Ascorbic acid does not reduce the anticancer effect of radiotherapy

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Abstract. The present study hypothesized that the therapeutic use of ascorbic acid (AsA) in combination with radiation may reduce therapy-related side effects and increase the antitumor effects. The aim of the study was to examine the association between the scavenged activity of AsA and the biological anticancer effect of hydroxyl (OH) radicals generated by X-ray irradiation. Cell survival, DNA fragmentation of human leukemia HL60 cells and the amount of OH radicals were investigated following X-ray irradiation and AsA treatment. The number of living cells decreased, and DNA fragmentation increased at AsA concentrations >1 mM. Electron spin resonance spectra revealed that X-ray irradiation generated OH radicals, which were scavenged by AsA at concentrations >75 μM. The AsA concentration inside the cell was 75 μM when cells underwent extracellular treatment with 5 mM AsA, which significantly induced HL60 cell death even without irradiation. No increase in the number of viable HL60 cells was observed following AsA treatment with irradiation when compared to irradiation alone. In conclusion, the disappearance of the radiation anticancer effects with AsA treatment in combination with radiotherapy for cancer treatment is not a cause for concern.

Introduction

It is well known that ascorbic acid (AsA) has anticancer effects due to its antioxidant activity, and the results of epidemiological surveys have demonstrated that AsA intake is inversely correlated with carcinogenicity (1). Almost 30 years ago, the study by Cameron and Pauling was the first to demonstrate the antitumor effect of AsA and this finding was applied to a clinical setting (2). A few years earlier, the effectiveness of combining cancer treatment with AsA was reported, but the anticancer activity of AsA was not confirmed in clinical tests at the Mayo Clinic (3).

Recent studies have shown that high plasma concentrations of AsA can only be achieved when AsA is administered intravenously or intraperitoneally as the rate of absorption from the gut is limited with oral administration (4). The plasma concentrations of AsA in humans following high-dose intravenous AsA is ~200 times higher in comparison to oral administration (5). Therefore, we hypothesized that the therapeutic use of AsA in combination with radiation may reduce therapy-related side effects and increase the antitumor effects (6). It has been reported that pharmacological ascorbate sensitized cells to the effects of ionizing radiation (7,8).

Ionizing radiation is known to damage numerous cell organelles through the production of free radicals (9). The hydroxyl (OH) radical ($\cdot$OH) is considered to have a major role in radiation-induced cell damage and induces apoptotic cell death through activation of a pro-apoptotic pathway (10). However, AsA is a known scavenger of reactive radical species, and it is possible that AsA may reduce the anticancer effect of radiation. The aim of the present study was to examine the association between the scavenged activity of AsA and the biological anticancer effect of OH radicals generated by X-ray irradiation.

Materials and methods

Cell culture, extracellular AsA treatment and X-ray irradiation. Human promyelocytic leukemia HL60 cells were purchased from the RIKEN Bioresource Center (Tsukuba, Japan). The cells were grown in 100-mm culture dishes containing Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The cells were passaged every third day, and the density in the culture was ≤1x10^6 cells/ml. The cell concentration was adjusted to 5x10^5 cells/ml prior to AsA treatment and X-ray irradiation. The AsA solution was titrated with NaOH at pH 7.4. HL60 (5x10^5 cells/ml) in 50-mm culture dishes were irradiated 1 h after adjusting each experimental AsA dose. The radiation was administered with Sofron BST 1505 CX (Sofron X-ray Industry Corporation, Ltd., Tokyo, Japan) under the following conditions: Tube voltage, 80 kVp

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(effective 62.3 kV); tube current, 5 mA; focus surface distance, 30 cm; dose rate, 16 Gy/h; filter, 1-mm aluminum; and temperature, room temperature.

Electron spin resonance (ESR) measurement. OH radicals generated from irradiated phosphate-buffered saline (PBS) were measured by ESR (JES-FR80; JEOL, Tokyo, Japan). The spin-trap technique was used with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Labotec Corp., Ltd., Tokyo, Japan). The PBS sample (200 μl) contained 150 μl of PBS with or without AsA, 35 μl of diethylenetriaminepentaacetic acid (Labotec Corp. Ltd.) and 15 μl of DMPO. Radicals generated in this sample by X-ray irradiation under the aforementioned condition were measured within 1 min of irradiation. The ESR spectrometer (JES-FR80) was used under the following conditions: Frequency, 9.475 GHz; power, 4 mW; center field, 334.5 mT; sweep width, ±10.0 mT; modulation width, 5x0.1 m; receiver gain, 0.4x100; time constant, 0.1 sec; sweep time, 2.0 min; and temperature, room temperature. The relative signal intensity (RSI) of OH radicals was defined as the ratio of the peak height of OH radicals and a manganese oxide standard marker on the ESR spectra. Each sample was measured three to five times and the results are shown as average values of RSI.

Cell viability assay and quantitative detection of apoptosis. The cells were stained with 2% trypan blue for 1 min and counted per 1 mm^3 in each experiment to estimate the number of unstained cells within 2 min. The number of unstained cells was considered to be the number of viable cells.

Apoptosis was detected using a cellular DNA fragmentation enzyme-linked immunosorbent assay kit (Roche Diagnostics, Basel, Switzerland). The cells were labeled with bromodeoxyuridine (BrdU) (Roche Diagnostics) on the day prior to treatment. Following AsA treatment and/or 4 Gy X-ray irradiation, the cells were cultured for 6 h. After this incubation, 1.5x10^6 cells were collected and treated with a cell solution (0.05% Triton-X and 650 μM EDTA) (Research Organics, Cleveland, OH, USA) at 4°C for 30 min. The cells were subsequently centrifuged at 4°C and 12,000 x g for 30 min at 4°C, and 160 μl of the supernatant was used as a sample. This sample was plated on a microtiter plate coated with an anti-BrdU antibody (Roche Diagnostics), and color development caused by the tetramethylbenzidine (Roche Diagnostics) substrate reaction was measured using a spectrophotometer (450 nm).

Analysis of intracellular AsA concentration. The OxiSelect™Ascorbic Acid Assay kit (FRASC) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Cells (1.0x10^6 cells/ml) were lysed by multiple freeze-thaw cycles in four volumes of cold 1X assay buffer. The samples and AsA standard were added to 96-well plate wells. Deionized water (-AO) or diluted 1X ascorbate oxidase (+AO) was added to each well. The reaction reagent was added to all the wells, and the absorbance of each well was measured at a wavelength of 540 nm using a microplate reader (10043; Bio Rad, Tokyo, Japan). The AsA concentration was calculated from a standard curve created from the measured absorbance.

Statistical analysis. All the data are expressed as the mean ± standard deviation of ≥3 independent experiments.
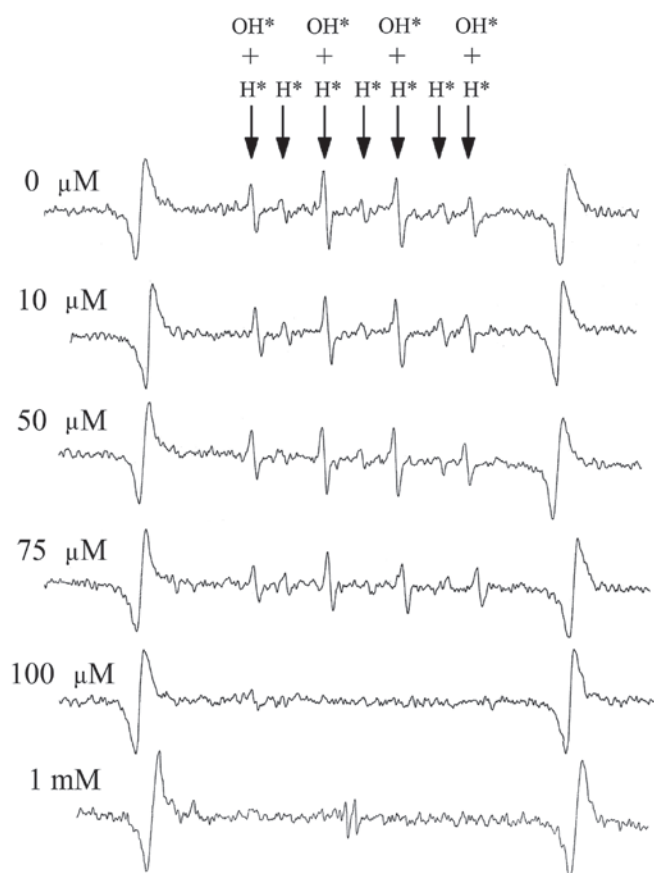

Figure 1. Electron spin resonance spectra in the case of 4 Gy X-ray irradiation with/without ascorbic acid (AsA). On the left, AsA concentrations in phosphate-buffered saline are shown. Arrows show the generation of hydroxyl radicals (OH*) and hydrogen radicals (H*). H* disappeared at 100 μM (0.1 mM) AsA and ascorbate radicals were observed.

Statistical comparisons between groups were performed by an analysis of variance and Student's t-test, where P<0.05 was considered to indicate a statistically significant difference.

Results

ESR spectra. The ESR spectra of PBS following irradiating with a 4 Gy X-ray are shown in Fig. 1. The spectra without AsA treatment shows the typical wave type of radicals generated by X-ray irradiation. There were two types, DMPO-OH (αN = αH = 1.49 mT) and DMPO-H (αN = 1.64, αH = 2.25 mT). However, there were unclear waveforms for the OH and H radicals in PBS with 100 μM (0.1 mM) AsA. With 1 mM AsA, the ascorbate radical in the middle of the ESR spectrum of the OH and H radicals disappeared.

RSIs of OH radicals. Fig. 2 shows the RSIs of the OH radicals generated from the 4 Gy X-ray irradiation with various concentrations of AsA in PBS. The RSIs of the OH radicals were similar for the AsA values <50 μM AsA, suggesting that OH radicals were not scavenged at concentrations <50 μM AsA. The RSIs of the OH radicals decreased with 75 μM AsA with similarly low RSIs at concentrations of 100 μM (0.1 mM) AsA.

Intracellular AsA concentration. The intracellular AsA concentrations in HL60 cells 1 h after extracellular AsA
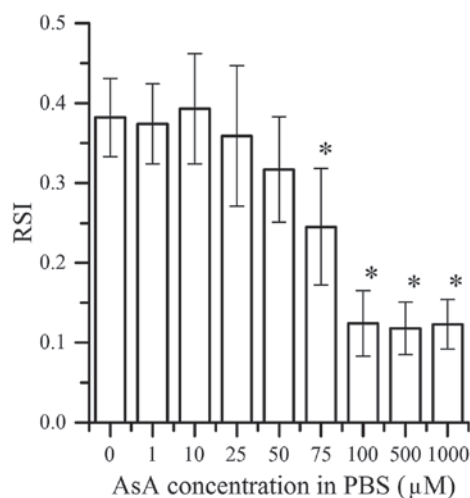
Figure 2. Relative signal intensities (RSIs) of hydroxyl (OH) radicals generated with 4 Gy X-ray irradiation in phosphate-buffered saline (PBS). RSIs of OH radicals were similar <50 μM ascorbic acid (AsA). RSI of OH radicals decreased at concentration of AsA >75 μM. *P<0.05 vs. 0 μM AsA.
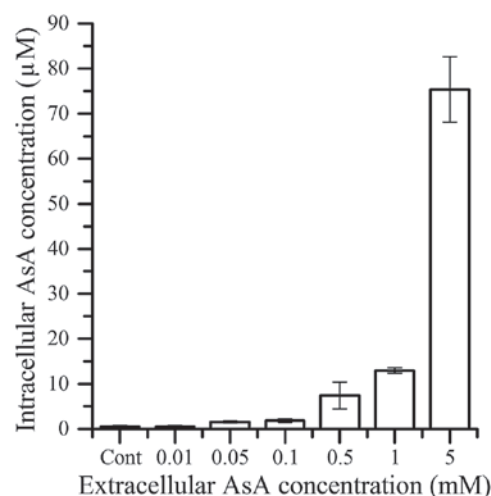

Figure 3. Intracellular ascorbic acid (AsA) concentration in HL60 cells following extracellular AsA treatment. Control (Cont) was 0 mM AsA. The intracellular AsA concentration was 75 μM after the 5 mM AsA treatment.

treatment are shown in Fig. 3. The intracellular concentration of AsA was <20 μM after treatment with 1 mM AsA. However, the intracellular AsA concentration was 75 μM after the 5 mM AsA extracellular treatment.

HL60 cell viability. Fig. 4A shows the cell viability of HL60 cells 24 h after AsA treatment alone. AsA treatment at concentrations >1 mM AsA decreased the number of living cells, and no effect was observed for concentrations <1 mM. The cell viability of HL60 cells 24 h after 4 Gy X-ray irradiation with/without AsA treatment is shown in Fig. 4B. At concentrations >1 mM AsA, combination AsA treatment and 4 Gy X-ray irradiation decreased the number of living cells compared to X-ray irradiation alone. The number of living cells with AsA treatment combined with 4 Gy X-ray irradiation was not higher than that for cells exposed to X-ray irradiation alone.

DNA fragmentation. DNA fragmentation following AsA treatment alone and combination AsA treatment with 4 Gy X-ray irradiation is shown in Fig. 5. AsA treatment increased DNA fragmentation at concentrations >1 mM in a concentration-dependent manner. DNA fragmentation with AsA treatment concentrations >5 mM in combination with 4 Gy X-ray irradiation was more than that of 4 Gy X-ray irradiation alone. When AsA was added to 4 Gy X-ray irradiation, a concentration that induced lower DNA fragmentation than 4 Gy irradiation alone was not identified.

Discussion

When ionizing radiation is delivered to living cells/animals, reactive oxygen species (ROS) such as ·OH, hydrogen radicals (·H) superoxide (O^{2-}), and hydrogen peroxide (H_2O_2) are produced. Initial generation of ·OH is via direct radiolysis of water. Highly reactive OH cannot travel long distances, but instead immediately reacts with other nearby molecules (11). For this reason, ·OH generated by ionizing irradiation near

DNA in cells have a major role in radiation-induced cell damage. Our previous study reported the significant association between the amount of intracellular ·OH and apoptotic cell death (12).

There is renewed interest in the use of high dose AsA (20 M in plasma) in the treatment of numerous different types of cancer (13-15). AsA has been shown to selectively kill cancer cells via the extracellular formation of H_2O_2 (16). It was hypothesized that the presence of a catalyst, such as ferric ion, within the extracellular matrix of tumors could oxidize AsA to ascorbate radicals, which could subsequently donate electrons to oxygen to form O^{2-}. O^{2-} can be converted by superoxide dismutase to the tumoricidal peroxide ion (5). In the present study, ascorbate radicals were detected on the ESR spectrum of 1 mM AsA PBS. The number of living cells decreased, and DNA fragmentation increased at concentrations >1 mM AsA. These results indicate that at concentrations >1 mM AsA treatment induces apoptosis and cell death of HL60 cells. Combination treatment with >1 mM AsA and X-ray irradiation decreased the number of living cells more than exposure to cells with X-ray irradiation alone. DNA fragmentation was also higher in cells treated with combination AsA treatment >1 mM and X-ray irradiation compared to that in cells treated with X-ray irradiation alone. The result confirmed that combination AsA treatment with X-ray irradiation increased apoptosis in HL60 cells.

AsA is present in aqueous compartments (such as cytosol, plasma and other body fluids) and has several metabolically important cofactor functions in enzyme reactions, particularly hydroxylation. AsA has been shown to efficiently scavenge O^{2-}, H_2O_2, hypochlorite, ·OH and peroxyl radicals (17). The radical scavenging effects of AsA are used to protect normal tissue from radiation injury (18). However, no increase in the number of viable HL60 cells following AsA treatment was observed with irradiation when comparing to irradiation alone. Therefore, the amount of ·OH scavenged by AsA was estimated. ·OH generated with 4 Gy X-ray irradiation were scavenged by AsA at concentrations >75 μM in the ESR analysis. The result suggests that AsA concentrations >75 μM in the fluid inside
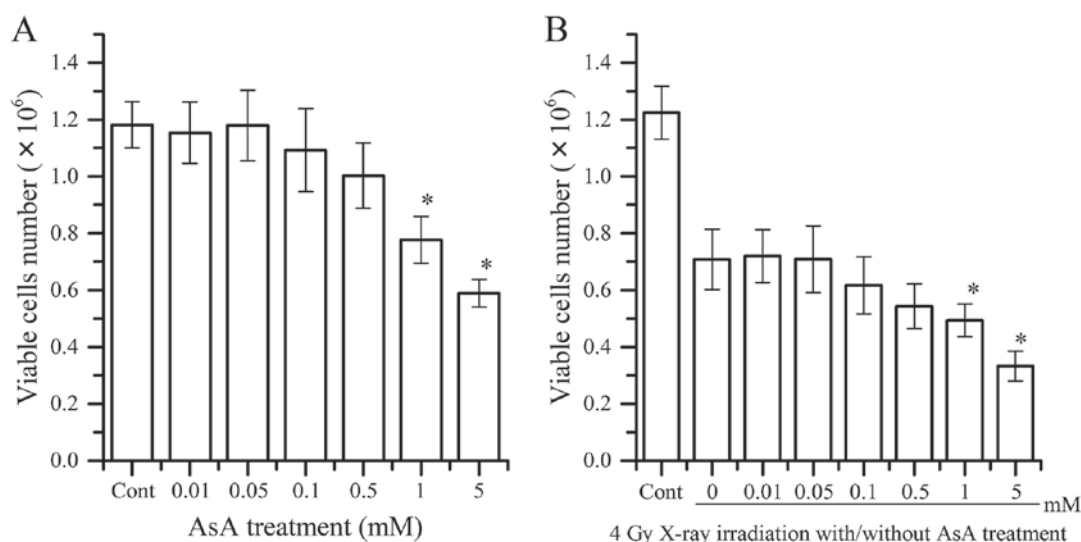
Figure 4. (A) Cell viability of HL60 cells following ascorbic acid (AsA) treatment alone and without X-ray irradiation. Control (Cont) was 0 mM AsA. Following AsA treatment alone, the number of living cells decreased >1 mM AsA; *P<0.05 vs. control. (B) Cell viability of HL60 cells 24 h after 4 Gy X-ray irradiation with/without AsA treatment. Following AsA treatment combined with 4 Gy X-ray irradiation, the number of living cells at concentrations >1 mM AsA was lower than those exposed to 4 Gy X-ray irradiation alone; *P<0.05 vs. 0 mM AsA (4 Gy X-ray irradiation alone).
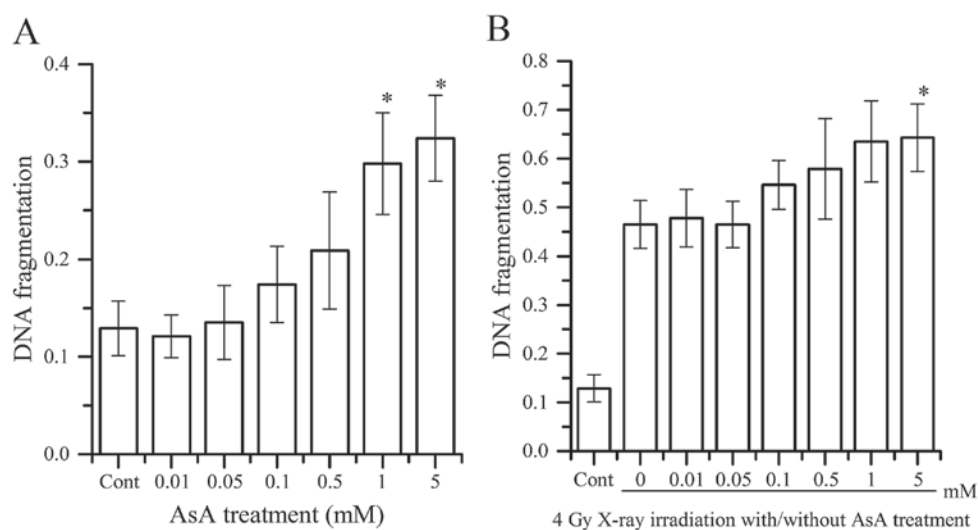

Figure 5. (A) DNA fragmentation 6 h after ascorbic acid (AsA) treatment alone. DNA fragmentation with AsA treatment alone increased in a concentration-dependent manner at concentrations >1 mM; *P<0.05 vs. control. (B) DNA fragmentation 6 h after combination AsA treatment with 4 Gy X-ray irradiation. DNA fragmentation with 4 Gy X-ray irradiation with AsA was not lower than with 4 Gy X-ray irradiation alone; *P<0.05 vs. 0 mM AsA (4 Gy X-ray irradiation alone).

the cell could scavenge ·OH generated with X-ray irradiation. The actual AsA concentration inside the cell was 75 μM when cells underwent extracellular treatment with 5 mM AsA. However, 5 mM AsA significantly induced HL60 cell death even without irradiation. The result was evidenced in the HL60 cells in the present study. However, our previous study reported that 5 mM AsA treatment suppressed the growth of epithelial cancer cells *in vitro* (6). It was suggested that extracellular H_2O_2 generated with >5 mM AsA mainly damaged the cell membrane due to passing more cascades than radiation alone (19,20). The current study revealed that caspase-3, -8 and -9 were activated 2 h after exposure to H_2O_2, whereas in irradiated cells activation of caspase-3 and -9 occurred 4 h after exposure; caspase-8 activation was not observed (19).

These high fluxes of H_2O_2 appear to have a limited effect on normal cells but can be detrimental to certain tumor cells (13).

In conclusion, treating cancer cells with high extracellular concentrations of AsA (>5 mM) could achieve an intracellular AsA concentration level of 75 μM, leading to scavenging of ·OH generated with X-ray irradiation. However, treatment with the high extracellular concentrations of AsA alone directly induced cancer cell death. As aforementioned, the disappearance of radiation anticancer effects with AsA treatment in combination with radiotherapy for cancer treatment is not a concern. Previously, clinical trials on the effects of high-dose intravenous AsA on patients with cancer have been performed (21,22). We hypothesize that a clinical trial to evaluate the high-dose intravenous AsA combined with

radiotherapy in patients with cancer will be launched in the near future.

Acknowledgements

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