

# Effect of ascorbic acid and X-irradiation on HL-60 human leukemia cells: The kinetics of reactive oxygen species

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**Abstract.** Ascorbic acid (AsA) treatment is expected to be a potential cancer therapy strategy with few side-effects that can be used alone or in combination with chemotherapy. However, the combination of AsA, a free radical scavenger, with radiation is not clearly understood; conflicting data are reported for cancer cell death. We conducted this study to determine the effect of AsA treatment combined with X-irradiation and the role of reactive oxygen species (ROS) in HL-60 human promyelocytic leukemia cells. Additive cytotoxic effects were observed when the cells were exposed to 2 Gy X-irradiation after 2.5 mM AsA treatment. When catalase was added to the culture with AsA alone, the cytotoxic effects of AsA disappeared. X-irradiation increased intercellular ROS levels and mitochondrial superoxide levels. By contrast, AsA alone and in combination with X-irradiation decreased ROS levels. However, in the presence of catalase neutralizing H<sub>2</sub>O<sub>2</sub>, AsA alone or in combination with X-irradiation only slightly decreased the intercellular ROS. Moreover, AsA decreased the mitochondrial membrane potential, which is commonly associated with apoptosis. These results suggest that the reduction of ROS did not result from ROS scavenging by AsA, and AsA induced apoptosis through a ROS-independent pathway. This study reports that a combination of AsA with radiation treatment is effective in cancer therapy when considering ROS in cancer cells.

## Introduction

Ascorbic acid (AsA) (also known as vitamin C) therapy has been considered a therapeutic option for cancer, and has few

side-effects when administered intravenously in pharmacologic concentrations (1). Chen *et al* reported that AsA is selectively toxic for some cancer cells but it is not toxic to normal cells (2). AsA exhibits cytotoxic effects in tumor cells, which have a low concentration of intracellular catalase that degrades hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (3). Studies have shown that tumor cells are easily damaged by H<sub>2</sub>O<sub>2</sub>, and production of the adenosine triphosphate (ATP) is decreased due to mitochondrial damage, thus leading to tumor cell death (3-7). H<sub>2</sub>O<sub>2</sub> is produced during radiation therapy and some antineoplastic drugs kill the tumor cell by its cytotoxic activity (8). The generation of reactive oxygen species (ROS) derived from H<sub>2</sub>O<sub>2</sub> is thought to be involved in the cytotoxicity. Vitamin C therapy can be used alone or in combination with chemotherapy (9-11). AsA in combination with radiation therapy is also expected to be effective in cancer therapy since it is considered to have few side-effects (12,13). Koyama *et al* reported that AsA does not inhibit the fatal effects of radiation, but inhibits carcinogenesis and mutation (30). Therefore, AsA may reduce the risk of a second cancer in normal cells during combined AsA and radiation therapy.

AsA is also known as a radical scavenger (14,15), and it scavenges O<sub>2</sub><sup>•-</sup>, •OH, <sup>1</sup>O<sub>2</sub>, and NO under *in vitro* conditions; thus, it can scavenge ROS generated by antineoplastic drugs or X-irradiation (16). Therefore, conflicting data exist regarding AsA inhibiting the cytotoxic effects generated by the action of antineoplastic drugs or X-irradiation (17-21).

The majority of cell deaths induced by X-irradiation depend on the production of intracellular ROS, which is generated during irradiation. Within several hours after irradiation, secondary ROS production occurs intracellularly, and it induces apoptosis (22,23). Mitochondria are well known as a major source of intracellular ROS and produce ROS during intracellular ATP synthesis. Therefore, the source of secondary ROS as a result of irradiation is thought to be the mitochondria (22-24). The generation of ROS from mitochondria and the loss of the mitochondrial membrane potential play an important role in inducing cell death (23,25,26).

In this study, we examined the mechanism underlying cell death caused by a combination treatment of AsA and X-irradiation from the viewpoint of ROS generation by using HL-60 human promyelocytic leukemia cells and we clarified that AsA does not inhibit the cytotoxic effects of X-irradiation.

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## Materials and methods

**Cell culture, X-irradiation, and drug treatment.** The HL-60 human promyelocytic leukemia cell line (RIKEN BioResource Center, Tsukuba, Japan) was used in these experiments. Cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and were maintained at 37°C with 95% air and 5% CO<sub>2</sub>. The passage duration was 3–5 days, and the density was not allowed to exceed 1×10<sup>6</sup> cells/ml. X-irradiation was delivered using an X-ray machine MBR-1520R-3 (Hitachi, Tokyo, Japan) at 150 kV and 20 mA through a 0.5-mm Al and 0.3-mm Cu filter at a dose rate of 1.0 Gy/min. L(+)-ascorbic acid was purchased from Wako (Tokyo, Japan). The AsA was dissolved in RPMI-1640 medium and deacidified with sodium hydrate before treatment. Catalase (Sigma Aldrich, St. Louis, MO, USA) was added to the culture to achieve a final concentration of 1,300 U/ml.

**Cell viability assay.** HL-60 cells (4.0×10<sup>5</sup> cells/ml) were cultured for 6 h. A final concentration of 0.01–10.0 mM AsA was then added to the culture and the number of viable cells was counted using trypan blue dye exclusion method after 24 h. For the next experiment, 4×10<sup>5</sup> cells/ml HL-60 cells were cultured with or without catalase for 6 h, and 1.0 or 2.5 mM AsA was added to the cells in combination with 2 Gy X-irradiation. The viable cells were counted by trypan blue dye exclusion method after 24 h.

**Measurement of intracellular ROS.** Subsequently, HL-60 cells (1.5×10<sup>5</sup> cells/ml) were cultured with or without catalase for 6 h, and 2.5 mM AsA was added to the cells in combination with 2 Gy X-irradiation. The intracellular ROS production was measured using a flow cytometer (Cytomics FC500, Beckman Coulter, Fullerton, CA) using the ROS-sensitive probe 2',7-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA; Molecular Probes, Invitrogen Corp., CA, USA) at the indicated times after exposure to X-irradiation. The cells were washed with phosphate buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> [PBS(-)], incubated at 37°C with 5 μM H<sub>2</sub>DCFDA in PBS(-) for 15 min, washed in PBS(-), and then resuspended in PBS(-) containing 5 mg/l propidium iodide (PI; Sigma Aldrich) to exclude dead cells. Sample data were analyzed using FlowJo software (Treestar, Inc., San Carlos, CA, USA). The median H<sub>2</sub>DCFDA fluorescence intensity of each sample was normalized to that of control sample to calculate the relative H<sub>2</sub>DCFDA intensity.

For the precise evaluation of ROS production immediately following X-irradiation, we labeled the cells with H<sub>2</sub>DCFDA prior to AsA and X-irradiation treatments (27,28). In brief, cells were incubated at 37°C with 5 μM H<sub>2</sub>DCFDA in PBS(-) for 15 min. After labeling, the cells were treated with AsA and/or X-irradiation in the presence of H<sub>2</sub>DCFDA, washed in PBS(-), and then resuspended in PBS(-) containing 5 mg/l PI. Samples were analyzed using a flow cytometer immediately after the treatment.

**Measurement of mitochondrial superoxide and mitochondrial membrane potential.** The cells were treated with AsA and/or X-irradiation as described above without the addition of catalase. The mitochondrial superoxide levels were measured using

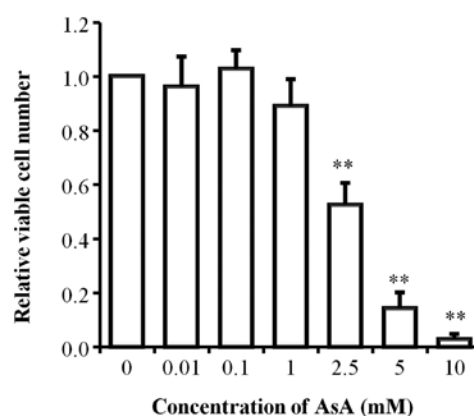


Figure 1. Relative viable cell number at 24 h after AsA treatment. \*\*p<0.01 vs. 0 mM. AsA showed cytotoxic effects on the growth of HL-60 cells in a dose-dependent manner from ~1 mM.

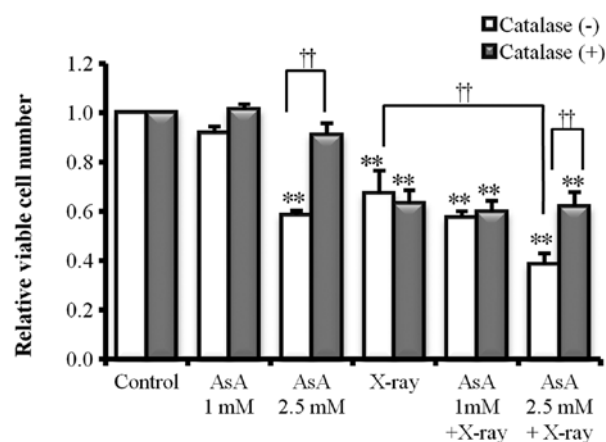


Figure 2. Relative viable cell number at 24 h after X-ray irradiation (2 Gy), AsA treatment (1 or 2 mM), and combined X-ray and AsA treatment (white bars, untreated with catalase; gray bars, treated with catalase). \*\*p<0.01 vs. 0 mM. ††p<0.01.

the flow cytometer with mitochondrial superoxide indicator MitoSOX Red (Molecular Probes, Invitrogen Corp.) at the indicated times after exposure to X-irradiation. The cells were washed with PBS(-), incubated at 37°C with 5 μM MitoSOX Red in Hanks' Balanced Salt Solutions (HBSS; with Ca<sup>2+</sup> and Mg<sup>2+</sup>) for the 15 min, and then washed and resuspended in PBS(-). Sample data were analyzed using the FlowJo software. The median MitoSOX Red fluorescence intensity of each sample was normalized to that of control sample to calculate the relative MitoSOX Red intensity.

The changes in the mitochondrial membrane potential were measured using the flow cytometer with 3,3'-diethyloxycarbocyanine iodide [DiOC6(3); Molecular Probes, Invitrogen Corp.] at indicated times after exposure to X-irradiation. The cells were washed with PBS(-), incubated at 37°C with 40 nM H<sub>2</sub>DCFDA in HBSS for 15 min, washed in HBSS, and then resuspended in serum-free RPMI-1640 medium containing 5 mg/l PI to exclude dead cells. Sample data were analyzed using FlowJo software. The median DiOC6(3) fluorescence intensity of each sample was normalized to that of control sample to calculate the relative DiOC6(3) intensity.

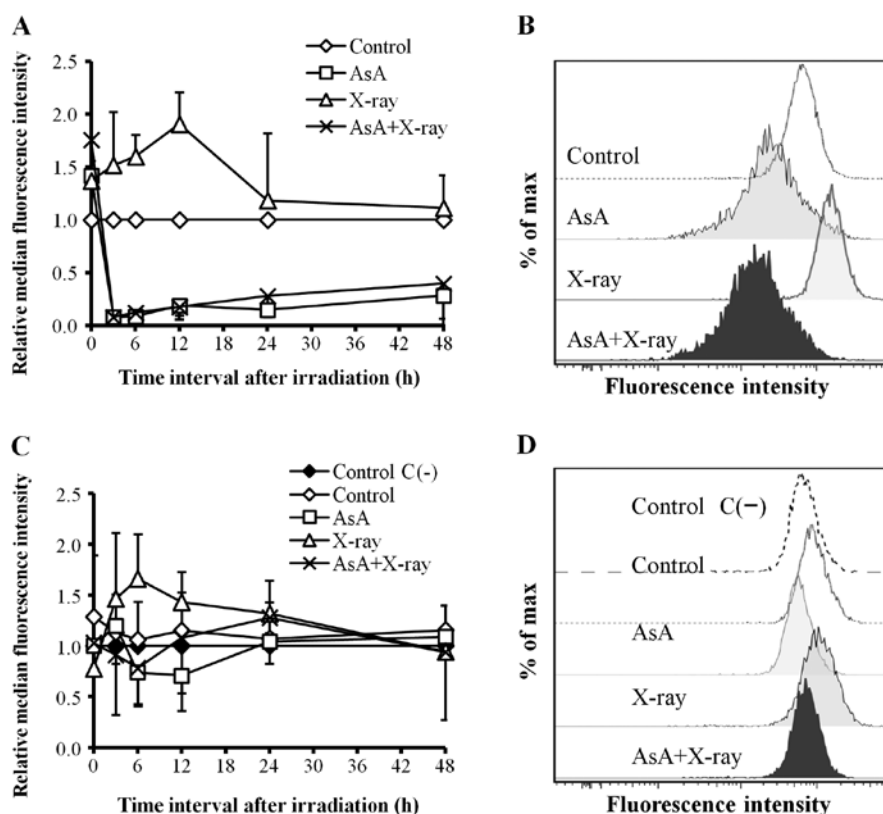


Figure 3. Kinetics of intracellular ROS production analyzed by flow cytometry using  $H_2DCFDA$ . Intracellular ROS levels without catalase (A) and with catalase (C) after X-irradiation. A representative flow cytometric histogram of  $H_2DCFDA$  fluorescence intensity at 12 h without catalase (B) and at 6 h with catalase (D) after X-irradiation. Control C(-) represents control in the absence of catalase.

**Statistical analysis.** Statistical comparisons were performed using the Tukey-Kramer test. All results are presented as the mean  $\pm$  SD from the results of at least three independent experiments.  $p$ -values  $>0.01$  or  $0.05$  were considered to indicate statistically significant differences. Statistical analysis was performed using the Excel 2007 software program (Microsoft, USA) with Statcel 2 add-in software (29).

## Results

**Cell death by AsA treatment and X-irradiation.** In the initial experiment, we examined the AsA sensitivity of HL-60 cells. After AsA treatment for 24 h, we counted the viable HL-60 cells by trypan blue dye exclusion method. AsA showed cytotoxic effects on the growth of HL-60 cells in a dose-dependent manner from  $\sim 1$  mM concentration (Fig. 1). Enhanced cell growth was not observed in AsA treatment at low concentrations (0.01 or 0.1 mM).

Additive cytotoxic effects were observed when the cells were exposed to 2 Gy X-irradiation after 2.5 mM AsA treatment (Fig. 2). When 2.5 mM AsA and 2 Gy X-irradiation were used in combination, a significant decrease in the relative viable cell number was observed when compared to application of 2 Gy X-irradiation alone ( $p < 0.05$ ). Fig. 2 also shows the cytotoxic effect of AsA alone and in combination with X-irradiation in the presence of catalase. When AsA was added to the culture in the presence of catalase, the cytotoxic effects of AsA disappeared. Moreover, the additive cytotoxic effects of 2.5 mM AsA and 2 Gy X-irradiation in combina-

tion decreased to the same level as those obtained by 2 Gy X-irradiation alone.

**Kinetics of intracellular ROS.** Fig. 3A shows the changes in intracellular ROS levels as analyzed using a flow cytometer. In cells treated with X-irradiation alone (2 Gy), the intracellular ROS levels increased and reached a peak at 12 h ( $p < 0.01$ ) compared to control, and decreased slowly thereafter. By contrast, treatment with AsA alone (2.5 mM) and in combination with X-irradiation (2 Gy) significantly decreased intracellular ROS levels at 3–24 h after X-irradiation. A representative histogram of DCF fluorescence intensity at 12 h is shown in Fig. 3B.

Fig. 3C shows the changes in the intracellular ROS levels in the presence of catalase. The intracellular ROS levels of X-irradiated cells increased slightly and reached a peak after 6 h, but there was no significant difference when compared to control cells. The ROS levels of cells treated with AsA alone and in combination with X-irradiation were also not significantly altered compared to those of the control cells. A representative histogram of DCF fluorescence intensity at 6 h is shown in Fig. 3D.

In the absence of catalase, the ROS levels of cells treated with a combination of AsA and X-irradiation were significantly higher than those of the control cells at 0 h after X-irradiation, and this difference was statistically significant ( $p < 0.05$ ). For the precise evaluation of ROS production immediately following X-irradiation, we labeled the cells with  $H_2DCFDA$  prior to AsA and X-irradiation treatment. The ROS levels of cells

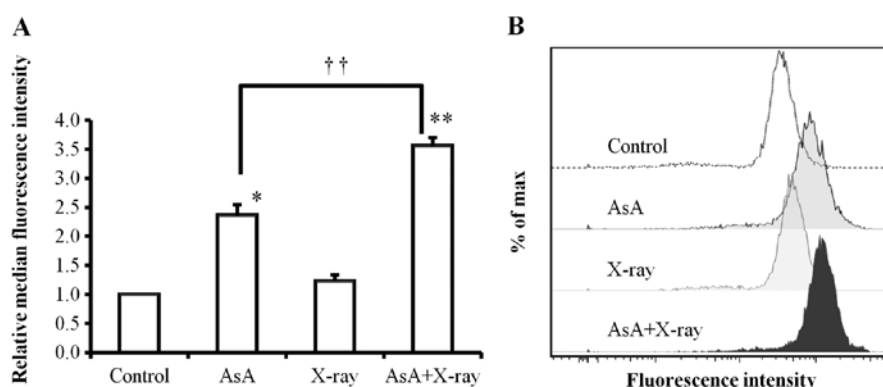


Figure 4. Evaluation of ROS production soon after X-irradiation. We labeled the cells with H<sub>2</sub>DCFDA before AsA and X-irradiation treatment. After AsA and/or X-irradiation, samples were run on a flow cytometer immediately. (A) ROS production immediately following X-irradiation. (B) A representative flow cytometric histogram of H<sub>2</sub>DCFDA fluorescence intensity immediately following X-irradiation. \**p*<0.05 vs. 0 mM, \*\**p*<0.01 vs. 0 mM. ††*p*<0.01.

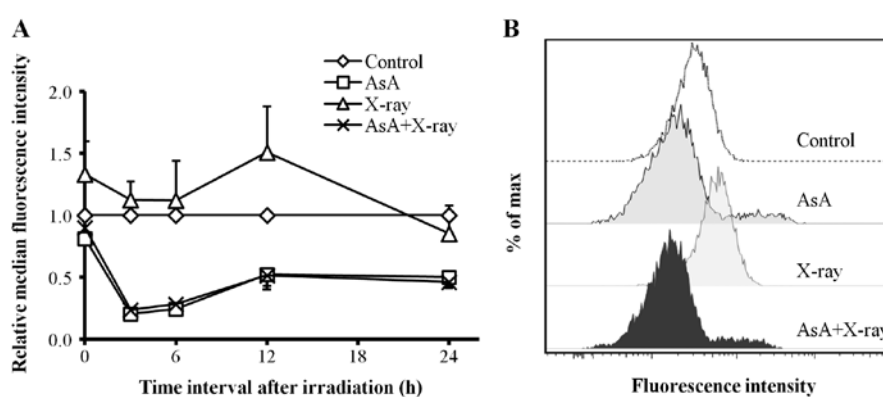


Figure 5. Mitochondrial superoxide analysis by flow cytometry using MitoSOX Red. (A) Kinetics of mitochondrial superoxide. (B) A representative flow cytometric histogram of MitoSOX Red fluorescence intensity at 12 h after X-irradiation.

treated with AsA alone and in combination with X-irradiation significantly increased immediately following X-irradiation, compared to control cells (Fig. 4A and B).

**Kinetics of mitochondrial superoxide and mitochondrial membrane potential.** The changes in mitochondrial superoxide levels were analyzed by flow cytometry (Fig. 5A). When X-irradiation was used alone, mitochondrial superoxide levels increased slightly and reached a peak at 12 h (*p*<0.01) compared to control (Fig. 5B). By contrast, AsA alone and in combination with X-irradiation significantly decreased the mitochondrial superoxide levels at 3–24 h after X-irradiation. These changes were similar to the changes in the intracellular ROS levels.

In order to investigate whether AsA scavenged intracellular ROS or whether generation of ROS from mitochondria was decreased, we used DiOC6(3) (a carbocyanine dye that accumulates in active mitochondria) to measure the mitochondrial membrane potential by flow cytometry. When X-irradiation was used alone, the mitochondrial membrane potential increased slightly and reached a peak at 12 h (*p*<0.01) compared to control (Fig. 6A). Contrary to this, in the presence of only AsA or in combination with X-irradiation, the mitochondrial membrane potential gradually decreased and became ~40% of control value at 12 h after X-irradiation (Fig. 6B).

## Discussion

In the present study, we described the potential of AsA and X-irradiation combination treatment, particularly against intracellular ROS in HL-60 cells. We demonstrated that AsA, a radical scavenger, did not inhibit the cytotoxic effect when used in combination with X-irradiation although it resulted in intracellular ROS reduction.

In this study, additive cytotoxic effects were observed when the cells were exposed to 2 Gy X-irradiation after 2.5 mM AsA treatment. When a combination of 1 mM AsA and 2 Gy X-irradiation was applied, the protective effect of AsA against 2 Gy X-irradiation was not observed and no significant cytotoxic effects were found. These results are consistent with the studies that AsA does not inhibit the fatal effects of radiation (12,30). When AsA was added to the culture in the presence of catalase, the cytotoxic effects of AsA disappeared. Moreover, the additive cytotoxic effects decreased to the same level obtained by X-irradiation alone. These results suggest that the action pathway of hydroxyl radicals derived from H<sub>2</sub>O<sub>2</sub> is different in AsA and X-irradiation treatments. Catalase, being a large protein, does not penetrate cell membranes and, therefore, it is not taken up by cells. Catalase neutralizes the H<sub>2</sub>O<sub>2</sub> derived from AsA in the extracellular fluids. Therefore, it is considered that the cytotoxic effect of extracellular H<sub>2</sub>O<sub>2</sub>

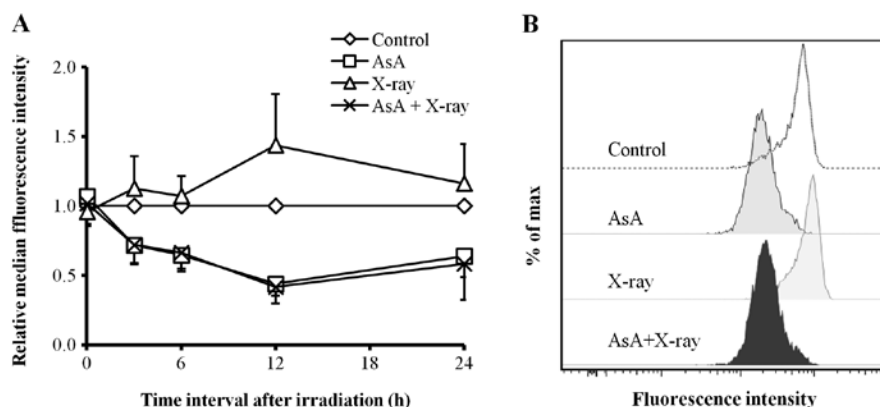


Figure 6. Mitochondrial membrane potential analyzed by flow cytometry by using DiOC6(3). (A) Kinetics of mitochondrial membrane potential. (B) A representative flow cytometric histogram of DiOC6(3) fluorescence intensity at 12 h after X-irradiation.

that AsA generates is much more effective than the cytotoxic effect of intracellular AsA (3,31).

Our present study showed that AsA significantly decreased intercellular ROS production. Hence, such a result might indicate that combined AsA and X-irradiation treatment may not be effective as cell death due to signal transduction by ROS is inhibited (20,21). In AsA treatment, a significant change in the intercellular ROS level was not observed in the presence of catalase as compared with the significant reduction of intracellular ROS in the absence of catalase. For this reason, our results suggest that the slight change in intracellular ROS is due to the neutralizing effect of extracellular  $H_2O_2$  by catalase in preference to scavenging intercellular ROS by AsA. It is thought that extracellular  $H_2O_2$  generated by AsA treatment might mainly decrease intercellular ROS or both cytotoxic effects and radical scavenger are necessary for significant reduction of ROS. Some studies have reported that antineoplastic drugs exhibit cytotoxic effects with reduction of ROS; hence, it showed the ability of scavenging ROS such as AsA (32,33). It was also reported that intercellular ROS slightly decreased when cells were treated with  $H_2O_2$  (34).

When AsA alone and in combination with X-irradiation was used, large quantities of intracellular ROS were observed immediately following AsA and X-irradiation treatment, which was observed by labeling the cells with  $H_2DCFDA$  prior to treatment. It is reported that  $H_2O_2$  generation was dependent on the presence of trace amounts of serum in media (2). When the cells were treated with AsA and/or X-irradiation in the presence of  $H_2DCFDA$  in 10% FBS growth medium, but not in PBS, ~12-fold ROS production in the control was observed for AsA alone and in combination with X-irradiation (data not shown). Since  $H_2O_2$  can easily permeate cell membranes (35), large quantities of  $H_2O_2$  derived from AsA might damage HL-60 cells immediately following AsA treatment, after which the intercellular ROS production is decreased. Frömberg *et al* showed that AsA or dehydroascorbate (DHA) is important for cytotoxic efficiency in the redox state of vitamin C, and they report higher therapeutic efficacy of AsA over DHA in various cell lines (31). Furthermore, it was reported that tumor cells take up DHA, but not AsA, in large quantities. However, a moderate change in the intracellular ROS levels was observed in the order of mM DHA (18), although DHA turn into AsA in cells (16), contrary to our results of AsA treatment. Therefore,

the redox state of vitamin C agents may lead to contradictory results in vitamin C treatment.

Mitochondria release cytochrome *c*, which activates caspase for apoptosis, leading to changes in mitochondrial respiratory chain, and the mitochondrial membrane potential is depolarized. Therefore, mitochondrial membrane potential is used as an indicator for evaluating cell life and death (36). In our study, mitochondrial membrane potential gradually disappeared in cells treated with AsA. However, it is reported that an increase in intracellular ROS is observed when the mitochondrial membrane potential is decreased by several antineoplastic agents (37). Some studies reported the existence of ROS-independent mitochondrial pathway, since reduction of the mitochondrial membrane potential is observed without an increase in intracellular ROS (32,33,38,39). AsA might induce apoptosis in HL-60 cells through a ROS-independent mitochondrial pathway as intracellular ROS was significantly decreased as compared with the control, and a reduction in the mitochondrial membrane potential was observed in AsA treatment.

Some studies reported that the antineoplastic agent that has the ability of scavenging ROS induces apoptosis through a ROS-independent mitochondrial pathway with reduction in ROS (32,33). Furthermore, an increase in intracellular ROS and superoxide levels derived from mitochondria was reported and mitochondrial membrane potential hyperpolarization was observed after X-ray irradiation. It is thought that X-irradiation arrests cell cycle, inhibits cell division and increases in the mitochondrial content, leading to the activation of mitochondrial respiratory chain, resulting in the increase of mitochondrial ROS (24,40). Our data are consistent with these studies, but changes in the X-ray irradiated cells were contrary to the changes in cells treated with AsA. When a combination of AsA and X-irradiation was used, ROS levels decreased to the same level obtained by AsA alone, but AsA did not inhibit the cytotoxic effects of X-irradiation. While considering ROS generation, these indicate that additive cytotoxic effects were observed since AsA and X-irradiation follow different signaling pathways. Shinozaki *et al* reported that the involvement of Bax and caspase 8 were different following X-irradiation or AsA treatment alone as compared with those following combined X-irradiation and AsA treatment against the apoptosis mechanism (12).

In the present study, we examined the mechanism underlying cell death caused by a combination of AsA and X-irradiation from the viewpoint of ROS generation by HL-60 cells to reveal the clinical possibility of a combination therapy. The combination decreased intracellular ROS generation, but additive cytotoxic effects and reduction of mitochondrial membrane potential were observed in cells. These results suggest that AsA, which is a radical scavenger, did not exert protective effects against ROS production by X-irradiation and the signaling pathway in mitochondria was different for AsA and X-irradiation. Our results suggest that combination therapy of AsA and X-irradiation does not have an effect on cancer cell death while considering ROS generation.

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