

Involvement of Reactive Oxygen Species (ROS) in the Induction of Genetic Instability by Radiation

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Ascorbic acid phosphate (APM)/Reactive oxygen species (ROS)/Genetic instability/Delayed reproductive cell death.

Radiation generates reactive oxygen species (ROS) that interact with cellular molecules, including DNA, lipids, and proteins. To know how ROS contribute to the induction of genetic instability, we examined the effect of the anti-ROS condition, using both ascorbic acid phosphate (APM) treatment or a low oxygen condition, on the induction of delayed reproductive cell death and delayed chromosome aberrations. The primary surviving colonies of mouse m5S-derived cl. 2011–14 cells irradiated with 6 Gy of X-rays were replated and allowed to form secondary colonies. The anti-ROS treatments were applied to either preirradiation culture or postirradiation cultures for primary or secondary colony formation. Both anti-ROS conditions relieved X-ray-induced acute cell killing to a similar extent. These anti-ROS conditions also relieved genetic instability when those conditions were applied during primary colony formation. However, no effect was observed when the conditions were applied during preirradiation culture and secondary colony formation. We also demonstrated that the amounts of ROS in X-ray-irradiated cells rapidly increase and then decrease at 6 hr postirradiation, and the levels of ROS then gradually decrease to a baseline within 2 weeks. The APM treatment kept the ROS production at a lower level than an untreated control. These results suggest that the cause of genetic instability might be fixed by ROS during a 2-week postirradiation period.

INTRODUCTION

Ionizing radiation generates reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radical that show high reactivity to a variety of cellular macromolecules materials, including DNA, lipids, and proteins.^{1,2)} ROS are implicated to play a crucial role in cellular radiation responses, including signal transduction.^{3–5)}

It has been well documented that radiation induces genetic instability that leads to delayed biological effects, including cell death, chromosome aberration, and gene mutation.^{6,7)} Although the cause to induce genetic instability is still poorly

understood, it is shown that ROS is involved in the induction of genetic instability.⁸⁾

Ascorbic acid (AsA), carotenes, and tocopherols are natural products having the ability to protect cells from radiation-induced genotoxic damage. AsA, a water-soluble dietary antioxidant that plays an important role as a first defense against ROS, reduces cellular damage produced by ionizing radiation and H₂O₂ *in vivo*^{9,10)} and *in vitro*.¹¹⁾ A supplementation of the diet with AsA decreases endogenous and induced levels of DNA damage in human lymphocyte.¹¹⁾ These protective effects of AsA are believed to be due to its scavenging ability of ROS before they attack cellular macromolecules.

We have previously reported that AsA suppresses radiation-induced gene mutation at hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) locus.¹²⁾ Although long-term processing is impossible because of AsA toxicity, a long-term treatment can be attained by the use of ascorbic acid phosphate (APM), a nontoxic reagent.⁵⁾

In the present study, we examined the effects of the anti-ROS condition, using APM treatment and a low oxygen (2%) condition, on the induction of delayed reproductive cell death and delayed chromosome aberrations.

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Abbreviations: ROS, reactive oxygen species; APM, ascorbic acid phosphate magnesium salt; AsA, Ascorbic acid; H₂DCFDA, 2',7'-dichlorofluorescein diacetate; DCF, The fluorescence of the 2',7'-dichlorofluorescein.

MATERIALS AND METHODS

Cells and cell culture

The cells used were derived from a cell line (cl. 2011–14) isolated from immortalized mouse m5S cells, which retain near-diploid karyotype and were transferred with a human chromosome 11 tagged with a blasticidine-resistant gene. The cells were cultured in α -modified minimum essential medium (α -MEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES, penicillin (100 units/ml), streptomycin (100 μ g/ml), and blasticidin-S-hydrochloride (3 μ g/ml) at 37°C in a humidified atmosphere with 5% CO₂. Under this culture condition, the average cloning efficiency of cl.2011–14 cells was around 0.75. For the low oxygen culture condition, they were cultured in an incubator (Espec BNA-121D, Tabai, Osaka) with 5% CO₂, 2% O₂, and 93% N₂ maintained by supplying nitrogen gas from a nitrogen gas generator (M3NT-8, Kojima, Kyoto).

X-irradiation

The cells were irradiated with X-rays by use of an X-ray generator (M-150WE, Softex, Osaka) at 150 kVp and 5 mA with a 0.1 mm Cu filter at room temperature, yielding an absorbed dose rate of 0.46 Gy/min.

Assay for cell survival

The killing effect of X-rays was determined by a colony formation assay. The cells were irradiated with graded doses of X-rays, inoculated into 100 mm dishes, and incubated for 2 weeks. Colonies containing more than 50 cells were scored as survivors.

To examine delayed reproductive cell death, we harvested and again replated and incubated primary surviving colonies irradiated with 6 Gy of X-rays, which is equivalent to a 10% survival dose, for another 2 weeks as secondary colonies.

Application for a radical scavenger

L-ascorbic acid 2-phosphate magnesium salt (APM; WAKO Chemicals, Osaka) was used as a radical scavenger. In an assay for acute lethal damage, the cells were treated with 200 μ M APM for 2 weeks before (pretreatment) or after (post-treatment) X-irradiation. In an assay for delayed lethal damage, the cells were examined three different ways with regard to treatment with 200 μ M APM, i.e., pretreatment (2 weeks), posttreatment in primary colony formation (2 weeks), and posttreatment in secondary colony formation (2 weeks). APM is nontoxic under the disposal condition used by this experiment (5).

Delayed chromosome aberrations

Cl. 2011–14 cells were irradiated with 6 Gy of X-rays, replated into a 100-mm ϕ plastic dish and allowed to form primary colonies for 2 weeks in the presence of 200 μ M APM.

The primary surviving colonies were then harvested, again replated, and incubated for another 2 weeks as secondary colonies in the absence of APM. Chromosome samples were prepared from the cells collected from secondary colonies. To detect the delayed instability of a human chromosome 11 in cl. 2011–14 cells, we performed whole chromosome painting as described previously.¹³⁾ Briefly, the slide was immersed in 2 \times SSC/0.5%NP-40 (pH 7.0) at 37°C for 30 min, dehydrated by successive treatments with 70%, 85%, and 100% ethanol for each 2 min, and dried with an air jet. The slide was then immersed in denaturing solution (70% formamide in 2 \times SSC, pH 7.3) at 72°C for 3 min. After dehydration and air drying as described above, FITC-labeled DNA probe (5 μ l) that was specific for human chromosome 11 (Q-BIOgene) was denatured at 72°C for 10 min and applied to a chromosome slide. The slide was covered with the probe mixture and a cover glass and sealed with rubber cement to avoid evaporation. The hybridization was performed at 37°C for 12–16 h in a humidified atmosphere. After hybridization, the cover glass was removed, and the slide was incubated at 43°C in 50% formamide in 2 \times SSC (pH 7.0) for 15 min, followed by incubation at 60°C in 0.1 \times SSC (pH 7.0) for 15 min. Ten μ l of 4, 6-diamidino-2-phenylindone (DAPI; Vysis, USA) in antifade solution were then applied on the target area of the slide for counter stain. Whole-chromosome painting was visualized with a fluorescent microscope (Olympus, Tokyo), and digital images were recorded with a CCD camera (Photometrics, USA.).

Measurement of intracellular ROS generation

The cells were grown in a 100 mm dish in the presence or absence of 200 μ M APM after X-radiation. They were rinsed with PBS(–) twice and incubated for an hour in serum-free α -MEM containing 10 μ M 2',7'-dichlorofluorescein diacetate (H₂DCFDA; Molecular Probes, OR, USA). After incubation, the cells, harvested by scraper, were washed twice by centrifugation with PBS(–), lysed in RIPA buffers (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride, and centrifuged at 15,000 rpm for 10 min at 4°C to collect the supernatant. The protein concentration in the supernatant was determined by the BCA (bicinchoninic acid) protein assay (Pierce, IL, USA). The fluorescence intensity of the 2',7'-dichlorofluorescein contained in the supernatant was measured with a fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan). The excitation and emission wavelengths used were 503 nm and 524 nm, respectively.

Fluorescence staining of intracellular ROS generation

The cells were grown on a cover glass (22 mm \times 22 mm) in a 35 mm dish in the presence or absence of 200 μ M APM after X-radiation. They were rinsed with PBS(–) twice and incubated for an hour in serum-free α -MEM containing 10

μM 2',7'-dichlorofluorescein diacetate (H₂DCFDA; Molecular Probes, OR, USA). Then 10 μM MitoTracker Red CMXRos (Molecular Probes, OR, USA) was added to the medium, and the cells were incubated for another 10 min, washed with PBS(–) twice, and fixed with ethanol. The cover glass was mounted on a slide and the cells were visualized with a fluorescence microscopy.

RESULTS

Effects of APM treatment and the low oxygen culture on radiosensitivity

To determine the effects of the anti-ROS condition on cell survival after X-irradiation, we performed an *in vitro* colony formation assay. Figure 1 shows the protective effects of the anti-ROS treatments, such as APM or the low oxygen (2%), on the clonogenic survival of cl. 2011–14 cells irradiated with graded doses of X-rays. Pretreatment with APM was found to be more effective than the posttreatment with regard to the

survival of X-irradiated mouse cl. 2011–14 cells. The D_{10} value, the dose producing 10% survival, of the cells treated with APM for 2 weeks before irradiation was increased to 8 Gy from the 6 Gy for the untreated cells (Fig. 1A); in contrast, in the low oxygen culture, it was more effective to increase the dose to about 9 Gy (Fig. 1B). In contrast to the pretreatment, posttreatment with APM for 2 weeks gave no significant change in the radiosensitivity of the cells (Fig. 1C). Similarly, the posttreatment culture cells in the low oxygen condition for 2 weeks after X-irradiation showed a less protective effect on the cell survival than the preculture condition did, and the APM treatment was no more effective (Fig. 1D). These results indicate that pretreatment rather than posttreatment of APM in the normal oxygen condition and preculture in the low oxygen condition give rise to a protective effect against acute lethal damage.

Effects of APM treatment and the low oxygen culture on delayed reproductive cell death

We examined the effects of pretreatment and posttreatment with APM combined with the culture in the low oxygen culture on radiation-induced delayed reproductive cell death in cl. 2011–14 cells. The posttreatment was separated into two periods, namely, primary colony formation and secondary colony formation.

As shown in Fig. 2, in the normal oxygen condition it is obvious that posttreatment with APM during primary colony formation significantly increased the cloning efficiency (CE) and that neither pretreatment nor posttreatment during sec-

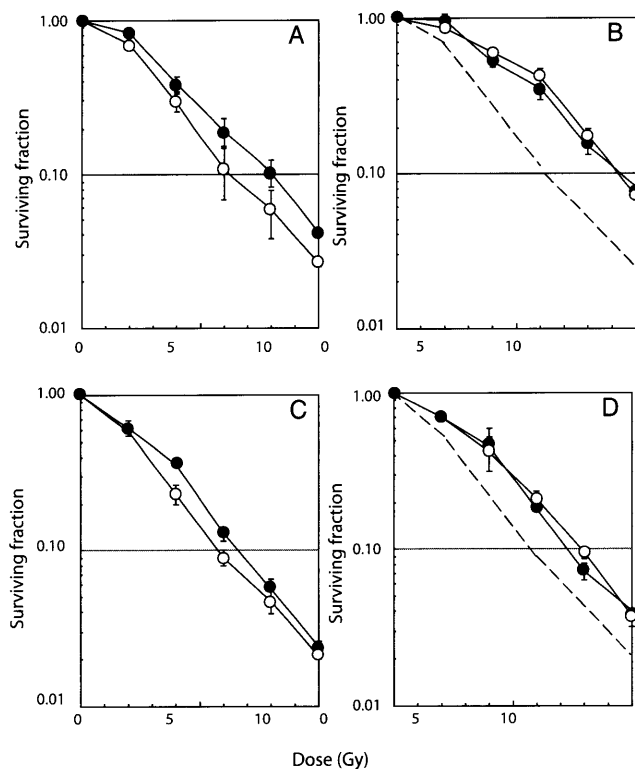


Fig. 1. Effects of antioxidant condition on radiation-induced acute cell death in cl. 2011–14 cells. Pretreatment of 200 μM APM for 2 weeks (A) in a normal oxygen condition and (B) in a low oxygen (2%) condition; and posttreatment of 200 μM APM for 2 weeks (C) in a normal oxygen condition and (D) in a low oxygen condition. The results were represented as mean \pm standard errors from three independent experiments. Open symbols (open circles) and closed symbols (solid circles) represent groups untreated and treated with 200 μM APM, respectively. Dashed lines represent a survival curve of untreated control cells in a normal oxygen condition.

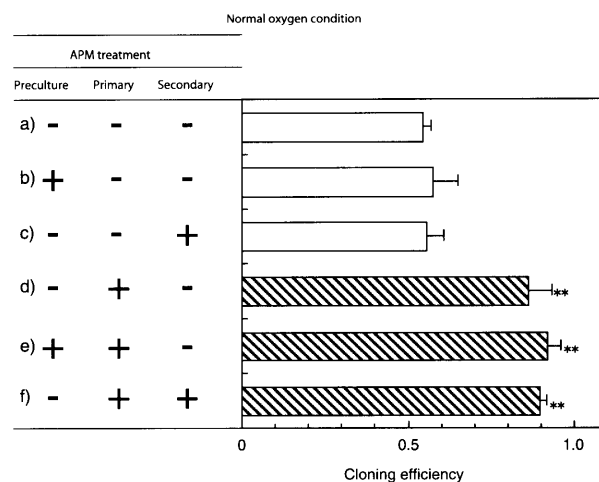


Fig. 2. Effect of APM on radiation-induced delayed reproductive cell death in cl. 2011–14 cells in a normal oxygen condition. We applied 200 μM APM in three periods; preculture for 2 weeks, primary colony formation for 2 weeks, and secondary colony formation for 2 weeks. Plus (+) and minus (–) symbols represent the presence and absence of 200 μM APM treatment, respectively. The results are represented as mean \pm standard errors from three independent experiments. The asterisks (*) indicate a significant difference from control by Student's *t*-test analysis. * $p < 0.01$, ** $p < 0.005$.

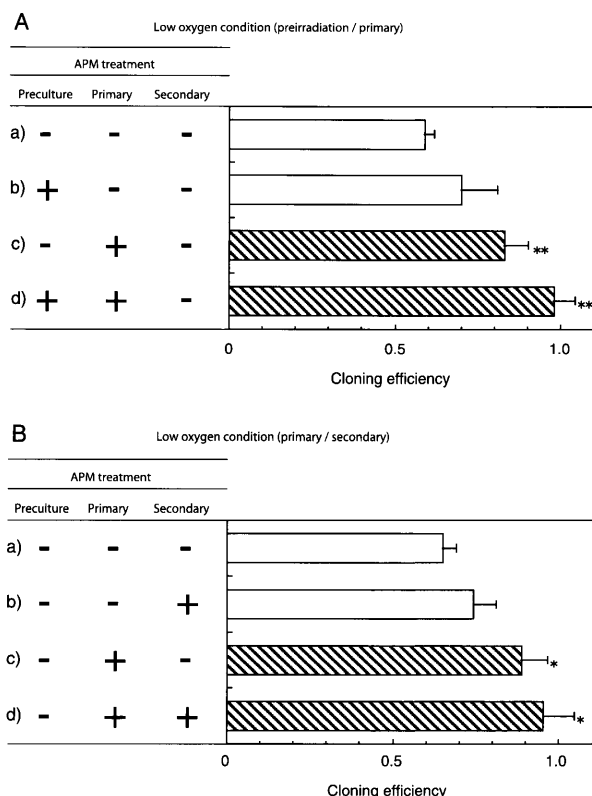


Fig. 3. Effect of APM on radiation-induced delayed reproductive cell death in cl. 2011–14 cells in a low oxygen condition. We applied 200 μ M APM in three periods; preculture for 2 weeks, primary colony formation for 2 weeks, and secondary colony formation for 2 weeks. Plus (+) and minus (–) symbols represent the presence and absence of 200 μ M APM treatment, respectively. The results are represented as mean \pm standard errors from three independent experiments. The asterisks (*) indicate a significant difference from control by Student's *t*-test analysis. * $p < 0.01$, ** $p < 0.005$

ondary colony formation enhanced the CE. This result indicates that oxidative stress-sensitive damages that lead to the induction of delayed reproductive cell death remain in a period for primary colony formation and disappear in a period for secondary colony formation.

For the low oxygen condition, two assay protocols were examined; one was that the cells were kept in the low oxygen condition during preirradiation culture and primary colony formation followed by culturing in the normal oxygen condition during secondary colony formation (Fig. 3A). The other is that the cells were kept in the normal oxygen condition during preirradiation culture followed by culturing in the low oxygen culture during primary and secondary colony formations (Fig. 3B). As already seen in Fig. 2, APM treatment during primary colony formation significantly enhanced the CE in both assay protocols as shown in Figure 3. In contrast, APM treatments during preirradiation culture and secondary colony formation were less effective (Fig. 3).

Without APM treatment, the low oxygen culture of proto-

Table 1. Relative values of protective effect of the anti-ROS condition on radiation-induced delayed reproductive cell death during preculture and postculture for primary colony formation.

Preculture condition	Primary colony formation			
	untreated	APM	2% O ₂	APM/2% O ₂
Untreated	1.0 ^{a)}	1.6	1.3	1.5
APM	1.0	1.6	1.1	1.8
2% O ₂	1.0	1.6	1.1	1.6
APM/2% O ₂	1.2	1.6	1.4	1.8

^{a)}The extent of each protective effect is represented as a relative value in comparison with that of the untreated control.

Table 2. Relative values of protective effect of the anti-ROS condition on radiation-induced delayed reproductive cell death during postcultures for primary and secondary colony formations.

Primary colony formation	Secondary colony formation			
	Untreated	APM	2% O ₂	APM/2% O ₂
Untreated	1.0	1.1	1.0	1.1
APM	1.5	1.6	1.6	1.6
2% O ₂	1.2	1.2	1.3	1.3
APM/2% O ₂	1.6	1.6	1.6	1.7

^{a)}The extent of each protective effect is represented as relative value in comparison with that of the untreated control.

col B showed a 20% increase in the CE ($p < 0.01$) compared with the normal oxygen condition (Figs. 2a and 3Ba). It is interesting that a slight, but not significant, increase in the CE by the low oxygen culture was also observed when APM treatment was applied during preirradiation culture (Fig. 3Ab) or secondary colony formation (Fig. 3Bb) in comparison with each counterpart (Fig. 2b and c). However, the low oxygen culture was no more effective in enhancing the CE when the APM treatment was applied during primary colony formation (Figs. 2 and 3). To compare the effect of each treatment on delayed reproductive cell death, we calculated the relative values of cloning efficiencies, and they are shown in Tables 1 and 2. They are evidence that the relative value of the CE in the cells treated with APM during primary colony formation was 1.6 or more, indicating that this treatment relieved delayed reproductive cell death. However, less effect was observed when the conditions were applied during preculture and secondary colony formation. These results indicate that the period for primary colony formation is the most sensitive period in which to reduce radiation-induced delayed reproductive cell death by the APM treatment.

Effect of APM treatment on delayed chromosome aberrations

Because delayed chromosome aberrations were well documented as a biological marker for radiation-induced genetic instability, we investigated the instability of a human chromosome 11 in cl. 2011–14 cells collected from secondary colo-

Table 3. Effect of APM on radiation-induced delayed chromosome aberrations in cl. 2011–14 cells.

Dose (Gy)	APM treatment	No. of cells examined	No. of cells with delayed chromosome aberrations ^{a)}
0	–	100	0
0	+	100	0
6	–	100	21 ^{b)}
6	+	100	10

^{a)}Aberrations of human chromosome 11 in cl. 2011–14 cells were detected by whole chromosome painting. ^{b)} $p < 0.05$ by χ^2 test.

nies. The instability was detected by whole chromosome painting by the use of a probe specific for human chromosome 11. As shown in Table 3, 6 Gy-irradiation induced a delayed instability of human chromosome 11 in cl. 2011–14 cells, whereas the human chromosome 11 was stable in the unirradiated cells. The APM treatment during primary colony formation significantly reduced the induction of delayed chromosome aberrations ($p < 0.05$), confirming the reduction of delayed reproductive death by the posttreatment with APM.

Effect of APM treatment on ROS generation postirradiation

To determine the effects of APM on ROS generation after radiation, we performed the DCFH-DA assay. Figure 4 shows the time course of the ROS generation after radiation. The results demonstrated a rapid increase and then a decrease in the amount of ROS in X-ray-irradiated cells for 6 hr postirradiation, followed by a gradual decrease in the ROS generation to a basal level within 2 weeks. An addition of APM accelerated to decrease the residual ROS to the basal level (Fig. 4A). After 6 h, the reduction of ROS became very slow, but it still continued for 14 days (Fig. 4B). An addition of APM was still effective to keep the ROS level of the treated cells lower than that of the untreated cells for 14 days, though the difference was very small (Fig. 4B).

The accelerated reduction of the residual ROS by APM treatment was confirmed by visualization with fluorogenic dyes, where the cells were stained with H_2DCFDA and counterstained with mitochondrion-selective Mito Tracer (Fig. 5). These fluorogenic dyes became fluorescent after oxidation. As shown in Fig. 5, intracellular oxidative stress was enhanced by X-irradiation (Fig. 5E and I), and APM accelerated the reduction of intracellular oxidative stress (Fig. 5J, K, and L).

The reason for a delayed induction of genetic instability in irradiated surviving cells is still obscure. One possibility to explain this phenomenon is that the persistent production of ROS in irradiated cells might be involved in destabilizing the genome of the progeny of irradiated cells. If that is so, we can expect that posttreatment with a certain antioxidant agent may

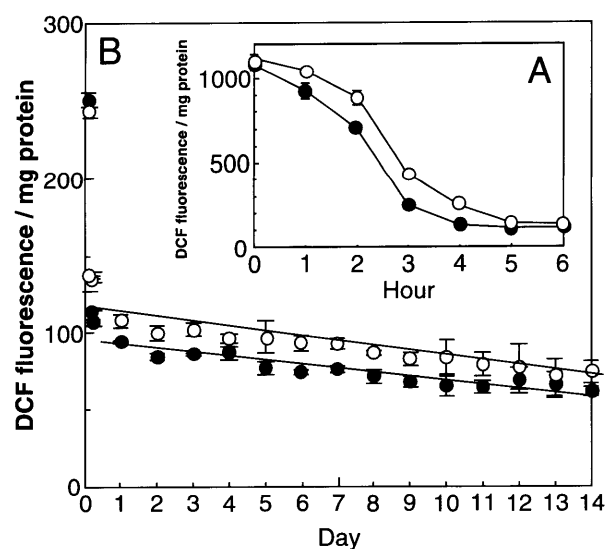


Fig. 4. Reduction of radiation-induced ROS by posttreatment with APM. Fluorescence intensity from fluorogenic dye was quantified for 14 days (A: 0 h–6 h; B: day 0–day 14). Open symbols (open circles) and closed symbols (solid circles) represent groups untreated and treated with 200 μM APM, respectively. The results are represented as mean \pm standard errors from three independent experiments.

relieve the induction of genetic instability by radiation.

Based on this idea, we applied APM, the derivative of AsA, to relieve radiation-induced genetic instability in the present study. Our most significant finding is that APM treatment during primary colony formation after irradiation significantly reduces delayed reproductive cell death and delayed chromosome aberrations by radiation. In contrast, APM treatments during preirradiation culture for 2 weeks and secondary colony formation are not effective in relieving the induction of delayed reproductive cell death by radiation. These results suggest that a certain type of radicals that can be scavenged by APM are involved in radiation-induced genetic instability and that a critical period of this phenomenon is restricted in 2 weeks of postirradiation.

The idea that the increase of intracellular free radicals is involved in the induction of genetic instability by radiation has been described in former studies.^{14–16} Limoli *et al.*^{15,16} demonstrated that genetically unstable cells that survived exposure to ionizing radiation had elevated levels of ROS compared with stable counterparts. They also indicated that these elevated levels of oxidative stress were due to an elevated number of dysfunctional mitochondria. These results suggest that the production ROS in mitochondria might be partially responsible for the induction of genetic instability by radiation.¹⁶ In the present study, we stained the irradiated cells with a mitochondrion-selective dye that became fluorescent by oxidation. However, our study showed no clear evidence to indicate a dysfunction of mitochondria in the

irradiated cells (Fig. 5).

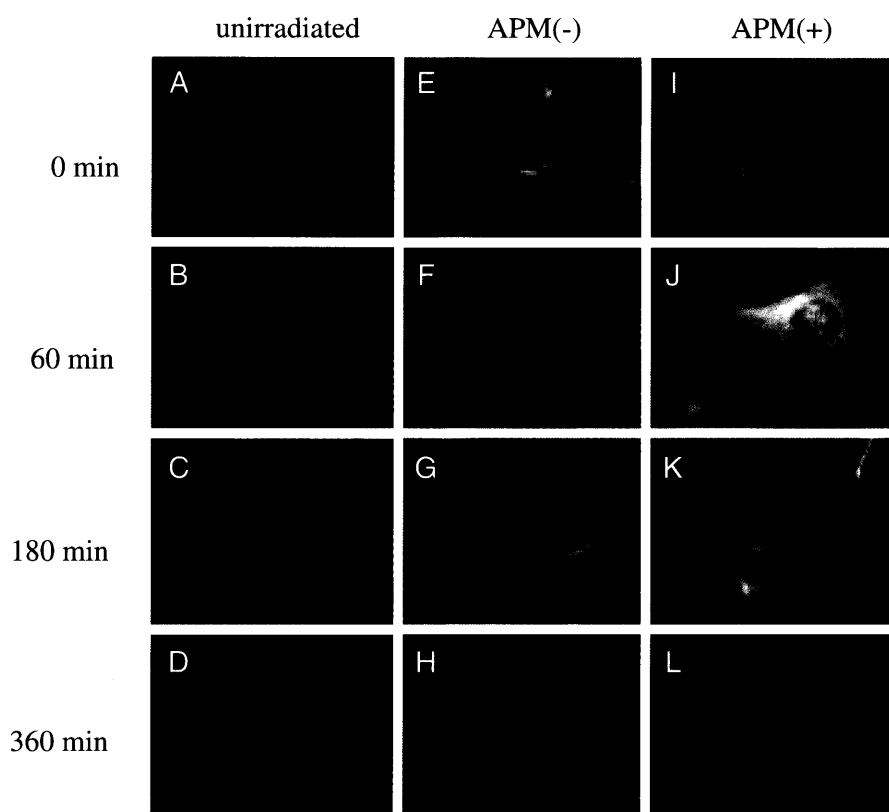


Fig. 5. Reduction of intracellular oxidative stress by posttreatment with APM. The cells were dually stained with H₂DCFDA and mitochondrion-selective MitoTracker, which became fluorescent in green and red, respectively, after oxidation. A–D: unirradiated controls; E–H: cells were irradiated with 6 Gy of X-rays without posttreatment with APM; I–L: cells were irradiated with 6 Gy of X-rays and posttreated with 200 μ M APM.

A recent report concerning a mechanism for oncogene-induced genetic instability demonstrated that c-Myc overexpression could induce DNA damage mediated by increasing ROS levels and that this DNA damage induced genetic instability.¹⁷⁾ Thus this result indicates that the elevated production of ROS is involved in oncogene-induced genetic instability. A similar involvement of ROS in the induction of chromosomal instability has been observed in DNA double-strand break repair mutants.¹⁸⁾ Based on these findings, we propose that one of the potential causes of genetic instability by radiation is the elevated and persistent production of endogenous free radicals after irradiation.

However, the radicals that are responsible for inducing genetic instability remain unidentified. We measured the level of intracellular ROS by their ability to oxidize a fluorogenic dye to examine a potential action of APM for reducing oxidative stress. The result indicated that APM kept the level of intracellular ROS of the treated cells lower than that of the untreated cells for 2 weeks after irradiation (Fig. 4). Although the difference between them is very small, it should be noticed that a level of oxidative stress of APM-treated cells is lower than the level of the untreated cells at any time of day during the 2 weeks following irradiation. We previously identified long-lived radicals (LLRs) induced by ionizing radiation,¹²⁾ which consist of sulfinyl radicals¹⁹⁾ and cause gene

mutation and malignant transformation. However, it is unlikely that these LLRs are involved in the induction of delayed reproductive cell death and delayed chromosome aberrations observed in the present study because LLRs are not associated with cell death and chromosome aberrations as shown previously.¹²⁾

We formerly demonstrated that postirradiation incubation in a low oxygen (2%) culture significantly reduced the delayed effects of radiation such as reproductive cell death, giant cell formation, and chromosome aberrations in normal human embryo cells.²⁰⁾ In the present study, we confirmed a significant diminishment of delayed reproductive cell death by an application for the low oxygen (2%) culture condition during primary and secondary colony formations (Figs. 2a and 3Ba). This implies that the reduction of intracellular oxidative stress postirradiation is generally important to relieve genetic instability by radiation. In contrast, as a relief of radiation-induced acute lethal damage, the preculture in the condition that reduces intracellular oxidative stress (APM treatment or a low-oxygen culture) is more effective than the postculture treatment (Fig. 1). Therefore the postculture condition rather than the preculture condition is crucial for the induction of genetic instability. Thus the present study emphasizes the biological significance of epigenetic effects such as intracellular oxidative stress after irradiation in radiation-induced genetic

instability.

Conversely, other studies have shown that under certain conditions, AsA functions as a pro-oxidant and can increase DNA damage.^{21,22)} It is well known that AsA acts as a prooxidant in the presence of free transition metals (Cu^{2+}).²³⁾ A recent study has pointed to the potential pro-oxidant properties of AsA, which are independent of free transition metals, based on the decomposition of lipid hydroperoxides induced by AsA *in vitro*.²⁴⁾ However, APM may be an effective antioxidant without injuring the cell membrane as a result of the lack of an antioxidative effect of extracellular APM.^{25,26)} Because APM is a stable form of AsA with low toxicity, it is expected to enrich the intracellular AsA concentrations in APM-treated cells. In fact, we found that the treatment of primary normal human embryo (HE49) cells with a low concentration (200 μM) of APM for 6 h increased the concentrations of intracellular AsA. Moreover, these concentrations were maintained even after 6 days (data not shown), indicating that APM can function as an antioxidant to relieve intracellular oxidative stress.

In summary, the present study demonstrates that a persistent induction of intracellular ROS in irradiated cells plays a significant role in radiation-induced genetic instability and that the cause of instability is almost fixed during primary colony formation after irradiation. Further study is needed to identify radicals responsible for this phenomenon.

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