Effects of arsenic trioxide on cell death, reactive oxygen species and glutathione levels in different cell types

YONG HWAN HAN, HWA JIN MOON, BO RA YOU, SUNG ZOO KIM, SUHN HEE KIM and WOO HYUN PARK

Department of Physiology, Medical School, Centers for Healthcare Technology Development
Institute for Medical Sciences, Chonbuk National University, JeonJu 561-180, Republic of Korea

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Abstract. Arsenic trioxide (ATO) can regulate many biological functions such as apoptosis and differentiation. We evaluated the effects of ATO on various cell types such as cervical cancer HeLa cells, pulmonary adenocarcinoma Calu-6 and A549 cells, calf pulmonary artery endothelial cells (CPAEC), human umbilical vein endothelial cells (HUVEC) and human pulmonary fibroblast (HPF) cells in relation to cell growth, cell death and reactive oxygen species (ROS) and glutathione (GSH) levels. The growth of HeLa and Calu-6 cells was inhibited by ATO with an IC_{50} of ~15 μM at 24 h. A549 cell growth was not inhibited by 15 μM ATO. The susceptibility to ATO in CPAEC and HUVEC was similar to that in HeLa cells. The IC_{50} of ATO in HPF cells was ~40 μM. ATO induced apoptosis in HeLa, CPAEC and HUVEC, which was accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$). However, ATO did not strongly trigger apoptosis in Calu-6, A549 and HPF cells. ATO increased or decreased the ROS level including $O_2^{\bullet-}$ and GSH levels depending on the incubation dose and cell type. In conclusion, ATO differentially affected cell growth inhibition and death depending on the incubation dose and cell type. The changes in ROS and GSH levels by ATO were not tightly correlated with the level of cell death. Our present data provide useful information for the action of ATO in various cell types in relation to cell growth, cell death, ROS and GSH levels.

Correspondence to: Dr Woo Hyun Park, Department of Physiology, Medical School, Chonbuk National University, JeonJu 561-180, Republic of Korea
E-mail: parkwh71@chonbuk.ac.kr

Abbreviations: ATO, arsenic trioxide (As_2O_3); CPAEC, calf pulmonary arterial endothelial cells; HUVEC, human umbilical vein endothelial cells; HPF, human pulmonary fibroblast; ROS, reactive oxygen species; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate

Key words: arsenic trioxide, cell, apoptosis, reactive oxygen species, glutathione

Introduction

Arsenic trioxide (ATO; As_2O_3) has been reported to induce complete remission without severe marrow suppression in patients with relapsed or refractory acute promyelocytic leukemia (APL) (1). The antiproliferative effect of ATO is not limited to APL cells, but can also be observed in a variety of hematological malignancies (2-4). Accumulating evidence indicates that ATO can affect many biological functions such as cell proliferation, apoptosis, differentiation, and angiogenesis in cell lines derived from renal (5), head and neck (6), ovarian (7), prostate (7), hepatoma (8,9), bladder (10), colon (11), lung (12), breast (13), cervical (14) and gastric (15) cancer cells. ATO is a mitochondrial toxin and induces loss of the mitochondrial transmembrane potential (2,5,16). Therefore, it is believed to induce apoptosis in tumor cells by affecting mitochondria and the production of reactive oxygen species (ROS).

ROS include hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$). These molecules have recently been implicated in many important processes such as transcription factor activation, gene expression, differentiation and cellular proliferation (17-19). Glutathione (GSH) is the main non-protein antioxidant in cells; it provides electrons for enzymes such as glutathione peroxidase, which reduce H_2O_2 to H_2O. GSH has been shown to be crucial for regulation of cell proliferation, cell cycle progression and apoptosis (20,21), and it is known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and ROS (22).

In the present study, we evaluated the effects of ATO on various cell types such as cervical cancer HeLa cells, pulmonary adenocarcinoma Calu-6 and A549 cells, CPAEC, HUVEC and HPF cells in relation to cell growth, cell death and ROS and GSH levels.

Materials and methods

Cell culture. Human cervix adenocarcinoma HeLa cells and human pulmonary adenocarcinoma Calu-6 and A549 cells from the ATCC (American Type Culture Collection), calf

pulmonary artery endothelial cells (CPAEC) from KCLB (Korean Cell Line Bank), primary human umbilical vein endothelial cells (HUVEC) and human pulmonary fibroblast (HPF) cells from PromoCell GmbH (Heidelberg, Germany) were maintained in a humidified incubator containing 5% CO_2 at 37˚C. HeLa, Calu-6 and A549 and CPAEC were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY). These cell lines were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA (Gibco BRL) while in a logarithmic phase of growth. HUVEC and HPF cells were cultured in complete endothelial cell growth medium and fibroblast growth medium 2 (PromoCell), respectively. HUVEC and HPF cells were washed and detached with HepesBSS (30 mM Hepes), trypsin-EDTA and trypsin neutralization solution (Promo Cell). HUVEC and HPF cells were used between passages four and six.

Reagents. ATO purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) was dissolved in 1.65 M NaOH at 100 mM as a stock solution, which was wrapped in foil and kept at -4˚C.

Cell growth assay. The effect of ATO on cell growth was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (2). In brief, cells ($5x10^4$/well) were seeded in 96-well microtiter plates (Nunc) for an MTT assay. After exposure to the indicated amounts of ATO for 24 h, 20 μl of MTT (Sigma) solution (2 mg/ml in PBS) was added to each well of 96-well plates. The plates were incubated for an additional 4 h at 37˚C. MTT solution in the medium was aspirated off, and 200 μl of DMSO was added to each well to solubilize the formazan crystals formed in the viable cells. Optical density was measured at 570 nm using a microplate reader (Spectra Max 340, Molecular Devices Co., Sunnyvale, CA).

Annexin V staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em=488 nm/519 nm) as previously described (23). In brief, $1x10^6$ cells in a 60-mm culture dish (Nunc) were incubated with the indicated amounts of ATO for 24 h. Cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. Five microliters of annexin V-FITC (Pharmingen, San Diego, CA) was then added to the cells, which were analyzed with a FACStar flow cytometer (Becton Dickinson).

Measurement of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured by the Rhodamine 123 fluorescent dye (Ex/Em=485 nm/535 nm; Sigma) as previously described (24). In brief, $1x10^6$ cells in a 60-mm culture dish were incubated with the indicated amounts of ATO for 24 h. Cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml; Sigma) at 37˚C for 30 min. Rhodamine 123 staining intensity was determined by a FACStar flow cytometer. Rhodamine 123-negative cells indicate the loss of MMP ($\Delta\Psi_m$) in cells.

Detection of intracellular ROS and O2•- levels. Intracellular ROS such as H_2O_2 and •OH were detected by means of an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Ex/Em=495 nm/529 nm; Invitrogen Molecular Probes, Eugene, OR) (25). H_2DCFDA is poorly selective for $O_2^{\bullet-}$. In contrast, dihydroethidium (DHE, Ex/Em=518 nm/605 nm; Invitrogen Molecular Probes) is highly selective for $O_2^{\bullet-}$ among ROS. In brief, $1x10^6$ cells in a 60-mm culture dish were incubated with the indicated amounts of ATO for 24 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min according to the manufacturer's instructions. DCF and DHE fluorescence was detected using a FACStar flow cytometer. ROS and $O_2^{\bullet-}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Detection of the intracellular GSH. Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Ex/Em=522 nm/595 nm; Molecular Probes) as previously described (25). In brief, $1x10^6$ cells in a 60-mm culture dish were incubated with the indicated amounts of ATO for 24 h. Cells were then washed with PBS and incubated with 5 μM CMFDA at 37˚C for 30 min. CMF fluorescence intensity was determined using a FACStar flow cytometer. CMF levels in viable cells without GSH-depleted cells were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Results

Effects of ATO on the growth of various cell types. We examined the effect of ATO on the growth of different cell types at 24 h by an MTT assay (Fig. 1). Treatment with ATO differentially inhibited cell growth depending on the incubation dose and cell type. The growth of cervical cancer HeLa cells was dose-dependently inhibited by ATO with an IC_{50} of ~15 μM (Fig. 1A). The growth of Calu-6 lung cancer cells was also dose-dependently reduced by ATO with an IC_{50} of ~15 μM (Fig. 1B) whereas that of A549 lung cancer cells was not inhibited by 15 μM ATO (Fig. 1C). Even 10-20 μM ATO slightly increased A549 cell growth, and only 50 μM ATO mildly inhibited A549 cell growth (Fig. 1C). ATO inhibited the growth of normal CPAEC in a dose-dependent manner with an IC_{50} of ~15 μM (Fig. 1D). In relation to primary normal cells, ATO dose-dependently inhibited the growth of HUVEC with an IC_{50} of ~20-30 μM (Fig. 1E) whereas 1-10 μM ATO significantly increased the growth of HPF cells (Fig. 1F). The IC_{50} of ATO in HPF cells was ~40 μM (Fig. 1F).

Effects of ATO on cell death and MMP ($\Delta\Psi_m$) of various cell types. Next, we determined whether ATO induces apoptosis in cells using an annexin V-staining assay. The numbers of annexin V-stained cells were dose-dependently increased in

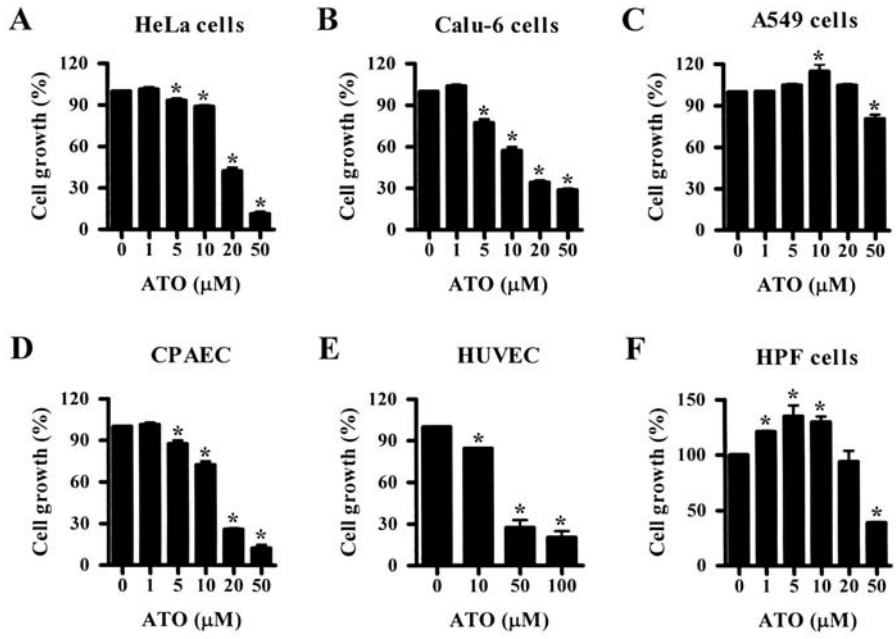

Figure 1. Effects of ATO on the growth of various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. Cell growth was assessed by an MTT assay. The results shown represent the mean of at least three experiments; bar, SD. The Student's t-test was used for parametric data. The statistical significance was defined as $^{*}p<0.05$, compared with the ATO-untreated control group.

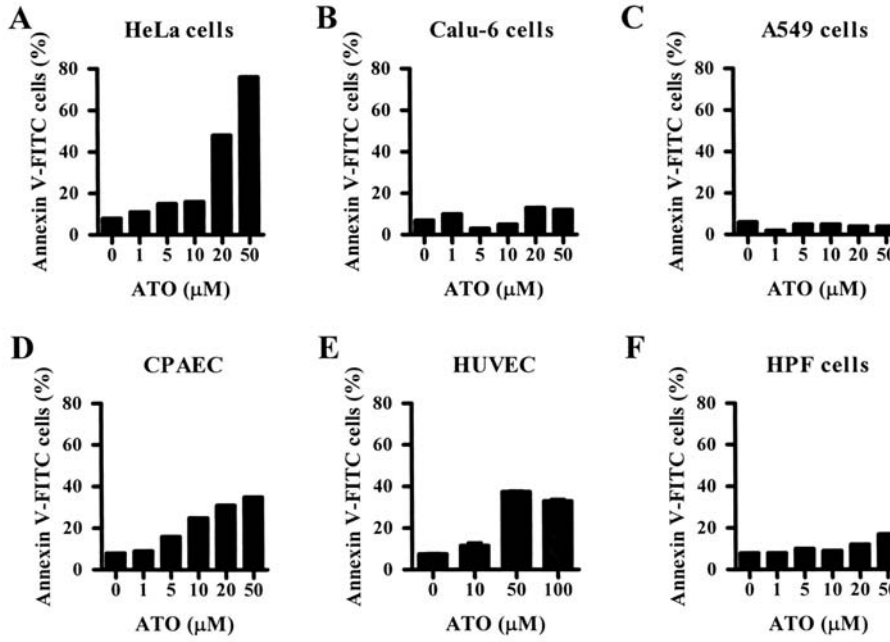

Figure 2. Effects of ATO on the cell death of various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. The percentages of annexin V-positive-stained cells were measured using a FACStar flow cytometer.

ATO-treated HeLa cells (Fig. 2A). At a 50-μM dose of ATO, the number of annexin V-stained cells was increased ~65% compared with the ATO-untreated control HeLa cells (Fig. 2A). Treatment with 20 or 50 μM ATO seemed to increase the number of annexin V-stained cells in Calu-6 cells, but the number was not large (Fig. 2B). In ATO-treated A549 cells, the number of annexin V-stained cells was not affected (Fig. 2C). ATO increased the number of annexin V-stained cells in both CPAEC and HUVEC (Fig. 2D and E). At a 50-μM dose of ATO, the number of annexin V-stained cells in both cells was increased ~30% compared with the ATO-untreated control cells (Fig. 2D and E). In ATO-treated HPF cells, only 50 μM ATO slightly increased the number (Fig. 2F).

Since apoptosis is closely related to the collapse of MMP ($\Delta\Psi$) (26), we assessed the effect of ATO on MMP ($\Delta\Psi_m$) in cells using Rhodamine 123. Treatment with >5 μM ATO induced the loss of MMP ($\Delta\Psi_m$) in HeLa and Calu-6 cells (Fig. 3A and B). At a 50-μM dose of ATO, the percentages

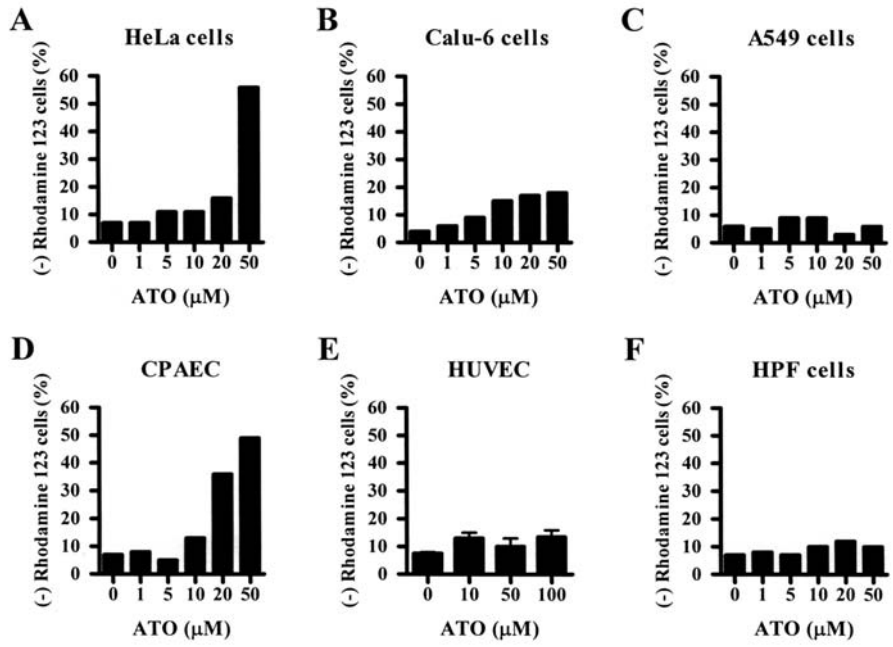

Figure 3. Effects of ATO on MMP ($\Delta\Psi_m$) in various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. The percentages of Rhodamine 123-negative [MMP ($\Delta\Psi_m$) loss] cells were measured using a FACStar flow cytometer.

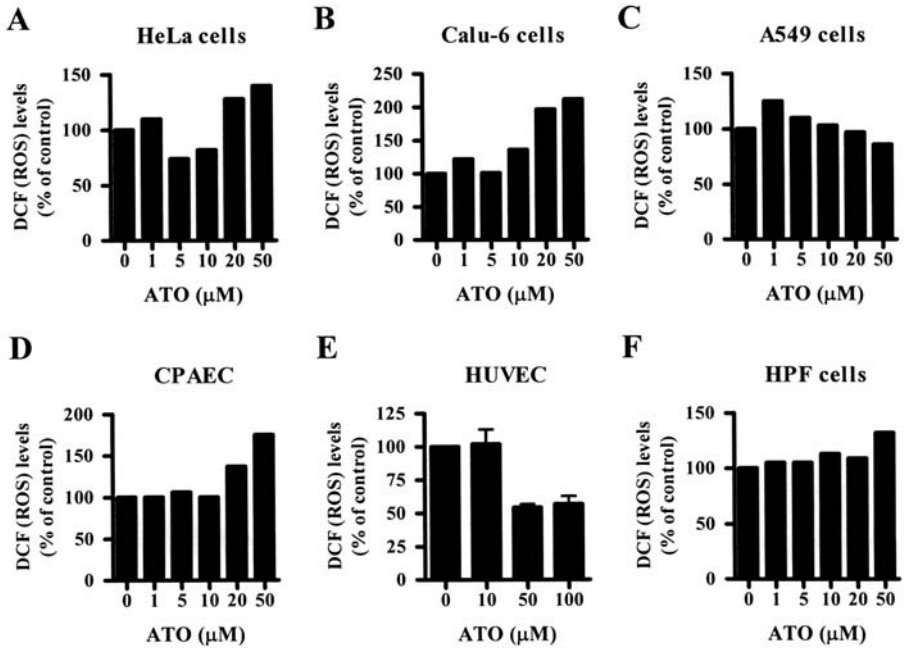

Figure 4. Effects of ATO on ROS levels in various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. ROS (DCF) levels in cells were measured using a FACStar flow cytometer. Graphs indicate DCF (ROS) levels (%) in cells compared with each group of control cells.

of MMP ($\Delta\Psi_m$) loss were 50 and 13% in HeLa and Calu-6 cells compared with ATO-untreated control cells, respectively (Fig. 3A and B). ATO did not affect the MMP ($\Delta\Psi_m$) loss in A549 cells (Fig. 3C). Treatment with >10 μM ATO seemed to induce the loss of MMP ($\Delta\Psi_m$) in both CPAEC and HUVEC (Fig. 3D and E). However, ATO did not strongly induce MMP ($\Delta\Psi_m$) loss in HUVEC (Fig. 3E). In ATO-treated HPF cells, ATO did not affect the MMP ($\Delta\Psi_m$) loss (Fig. 3F).

Effects of ATO on intracellular ROS in various cell types. To assess levels of intracellular ROS in ATO-treated cells at 24 h, we used H_2DCFDA and DHE. Treatment with ATO differentially affected ROS levels depending on the incubation

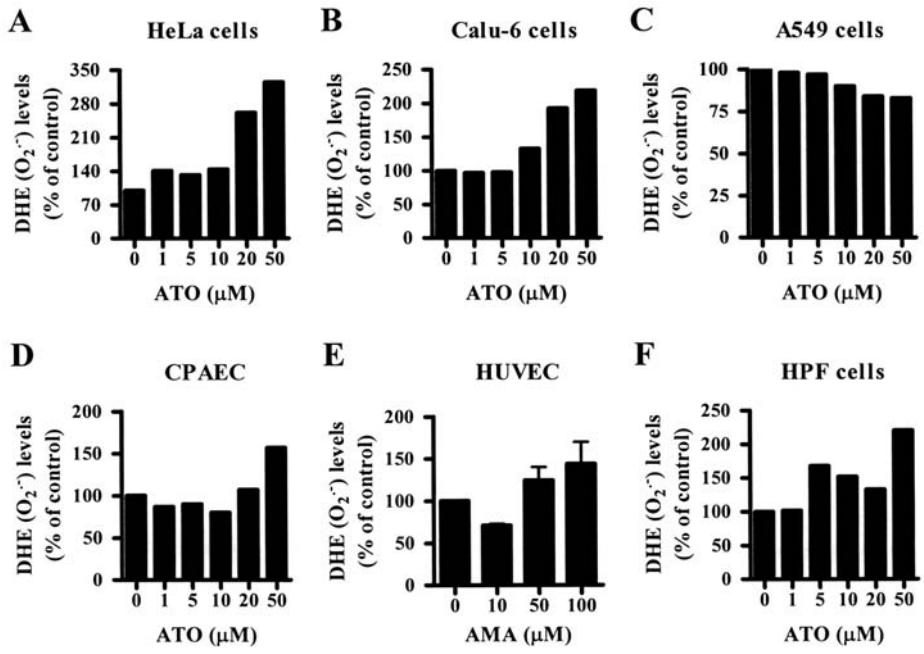

Figure 5. Effects of ATO on $O_2^{\bullet-}$ levels in various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. DHE ($O_2^{\bullet-}$) levels in cells were measured using a FACStar flow cytometer. Graphs indicate DHE ($O_2^{\bullet-}$) levels (%) in cells compared with each group of control cells.

dose and cell type (Fig. 4). While 5 or 10 μM ATO decreased ROS in HeLa cells, 20 or 50 μM ATO increased the level of ROS (Fig. 4A). Treatment with ATO generally increased the ROS level in Calu-6 cells (Fig. 4B). Treatment with 1 μM ATO increased the ROS level in A549 cells whereas 50 μM ATO decreased the ROS level (Fig. 4C). The relatively higher doses of ATO increased the ROS level in CPAEC (Fig. 4D) and decreased the ROS level in HUVEC (Fig. 4E). In addition, the higher dose of 50 μM ATO increased the ROS level in HPF cells (Fig. 4F). The level of red fluorescence derived from DHE, which reflected $O_2^{\bullet-}$ accumulation, was increased in ATO-treated HeLa and Calu-6 cells (Fig. 5A and B). In contrast, all the tested doses of ATO decreased $O_2^{\bullet-}$ levels in A549 cells (Fig. 5C). While the dose of 10 μM ATO decreased $O_2^{\bullet-}$ levels in both CPAEC and HUVEC, higher doses of ATO increased the levels (Fig. 5D and E). Treatment with >5 μM ATO increased $O_2^{\bullet-}$ levels in HPF cells but the increase was not dose-dependent (Fig. 5F).

Effects of ATO on the intracellular GSH levels in various cell types. Next, we analyzed the changes in GSH levels in cells without GSH-depleted cells using CMF fluorescence dye. Treatment with ATO increased or decreased GSH levels depending on the incubation dose and cell type (Fig. 6). Treatment with <20 μM ATO increased GSH levels in HeLa cells, and 50 μM ATO deceased the level (Fig. 6A). In contrast, lower doses of ATO decreased GSH levels in Calu-6 cells whereas higher doses of ATO increased the levels (Fig. 6B). In ATO-treated A549 cells, the GSH level was not strongly altered (Fig. 6C). Treatment with >20 μM ATO decreased GSH levels in CPAEC (Fig. 6D), and >10 μM ATO decreased the levels in HUVEC (Fig. 6E). The relatively lower doses of 1-10 μM ATO increased GSH levels in HPF cells, while higher doses of ATO did not change the level (Fig. 6F).

Discussion

In the present study, we focused on evaluating the effects of ATO on various cell types in relation to cell growth, cell death, and ROS and GSH levels, even though there have been many theories and proposed potential targets regarding the mechanism of the anticancer effect in response to ATO. The growth of HeLa and Calu-6 cells was dose-dependently inhibited by ATO with an IC_{50} of ~15 μM. The susceptibility to ATO in HeLa and Calu-6 cells is generally lower than that in leukemia and myeloma cell lines (2,27), and is similar to that in other solid tumors such as ovarian cancer, colon cancer, cervical cancer, renal cell carcinoma and breast cancer cell lines (5,28). However, A549 cell growth was not inhibited by 15 μM ATO at 24 h. Even at 10-20 μM, ATO slightly increased A549 cell growth. A549 cells were considered to be very resistant to ATO. In relation to normal and primary cells, the susceptibility to ATO in CPAEC and HUVEC was similar to that shown in HeLa and Calu-6 cells. Notably, 1-10 μM ATO significantly increased the growth of HPF cells, and the IC_{50} of ATO in HPF cells was ~40 μM. The differential susceptibility to ATO of the various cell types depended on the incubation dose and cell type. Even the susceptibility to ATO was different in cells derived from tissues of the same origin (Calu-6, A549 and HPF) and was also diverse in normal and primary cell types (CPAEC, HUVEC and HPF). Therefore, before using ATO as a therapeutic agent in cancer patients, we must consider the differential mechanisms involving the anticancer effect of ATO and the specificity of the target tumor.

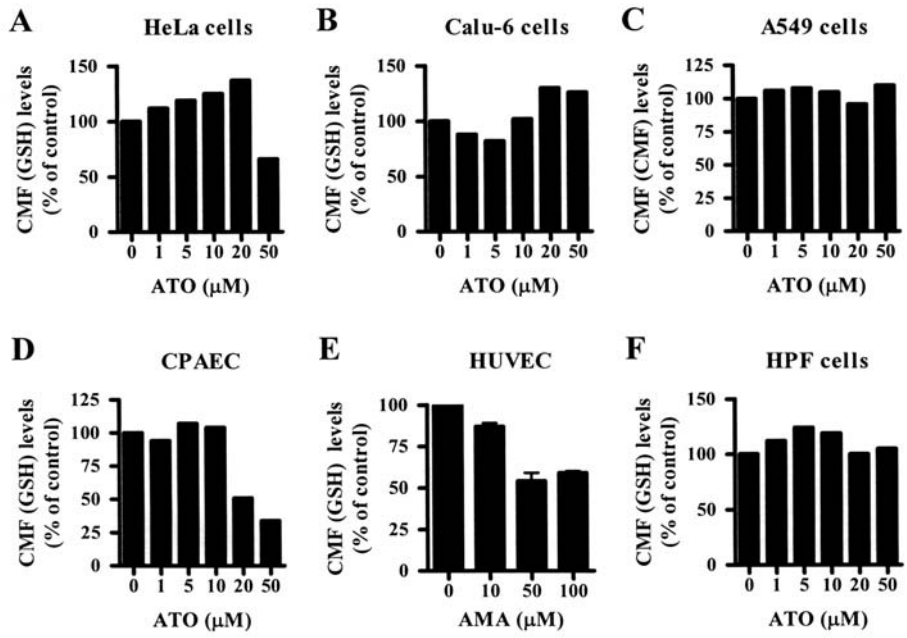

Figure 6. Effects of ATO on GSH levels in various cell types (A-F). Exponentially growing cells were treated with the indicated concentrations of ATO for 24 h. GSH (CMF) levels in cells were measured using a FACStar flow cytometer. Graphs indicate GSH (CMF) levels (%) in cells except (-) CMF (GSH-depleted) cells compared with each group of control cells.

ATO dose-dependently induced apoptosis in HeLa, CPAEC and HUVEC as evidenced by the annexin V staining of cells. However, ATO did not strongly trigger apoptosis in Calu-6, A549 and HPF cells. These results seem to correlate with the results of the growth inhibition by ATO. However, ATO-treated Calu-6 cells showed dissimilar results. Since suppression of cell growth by ATO can be explained by its capacity to affect arrest during the cell cycle, we examined cell cycle distributions in ATO-treated Calu-6 cells and observed that ATO induced a G2 phase arrest of the cell cycle (data not shown), which could be considered a major pathway by which the growth of Calu-6 cells is suppressed. However, we could not exclude other cell death pathways such as necrosis in ATO-treated Calu-6 cells. Apoptosis is closely related to the collapse of MMP ($\Delta\Psi_m$) (26), and ATO can cause a breakdown in MMP ($\Delta\Psi_m$) (29-31). Correspondingly, ATO induced the loss of MMP ($\Delta\Psi_m$) in HeLa, CPAEC and HUVEC. The loss of MMP ($\Delta\Psi_m$) in ATO-treated HUVEC was less than that in CPAEC. As expected from the annexin V assay results, MMP ($\Delta\Psi_m$) loss in A549 and HPF cells was not triggered by ATO. Notably, ATO-treated Calu-6 cells showed some loss in MMP ($\Delta\Psi_m$) without the strong induction of apoptosis. These results imply that ATO first damages mitochondria in HeLa, Calu-6 and CPAEC in order to proceed to the next step of apoptosis, but ATO does not initially destroy mitochondria in HUVEC. In addition, these results suggest that the differences in sensitivity to ATO in various cell types in relation to apoptosis and MMP ($\Delta\Psi_m$) are probably due to the different basal activities of mitochondria depending on cell type, tissue origin and species (32).

ATO can disturb the natural oxidation and reduction equilibrium in cells, leading to an increase in ROS by a variety of redox enzymes, including flavoprotein-dependent superoxide-producing enzymes such as nicotine adenine diphosphate oxidase (33,34). The increased intracellular ROS is observed in ATO-treated cervical cancer cells (35), APL cells (36), hepatocellular carcinoma HepG2 (37), and glioblastoma A172 cells (16). These results suggest that ATO-induced cell death is related to ROS accumulation. However, according to our results, 5 or 10 μM ATO triggering apoptosis in HeLa cells decreased the ROS level whereas 20 or 50 μM ATO increased the ROS level. ATO commonly increased the ROS level in Calu-6 cells without the convincible induction of apoptosis. Treatment with 1 μM ATO increased the ROS level without apoptosis in A549 cells whereas 50 μM ATO decreased the ROS level. The relatively higher doses of ATO increased the ROS level in CPAEC but decreased the ROS level in HUVEC. In addition, the higher dose of 50 μM ATO increased the ROS level but slightly induced apoptosis in HPF cells. Similarly, results from Haga *et al* showed that ROS accumulation was detected in ATO-treated glioblastoma T98G cells but apoptosis did not occur in these cells (16). We also prevously showed that ATO reduced intracellular ROS levels in As4.1 juxtaglomerular cells (24). In relation to the $O_2^{\bullet-}$ level in ATO-treated cells, an increased pattern in $O_2^{\bullet-}$ levels following treatment with ATO was reported in esophageal cancer SHEE85 (38) and As4.1 juxtaglomerular cells (24), but this pattern was not observed in ATO-treated acute myelogenous leukemia HL-60 (39) and renal cell carcinoma ACHN cells (40). According to our results, the $O_2^{\bullet-}$ level was increased in ATO-treated HeLa and Calu-6 cells. In contrast, all the tested doses of ATO decreased $O_2^{\bullet-}$ levels in A549 cells. While the dose of 10 μM ATO decreased $O_2^{\bullet-}$ levels in both CPAEC and HUVEC, higher doses of ATO increased the levels. Treatment with >5 μM ATO increased $O_2^{\bullet-}$ levels in HPF cells, but the increase was not dose-dependent. Taken together, these results imply that treatment

with ATO differentially affects ROS levels depending on the incubation dose and cell type, and cell death by ATO is not tightly correlated to changes in ROS levels.

In relation to the GSH level in ATO-treated cells, ATO decreases GSH levels in certain APL cells (41). Likewise, relatively higher doses of ATO inducing apoptosis in HeLa cells, CPAEC and HUVEC deceased the GSH level. However, 10 or 20 μM ATO triggering apoptosis in HeLa cells increased the GSH level, and 5 or 10 μM ATO did not reduce the GSH level in CPAEC. In addition, lower doses of ATO decreased GSH levels in Calu-6 cells whereas higher doses of ATO increased these levels. In ATO-treated A549 cells, the GSH level was not strongly altered. The relatively lower doses of 1-10 μM ATO increased GSH levels in HPF cells, and higher doses of ATO slightly inducing apoptosis did not change the level. These results also suggest that ATO increased or decreased GSH levels without the tight relationship with changes in the ROS level depending on the incubation dose and cell type.

In conclusion, we demonstrated that ATO differentially affected the growth inhibition and cell death in the tested cells depending on the incubation dose and cell type. ATO also increased or decreased ROS and GSH levels in the tested cells, and these changes in levels were not tightly correlated with the level of cell death. Our present data provide useful information regarding the action of ATO in various cell types including normal cells in relation to cell growth, cell death, ROS and GSH levels.

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