Enhancement of radiation response in human cervical cancer cells in vitro and in vivo by arsenic trioxide (As$_2$O$_3$)

Yong-Jin Chun[a], In-Chul Park[b], Myung-Jin Park[b], Sang-Hyeok Woo[b], Seok-Il Hong[b],
Hee Yong Chung[c], Tae-Hwan Kim[a], Yun-Sil Lee[a], Chang-Hun Rhee[b,1], Su-Jae Lee[a,1,*]

[a]*Laboratory of Radiation Effect, Korea Cancer Center Hospital, Gongneung-Dong, Nowon-Ku, Seoul 139-706, South Korea*
[b]*Laboratory of Cell Biology, Korea Cancer Center Hospital, Seoul 139-706, South Korea*
[c]*Department of Microbiology, College of Medicine, Han-Yang University, Seoul 133-791, South Korea*

Abstract Arsenic trioxide (As$_2$O$_3$) inhibits cell growth and induces apoptosis in certain types of cancer cells including acute promyelocytic leukemia, prostate and ovarian carcinomas, but its effect on response of tumor cells to ionizing radiation has never been explored before. Here we demonstrate that As$_2$O$_3$ can sensitize human cervical cancer cells to ionizing radiation both in vitro and in vivo. As$_2$O$_3$ in combination with ionizing radiation have a synergistic effect in decreasing clonogenic survival and in the regression of established human cervical tumor xenografts. Pretreatment of the cells with As$_2$O$_3$ also synergistically enhanced radiation-induced apoptosis. Apoptosis of the cells by combined treatment of As$_2$O$_3$ and radiation was associated with reactive oxygen species generation and loss of mitochondrial membrane potential, resulting in the activation of caspase-9 and caspase-3. The combined treatment also resulted in an increased G2/M cell cycle distribution at the concentration of As$_2$O$_3$ which did not alter cell cycle when applied alone. These results indicate that As$_2$O$_3$ can synergistically enhance radiosensitivity of human cervix carcinoma cells in vitro and in vivo, suggesting a potential clinical applicability of combination treatment of As$_2$O$_3$ and ionizing radiation in cancer therapies. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Arsenic trioxide; Radiosensitivity; Cervical cancer cells; Reactive oxygen species; Mitochondrial membrane potential

1. Introduction

Cervical cancer is one of the most common cancers, accounting for almost 6% of all malignancies in women. Each year, there are an estimated 16 000 new cases of invasive cancer of the cervix and 5000 deaths in the United States. Worldwide, cervical cancer is second only to breast cancer as the most common malignancy in both incidence and mortality [1].

Arsenic agents have been used as anti-cancer agents in tra-

ditional Chinese medicine [2]. The clinical efficacy of arsenic trioxide (As$_2$O$_3$) in acute promyelocytic leukemia (APL) has been confirmed even in patients resistant to conventional chemotherapy [3,4]. As$_2$O$_3$ is recently reported to be an effective inducer of apoptosis in certain cancer cells including APL, other myeloid leukemic cells, esophageal, prostate and ovarian carcinomas [5,6,7]. Although the mechanism for the induction of apoptosis has not fully been characterized, several studies demonstrated that As$_2$O$_3$ might be an oxidative agent that induced DNA damage and caused apoptosis [8,9]. Evidences also indicate that As$_2$O$_3$ induces apoptosis in leukemia and melanoma cells by modulating expression of Bcl-2 protein family [10,11], or by activating caspases [12,13].

One of the important points in the development of a new anti-cancer drug is the understanding of its potential for inclusion in combination treatment regimens. Recent studies demonstrated additive effects of As$_2$O$_3$ with conventional chemotherapeutic agents such as cisplatin, adriamycin, and etoposide, but no synergism [5,14]. On the other hand, the combined effect of As$_2$O$_3$ on response of cancer cells to ionizing radiation (IR) has never been examined before. In the present study, we demonstrated for the first time that As$_2$O$_3$ sensitize human cervix carcinoma cells to IR, suggesting that combination treatment of As$_2$O$_3$ with IR may be a new and more effective means of cancer treatment.

2. Materials and methods

2.1. Cell culture
HeLa human cervical carcinoma was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

2.2. Materials
Arsenic trioxide (As$_2$O$_3$) was obtained from Sigma. Polyclonal antibodies to caspase-3 and poly ADP-ribose polymerase (PARP) were purchased from Cell Signaling Technology, and caspase-9 was obtained from Santa Cruz.

2.3. Clonogenic survival assay
Cells were seeded into 60-mm dishes at a density to produce approximately 200 colonies per dish in the controls and were incubated for 10–14 days. Colonies were fixed with 75% ethanol and 25% acetic acid, and stained with trypan blue. The number of colonies consisting of more than 50 cells was counted.

2.4. Flow cytometric analysis of apoptosis
Apoptosis was identified and quantified by flow cytometry with propidium iodide (PI) staining. Both adherent and floating cells

*Corresponding author. Fax: (82)-2-970-0381.
E-mail address:* sjlee@mail.kcch.re.kr (S.-J. Lee).

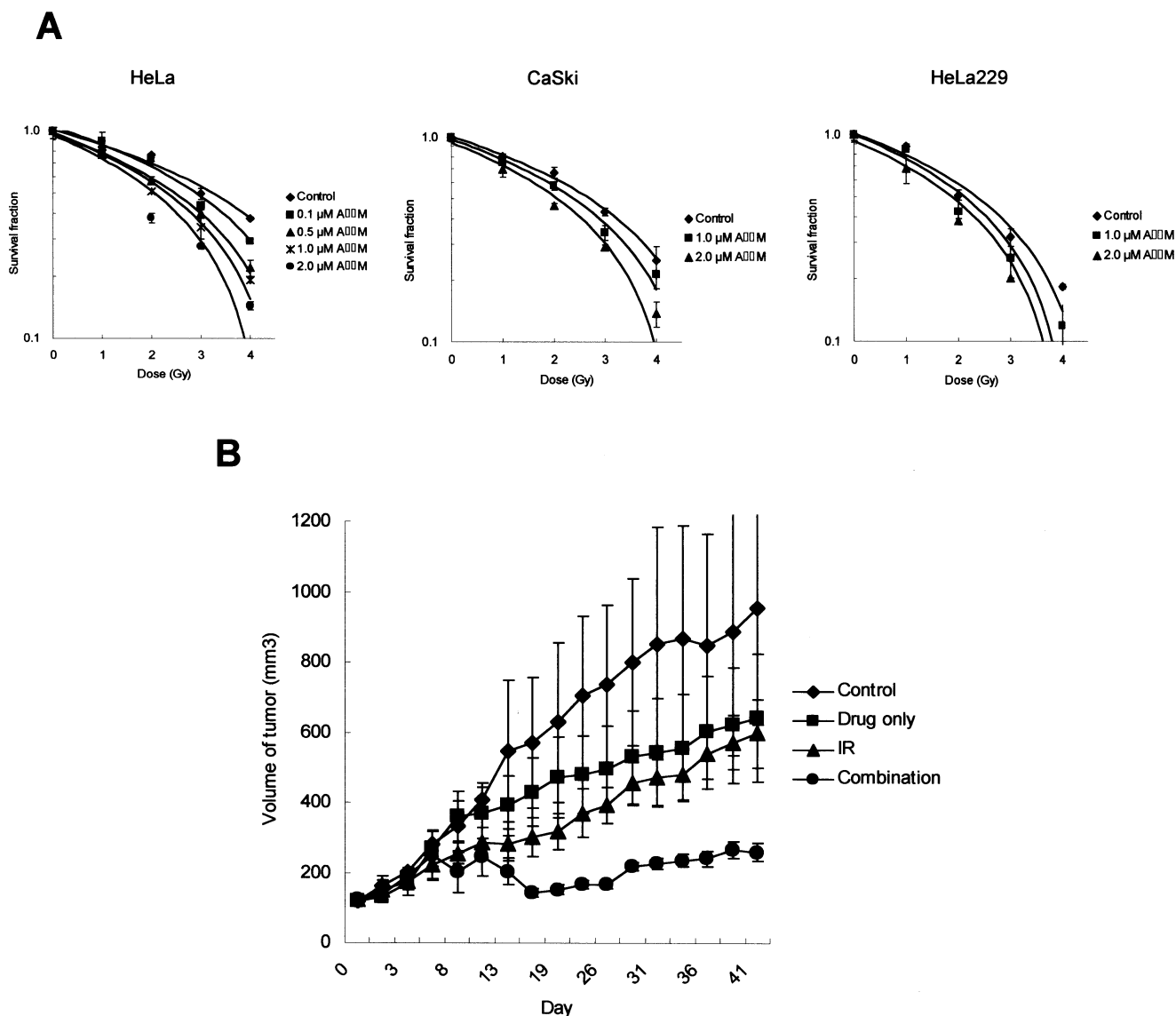[1] These authors contributed equally to this work.

A

B

Fig. 1. Radiosensitizing effect of As_2O_3 in vitro and in vivo. A: Cells were treated with various concentrations of As_2O_3 and irradiated with increasing doses of γ-radiation 1 h after drug addition. Cells were allowed to grow for 10–14 days and were stained with trypan blue and scored for colony formation. Results are given as means ± S.D. of three independent experiments. B: Exponentially growing HeLa cells were injected into the hind leg of nude mice. Treatment with As_2O_3 (3×20 mg/kg) alone, γ-radiation (1×7.5 Gy) alone or combination with As_2O_3 and γ-radiation (3×20 mg/kg of As_2O_3 combined with 1×7.5 Gy of γ-radiation) was started after tumor reached a minimal volume of 120 mm³.

were collected 24 or 48 h after the different regimen treatment, washed with ice-cold PBS and fixed with 70% ice-cold ethanol overnight at 4°C. Fixed cells were washed twice with PBS and treated with 1 mg/ml RNase for 30 min at 37°C. Cellular DNA was stained with 50 μg/ml PI in PBS containing 0.05% Nonidet P-40. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). From the analysis of DNA histograms, the percentage of cells in different cell cycle phases was evaluated. Cells with DNA content less than the cells in the G1 phase (sub-G1) were taken as apoptotic cells.

To determine cell death was attributable to apoptosis, whole cells were stained with FITC-conjugated Annexin V (Pharmingen) and PI according to manufacturer's protocol. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Western blot analysis

Cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. Proteins were separated by SDS–

PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence procedures (NEN) according to the manufacturer's recommendation.

2.6. Fluorescent measurement of intracellular reactive oxygen species (ROS)

The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was used for the assessment of intracellular ROS. For fluorocytometrical analysis, cells were plated in 60-mm dishes (1×10^5 cells/dish) and loaded for 30 min at room temperature with 10 μM DCFH-DA in 5 ml PBS. Unincorporated DCFH-DA was removed by two washes in PBS. DCFH-DA-loaded cells were stimulated with As_2O_3 or radiation alone, or in combination. Fluorescence was measured using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Measurement of mitochondrial membrane potential

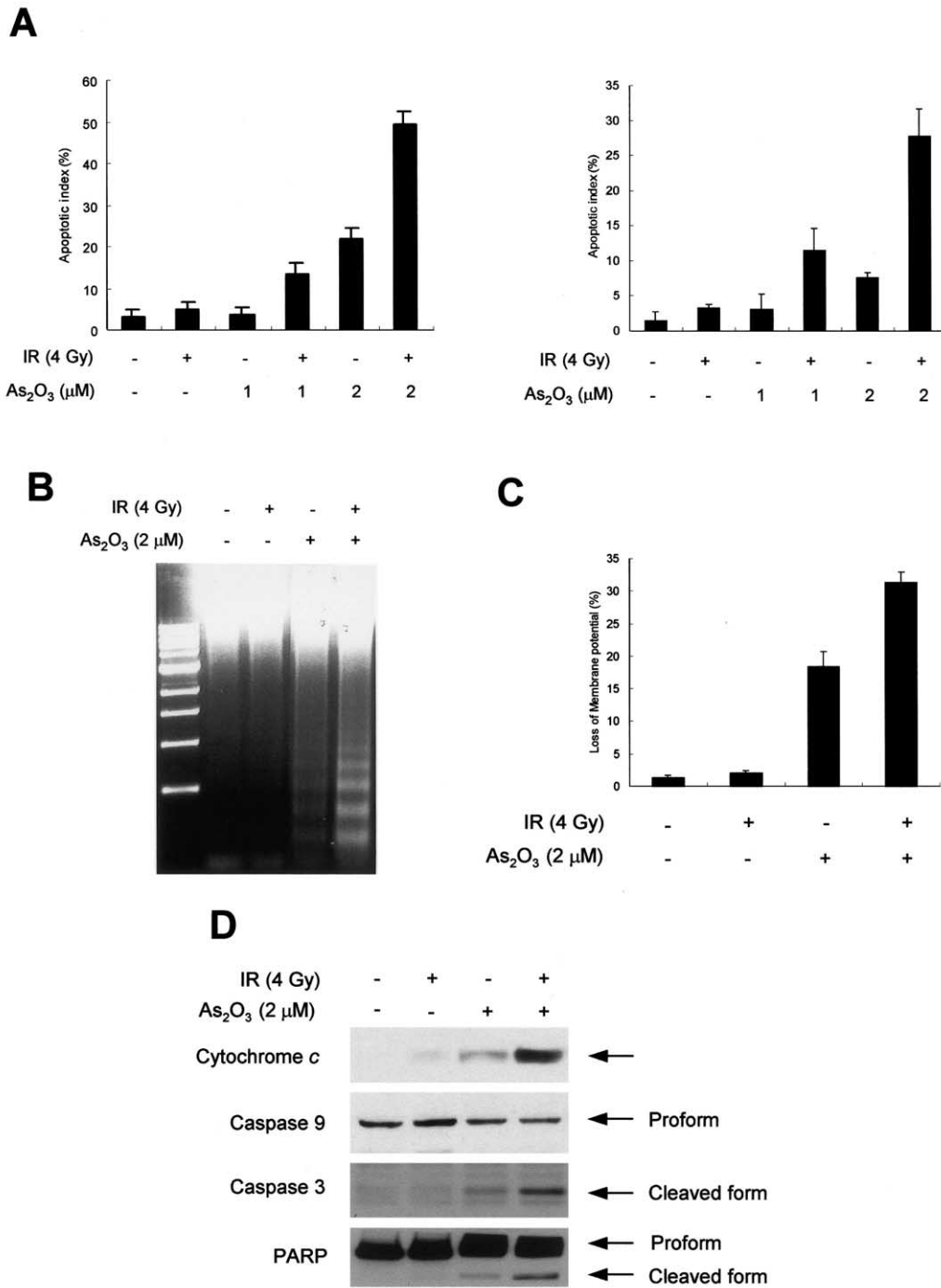Mitochondrial membrane potential was determined as the retention

A

B

C

D

Fig. 2. Enhancement of IR-induced apoptosis by As_2O_3. Cells were treated with As_2O_3 alone (1 or 2 μM), γ-radiation alone (4 Gy) or combination of both As_2O_3 and γ-radiation (1 or 2 μM of As_2O_3 combined with 4 Gy of γ-radiation). A: After 48 h, apoptosis was determined by Annexin V staining (left) and flow cytometric analysis after PI staining (right). The data represent average values of triplicate experiments with standard deviation. B: To analyze DNA ladder formation, the DNAs were extracted from cells as described in Section 2. The DNAs were subjected to agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. A representative of three independent experiments is shown. C: Cells were incubated with 30 nM $DiO_6(3)$ during the last 30 min of treatment. Forty-eight hours after treatment, the amount of retained $DiO_6(3)$ was measured with flow cytometry. The data represent average values of triplicate experiments with standard deviation. D: After 48 h, cell lysates were prepared and Western blot analysis was performed using anti-cytochrome c, caspase-9, caspase-3 and PARP antibodies. The data represent a typical experiment conducted three times with similar results.

of the mitochondria-specific dye $DiO_6(3)$. Cells were loaded with 30 nM $DiO_6(3)$ during the last 30 min of radiation and/or As_2O_3 treatment. After removal of the medium, the cells were washed twice with PBS, and the concentration of retained $DiO_6(3)$ was measured using flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA).

2.8. Tumor xenografts in nude mice

Human cervix carcinoma cells (HeLa) were injected into the hind leg of 5–6 week old athymic nude mice, and tumor volumes were determined according to the formula $(L \times l^2)/2$ by measuring tumor length (L) and width (l) with a caliper.

3. Results and discussion

3.1. Radiosensitizing effect of As₂O₃ in vitro

Earlier studies demonstrated an additive effect of As_2O_3 on apoptosis induced by chemotherapeutic agents such as cisplatin, adriamycin and etoposide in certain types of cancer [5,14]. In the present study, employing human cervix carcinoma cells, clonogenic survival assays were performed with increasing doses (0.5, 1, 2, 3 or 4 Gy) of IR alone and in combination with increasing concentration of As_2O_3 (0.1, 0.5, 1 or 2 μM). As shown in Fig. 1A, clonogenic survival of HeLa cells was synergistically reduced, when IR treatment was combined with As_2O_3. The pretreatment of cells with As_2O_3 alone did not significantly affect clonogenic survival at the dose levels of 0.1 and 0.5 μM, however As_2O_3 in combination with IR synergistically reduced clonogenic survival. The cooperative effect of As_2O_3 and IR was further

enhanced when higher concentrations of As_2O_3 were applied, resulting in reduced clonogenic survival. Similar radiosensitizing effects by pretreatment with As_2O_3 (1 or 2 μM) were also observed in other human cervical cancer cell lines such as HeLa229 and CaSki (Fig. 1A). These data indicate that As_2O_3 can sensitize human cervical cancer cells to IR in vitro.

3.2. Radiosensitizing effect of As₂O₃ on tumor xenografts

To determine whether the in vitro effects of As_2O_3 can be extended to an in vivo xenografts model, the effect of combined treatment of As_2O_3 and IR was tested in vivo against tumor derived from HeLa cells injected into the hind leg of nude mice. Treatment with locoregional application of IR using a shielding device at a single dose of 7.5 Gy was started when tumors reached a minimal size of 130 mm³ ± 10% (days 14–18 after cell injection). Fig. 1B shows the effect of treat-
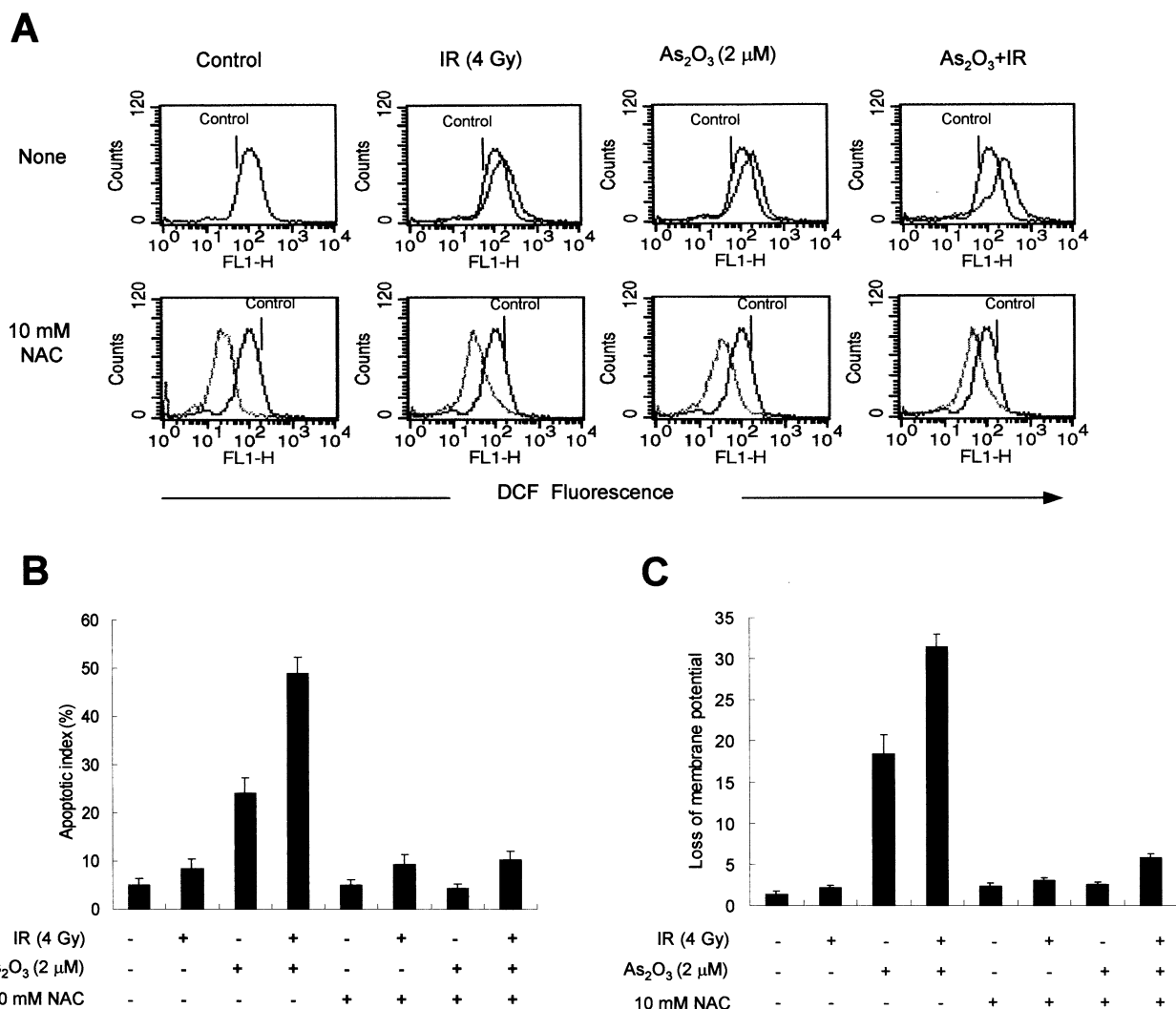

Fig. 3. Combined effect of As_2O_3 and IR on production of ROS. A: HeLa cells were loaded with DCFH-DA and further stimulated with As_2O_3 alone (2 μM), γ-radiation alone (4 Gy) or combination of both As_2O_3 and γ-radiation (2 μM of As_2O_3 combined with 4 Gy of γ-radiation) in the presence or absence of NAC. After 48 h, fluorescence was measured using flow cytometry. B: Cells were treated with As_2O_3 alone (2 μM), γ-radiation alone (4 Gy) or combination of both As_2O_3 and γ-radiation (2 μM of As_2O_3 combined with 4 Gy of γ-radiation) in the presence or absence of NAC. After 48 h, cells were stained with Annexin V-FITC. Fluorescence was measured using flow cytometry. The data represent average values of three independent experiments with standard deviation. C: Cells were treated with As_2O_3 alone (2 μM), γ-radiation alone (4 Gy) or combination of both As_2O_3 and γ-radiation (2 μM of As_2O_3 combined with 4 Gy of γ-radiation) in the presence or absence of NAC. Cells were incubated with 30 nM $DiO_6(3)$ during the last 30 min of treatment. After 48 h, the amount of retained $DiO_6(3)$ was measured with flow cytometry. The data represent average values of triplicate experiments with standard deviation.
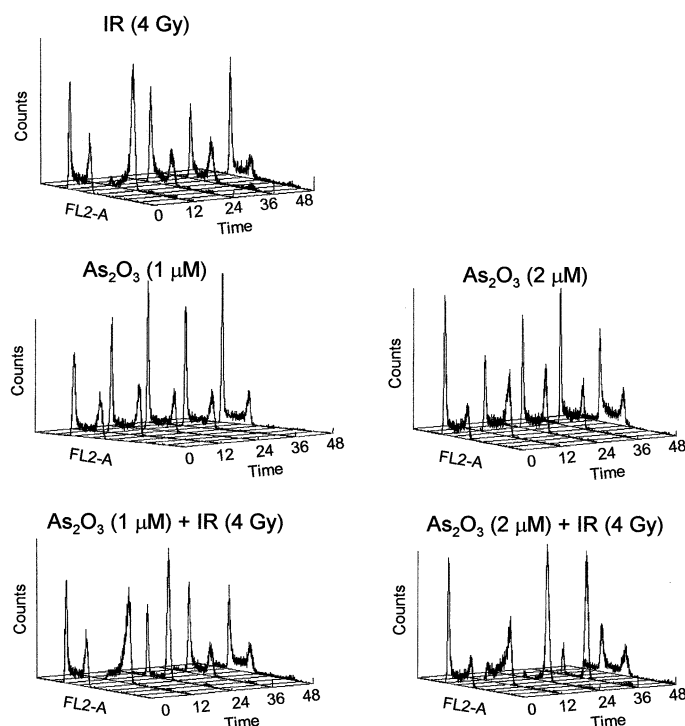
Fig. 4. As$_2$O$_3$ enhances IR-mediated increase of G2/M distribution. Cells were treated with As$_2$O$_3$ alone (1 or 2 μM), γ-radiation alone (4 Gy) or combination of both As$_2$O$_3$ and γ-radiation (1 or 2 μM of As$_2$O$_3$ combined with 4 Gy of γ-radiation). At times indicated, cells were harvested and subjected to flow cytometric analysis to determine cell cycle distribution.

ment of tumors with As$_2$O$_3$ (3×20 mg/kg) alone, γ-radiation (1×7.5 Gy) alone, and in combination (3×20 mg/kg As$_2$O$_3$ combined with γ-radiation 1×7.5 Gy). Treatment with As$_2$O$_3$ or γ-radiation alone resulted in a partial tumor growth delay, whereas combined treatment with As$_2$O$_3$ increased the anti-tumor effect of IR, which resulted in extended tumor growth delay.

3.3. Enhancement of radiation-induced apoptosis by As$_2$O$_3$

We next investigated whether the reduced clonogenic survival by combined treatment of As$_2$O$_3$ and γ-radiation was associated with induction of apoptosis. Therefore, Annexin V staining, flow cytometric cell death analysis and DNA laddering were performed to evaluate the extent of apoptosis. As seen in Fig. 2A, pretreatment with As$_2$O$_3$ at 1 μM concentration, which induced only minimal apoptotic effect (less than 5%), synergistically enhanced IR-induced apoptosis. The combined effect of As$_2$O$_3$ and IR was further enhanced, when an increased concentration of As$_2$O$_3$ (2 μM) was applied. The extent of apoptosis of HeLa cells in response to the different regimen treatment was further confirmed by DNA laddering analysis (Fig. 2B): cells were treated with As$_2$O$_3$ (2 μM) in combination with IR (4 Gy) and were analyzed for DNA laddering formation 48 h after the treatment. Taken together, these results demonstrate that As$_2$O$_3$ augments apoptotic cell death of human cervical cancer cells in response to the IR.

3.4. Loss of mitochondrial membrane potential and caspase activation are involved in As$_2$O$_3$-induced radiosensitization

To assess the induction of apoptosis at the biochemical level, changes in mitochondrial membrane potential, activation of caspase-9, the initiator caspase, and caspase-3, the major effector caspase of the apoptotic machinery, were determined after treatment with As$_2$O$_3$ alone, IR alone or in combination. Treatment with 2 μM As$_2$O$_3$ alone decreased DiO$_6$(3) retention, which reflects loss of mitochondrial membrane potential. The loss of mitochondrial membrane potential was further enhanced by the combined treatment with As$_2$O$_3$ and radiation (Fig. 2C). We also observed enhanced activation of caspase-9 and caspase-3, and PARP cleavage on treatment with As$_2$O$_3$ in combination with radiation compared to the groups treated with either reagent alone (Fig. 2D). These findings suggest that γ-radiation in combination with As$_2$O$_3$ modulates caspase-3 activity following the loss of mitochondrial membrane potential, thereby enhancing apoptotic cell death.

3.5. Combined effect of As$_2$O$_3$ and IR on ROS generation

Recently, the generation of ROS has been shown to regulate As$_2$O$_3$-induced apoptosis [15–17]. To investigate the relationship between ROS production and enhancement of radiation-induced apoptosis by As$_2$O$_3$, the ROS-sensitive dye DCFH-DA was used in the flow cytometric analysis to detect ROS generation (Fig. 3A). The production of ROS was synergistically induced by the combined treatment of As$_2$O$_3$ and IR even at doses that induce minimal ROS generation upon treatment with either reagent alone. N-acetylcysteine (NAC) which is a well known inhibitor of ROS completely protected HeLa cells from apoptosis as well as ROS generation induced by the combined treatment (Fig. 3A,B). NAC also attenuated the loss of mitochondrial membrane potential (Fig. 3C) and caspase-3 activation (data not shown). These results indicate that ROS generated by combined treatment of As$_2$O$_3$ and IR regulates apoptotic process including intracellular caspase signaling.

3.6. As2O3 enhances IR-mediated increased G2/M distribution

In addition to apoptosis, As_2O_3 and IR can also modulate cell cycle progression. Therefore, we examined cell cycle alterations as an another possible explanation for the observed cooperative effect on the clonogenic cell survival. As shown in Fig. 4, IR alone induced accumulation of cells at G2/M phase on 12 h after radiation and cells then resume cell cycle progression after 24 h. At the concentration of 1 μM As_2O_3 which, had no effect on cell cycle distribution in HeLa cells, in combination with IR increased the G/M arrested cells up to 80% of the total cell population that lasted for 36 h. These data indicate that As_2O_3 delay cell cycle progression after irradiation. There have been reports suggesting a correlation between radiosensitivity and G2/M phase delay [18,19]. For example, a radiosensitive cell line shows longer delay at G2/M phase than the resistant cell lines upon γ-irradiation [20]. However, it is still possible that the accumulation of cells at G2/M phase induced by the combined treatment is not a direct consequence or the primary cause of increased radiosensitivity.

Combination of different anti-tumoral treatment modalities is advantageous in limiting non-specific toxicity often observed by an exceedingly high dose of single regimen. The data reported in this study demonstrate that a clinically promising new anti-cancer drug, As_2O_3, potentiates the response of human cervical carcinoma cells to IR both in vitro and in vivo. As_2O_3 decreases survival of cancer cells via sensitizing cells to radiation-induced apoptosis. The apoptosis induced by the combined treatment in human cervical cancer cells was associated with loss of mitochondrial membrane potential, enhanced generation of ROS, and activation of caspase-9 and caspase-3.

Several studies have already demonstrated that As_2O_3 has an anti-cancer effect in different types of cancer cells. Here, we showed that As_2O_3 can enhance the efficacy of radiation-induced apoptosis of human cervical cancer cell line. Significantly, the combination of As_2O_3 and radiation can induce a complete regression of tumor xenografts established in nude mice. Taken together, our results indicate that combining radiation and As_2O_3 may be a new and more effective means of cancer treatment.

In conclusion, As_2O_3 in combination with irradiation revealed a synergistic anti-tumoral effect in vitro and in vivo in human cervical cancer cells, suggesting potential usefulness of combined therapy of As_2O_3 together with IR for cervical cancer treatment.

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