

Ascorbic Acid Enhances Radiation-induced Apoptosis in an HL60 Human Leukemia Cell Line

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Ascorbic acid/Radiation/Apoptosis.

This study was conducted to examine the utility of the combined use of ascorbic acid (AsA) and radiation in clinical applications. We investigated cell survival, DNA fragmentation, and caspase activation after X-ray irradiation and AsA treatment of human leukemia HL60 cells. The number of living cells decreased after combined X-ray irradiation and AsA treatment (2 Gy + 5 mM) in comparison with that after X-ray irradiation (2 Gy) or AsA treatment (5 mM) alone. DNA fragmentation was more in the cells subjected to combined X-ray irradiation and AsA treatment than in those subjected to X-ray irradiation alone. Caspase-3, caspase-8, and caspase-9 were highly activated following combined X-ray irradiation and AsA treatment, but caspase-8 activity was not markedly increased after X-ray irradiation alone. Bax levels in the mitochondrial membrane fractions were increased after AsA treatment alone and after combined X-ray irradiation and AsA treatment. However, there was no apparent increase in the Bax levels after X-ray irradiation treatment alone. Thus, this study confirmed that supplementing X-ray irradiation with AsA treatment results in increased apoptosis in HL60 cells. With regard to the apoptosis-inducing factors, we hypothesized that Bax and caspase-8 were activated after combined X-ray irradiation and AsA treatment compared with either treatment alone.

INTRODUCTION

Highly precise irradiation methods in which the irradiation is localized to a malignant tumor have been continually improving owing to the steady progress in engineering technologies, and radiation treatment is an important component of present-day cancer treatment.¹⁾ An advantage of radiation treatment is that it has better aesthetical results than surgery, but the fact of the matter is that sufficient local control has not been achieved.²⁾ Accordingly, concurrent chemoradiation therapy is actively pursued in clinical trials to improve the local control rate without operation, and several reports have demonstrated that the local control rate and survival

rate are improved after these treatments.^{3,4)} However, when simultaneously administering an anticancer agent, strict patient care is required during the acute phase because adverse events occur during this period, and the frequency of late adverse events can increase thereafter.^{5,6)}

It is known that ascorbic acid (AsA) has anticancer effects due to its antioxidative activity, and the results of epidemiological surveys have demonstrated that AsA intake is inversely correlated with carcinogenicity.⁷⁾ Further, cytotoxic activity of AsA against malignant cell lines has been reported.⁸⁾ Sakagami and Satoh (1997) reported that AsA induces apoptotic cell death in HL60 line.⁹⁾ AsA is directly used for cancer treatment, and Padayatty *et al.* (2006) reported 3 cases of malignant tumors which were controlled primarily by treatment with AsA.¹⁰⁾ Furthermore, Padayatty *et al.* (2009) reported side effects, mostly minor, in only 101 patients of the 9,328 patients treated with intravenously administered AsA.¹¹⁾ Therefore, we hypothesized that therapeutic use of AsA in combination with radiation may reduce therapy related side effects and increase the antitumor effects of these treatments. This study was conducted to examine the effect of the combined use of AsA and radiation with the goal of establishing this regimen in the clinics.

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MATERIALS AND METHODS

Cell culture and cell viability assay

Human promyelocytic leukemia HL60 cells were purchased from the RIKEN Bio Resource Center (Tsukuba, Japan). The cells were grown in 100 mm culture dishes containing Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂ atmosphere. They were passaged every third day, and the density in culture was not allowed to exceed 1 × 10⁶ cells/mL. Cell concentration was adjusted to 5 × 10⁵ cells/mL prior to irradiation or AsA treatment. The cells were stained with 2% trypan blue for 1 minute and counted per 1 mm³ in each experiment to estimate the number of unstained cells within 2 minutes. The number of unstained cells was considered to be the viable cell number.

X-rays irradiation and AsA treatment

Dishes or microtiter plates containing HL60 cells were exposed to X-rays from a soft X-ray machine (SOFRON BST-1500 CX; Sofron, Tokyo, Japan) under the following conditions: tube voltage, 120 kVp; tube current, 5 mA; FSD, 30cm; dose rate, 1 Gy/minute; room temperature. focus-to-surface (FSD) was determined as the distance from the focus to the surface of the medium. The dose and dose rate were estimated with the Fricke chemical dosimeter. AsA was dissolved in RPMI-1640 medium. The AsA solution with 0.01–100 mM AsA, was titrated with NaOH at pH 7.4. Cells concentration was adjusted to 5 × 10⁵ cells/mL for the treatments.

Colony assay (Plasma clot culture)

To analyze the suppressive potential and radiation-and/or AsA sensitizing effects in the long term, the clonogenic potential of HL60 cells was assessed. The cells were assayed for colony formation by using a plasma clot technique with human platelet-poor human plasma. Human platelet-poor AB plasma (15%), penicillin (100 U/mL), and streptomycin (100 µg/ml) were added to the culture medium based on RPMI-1640. After AsA treatment and/or X-ray irradiation, each the cells were cultured in the medium at a concentration of 1,000 cells/mL. This baseline culture was plated onto a 24-well plate (Iwaki, Chiba, Japan) at 0.3 mL/well (300 cell/well) and incubated at 37°C for 8 days in a humidified atmosphere with 5% CO₂. The colonies consisting of more than 50 cells were counted using an inversion microscope.

Morphological observation

Treated HL60 cells were subjected to centrifugation at 1,500 × g for 10 minutes together with the culture fluid, fixed with 25% glutaraldehyde after removing the superna-

tant, and then fixed with OsO₄. The cells were embedded in Epon 812 (TAAB, Aldermaston, England) after dehydration in an ascending series of alcohol later replaced with propylene oxide. The sample was cut into 1 micrometer thick semi-ultrathin sections which were stained with toluidine-blue and observed by light microscopy. From these sections, 100 cells were randomly selected and the number of apoptotic-like cells was counted. This number was determined as the standard for apoptotic cells with either of the following observations: 1) aggregation of unevenly distributed chromatin around the nuclear membrane, or 2) fragmentation of a concentrated nuclear membrane.

Quantitative detection of apoptosis

Apoptosis was detected using a cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Roche, Basel, Switzerland). The cells were labeled with bromodeoxyuridine (BrdU) (Roche) a day prior to treatment and then subjected to X-ray irradiation and AsA treatment, and cultured for a given length of time. There after, 1.5 × 10⁶ cells were collected and treated with a cell solution (0.05% Triton-X and 650 µM EDTA) (Research Organics, Cleveland, OH) at 4°C for 30 minutes. The cells were then centrifuged at 4°C and 12,000 × g for 30 minutes 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), and color development caused by the tetramethylbenzidine (TMB, Roche) substrate reaction was measured using a spectrophotometer (450 nm).

Caspase activity assay

The activities of caspases-3, caspase-8, and caspase-9 were measured using colorimetric assay kits (BioVision, Palo Alto, CA) according to the manufacturer's instructions. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate that recognizes the optimal tetrapeptide sequence of the individual activation site. In brief, HL60 cells (6 × 10⁶) exposed to X-rays or treated with AsA were incubated for the indicated times before harvesting. The cells were lysed in a cell lysis buffer for 10 minutes on ice and centrifuged at 10,000 × g for 1 minute at 4°C. Cell lysates (200-µg protein/100-µL reaction buffer) were incubated with 5 µL of 4 mM substrates (final concentration, 200 µM) specific for caspases-3, caspase-8, and caspase-9 for 2 hours at 37°C. The specific substrate was Asp-Glu-Val-*p*NA (DEVD-*p*NA) for caspase-3, Ile-Glu-Thr-Asp-*p*NA (IETD-*p*NA) for caspase-8, and Leu-Glu-His-Asp-*p*NA (LEHD-*p*NA) for caspase-9. The *p*NA light emission was quantified at 405 nm, using a microtiter plate reader.

Specific inhibitors of caspases

To determine the involvement of caspases-3, caspase-8, and caspase-9 in proapoptotic activity, a specific inhibitor

(2 μ M) of each caspase, Z-Leu-Glu-His-Asp-FMK (Z-DEVD-FMK) (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) for caspase-3, Carbobenzoxy-IETD-fluoromethyl ketone (Z-IETD-FMK) (Kamiya Biochemical, Seattle, WA) for caspase-8, and Z-Asp-Glu-Val-Asp-FMK (Z-LEHD-FMK) (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) for caspase-9 was added just prior to irradiation or AsA treatment. For the controls, a vehicle of DMSO was added at a final concentration of 0.1 μ M. The effects of the inhibitors were estimated on the basis of DNA fragmentation up to 8 hours and cell viability 24 hours after treatment, as described above.

Western blot analysis

HL60 cells exposed to radiation or treated with AsA were chased up to 6 hours and were subjected to immunoblot analysis. The cells were collected by centrifugation and were resuspended in 500 μ L of isotonic lysis buffer (210 mM sucrose, 70 mM mannitol, 10 mM HEPES pH 7.4, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail. After homogenization with a sonicator, cell lysates were centrifuged at $800 \times g$ to removed the cell debris. Supernatants were centrifuged at $1,000 \times g$ to discard the nuclei. The postnuclear supernatant was centrifuged at $10,000 \times g$ for 10 minutes at 4°C to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at $100,000 \times g$ for 20 minutes at 4°C to isolate the cytosolic fraction. The membrane fractions were resuspended in 30 μ L of 0.5% Triton X-100 in the isotonic lysis buffer containing protease inhibitors for 10 minutes at 4°C to release the membrane- and organelle-bound soluble proteins, including mitochondrial cytochrome c. The protein concentrations were determined by using a protein assay kit (Bio-Rad, Hercules, CA).

The proteins (10 μ g/lane) were loaded onto 10% SDS-polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in a tank blotter. The membrane was blocked overnight at 4°C with Block Ace (Dainippon Pharmaceuticals, Osaka, Japan) containing 0.1% Tween-20 and then incubated with the following primary antibodies (diluted to 1:1,000 with 10% Block Ace) for 2 hours at room temperature, rabbit anti-human Bax polyclonal antibody (PharMingen International, San Diego, CA), mouse anti-human cytochrome c monoclonal antibody (ProSci Inc, Poway, CA), and rabbit anti-human Bid monoclonal antibody (Sigma-Aldrich, Tokyo, Japan). The blots were rinsed with 10% Block Ace (10 minutes \times 5), incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) (diluted to 1:5,000 with 10% Block Ace) for 60 minutes at room temperature, and rinsed again. The immunoreactive bands were visualized with an ECL detection kit (Amersham Biosciences, Buckinghamshire, England). Densities of the blot bands

were finally determined using a computer-assisted densitometer, DD-system (R-TECH Inc, Tokyo, Japan).

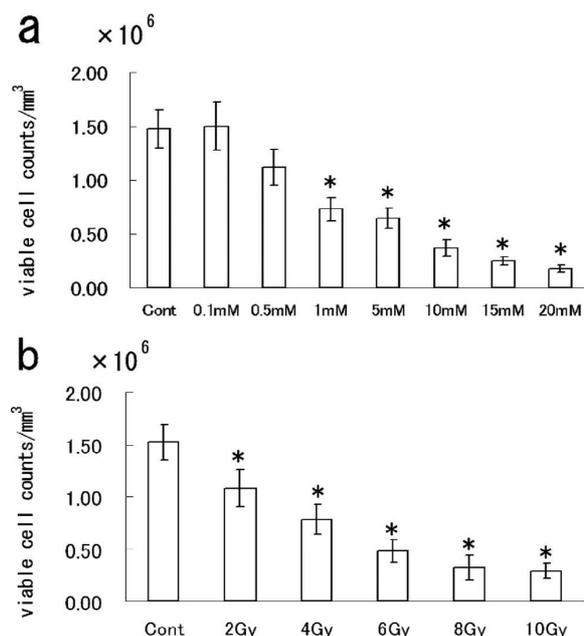


Fig. 1. Number of viable cells 24 hours after (a) AsA treatment and (b) X-ray irradiation. Viability of HL60 cells are depended on the radiation dose and AsA concentration. * $p < 0.05$ vs. control (Cont).

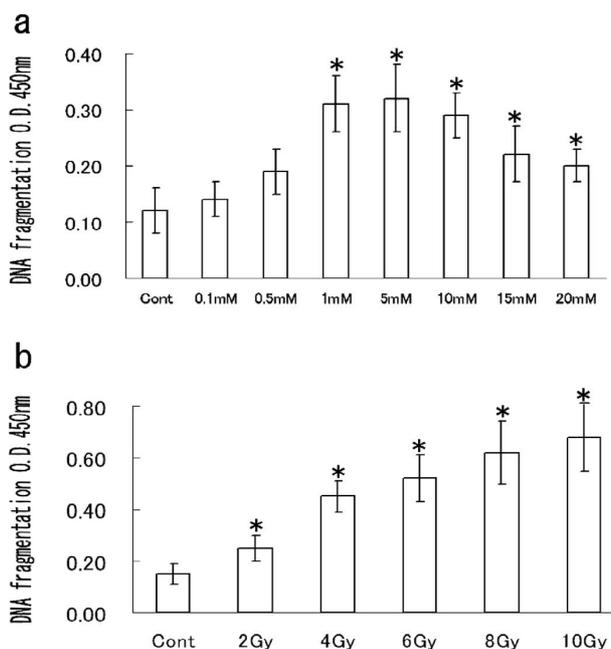


Fig. 2. DNA fragmentation 6 hours after (a) AsA treatment and (b) X-ray irradiation. DNA fragmentation depended on the radiation dose and, to a particular extent, on AsA treatment. However, it decreased at AsA concentrations over the 5 mM. * $p < 0.05$ vs. control (Cont).

Statistical analysis

All data are expressed as the means \pm standard deviation (SD) of at least 3 independent experiments. Statistical comparisons between groups were performed by an analysis of variance (ANOVA) and Student's t-test ($p < 0.05$).

RESULTS

Cell death by X-ray irradiation and AsA treatment

Cell death by X-ray irradiation alone, AsA treatment

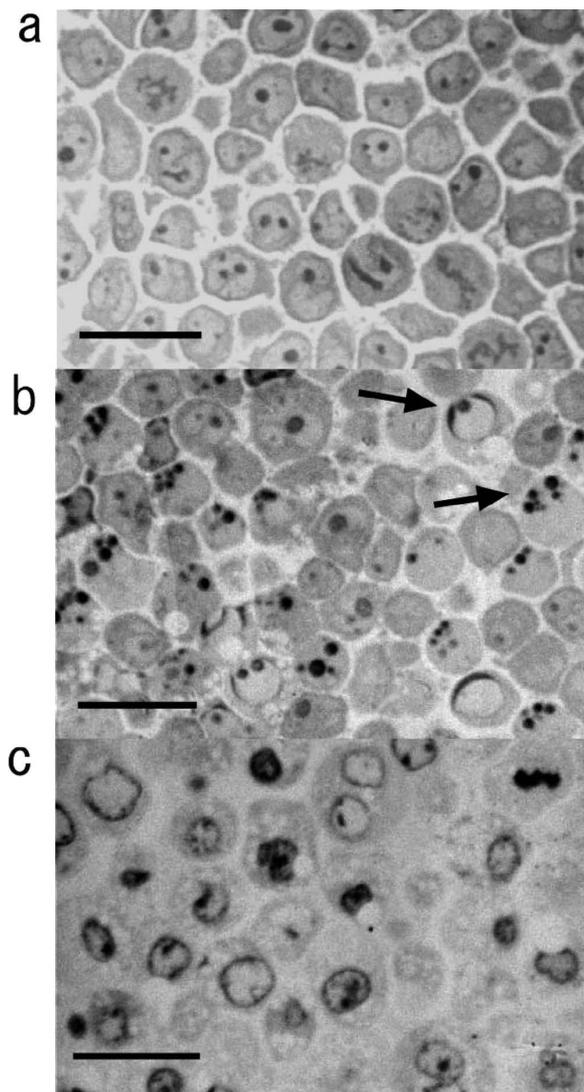


Fig. 3. Morphological findings of semi-ultrathin sections (stained with toluidine-blue). (a) Control HL60 cells. (b) HL60 cells treated with 5 mM AsA alone, there are many apoptotic-like cells showing nuclear fragmentation (lower arrow) and aggregation of chromatin (upper arrow). (c) HL60 cells treated with 20 mM AsA alone, cell membrane and cell organelle are unclear and typical apoptotic-like cells are not found. The horizontal bar in the lower left side represents of 5 μ m.

alone and combination treatment was examined. For a dose (in X-ray irradiation) or a concentration (in AsA treatment), living cells were examined after 24 hours and DNA fragmentation was examined six hours after treatment. The results are shown in Figs. 1 and 2. Following X-ray irradiation treatment, the number of living cells decreased and DNA fragmentation increased in a dose-dependent manner (Figs. 1b and 2b, respectively). On the other hand, although treatment with AsA decreased the number of living cells in a concentration-dependent manner, DNA fragmentation increased in a concentration-dependent manner only up to 5 mM (Figs. 1a and 2a). Treatment with AsA at concentrations more than 5 mM decreased DNA fragmentation (Fig. 2a). The morphological changes observed in the semi-ultrathin sections of HL60 cells are shown in Fig. 3. After treatment with 5 mM AsA, the sections showed morphological changes consistent with apoptosis, such as unevenly distributed chromatin around the nucleus and nuclear fragmentation. Conversely, after treatment with 20 mM AsA, opaque cell membranes and unclear organelles were seen. The results of the quantification of apoptotic-like cells are shown in Fig. 4. After X-ray irradiation with a dose of 10 Gy or less, many apoptotic-like cells were observed. Although treatment with 5 mM AsA yielded many apoptotic-like cells, few apoptotic-like cells were found after treatment with 20 mM AsA. Compared with the control cells, there were statistically significant differences ($p < 0.05$) in the number of apoptotic-like cells after treatment with 2-Gy X-ray irradiation, 10-Gy X-ray irradiation, and 5 mM AsA treatment, but there was no significant difference in the number of apoptotic-like cells after 20 mM AsA treatment.

Cell death and DNA fragmentation after combined X-ray irradiation and AsA treatment

Cell viability and DNA fragmentation following X-ray

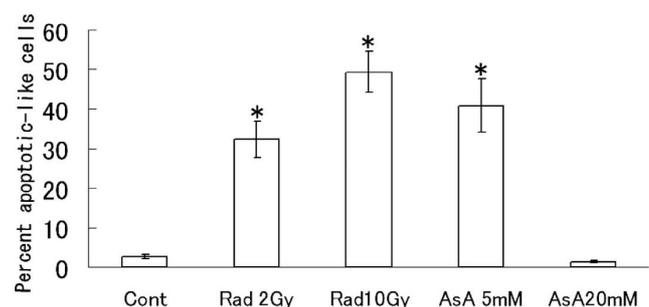


Fig. 4. Number of apoptotic-like cells 24 hours after X-ray irradiation or AsA treatment alone. From the semi ultrathin sections, 100 cells were randomly selected, and the number of apoptotic-like cells was counted after X-ray irradiation alone (Rad) or AsA treatment alone (AsA). Many apoptotic-like cells were seen after exposure to 2 Gy and 10 Gy X-ray irradiation. Further many apoptotic-like cells were seen after 5 mM AsA treatment, however very few apoptotic-like cells were observed after 20 mM AsA treatment. * $p < 0.05$ vs. control (Cont).

irradiation alone (2 Gy), AsA treatment alone (5 mM), and combined X-ray irradiation and AsA treatment (2 Gy + 5 mM) are shown in Figs. 5 and 6. The number of living cells and colonies decreased after combined X-ray irradiation and AsA treatment in comparison with that X-ray irradiation or

AsA treatment alone (Fig. 5). Compared with either treatment alone, the combined X-ray irradiation and AsA treatment induced statistically significant differences in cell death ($p < 0.05$). However, the colony assay revealed that

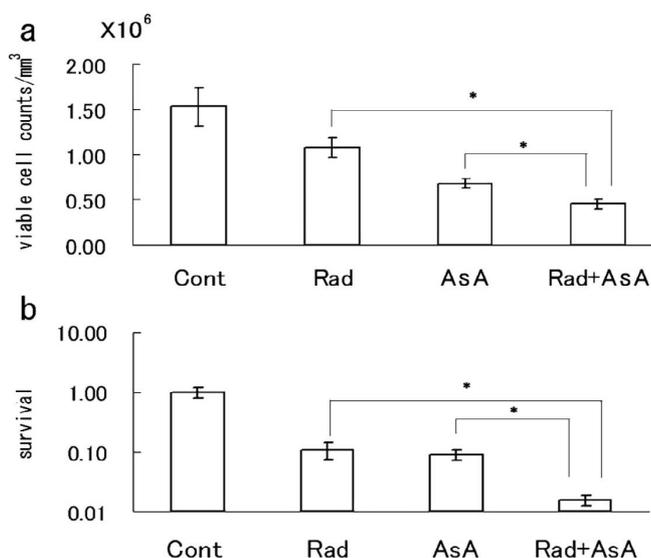


Fig. 5. Cell viability after X-ray irradiation alone (2 Gy, Rad), AsA treatment alone (5 mM, AsA), and combined X-ray irradiation and AsA treatment (2 Gy + 5 mM, Rad + AsA). (a) Number of viable cells and (b) the number of colonies after combined X-ray irradiation and AsA treatment decreased in comparison with those after X-ray irradiation alone. The plating efficiencies are as follows: control 9.07%, Rad 0.50%, AsA 0.42%, Rad + AsA 0.04%. Statistically significant difference ($p < 0.05$) was seen in cell viability between X-ray irradiation alone and combined X-ray irradiation and AsA treatment.

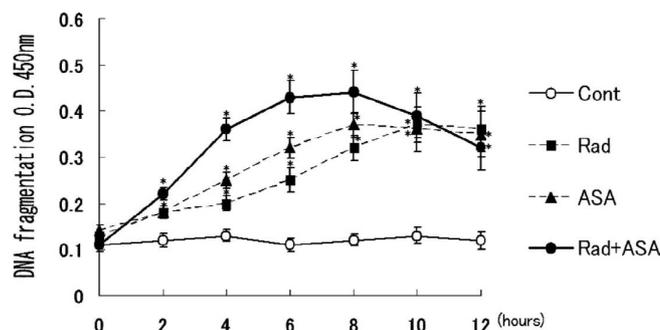


Fig. 6. DNA fragmentation after X-ray irradiation alone (2 Gy, Rad), AsA treatment alone (5 mM, AsA), and combined X-ray irradiation and AsA treatment (2 Gy + 5 mM, Rad + AsA). The data show more DNA fragmentation in the cells subjected to combined X-ray irradiation and AsA treatment cells than in those subjected to X-ray irradiation alone. A tendency of early induction of DNA fragmentation by combined X-ray irradiation and AsA treatment was observed. * $p < 0.05$ vs. control (Cont).

Table 1. Analysis of colony assay results

Colony formation rate	Treatment	Average	SD
A	Cont/Rad	0.173	0.041
B	(AsA + Rad)/AsA	0.341	0.069

SD, standard deviation; Cont, control; Rad, X-ray irradiation alone (2 Gy); AsA, AsA treatment alone (5 mM); AsA + Rad, combined AsA and X-ray irradiation treatment (5 mM + 2 Gy).

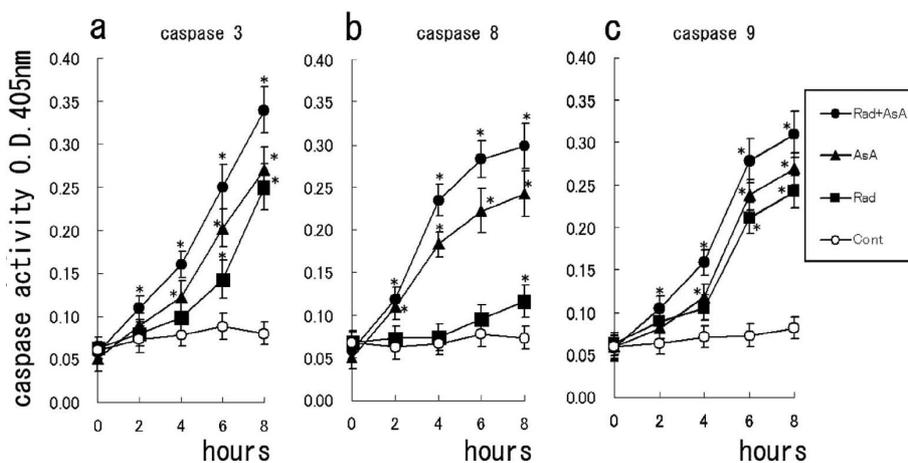


Fig. 7. Time-course changes in caspase-3, caspase-8 and caspase-9 activity. (a) Caspase-3 activity, (b) caspase-8 activity and (c) caspase-9 activity in HL60 cells 2-8 hours after treatment. The data show that activation of caspase-8 occurred at an early stage after AsA treatment alone (5 mM, AsA) and combined X-ray irradiation and AsA treatment (2 Gy + 5 mM, Rad + AsA). However, no remarkable increase in caspase-8 activity was observed after X-ray irradiation alone (2 Gy, Rad) * $p < 0.05$ vs. control (Cont).

colony formation rate B (combination/AsA) did not considerably exceed rate A (Cont/Rad) (Table 1). This result showed that the effect of AsA was additive and not synergistic. DNA fragmentation peaked approximately 8–10 hours both after either treatment alone and after combined AsA and X-ray irradiation treatment, and it tended to occur earlier following damage after combined AsA and X-ray irradiation treatment (Fig. 6).

Caspase activity and apoptosis-related proteins

Time-course changes in caspase activity in cells determined using colorimetric assay kit is shown in Fig. 7. Caspases-3, caspase-8, and caspase-9 activity increased after

combined X-ray irradiation and AsA treatment. Caspase-3 activity and caspase 9 activity considerably increased with time following all the treatment; while caspase-8 activity considerably increased after combined X-ray irradiation and AsA treatment, but was not markedly increased following X-ray irradiation alone. The levels of DNA fragmentation after

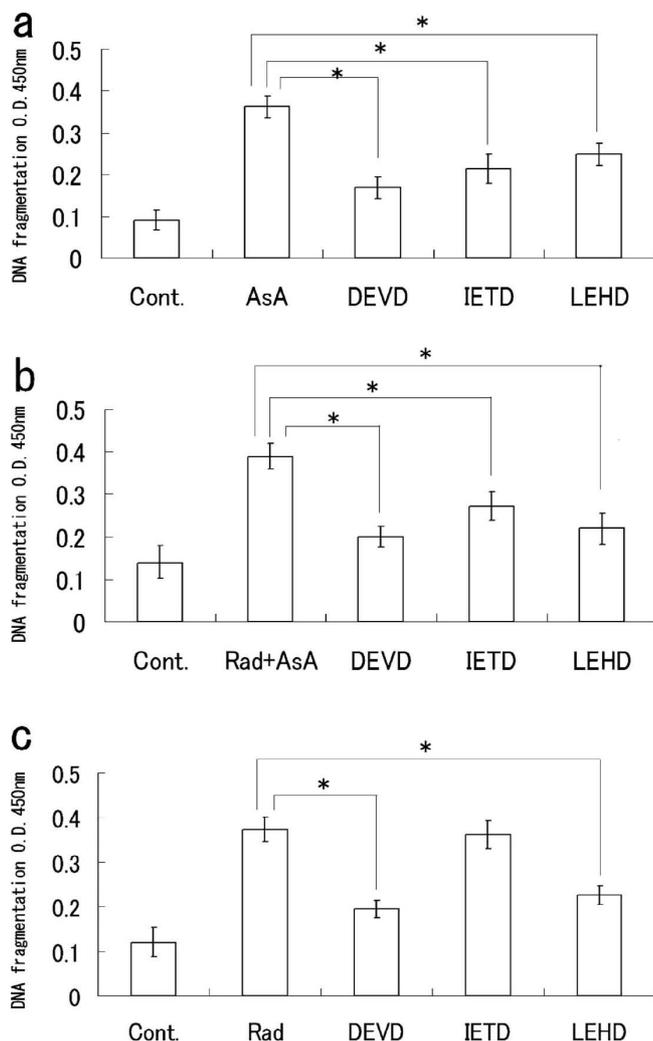


Fig. 8. Effects of caspase inhibitors. (a) DNA fragmentation in HL60 cells after 5 mM AsA treatment alone. (AsA) (b) DNA fragmentation in HL60 cells after 2-Gy X-ray irradiation alone (Rad). (c) DNA fragmentation in HL60 cells after combined 2 Gy X-ray irradiation and 5 mM AsA treatment (AsA). DEVD inhibitor of caspase-3, IETD inhibitor of caspase-8, LEHD inhibitor of caspase-9. * $p < 0.05$.

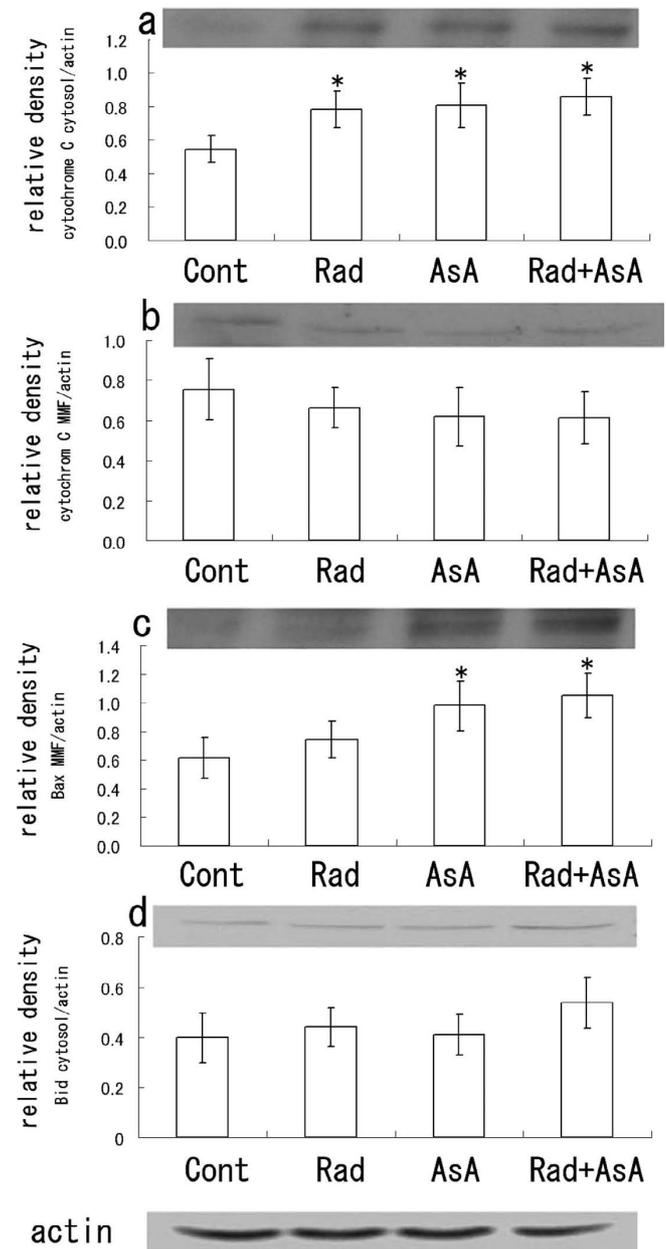


Fig. 9. Immunoblots and relative densities (protein/actin). (a) Cytochrome c in cytosolic fraction, (b) cytochrome c in mitochondria-enriched heavy membrane fraction (MMF), (c) Bax in MMF and (d) full length of Bid protein in cytosolic fraction. * $p < 0.05$ vs. control (Cont). Rad, X-ray irradiation alone (2 Gy); AsA, AsA treatment alone (5 mM); Rad + AsA, combined X-ray irradiation and AsA treatment (2 Gy + 5 mM).

10 hours after treatment with the inhibitors of caspase-3, caspase-8, and caspase-9 are shown in Fig. 8. When a caspase-3 or caspase-9 inhibitor was present, DNA fragmentation in cells decreased in all the treatment group. On the other hand, treatment with a caspase-8 inhibitor caused a decreased in DNA fragmentation in cells in all the treatment groups, except in the case of X-ray irradiation alone. In addition, no statistically significant difference in DNA fragmentation was observed on comparing the cells subjected to X-ray irradiation in the presence or absence of a caspase-8 inhibitor. The results of Western blot analysis of fractionated cells 6 hours after treatment are shown in Fig. 9. In comparison with the control cells, the treated cells showed an efflux of cytochrome c to the cytosolic fractions, conversely, cytochrome c present in the mitochondrial membrane fractions decreased in the treated cells. Increased Significant increase in Bax levels was observed in the mitochondrial membrane fractions following AsA treatment alone and combined X-ray irradiation and AsA treatment. However, no marked increase in the Bax levels was observed after X-ray irradiation alone. In addition, there were no significant differences in the levels of Bid between the treatment groups compared with the control.

DISCUSSION

Cameron and Pauling (1987) first observed that AsA has antitumor activity and applied this finding to a clinical setting, and their studies date back approximately 30 years.¹²⁾ At that time, they touted the effectiveness of AsA for other purposes in addition to cancer treatment, but the anticancer activity of AsA was not confirmed by clinical tests at the Mayo Clinic, and AsA had not been considered for primary clinical therapies until recently.¹³⁾ However, it is possible to administer a high intravenous dose of AsA, and there have been effects to apply AsA to cancer treatment.¹⁴⁾ Although the maximal blood AsA concentration following oral AsA administration is limited to approximately 1 mM, it can be raised approximately 10-fold by an intravenous injection, although this achievable concentration is temporary. Hoffer *et al.* (2008) reported that the blood AsA concentration can be maintained at approximately 5–10 mM for several hours by an intravenous injection of AsA at a dose of 1.5 g/kg as demonstrated in a one-phase clinical test on 24 subjects.¹⁵⁾

Our results showed that after treatment with AsA, the number of living cells decreased with increasing concentration, but DNA fragmentation, which is an indicator of apoptosis, decreased at AsA concentrations above 5 mM. Morphologically, many apoptotic cells were observed at AsA concentrations of 5 mM, but were rare events after treatment with 20 mM AsA. It is likely that 5–10 mM is the upper limit of AsA concentration for inducing apoptosis, and concentrations exceeding this cause necrosis. This concentration is in agreement with the blood AsA concentration

described in the clinical study by Hoffer *et al.*¹⁵⁾ In addition, Padayatty *et al.* stated that 1 mM or higher blood AsA concentration by an intravenous injection is necessary for cancer treatment and an blood AsA concentration of approximately 5 mM is optimal.¹⁰⁾

Radiation is widely used for local cancer treatment, and cell death by radiation is classified into reproductive and interphase deaths.¹⁶⁾ Interphase death is a morphologic type of cell death in which the cell undergoes apoptosis in a short time following irradiation. However, reproductive death is not an entirely accurate term: molecular biological studies have demonstrated that a cell does not die biologically but only halts its cell cycle.¹⁷⁾ Because there is a possibility of recurrence following radiation treatment in the case of reproductive death, a desirable result of the treatment is interphase death, which is cell death from apoptosis.¹⁸⁾ In order to assess this radiation-induced apoptosis, HL60 cells were used in this study as a model system that is prone to apoptosis.¹⁹⁾ Our results showed that, at a doses up to 10 Gy, the primary cause of HL60 cell death was apoptosis. One dose is in standard radiation treatment is 2 Gy; therefore, even this dose can induce interphase death in HL60 cells by apoptosis.²⁰⁾

These studies reveal the clinical possibility that, if AsA is used at a final blood concentration of 5 mM, apoptosis will be optimally increased and the effects of radiation treatment will be improved in comparison with radiation treatment alone. In the clinical tests conducted by Hoffer *et al.*,¹⁵⁾ even when AsA was administered at a blood concentration of 5 mM, no side effects were found in humans. From these results, it is considered that this method is worth examining to identify its clinical applications, one of which may be combined radiation treatment, which has few side effects and the strongest antitumor effect.

Observed in this study, the peak times of apoptosis were 8–10 hours after treatment in all the cases. We examined signal transduction in the initial phases of apoptosis, within 10 hours following the treatments. Moreover, we specifically examined caspase activity and apoptosis-related proteins. We observed that caspase-8 activity and caspase-9 activity increased at earlier time points, and caspase-3 activity increased at a later stage. This result is consistent with those of other studies because caspase-8 and caspase-9 are initiator caspases, which activate effector caspases, and caspase-3 is an effector caspase, which ultimately induces DNA fragmentation.²¹⁾ Following X-ray irradiation, however, both the caspase-9 activity and caspase-3 activity increased but no remarkable increase was observed in caspase-8 activity. In the presence of a caspase-8 inhibitor, DNA fragmentation decreased after AsA treatment but no change in DNA fragmentation was observed after X-ray irradiation. We considered that AsA induction of apoptosis activates caspases-3, caspase-8, and caspase-9, while X-ray irradiation-induced apoptosis is dependent on caspases-3 and caspase-9 but not

on caspase-8.

It is known that activation of an effector caspase and fragmentation of poly (ADP-ribose) polymerase (PARP), an effector caspase substrate, follows cytochrome c release from mitochondria, which is Bax dependent.²³⁾ Cytochrome c binds to apoptosis protease-activation factor-1 (Apaf-1), forms an Apaf-1/cytochrome c complex, and activates caspase-9.²⁰⁾ According to our western blot analysis results, an outflow of cytochrome c was observed in the cytosolic fractions of the treated cells, and conversely, cytochrome c decreased in the mitochondrial membrane fractions of all the treated cells; cytochrome c was present at higher levels in the cytosol after combined X-ray irradiation and AsA treatment, and caspase-9 activity increased thereafter. An increase in the Bax levels was observed in the mitochondrial membrane fractions after treatment alone and after combined X-ray irradiation and AsA treatment, however, no major effects were observed after X-ray irradiation alone. This suggested that Bax is not involved in the mechanism of apoptosis following irradiation. However, Bid contributes to and promotes the induction of apoptosis in an extracellular manner, and it is partially reduced by caspase-8. Furthermore, Bid fragments induce cytochrome c outflow from the mitochondria.²⁴⁾ Both Bax and Bid induce cytochrome c outflow by increasing the mitochondrial membrane permeability to facilitate apoptosis, and although it was previously hypothesized that Bax and Bid interact, recent reports have found that they do not associate with each other. It will be necessary to study this mechanism in the future.^{25,26)}

It is known that the cytotoxic activity of AsA against tumor cell lines is inhibited by catalase, an enzyme that degrades hydrogen peroxide (H₂O₂).⁹⁾ Ascorbate-mediated cell death is suggested to be caused by protein-dependent extracellular H₂O₂ generation, via ascorbate radical formation by electron donation by ascorbate.²⁷⁾ On the other hand, AsA is known to scavenge reactive radical species. In our *in vitro* ESR study, OH radicals generated in medium by radiation were scavenged by added AsA, however, we did not observe an increase in the number of viable HL60 cells on comparing AsA addition with irradiation alone (data not shown). Our findings suggest that AsA does not exert a biologically protective effect against the radical species generated by radiation. It will therefore be necessary to study this mechanism further in the future.

This study experiments confirmed that combination of AsA treatment with X-ray irradiation results in increased apoptosis in HL60 cells. Regarding the apoptosis mechanism, it was considered that the involvement of Bax and caspase 8 were different following X-ray irradiation or AsA treatment alone as compared with those following combined X-ray irradiation and AsA treatment. It has been confirmed that the AsA concentration used in this study does not produce any serious side effects. Recently, a similar AsA concentration was administered to cancer patients for treatment.

The possibility of clinical applications will be examined in the future.

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