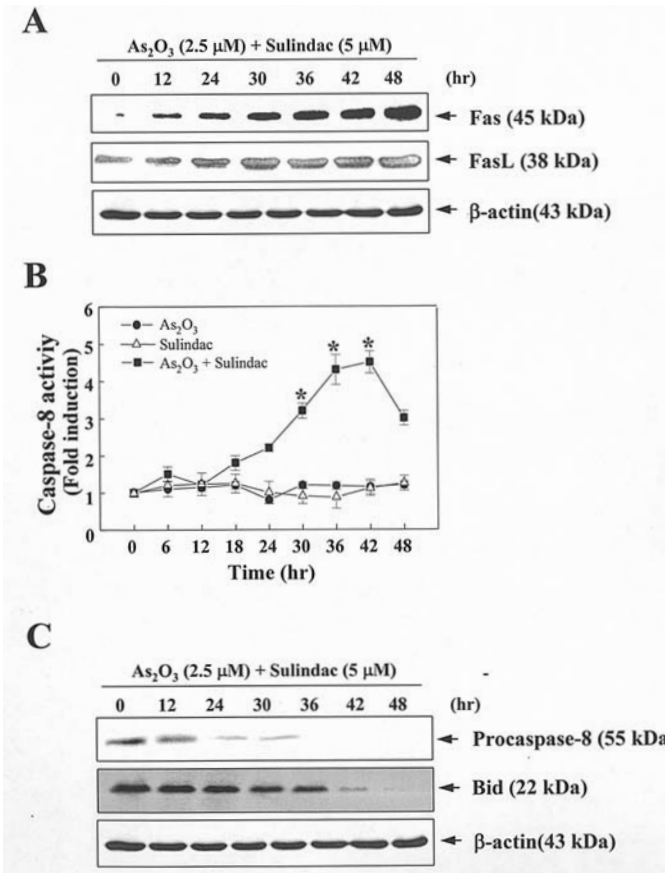

Figure 2. Combination treatment with As_2O_3 and sulindac induced the activation of caspase-8. (A) Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h and lysate was subjected to 12.5% SDS-PAGE to measure the expression of Fas and FasL proteins. (B) After treatment of cells with As_2O_3 (2.5 μM) only, sulindac (5 μM) only, or combination of both for up to 48 h, lysate was used to measure the catalytic activity of caspase-8 by using a fluorogenic substrate. The data represent the mean ± SD of triplicate experiments. *p<0.05. (C) Lysate was used to measure the expression of pro-caspase-8 and Bid protein.

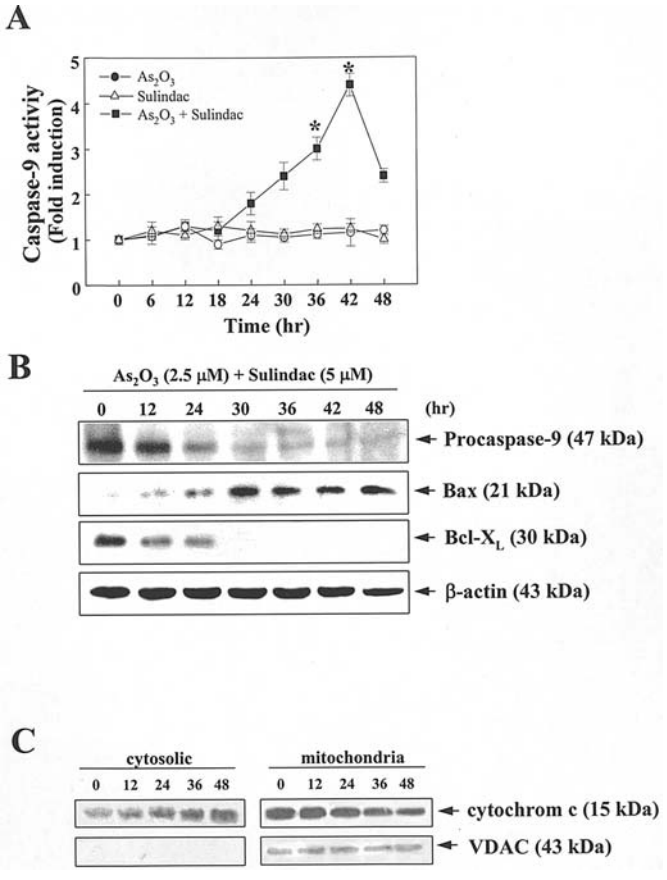

Figure 3. Combination treatment with As_2O_3 and sulindac induced the activation of caspase-9. (A) Cells were treated with As_2O_3 (2.5 μM) only, sulindac (5 μM) only, or combination of both for up to 48 h, lysate was used to measure the catalytic activity of caspase-9 by using a fluorogenic substrate. The data represent the mean ± SD of triplicate experiments. *p<0.05. (B) Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h and lysate was subjected to 12.5% SDS-PAGE to measure the expression of pro-caspase-9, Bax, and Bcl-X_L. (C) Lysate was fractionated into cytosolic and mitochondrial portions and proteins were separated on 15% SDS-PAGE to immunoblot for cytochrome c. The purity of the mitochondrial fraction was determined with anti-VDAC antibody.

activity of caspase-8, a downstream target of Fas/FasL, in cells treated with As_2O_3 and sulindac for 48 h (Fig. 2B). The enzymatic activity of caspase-8 started to increase at 18 h, attained its peak at 36-42 h, and subsequently decreased in cells treated with As_2O_3 and sulindac. However, single application with either As_2O_3 or sulindac did not affect the catalytic activity of caspase-8 in cells. Consistent with the enzymatic activity, the expression level of pro-caspase-8 was decreasing at 24 h and was undetectable at 36 h after combination treatment with As_2O_3 and sulindac. Furthermore, a time-dependent study of Bid expression demonstrated that Bid expression was gradually decreased by combination treatment with As_2O_3 and sulindac (Fig. 2C). These data indicate that combination treatment with As_2O_3 and sulindac activates the signaling pathway of extrinsic caspase, including Fas/FasL, caspase-8, and Bid truncation, which may target mitochondrial dysfunction.

Combination treatment with As_2O_3 and sulindac increased the catalytic activity of caspase-9. To confirm the implication of intrinsic caspase cascade, including caspase-9, in apoptotic signaling of H157 cells after combination treatment with As_2O_3 and sulindac, cells were subjected to the catalytic activity assay for caspase-9 (Fig. 3A). Also, activation of caspase-9 was further confirmed by a gradual decrease in expression level of pro-caspase-9 according to time after combination treatment with As_2O_3 and sulindac.

We were further interested in the expression of anti- or pro-apoptogenic Bcl-2 family proteins, which is known to affect mitochondrial functions (Fig. 3B) as a downstream modulator of caspase. Combination treatment with As_2O_3 and sulindac resulted in a gradual increase of pro-apoptogenic Bcl-2 protein whereas it decreased the level of anti-apoptogenic Bcl-X_L in a time-dependent fashion. This result led us to test whether mitochondrial dysfunction occurred in H157 cells treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h. Thus, the cell lysate was fractionated into mitochondrial and cytosolic parts, which were used to measure the expression of cytochrome c by Western blotting (Fig. 3C). Cytochrome c in the cytosolic fraction was markedly increased at 48 h whereas mitochondrial cytochrome c was time-dependently diminished in expression. The purity of mitochondrial fraction was verified by Western blotting with anti-VDAC antibody.

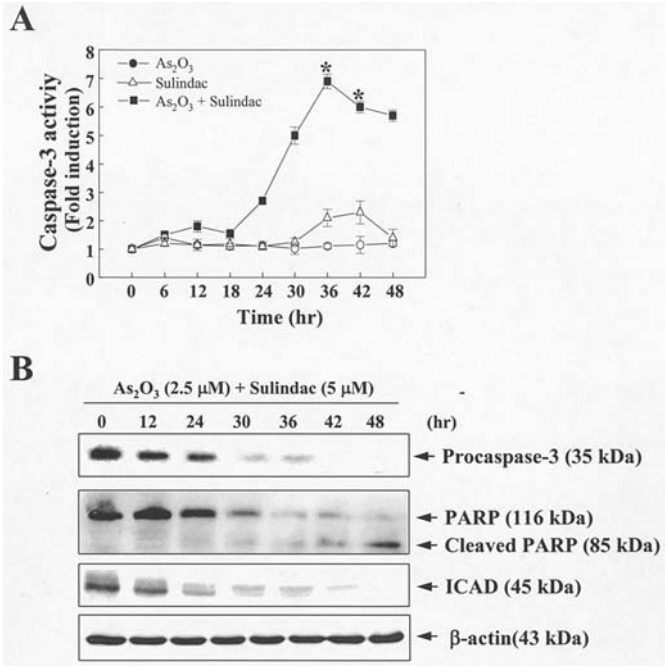

Figure 4. Combination treatment with As_2O_3 and sulindac induced the activation of caspase-3. (A) Cells were treated with As_2O_3 (2.5 μM) only, sulindac (5 μM) only, or combination of both for up to 48 h. Lysate was used to measure the catalytic activity of caspase-3 by using a fluorogenic substrate. The data represent the mean ± SD of triplicate. *p<0.05. (B) Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h and lysate was subjected to 12.5% SDS-PAGE to measure the expression of pro-caspase-3, PARP, and ICAD.

Combination treatment with As_2O_3 and sulindac increased the catalytic activity of caspase-3. To gain the downstream event of activation of caspases, including caspase-8 and -9, H157 cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for up to 48 h, and the proteolytic activity of caspase-3 was determined (Fig. 4A). The enzymatic activation of caspase-3 started after 24 h, attained its peak at 36 h, and was sustained at that level for 48 h in cells treated with As_2O_3 and sulindac. However, single application with either As_2O_3 or sulindac did not affect the catalytic activity of caspase-3 of H157 cells. To further confirm the activation of caspase-3, the cleavage of pro-caspase-3 and intracellular biosubstrates, including PARP and ICAD, was measured by Western blot analysis (Fig. 4B). Degradation of pro-caspase-3 by combination treatment with 2.5 μM As_2O_3 and 5 μM sulindac occurred at 30 h and the immunoreactive band was hardly detectible 42 h after treatment. Cleavage of PARP from 116 to 85 kDa was clearly demonstrated at 30 h after combination treatment with As_2O_3 and sulindac. Also, ICAD expression started to decrease at 24 h in a time-dependent manner by combination treatment with As_2O_3 and sulindac.

Antioxidants antagonized the synergistic cytotoxicity of As_2O_3 and sulindac in H157 cells. To investigate the synergistic mechanism of As_2O_3 and sulindac, we tested the effect of antioxidants, including NAC and GSH, in H157 cells. Cells were pretreated with either NAC or GAH, followed by the addition of 2.5 μM As_2O_3 and 5 μM sulindac for 48 h, and

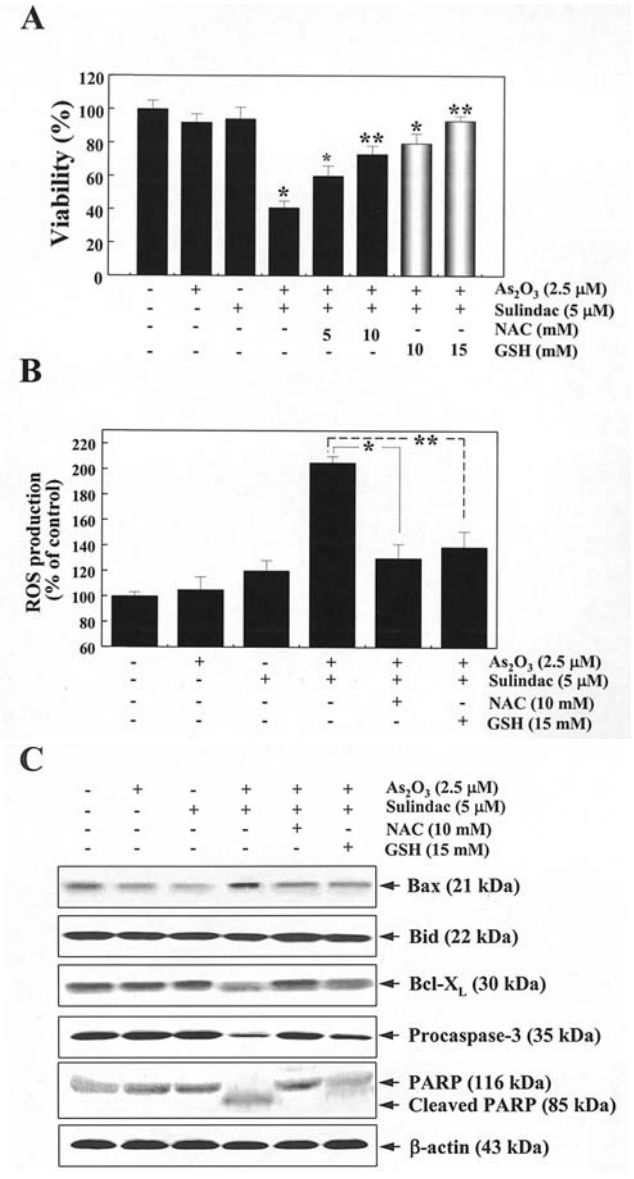

Figure 5. Antioxidants antagonized the synergistic cytotoxicity of As_2O_3 and sulindac in H157 cells. Cells were pretreated with GSH or NAC, followed by the addition of As_2O_3, sulindac, or combination of both for 48 h. (A) Cell viability was determined by MTT assay. *p<0.05; **p<0.01. (B) Measurement of intracellular H_2O_2 was determined fluorimetrically using scopoletin-horseradish peroxidase assay. The data represent the mean ± SD of three independent experiments. *p<0.05; **p<0.01. (C) Under the same experimental conditions, lysate was subjected to 12.5% SDS-PAGE to measure the expression of Bax, Bid, Bcl-X_L. pro-caspase-3, and PARP.

viability was measured by MTT assay. As shown in previous data, combination treatment with As_2O_3 and sulindac resulted in a significant decrease in cell viability (58% of control cells) even though single application of either one did not exert any notable change in cell viability. However, pretreatment with either NAC or GSH significantly inhibited the synergistic cytotoxicity of As_2O_3 and sulindac in a dose-dependent manner (Fig. 5A). To verify the direct involvement of reactive oxygen species (ROS), we measured the generation of intracellular hydrogen peroxide (H_2O_2) by scopoletin-horseradish peroxidase (HRP) assay (Fig. 5B). The data revealed that combination treatment with As_2O_3 and sulindac induced the

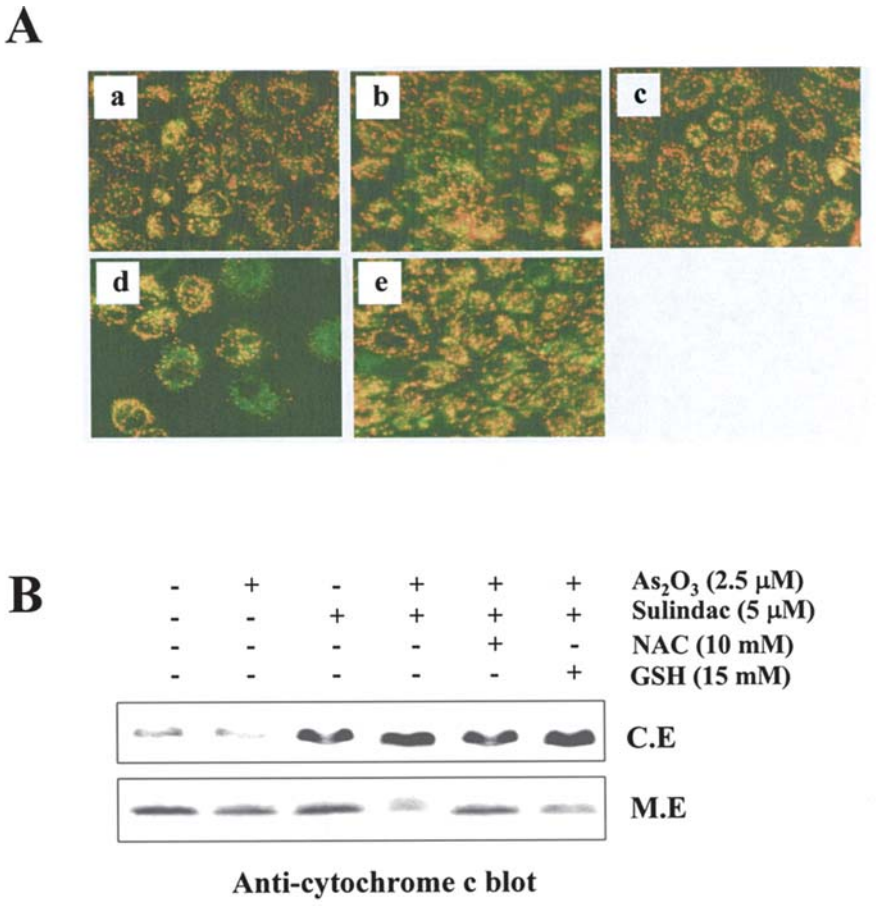

Figure 6. Antioxidants prevented the mitochondrial membrane potential transition (MPT) and cytosolic release of cytochrome c by combination treatment with As_2O_3 and sulindac. (A) Cells were treated with As_2O_3 and/or sulindac in the presence or absence of antioxidants, including NAC and GSH. Then, cells were stained with 10 μg/ml of JC-1 and observed under fluorescent microscope. a, control cells; b, As_2O_3 only; c, sulindac only; d, As_2O_3 and sulindac; e, pretreatment of GSH plus As_2O_3 and sulindac. (B) Cells were treated with As_2O_3 and/or sulindac in the presence or absence of antioxidants. Lysate was fractionated into cytosolic and mitochondrial portions and proteins were separated on 15% SDS-PAGE to immunoblot for cytochrome c.

generation of H_2O_2 (2-fold), which returned to the level of that in control cells by pretreatment with antioxidants, including NAC and GSH.

To further confirm the implication of signaling components of apoptosis, cell lysate was used to perform Western blot analysis. Combination treatment with As_2O_3 and sulindac induced the expression of apoptogenic Bax whereas it resulted in a decrease in anti-apoptotic Bid and Bcl-X_L (Fig. 5C). Combination treatment with As_2O_3 and sulindac had no influence on the expression level of Bcl-2 (data not shown). However, the expression levels of Bax, Bid, and Bcl-X_L were demonstrated as similar to that of control cells in cells pretreated with NAC and GSH before combination treatment with As_2O_3 and sulindac. We next measured the effect of antioxidants on the activation of caspase-3. Pretreatment of antioxidants, including NAC and GSH, markedly suppressed the activation of caspase-3, evidenced by the decreased expression of pro-caspase-3 and cleavage of PARP in cells treated with As_2O_3 and sulindac.

Pretreatment of antioxidants prevented mitochondrial dysfunction by combination treatment with As_2O_3 and sulindac. To examine the upstream component of caspase-3 in apoptosis signaling, markers of mitochondrial dysfunction, including mitochondrial membrane potential transition (MPT) and cytosolic release of cytochrome c, were evaluated in cells treated with As_2O_3 and sulindac. Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for 48 h in the presence or absence of antioxidants, including 10 mM NAC and 15 mM GSH. Then, cells were used to measure the MPT by staining mitochondria with JC-1 under a fluorescence microscope. Mitochondria were mainly located in the cytosol as a punctuated form colored with orange in the control culture (Fig. 6A, a) and cells treated with As_2O_3 alone (Fig. 6A, b) and sulindac alone (Fig. 6A, c). Simultaneous exposure of H157 cells to As_2O_3 and sulindac caused a dramatic change in MPT, shown as a diffuse form and mainly located in the cytosol as well as the nuclei (Fig. 6A, d). However, pretreatment of GSH prevented the changes in MPT and morphological features as similarly demonstrated in control cells (Fig. 6A, e). To provide further evidence of mitochondrial dysfunction, cytosolic release of cytochrome c was examined by Western blotting in both mitochondrial and cytosolic fractions (Fig. 6B). Cytochrome c in the cytosolic fraction was markedly increased in cells treated with sulindac only as well as with combination treatment of both. Consistently, the immunoreactive band of cytosolic cytochrome c in cells pretreated with either NAC or GSH remained at a similar level in its intensity to

that of control cells. The purity of mitochondrial fraction was verified by Western blotting with anti-VDAC antibody.

Discussion

NSAIDs are known to enhance tumor cell responses toward various anticancer therapies, including biologic therapy, radiotherapy, and conventional anticancer chemotherapeutic agents. A combination of sulindac and EKI-569, an inhibitor of EGFR tyrosine kinase, remarkably protects intestinal cells from neoplastic transformation (22). NSAIDs also significantly increase the cytotoxicity of anthracyclines and vinca alkaloids in human lung cancer cells and leukemia cell lines (23). The combination of sulindac with paclitaxel and/or cisplatin afforded a synergistic augmentation of cytotoxicity toward lung cancer cell lines (24). In addition, sulindac enhanced cytotoxicity when combined with As_2O_3 in A549 human lung cancer cells, but the exact mechanism of synergism was not clearly defined (25).

Although As_2O_3 is known to be effective in the treatment of leukemic cells, especially in APL, there is little evidence of its pharmacologic efficacy in solid tumor cells. Bode and Dong demonstrated that As_2O_3 induced apoptosis of cancer cells through the consequences of oxidative stress, activation of caspase pathways, and mitochondrial dysfunction (26). Arsenic trioxide directly condenses mitochondrial matrix and decreases mitochondrial transmembrane potential (MMP) to trigger apoptosis via the release of cytochrome c from mitochondria and the subsequent activation of the caspase proteins (27,28). Also, treatment of As_2O_3 results in reduction of GSH and thereby increases the intracellular ROS level in certain APL cells (29).

In this study, we showed that combination treatment with As_2O_3 and sulindac enhanced the activity of intrinsic and extrinsic caspase cascades through ROS generation. Furthermore, combination treatment also resulted in loss of MMP, changes in expression of Bcl-2 family proteins and cytosolic release of cytochrome c. Mitochondria are known as an important regulator of apoptosis (30). It undergoes a series of consequence changes during apoptotic death of cells. A loss of MMP together with the permeability transition pore induces the cytosolic release of cytochrome c, which further activates caspase-3, following activation of caspase-9. In accordance with previous reports of apoptosis signaling, our data demonstrated that combination treatment resulted in perturbation of mitochondrial functions in H157 cells.

Recent evidence suggests that sulindac engages the mitochondrial pathway involving caspase-9 and Bax in induction of apoptotic effects. Similarly, As_2O_3 can induce apoptosis via upregulation of intracellular ROS, such as H_2O_2, which cause depolarization of the mitochondrial transmembrane and subsequent caspase-3 activation. These pathways may account for the interactions between As_2O_3 and sulindac in our experimental model. The molecular mechanism of synergism in the combination of As_2O_3 and sulindac was involved in the generation of ROS because the apoptotic death of H157 cells by combination treatment was inhibited by scavenging H_2O_2 with NAC and GSH, consequently restoring the MPT, which preceded the caspase-3 activation, DNA fragmentation, and morphologic features of apoptosis.

Consistent with the report of Finucane *et al* (31), we also speculate that the induction of apoptogenic Bcl-2 family protein, such as Bax, together with decreased levels of anti-apoptogenic Bcl-2 family protein, such as Bid and Bcl-X_L, may contribute the release of cytochrome c and generation of ROS in H157 cells treated with As_2O_3 and sulindac. Single treatment with sulindac could induce the cytosolic release of cytochrome c even though the MPT was not changed at all. This result may indicate the possibility that MPT is not always required for cytosolic release of cytochrome c or that cytosolic release of cytochrome c is not only a limiting condition for caspase-3 activation, followed by formation of apoptosome complex.

Up to 10-fold higher concentrations of As_2O_3 are required to induce apoptosis in non-APL tumor cells, which is generally unacceptable in the clinic because of higher toxicity. As a consequence, clinical use of As_2O_3 has thus far been largely limited to patients with APL. Our data suggest that combination treatment with As_2O_3 and sulindac may be useful in practical application in the treatment of solid tumors, including lung cancer, as an alternate anticancer strategy for circumventing the serious general toxicity of anticancer chemotherapeutic agents, such as As_2O_3.

Acknowledgements

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