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Micronuclei Formation Induced by X-ray Irradiation Does Not Always Result from DNA Double-strand Breaks

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Micronuclei/DNA double-strand breaks (DSBs)/Ascorbic acid (vitamin C).

X-ray induced formation of micronuclei is generally thought to result from DNA double-strand breaks (DSBs). However, DNA DSBs inhibit the cell cycle progression that is required for micronucleus formation. In order to reconcile this apparent discrepancy, we investigated whether DNA DSBs induced during the G1 phase could lead to micronucleus formation. We irradiated human embryonic (HE17) cells that had been treated with a radical scavenger, either DMSO or ascorbic acid (AsA), and determined the level of suppression of DNA DSBs or micronuclei. When DNA DSBs were evaluated using 53BP1 foci, treatment with 5 mM AsA did not inhibit the numbers of foci at various intervals after X-ray irradiation; however, treatment with 5 mM or 256 mM DMSO did have a significant inhibitory effect. By contrast, an assay of micronucleus numbers showed that treatment with 5 mM or 256 mM DMSO before X-ray irradiation resulted in almost no inhibition of micronucleus formation, but treatment with 5 mM AsA did have a significant inhibitory effect. These results clearly showed that AsA could suppress micronucleus formation, although it was not effective for suppression of DNA DSBs. Therefore, we conclude that DNA DSBs induced in the G1 phase do not directly lead to micronucleus formation.

INTRODUCTION

X-ray induced micronucleus formation is a well known phenomenon that has been used to investigate the biological effects of radiation exposure.[1–3] It is thought that DNA is the main target of X-rays in the cell and, therefore, it is a plausible scenario that X-ray induced micronucleus formation results from the fragmentation of chromosomes. In support of this idea, a defect in the repair of DNA double-strand breaks (DSBs) has been shown to lead to increased numbers of micronuclei in X-ray irradiated cells.[3,4] However, it was suggested recently that some micronuclei result from errors in chromosome segregation in mitosis, as a consequence of so-called "lagging chromosomes".[5] Although different mechanisms for micronucleus formation have been identified in irradiated cells,[6,7] these are not yet fully understood. One of the approaches that can be used to clarify these mechanisms is by investigating the contents of micronuclei using fluorescence *in situ* hybridization (FISH).[8,9]

X-ray induced DNA damage generally leads to cell death, although the pathway for this effect with respect to DNA DSBs is not yet completely understood. Recently, many researchers have used radiation-induced formation of specific types of foci, such phosphorylated H2AX,[10] phosphorylated ATM,[11] MDC1,[12] or 53BP1,[13] as biomarkers of the effects of radiation. Yamauchi *et al.*[14] reported that increase in the size of 53BP1 foci or other factors was sufficient for cell cycle arrest at G1. In addition, the permanent arrest of X-ray irradiated cells in G1 is thought to be due to senescence, an irreversible process.[15] Therefore, the DNA DSBs that are sufficient to induce cell cycle arrest, known as senescence-like growth arrest,[15] can be visualized as large foci. However, the relationship between formation of large foci and formation of micronuclei after cell cycle progression is as yet unknown.

We previously reported that DMSO and ascorbic acid (AsA) could suppress various biological effects occurring after X-ray irradiation.[16–20] We concluded that DMSO could suppress lethal effects, including those of DNA DSBs, whereas AsA could not. However, AsA could suppress non-lethal events, such as mutagenesis and tumorigenesis. In the present study, we selected these two compounds in order to determine which events, either lethal or non-lethal, were suppressed during X-ray induced micronucleus formation.

Here, we demonstrate that X-ray induced micronucleus

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formation is suppressed by AsA treatment but not by DMSO, suggesting that micronucleus formation does not result mainly from DNA DSBs and consequently does not lead to cell death.

MATERIALS AND METHODS

Cell culture and irradiation

In this study, human embryonic (HE17) fibroblast-like cells were used. Cells were isolated from embryonic abdomen tissues as described.[21] Cells were cultured in minimal essential Eagle's medium (MEM; Nissui, Tokyo, Japan) containing 10% fetal bovine serum in a 95% air/5% CO_2 incubator at 37°C. We seeded 1×10^6 cells in T-75 culture flasks (75 cm^2; Becton Dickinson, Franklin Lakes, NJ) and subcultured them every 3 days to maintain the exponentially cell growing state. Cells were irradiated using an M-150WE X-ray generator (Softex, Tokyo, Japan) at 150 kVp and 5 mA with a 0.1 mm copper filter and at a dose rate of 0.635 Gy/min.

Treatment with radical scavengers

To compare the radioprotective effect of DMSO with AsA, 5 mM Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and 5 mM ascorbic acid (AsA: vitamin C) (Sigma-Aldrich) were selected for use as radical scavengers. Although 256 mM of DMSO was an enough concentration for reduction of lethal effects, because lethal toxicity of AsA was very strong, we could not use AsA at 256 mM in this study.

Each compound was dissolved in culture medium, adjusted to pH 7.2 using 1 N NaOH or 1N HCl solution, and sterilized through a filter (pore size: 0.22 μm; Kurabo, Osaka, Japan). Cells were treated with the radical scavenger for 2 h, timed from when normal culture medium was replaced with medium containing the compound. The cells were X-ray irradiated for less than 10 min, depending on the dose given, before the end of the treatment period with the radical scavenger.

Colony formation assay

Three $\times 10^5$ HE17 cells were seeded in T-25 culture flasks (25 cm^2; Becton Dickinson). HE17 cells reached sub-confluent state three to four days after they were seeded. At 7 days after were seeded, all of them were in confluent state. At this point, it was confirmed by cell cycle analysis using FACScan that over 95% cells were G1 phase cells. Then cell were irradiated with 0.5, 1, 2, 4 or 6 Gy of X-rays. Then, the appropriate number of cells (100 cells for those not irradiated or given 0.5, 1 or 2 Gy, 500 cells for those given 4 Gy, and 5,000 cells for those given 6 Gy) was seeded in 100 mm diameter plastic dishes (P100; Becton Dickinson). After incubation for 2 weeks, the cells were washed with phosphate-buffered saline minus (PBS^-), fixed with methanol and stained with 5% Giemsa reagent (Sigma-Aldrich). Colonies that included more than 50 cells were counted.

Immunofluorescence staining

For immunofluorescence staining of 53BP1 foci, 1×10^5 cells were inoculated onto 22 mm × 22 mm coverslips in 35 mm diameter plastic dishes (P35; Becton Dickinson) and, after 7 days, the cells in confluent state (G1 phase; over 95%) were given 0.5, 1, 2, 4 or 6 Gy of X-rays. The cells were then incubated and, at 0.25, 1, 2, 4, 6, 12 or 24 h after X-ray irradiation, were washed with PBS^-. Next, the cells were fixed with 4% formaldehyde/PBS^- for 10 min and permeabilized with 0.5% Triton X-100/PBS^- on ice for 5 min. After permeabilization, the cells were incubated for 2 h at 37°C with a rabbit anti-53BP1 antibody (Bethyl Laboratories, Montgomery, TX) in TBS-DT (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, 5% skim milk). The cells were washed four times with PBS^- and then incubated for 1 h at 37°C with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes Inc., Eugene, OR) in TBS-DT. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 10 ng/ml in TBS-DT; Molecular Probes Inc.) for 30 min. The cells were then washed four times with PBS^-, and the coverslips were attached onto glass microscope slides with 10% glycerol in PBS^-. Cells with one or more foci were scored as 53BP1-positive cells.

Micronucleus formation assay

To measure the frequency of micronucleus formation, we used simple DAPI staining. We did not use the cytokinesis-block procedure so that the technical error between different

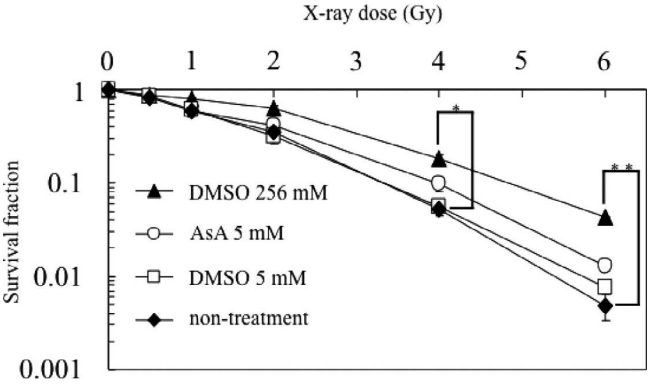

Fig. 1. Survival fractions after X-ray irradiation of cells treated with radical scavengers. Cells were treated with 5 mM AsA (open circle), 5 mM DMSO (open square) or 256 mM DMSO (filled triangle) for 2 hours. They were given 0.5, 1, 2, 4 or 6 Gy of X-ray irradiation at the end of the treatment period with the radical scavengers. Cells were incubated for 2 weeks after X-ray irradiation to allow colony formation. Data were obtained from at least three independent experiments and are shown as the mean ± S.E. Significant differences were observed in cells treated with 256 mM DMSO vs. cells not treated with radical scavengers (non-treatment) (*$*p < 0.005$, **$p < 0.001$*).

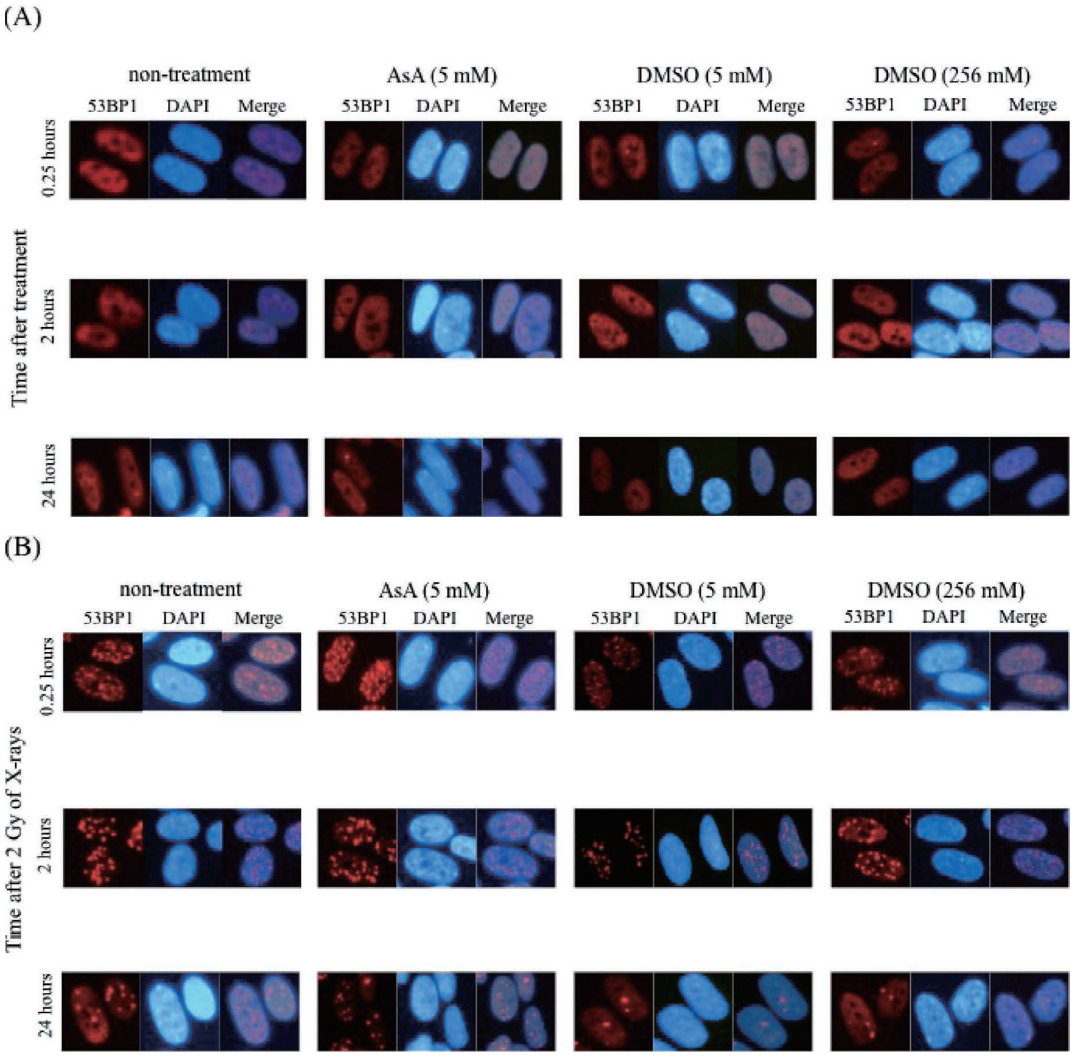

Fig. 2. Cells immunostained for 53BP1 and counterstained with DAPI. Cells were treated with 5 mM AsA, 5 mM DMSO or 256 mM DMSO for 2 hours. X-ray irradiation was performed during the final part of the treatment period with radical scavengers. Red indicates 53BP1 (focus) and blue indicates DAPI (nucleus). (Panel A) non-irradiated cells, (panel B) 2 Gy-irradiated cells.

experiments became minimal in this study.

Actually, 1×10^5 cells were inoculated onto 22 mm × 22 mm coverslips in 35 mm diameter plastic dishes (P35; Becton Dickinson) and, after 3 days, the cells were given 0.5, 1 or 2 Gy of X-rays. The cells were then incubated and, at 24 h after X-ray irradiation, were washed with PBS^-. Next, the cells were fixed with 4% formaldehyde/PBS^- for 10 min and permeabilized with 0.5% Triton X-100/PBS^- on ice for 5 min. After permeabilization, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 10 ng/ml in TBS-DT; Molecular Probes Inc.) for 30 min. The cells were then washed four times with PBS^-, and the coverslips were attached onto glass microscope slides with 10% glycerol in PBS^-. Nuclei with a diameter of less than 1/3 of normal nuclei were scored as micronuclei.

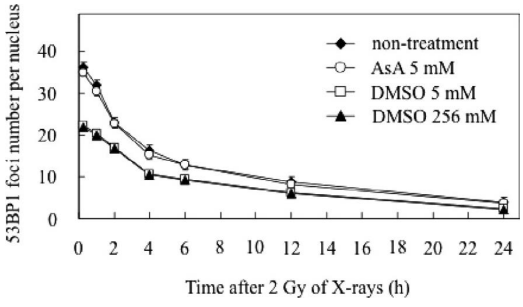

Fig. 3. The kinetics of 53BP1 focus number per nucleus from 0.25 to 24 hours after 2 Gy of X-rays. Cells were treated with 5 mM AsA, 5 mM DMSO or 256 mM DMSO for 2 hours. X-ray irradiation was performed during the final part of the treatment period with radical scavengers. The number of foci was counted in 200 nuclei per sampling point. At least three independent experiments were carried out and the data are shown as the mean ± S.E.

Large foci formation assay

The frequency of large foci formation was assessed using the same method as for the immunofluorescence staining assay. Foci with a diameter greater than 1.6 μm were scored as large foci.[14)]

FISH analysis of centromeres

The frequency of micronuclei with centromeric signals was determined using fluorescence *in situ* hybridization (FISH) with a directly labeled human pancentromeric probe. Cells that had been fixed in 5:1 methanol: acetic acid (Carnoy fluid) were attached to glass microscope slides, and the slides were allowed to air-dry for 2 days. The slides were then washed with 0.1% NP-40/20 × SSC (175.2 g NaCl, 88.2 g sodium citrate and 1 l distilled water, pH 7.0) at 37°C for 30 min, dehydrated through a graded ethanol series 70, 85, 100% for 2 min, air-dried for 1 min, placed in a denaturing solution of 70% formamide/2 × SSC at 72°C for 2 min, and air-dried for 1 min. The pancentromeric DNA probe (STAR*FISH 1695-Cy3-01; Cambio, Cambridge, UK) was denatured at 72°C for 5 min and chilled on ice. Eight microlitres of the probe were applied to each slide, prewarmed at 37°C, and the slides were then mounted with coverslips, sealed and hybridization was allowed to occur at 37°C overnight (16 h–18 h) in a moisture chamber. On the following day, the slides were washed in a solution of 0.1% Tween 20/2 × SSC at 65°C for 5 min, then in PBD (0.5% Triton X-100, 0.5% Tween 20, 0.5% NP-40/2 × SSC) at room temperature for 15 min, and air-dried for 1 min. Finally, the slides were counterstained in the dark with DAPI.

Images were acquired with an AX70 fluorescence microscope (Olympus, Tokyo, Japan) and analyzed using Image J software (Wayne Rasband National Institutes of Health, Bethesda, MD).

Statistical analysis

Statistical comparisons were performed using Student's *t*-test.

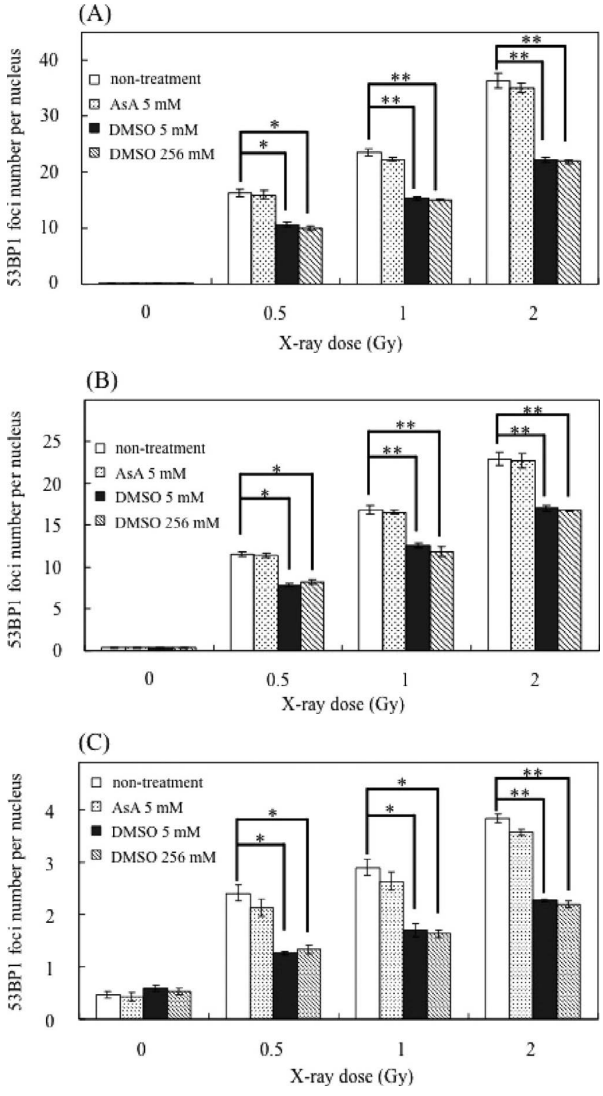

Fig. 4. Dose dependent increases in formation of 53BP1 foci and the suppressive effects of the radical scavengers DMSO or AsA after irradiation: panel A, 0.25 h; panel B, 2 h; panel C, 24 h. Cells were treated with 5 mM AsA, 5 mM DMSO or 256 mM DMSO for 2 hours. X-ray irradiation was performed during the final part of the treatment period with radical scavengers. The number of foci was counted in 200 nuclei per sampling point. At least three independent experiments were carried out and are shown as the mean ± S.E. Significant differences were observed in cells treated with 5 mM and 256 mM DMSO vs. cells not treated with radical scavengers (non-treatment) (*$p < 0.005$, **$p < 0.001$).

RESULTS

DMSO, but not AsA, can inhibit the death of HE17 cells following X-ray irradiation

The effect of radical scavengers on sensitivity to X-ray

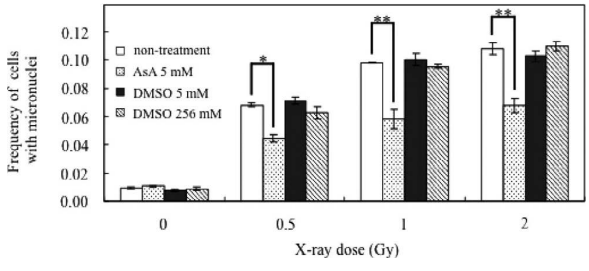

Fig. 5. The frequencies of cells with micronuclei after 0.5, 1 or 2 Gy of X-rays. Cells were treated with 5 mM AsA, 5 mM DMSO or 256 mM DMSO for 2 hours. X-ray irradiation was performed in the final part of the treatment period with radical scavengers. One thousand cells were screened for micronuclei at each sampling point. At least three independent experiments were carried out and the data are shown as the mean ± S.E. Significant differences were observed in cells treated with 5 mM AsA vs. cells not treated with radical scavengers (non-treatment) (*$p < 0.001$, **$p < 0.005$).

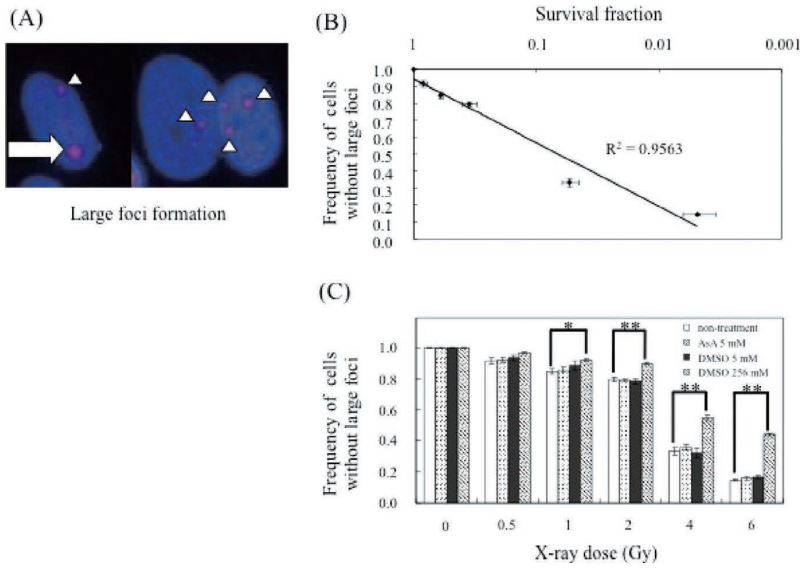

Fig. 6. (Panel A) Immunostaining of 53BP1 foci at 24 hours after 2 Gy of X-rays. The white arrow points to persistent foci with a diameter of 1.6 μm or more (large foci). The white triangles point to persistent foci with a diameter of less than 1.6 μm. The 53BP1 foci are stained red, and the nuclear DNA is counterstained blue with DAPI. (Panel B) Correlation of the frequency of cells without large foci and the survival fraction in cells untreated with radical scavengers and assessed by the colony formation assay. The frequency of cells without large foci was obtained by analysis of 200 cells per sampling point. (Panel C) The effect of radical scavengers on the frequency of cells without large foci after 0.5, 1, 2, 4 or 6 Gy of X-rays. Cells were treated with 5 mM AsA, 5 mM DMSO or 256 mM DMSO for 2 hours. X-ray irradiation was performed in the final part of the treatment period with radical scavengers. The frequency of cells without large foci was obtained by analysis of 200 cells per sampling point. At least three independent experiments were carried out and the data are shown as the mean ± S.E. Significant differences were observed in cells treated with 256 mM DMSO vs. cells not treated with radical scavengers (non-treatment) (*$p < 0.05$, **$p < 0.005$).

induced cell lethality was assessed using the colony formation method. Following X-ray irradiation, the rate of survival decreased in a dose-dependent manner (Fig. 1). Treatment with 256 mM DMSO increased the proportion of cells surviving, and this protective effect was significant after 4 or 6 Gy of X-rays compared to non-treated cells. By contrast, treatment of the cells with either 5 mM AsA or 5 mM DMSO did not increase cell survival rates (Fig. 1).

DMSO, but not AsA, inhibits X-ray-induced generation of DNA DSBs in HE17 cells

We used immunofluorescence staining of 53BP1 to assess the incidence of DNA DSBs at 0.25 to 24 h after X-ray irradiation. Although it has been suggested that 53BP1 foci may be present but not always mark DNA DSBs, they have sufficient reliability as the indicator of DSBs.[22,23] Non-irradiated cells were used as controls. Examples of 53BP1 foci in non-irradiated cells and in cells given 2 Gy of X-rays are shown in Figs. 2A and B, respectively. The changes with time in numbers of foci in irradiated cells treated with DMSO or AsA are shown in Fig. 3. At all time points after X-ray irradiation, treatment with either 5 or 256 mM DMSO significantly reduced the number of foci per nucleus, while treatment with AsA 5 mM had no effect. Next, we compared the abilities of the radical scavengers to suppress foci induc-

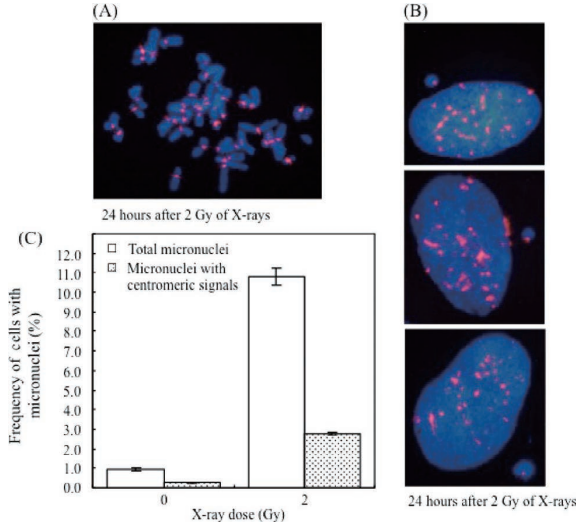

Fig. 7. FISH with pancentromeric DNA probes. Cells with centromeric signals, counterstained with DAPI, at 24 hours after 2 Gy of X-rays. (Panel A) Metaphase chromosomes with centromeric signals. (Panel B) Cells with micronuclei harboring centromeric signals. (Panel C) The percentage frequency of cells with micronuclei (total micronuclei) and that of cells with micronuclei harboring centromeric signals. Three thousand cells were screened for micronuclei harboring centromeric signals at each sampling point. At least three independent experiments were carried out.

tion at lower doses of X-rays (Fig. 4). The data displayed in Fig. 4 strongly support the conclusion that DMSO, but not AsA, can suppress focus formation. The rate at which the foci disappeared did not differ among the treatments suggesting that the repair kinetics were not affected by the treatments (Figs. 3 and 4).

AsA, but not DMSO, can inhibit X-ray-induced formation of micronuclei in HE17 cells

The frequency of micronucleus formation in HE17 cells was compared in non-irradiated control and X-ray irradiated cells at 24 h after treatment. A dose-dependent increase in the numbers of micronuclei was found using 0.5, 1 and 2 Gy (Fig. 5). Treatment with 5 mM AsA significantly reduced the frequency of micronucleus formation, whereas DMSO had no effect at either dose tested (Fig. 5).

DMSO, but not AsA, inhibits X-ray-induced generation of large foci in HE17 cells

Yamauchi *et al.*[14] have reported that cells harboring persistent large foci of 53BP1, defined as those with a diameter of 1.6 μm or more at 24 h after X-ray irradiation, did not enter S phase. These cells appear to be arrested at G1, suggesting that they cannot contribute to the colonies in the surviving fraction. In addition, these cells did not show micronucleus formation. We examined the frequency of cells without large foci at 24 h after X-ray irradiation in order to determine the effect of radical scavengers on suppression of large foci. First, we used a colony formation assay to determine whether there was a correlation between the frequency of cells without large foci and the rate of survival of cells in the absence of treatment with radical scavengers. We found that absence of large foci and survival rates were strongly correlated ($R^2 = 0.9563$; Fig. 6B), and we therefore concluded that cells with large foci arrested after X-ray irradiation. When irradiated cells were treated with 256 mM DMSO, the frequency of cells lacking large foci was significantly elevated compared to non-treated cells. However, irradiated cells treated with 5 mM DMSO or 5 mM AsA showed no change in the frequencies of cells without large foci (Fig. 6C). These results are consistent with those of the colony formation assay (Fig. 1). Putting these results together, we suggest that most X-ray irradiation-related DNA DSBs lead to cell cycle arrest due to the formation of large foci. We also conclude that the large foci assay provides a reliable and quantitative method for investigating cell survival after treatment with various radical scavengers.

X-ray induced micronuclei in HE17 cells contain centromeric DNA

We performed a FISH analysis to confirm the existence of centromeric DNA in micronuclei and determine the frequency of micronuclei with centromeric signals at 24 h after 2 Gy of X-rays to HE17 cells (Figs. 7A and B). Non-irradiated cells were used as controls. We found micronuclei with centromeric signals in irradiated cells (25.5% of the total X-ray induced micronuclei) (Fig. 7C).

DISCUSSION

X-ray induced DNA DSBs are thought to lead to cell death, which is manifested as a reduction in colony forming ability, or to an increase in the frequency of formation of micronuclei. However, the pathways leading to these outcomes are still not fully understood. It is generally accepted that cell viability after irradiation can be estimated using colony-forming ability. In addition, there is evidence showing that irradiation of G1 phase cells causes arrest by a p53-dependent pathway. In some cells, micronucleus formation may result from radiation-induced chromosome fragmentation or abnormal separation of chromosomes in mitosis. Many researchers have suggested that the main cause of micronucleus formation is chromosome fragmentation due to DNA DSBs, since these are efficiently induced by ionizing radiation. However, there is a problem with this suggested explanation in that irradiated cells arrest and do not progress through the cell cycle, whereas micronucleus formation requires cell cycle progression.

In the present study, we examined the ability of radical scavengers to suppress DNA DSBs after X-ray irradiation, and we found that DMSO, but not AsA, could suppress formation of DNA DSBs. However, we also found that AsA, but not DMSO, could suppress micronucleus formation. These results clearly show that the two radical scavengers have different suppressive effects. In addition, we found that formation of large 53BP1 foci were not correlated with micronucleus formation, since the two radical scavengers had quite opposite suppressive effects. We previously reported that AsA cannot suppress cell death or severe chromosome aberrations, but can suppress non-lethal effects such as the induction of mutations or transformation.[19] Therefore, we concluded that AsA might contribute to the suppression of tumorigenesis and mutagenesis. Our present results that AsA did not suppress formation of DNA DSBs, estimated using formation of 53BP1 foci, strongly supports this idea.

Two important points can be drawn from our data. First, micronucleus formation, which is frequently used as a biomarker of radiation, does not provide an accurate estimate of the real number of DNA DSBs as there is a possibility that a significant fraction of the micronuclei are not generated from DNA DSBs. This conclusion is also strongly supported by the fact that large 53BP1 foci were not suppressed by 5 mM AsA but were suppressed by 256 mM DMSO; consequently, the rates of formation of these foci did not correlate with micronucleus formation in the present study (Figs. 5 and 6C). Our data imply that micronucleus formation after X-ray irradiation should be considered as the result of abnormal chromosome segregation in mitosis. Even in non-

irradiated cells, the mechanism of micronucleus formation has been suggested to be "lagging chromosomes" in mitosis with the observation of higher frequency;[5] therefore, the contribution of this mechanism is likely to be much higher after irradiation. Second, estimation of the incidence of micronucleus formation may be a useful indicator of the likelihood of radiation-induced carcinogenesis, because AsA is an effective suppressor of tumorigenesis, as reported previously. We previously suggested that "long-lived radicals" might be involved in mutagenesis and tumorigenesis; AsA is believed to be an effective scavenger of this type of radical.[19] If micronucleus formation is caused by abnormal chromosome segregation in mitosis, then the production of aneuploid cells should be observed. Malsegregation of chromosomes is thought to be an important mechanism in tumorigenesis, and thus a higher rate of micronuclei after X-ray irradiation may be related to formation of aneuploid cells.

It is important to understand the mechanisms of formation of a biomarker such as micronuclei. Many researchers have used estimation of micronucleus frequencies as a marker of DNA DSBs. However, our results here clearly show that the suppressive effect of AsA on micronucleus formation is not mediated via DNA DSBs. Therefore, it is essential to clarify the types of damage involved in the formation of micronuclei and also the effects of radical scavengers on such damage. In addition to DNA, other cellular structures may be affected by ionizing radiation. One potential target is the mitochondrion, which is important in energy production and for the generation of reactive oxygen species (ROS). Thus, it will be necessary to determine the damaging effects of ionizing radiation on mitochondria and the protective effects of radical scavengers. Likewise, the centrosome and kinetochore, which have key roles in chromosome segregation in mitosis, are strongly implicated in the occurrence of "lagging chromosomes". Here, we found micronuclei with centromeric signals in irradiated cells (approximately 26% of the total X-ray induced micronuclei) (Fig. 7C). This result provides strong evidence in support of our speculation that micronuclei formation induced by X-ray irradiation does not always result from DNA double-strand breaks. However, the remaining approximately 74% of the total X-ray induced micronuclei does not have centromeric signals and we also found micronuclei with centromeric signals in non-irradiated cells.

In future studies, we hope to further clarify the mechanisms of micronucleus formation and the effects of radical scavengers.

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