

Effect of Medium on Chromatin Damage in Bystander Mammalian Cells

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In the present study, we examined the potential contribution of irradiated medium to the bystander effect using custom-made double-Mylar stainless steel rings. Exponentially growing human–hamster hybrid (A_L) cells were plated on either one or both sides of double-Mylar dishes 2–4 days before irradiation. One side (with or without cells) was irradiated with α particles using the track segment mode of a 4 MeV Van de Graaff accelerator at the Radiological Research Accelerator Facility of Columbia University. Since α particles can traverse only a very limited distance (around 23 μm in water), cells plated on the other side of a medium-filled Mylar dish will not be irradiated by the α particles. The results of the cytogenetic assay of unirradiated target cells that were attached to the top Mylar layer indicate that the number of chromatid-type aberrations was higher when there was a bottom layer of cells in the medium-filled chambers than with just medium alone. Furthermore, when the medium was transferred from these cell-irradiated dishes to fresh A_L cell cultures, chromatid-type aberrations were produced in the unirradiated fresh cells. In contrast, medium irradiated in the absence of cells had no effect on chromatid aberrations. These results suggest that certain unidentified modulating factors secreted from the irradiated cells on the bottom Mylar layer into the medium induce chromatin damage in the unirradiated bystander cells. © 2004 by Radiation Research Society

INTRODUCTION

In recent years, there has been evidence that low fluences of α particles induce biological effects such as mutation induction (1, 2), sister chromatid exchange (3, 4), micronucleus formation (5), and changes in gene expression (6, 7) in the cell population close to but not directly hit by α particles. These studies suggest that some signals induced by the α particles in directly hit cells might be transmitted to unirradiated neighbors by intercellular communication (2, 7) or through the release of cytokines or other soluble factors into the culture medium (8–10). Recent studies from

our laboratory have shown that α particles caused an increase in both mutation induction and chromatid-type breaks in unirradiated bystander cells, and that these bystander effects were significantly decreased by pretreatment with 40 μM lindane, which inhibits gap junction-mediated cell-to-cell communication (2, 11). Although gap junction communication appears to play an important role in mediating this bystander effect, we cannot completely rule out the possibility of a medium effect.

Previous studies have shown that medium from irradiated cultures upon transfer to unirradiated cultures can induce increased biological effects. Mothersill and Seymour first demonstrated a highly significant reduction in cloning efficiency in both unirradiated normal and unirradiated malignant epithelial cell lines that had received medium from cultures irradiated with ⁶⁰Co γ rays (12). These results suggested that irradiated cells secreted a cytotoxic factor into the culture medium that was capable of killing unirradiated cells. The authors also reported individual variations in bystander signals produced by the primary cultures of normal human uroepithelial cells (13). Furthermore, transferring medium from cultures exposed to low-LET radiation to unirradiated cells lead to increased levels of various bystander effects, including cell killing (8, 14–16), neoplastic transformation (16), and genomic instability (14). Interestingly, these responses often correlated with the early stages of the cascade leading to apoptosis, such as mobilization of intracellular calcium, loss of mitochondrial membrane potential, and increase in production of reactive oxygen species (15, 17).

Information on medium effects induced by high-LET radiation is limited. By transferring supernatants from α -particle-irradiated cells to unirradiated cells, Iyer and Lehnert demonstrated a decrease in the basal levels of TP53 and CDKN1A in the unirradiated cells (18). Furthermore, they suggested that the decrease in these cell cycle-regulating genes correlated with increases in intracellular reactive oxygen species and cell growth. A recent study by Zhou *et al.* demonstrated that culture medium irradiated with α particles in the presence of attached cells was cytotoxic to unirradiated bystander cells using the double-Mylar technique (19). The results showed that the surviving fraction among unirradiated cells was significantly lower than that of the controls after being exposed to irradiated medium

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for 48 h. However, there was no significant effect on the yield of spontaneous mutations in unirradiated cells. These results suggested that different bystander end points could involve a different mechanism. Although medium transfer of signaling molecules from irradiated cultures may be one possible mechanism for radiation-induced bystander effects, the detailed process is still unclear, notably in the case of high-LET radiation. Extending this work using the double-Mylar technique, we examined the potential contribution of irradiated medium to chromosomal damage in the bystander cells, a bystander end point that we showed previously using an α -particle microbeam (11).

MATERIALS AND METHODS

Cell Culture

A_L human-hamster hybrid cells, which contain a complete set of chromosomes of Chinese hamster ovary (CHO) cells and a single copy of human chromosome 11, were used in this study. Cells were cultured in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum, 25 $\mu\text{g/ml}$ gentamicin, and 2×10^{-4} M glycine at 37°C in a humidified 95% air/5% CO_2 incubator as described previously (20–22).

Irradiation Procedure

The irradiation procedure using the double-Mylar technique has been described previously (19). Briefly, exponentially growing A_L cells were inoculated on one side of the double-Mylar dishes (35 mm in diameter) at a concentration of 1.0×10^5 cells per dish 4 days before irradiation. Two days later, the medium was aspirated and the double-Mylar dishes were turned around, and another batch of exponentially growing cells was inoculated on the other side of the double-Mylar dishes at a concentration of 1.8×10^5 cells per dish. These target cells were then used to detect chromosomal damage. In experiments involving medium-irradiated dishes, the same number of target cells was also inoculated on one side of the double-Mylar dishes, which had no cells on the other side of the dishes. One side of the dishes with or without attached cells was irradiated with α particles (stopping power = 124 keV/ μm) using doses ranging from 0.1 to 100 Gy in the track segment mode of a 4 MeV Van de Graaff accelerator at the Radiological Research Accelerator Facility of Columbia University. The dose rate of the α -particle irradiations was 0.6–0.8 Gy/min for low-dose irradiation (0.1 and 1.0 Gy) and 12–15 Gy/min for high-dose irradiation (10 and 100 Gy). We calculated the residual range of the α particles to be around 23 μm in water, according to the data tables of Northcliffe and Schilling (23). Since α particles can traverse only a very limited distance, cells plated on the other side of a medium-filled Mylar dish would have no chance of being hit directly by α particles. However, a small number of the cells cultured on both sides of the Mylar film detached during our procedures. Unfiltered medium was also placed on a fresh cell population of cells during the medium transfer experiments. Although there is no cell-to-cell contact between attached cells on the two sides, cell-to-cell communication is available through either the medium or the small number of detached cells.

Detection of Chromatin Damage

The Calyculin A-mediated premature chromosome condensation (PCC) technique was used to detect chromatin damage as described previously (24). Briefly, the unirradiated target cells attached on the top Mylar layer were treated with Calyculin A (Wako Chemicals, Tokyo) at a final concentration of 50 nM for 30 min in a CO_2 incubator at 37°C. After treatment with Calyculin A, cells detached spontaneously or were easy to detach from Mylar films or culture flasks. Prematurely condensed chromosome samples were prepared according to a conventional cytogenetic

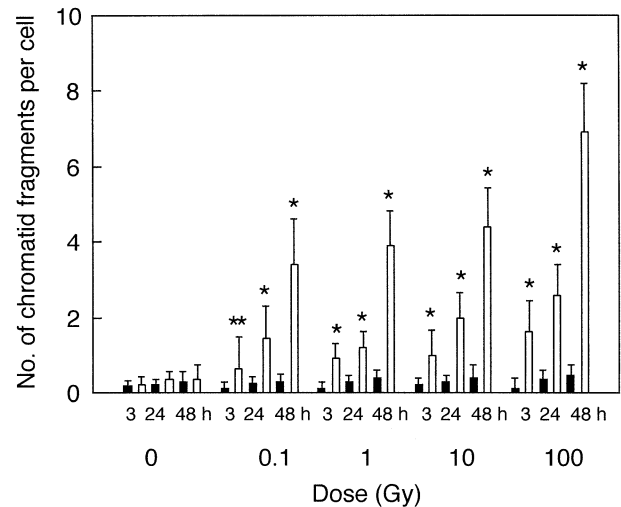
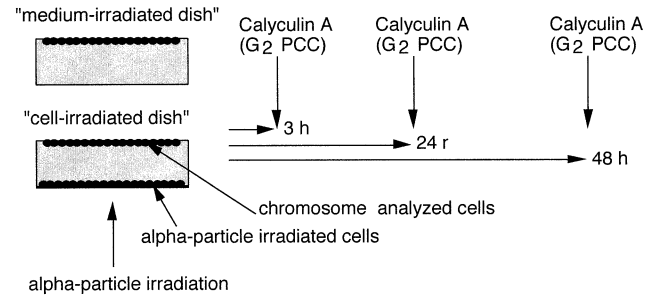


FIG. 1. The induction of chromatid fragments in unirradiated A_L cells attached to the top Mylar layer as a function of the dose of α particles delivered to either cells attached to the bottom Mylar layer (open bars; "cell-irradiated dishes") or medium only (closed bars; "medium-irradiated dishes"). The results are the means and 95% confidence intervals of two to three independent experiments. * $P < 0.001$, ** $P < 0.05$ compared to the data for medium-irradiated dishes for each dose and interval.

procedure. Cells were treated with a 75 mM KCl solution for 20 min at 37°C and fixed in 3:1 methanol:acetic acid. The cell suspension was dropped onto slides that had been cleaned with ethanol, air-dried and stained with a 5% Giemsa solution. Prematurely condensed chromosome samples of 100 G_2 phases were scored under a light microscope. The yield of induced chromatid-type aberrations was determined as reported previously by Savage (25). We scored chromatid breaks, isochromatid deletions, and acentric fragments as chromatid fragments.

RESULTS

Dependence of Dose and Incubation Time in Chromatid-Type Aberrations

Figure 1 shows the induction of chromatid fragments in unirradiated A_L cells attached to the top Mylar layer as a function of dose of α particles to the bottom Mylar layer with cells ("cell-irradiated dishes") or without cells ("medium-irradiated dishes"). The bystander cells were incubated in a CO_2 incubator for 3, 24 and 48 h after irradiation and then treated with Calyculin A to condense interphase chromatin. No chromatin fragments were induced in the unhit bystander cells attached to the top Mylar layer in the

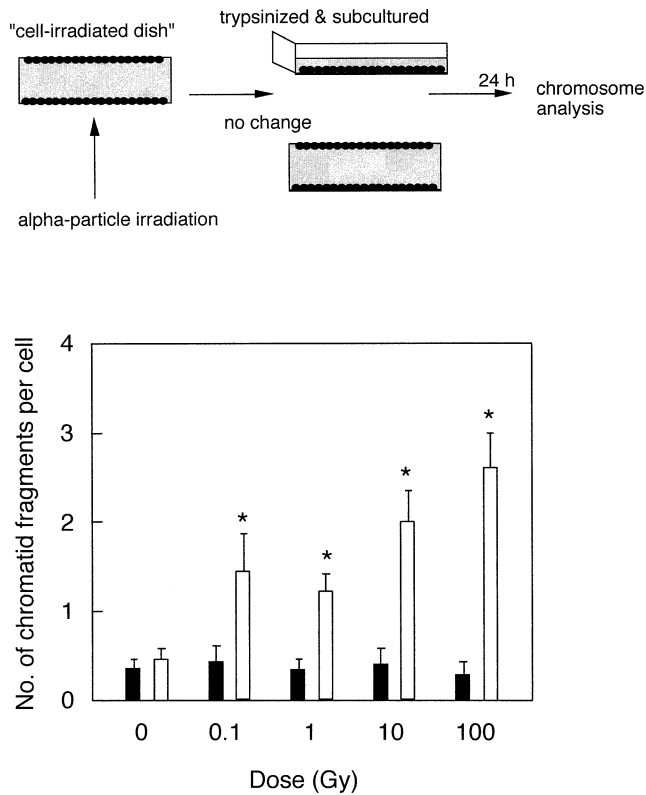


FIG. 2. The induction of chromatid fragments in unirradiated bystander A_L cells in cell-irradiated dishes upon trypsinization and subculturing into new flasks immediately after irradiation (closed bars). Open bars depict data from control flasks that were not subcultured in cell-irradiated dishes. The results are the means and 95% confidence intervals of two or three independent experiments. * $P < 0.001$ compared to the data for trypsinized and subcultured cells immediately after irradiation with each dose.

medium-irradiated dishes. On the other hand, the number of chromatid fragments per cell in the cell-irradiated dishes was significantly higher than that in medium-irradiated dishes, depending on the incubation time. These increased with increasing incubation periods ranging from 3 to 48 h. No clear dose dependence was observed for the bystander effect.

Effects of Trypsinization and Medium Replenishment

We attempted to clarify the effect of medium in the cell-irradiated dishes on the induction of chromatid fragments in the unirradiated top layer of cells. We trypsinized and replated this top layer of unirradiated cells into new flasks using fresh medium within 10–20 min after irradiation. One day after plating, these cells were processed for chromosome analysis. As shown in Fig. 2, there was no induction of prematurely condensed chromosome fragments in G_2 phase among these bystander cells. In contrast, in bystander cells that were not subcultured but were incubated for 24 h before processing for chromosomal analyses, there were significant increases in induction of prematurely condensed chromosome fragments in G_2 -phase cells. These results suggest that signaling molecules present in the irradiated

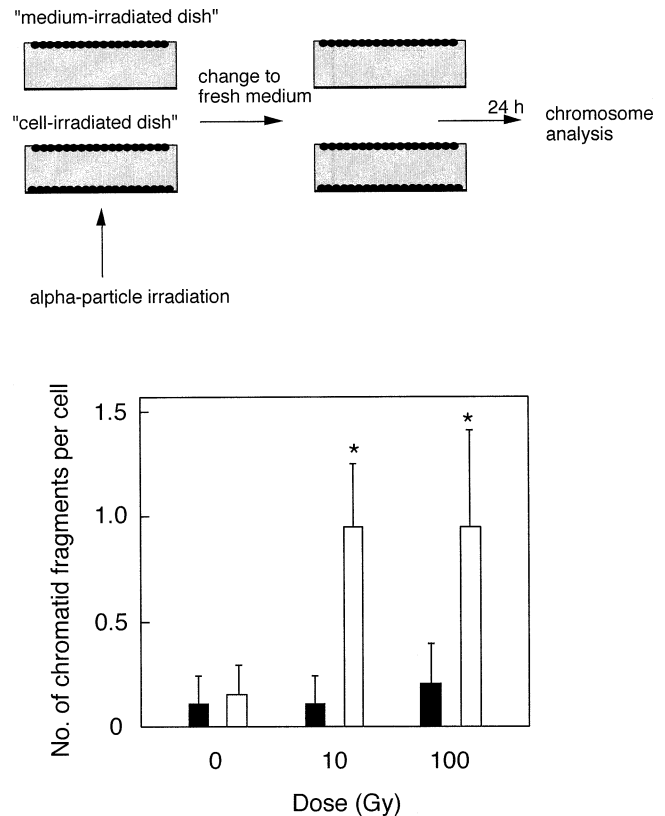


FIG. 3. The induction of chromatid fragments in unirradiated bystander A_L cells in either cell-irradiated (open bars) or medium-irradiated dishes (closed bars) when the medium was replenished with fresh medium immediately after irradiation. The results are the means and 95% confidence intervals of two or three independent experiments. * $P < 0.001$ compared to the data for medium-irradiated dishes for each dose.

medium are necessary in mediating the bystander chromatin damage in the unirradiated cells.

Effects of Irradiated Medium from either Cell-Irradiated or Medium-Irradiated Dishes

When the medium in either cell-irradiated or medium-irradiated dishes was changed to fresh medium in the same double-Mylar dish immediately after irradiation, the numbers of chromatid fragments in the unirradiated target cells in cell-irradiated dishes were found to be significantly higher than those in medium-irradiated dishes. In contrast, no chromatid alteration was observed in unirradiated cells in medium-only flasks without the bottom layer of cells (Fig. 3). These observations provide evidence that unknown modulating factors that can induce chromatid fragments in the bystander cells are produced and secreted from the irradiated bottom Mylar layer cells within 24 h after irradiation.

To ensure that an unknown modulating factor(s) was produced by and secreted from the irradiated cells, we turned the sealed double-Mylar dishes upside down and changed the medium immediately after irradiation in two different ways. In the first protocol, the medium was completely re-

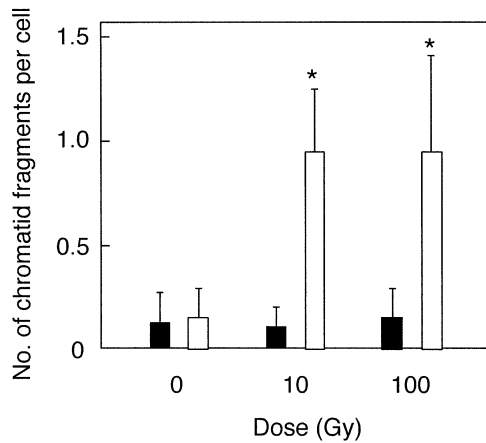
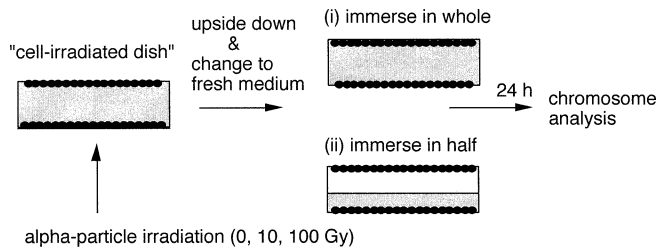


FIG. 4. The frequency of chromatid fragments in unirradiated bystander A_1 cells in cell-irradiated dishes when the medium was changed to fresh medium immediately after irradiation. Open bars: the upside down dishes were filled completely to immerse the irradiated cells. Closed bars: dishes were half filled. The results are the means and 95% confidence intervals for two or three independent experiments. * $P < 0.001$ compared to the data at the same dose for filling half of the dish dose.

plenished to fill and immerse the irradiated cells (now the top layer). In the second protocol, the dishes were only half filled so that the irradiated cells were not immersed in medium. After 24 h incubation, chromatid alterations were analyzed. As shown in Fig. 4, prematurely condensed chromosome fragments detected only in G_2 phase in the unirradiated bystander cells in dishes that were completely replenished, whereas no chromatid alteration was detected in those of the half-filled dishes.

When the medium was transferred from either cell-irradiated or medium-irradiated dishes to new target cells in a 25-cm² plastic flask (Falcon 353014) after 24 h of postirradiation incubation and culture for another 24 h, chromatid fragments were produced in the target cells added to the medium from cell-irradiated dishes, while no damage was observed in the cells added to the medium from medium-irradiated dishes (Fig. 5). The results clearly show that the life span of the unknown factor(s) may be at least 24 h.

DISCUSSION

Recent studies from our laboratory using the double-Mylar dishes showed that cells irradiated with α particles at doses ranging from 0.1 to 100 Gy released certain cytotoxic

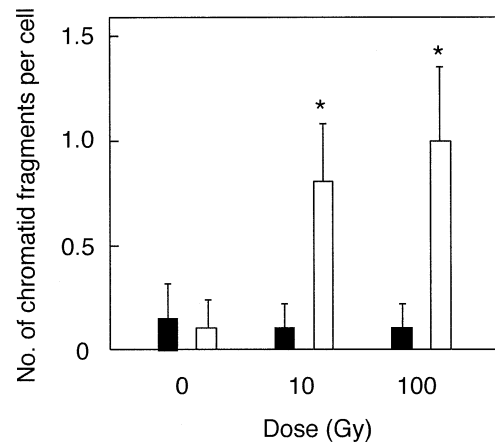
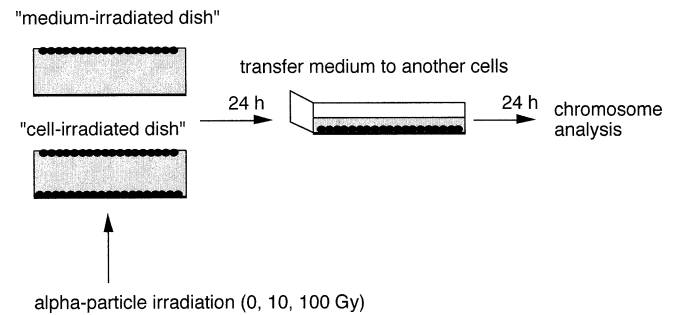


FIG. 5. The induction of chromatid fragments in either cell-irradiated (open bars) or medium-irradiated dishes (closed bars) when medium was transferred from either cell-irradiated or medium-irradiated dishes to new target cells in a 25-cm² plastic flask after 24 h postirradiation incubation and culture for another 24 h. The results are the means and 95% confidence intervals of two or three independent experiments. * $P < 0.001$ compared to the data for medium-irradiated dishes for each dose.

factors into the culture medium that were capable of killing unirradiated bystander cells. Such a factor(s), however, had little effect on mutation induction. There is evidence that irradiation of chromosomally unstable cells secreted certain death-inducing factor(s) into the medium and resulted in increased toxicity among unirradiated parental cells (26). However, the cytotoxic effect was not seen with medium transferred from irradiation of genetically stable cells. Taken together, our present findings suggested that an unknown factor(s) secreted from irradiated cells into the culture medium could induce both nonrepairable chromosomal changes and slight chromosomal aberrations (error-free type), resulting in cell killing and an increased incidence of prematurely condensed chromosome fragments in G_2 phase, but no mutagenesis in unirradiated bystander cells. Iyer *et al.* (18) reported that cell numbers in cultures that received the supernatants from α -particle-irradiated cells were ~135% higher than those in control cultures on assay day 2. Moreover, they observed decreased in basal levels of TP53 and CDKN1A in unirradiated cells. Also, there were many reports that a relatively low-level exposure to reactive oxygen species (ROS) can stimulate cell growth (27–29). We did not observe a clear difference in prolif-

eration in the cell population treated with cell-irradiated medium either directly or indirectly in which we analyzed chromatin damage. We suppose that most of the damage may be slight damage (error-free type), which may be repaired easily for up to 48 h, while the amount of damage increased up to 48 h (Fig. 1). We think there are two possible explanations: (1) an unknown factor(s) secreted from irradiated cells was capable of inducing damage at least during the first 48 h; (2) bystander cells (on the upper side of Mylar) induced genomic instability and chromatin damage during first 48 h.

The number of chromatid fragments per cell was dependent on incubation time from 3 to 48 h and not on radiation dose in cells attached on the bottom Mylar layer in the double-Mylar dishes (Fig. 1). We estimate that the number of α particles deposited per cm^2 of growth area was 5×10^5 particles at a dose of 0.1 Gy. Given that the growth area of the double-Mylar dish is 9.62 cm^2 , the total number of α particles per dish is estimated to be 4.8×10^6 particles at 0.1 Gy. With the number of cells attached on the Mylar at $\sim 10^6$ cells, we believe that enough α particles hit the cells on the bottom Mylar layer to release a sufficient amount of modulating factors such that the number of chromatid fragments per cell became saturated in the dose range of 0.1 to 100 Gy.

According to data showing a significant G_1 -phase delay in bystander cell populations (30, 31), our results show that a limited number of prematurely condensed G_2 -phase chromosomes was scored in unblocked cell populations at each radiation dose. If a G_1 block did not occur in our bystander cells, we could detect an increased number of G_2 cells with prematurely condensed chromosomes. If cells that were blocked in G_1 phase could be correlated with the quantity of chromosomal aberrations in G_2 cells with prematurely condensed chromosome fragments, which means there is a threshold number of chromosomal aberrations required for inducing G_1 block, our data on the numbers of prematurely condensed chromosomes per G_2 -phase cell may be the upper limit of the threshold for inducing G_1 block.

Based on our present findings, several pertinent observations can be made regarding the nature of the unknown modulating molecules:

1. There is evidence that these modulating factors are produced and secreted from the irradiated cells attached in the bottom Mylar layer from several minutes to 24 h after irradiation (Figs. 3 and 4).
2. The data suggest that the life span of these unknown modulating factors for inducing chromatin damage may be at least 24 h (Fig. 2).

Ionizing radiation can cause damage to biologically important molecules, such as DNA, by either direct or indirect effect. Radiation-induced radicals that originate in water, such as $\text{OH}\cdot$, $\text{H}\cdot$ and e_{aq}^- , have been found to play an important role in the indirect mechanisms of radiation biology since cells are composed of more than 80% water. Although

the half-lives of these radicals are very short (less than 200 ns), their reactivity with macromolecules is very high, and they are known to produce DNA strand breaks (32). However, our results shown in Fig. 2 indicate that short-lived radical species such as $\text{OH}\cdot$, $\text{H}\cdot$ and e_{aq}^- may not play an important role in inducing chromatin damage. The absence of any induction of prematurely condensed chromosome fragments in the G_2 -phase cells among the top layer of bystander cells as a result of 10–20 min exposure to irradiated medium from the cell-irradiated dishes suggests that bystander signaling is a time-dependent process (Fig. 1). Although we have no direct data using radical scavengers to show that these short-lived modification factors did not take part in inducing chromatin damage in the bystander cells, it is reasonable to assume that, if short-lived modification factors were involved, they would be effective within a very short time, and certainly within the half-lifetime of the radicals. The electron spin resonance (ESR) studies of Miyazaki *et al.* have shown that stable organic radicals with long lifetimes ($t_{1/2} > 20 \text{ h}$) are induced in X-irradiated mammalian cells (33). Moreover, using ESR spectroscopy, Kumagai *et al.* showed that long-lived mutagenic radicals were produced in proteins (34). There is also evidence that these radical species play an important role in radiation-induced biological effects (35, 36). Our results support the existence of such long-lived radicals. The unknown modulating factors that are capable of inducing chromatin damage in the unirradiated target cells attached on the top Mylar layer will continue their activity for at least 24 h. Here we report circumstantial evidence of the potential contribution of the medium from α -particle-irradiated cells to the induction of chromatin damage among bystander cells. The actual mechanism, however, remains unknown. Although small soluble proteins have been postulated to be involved in bystander signaling across gap junctions in confluent cultures (26, 37), it is difficult to envision the role of protein molecules in mediating the induction of chromatin damage through culture medium in the present study. It is possible, however, that receptor-mediated signaling processes are involved in the targeting of cells and the translocation of the signals from the cytosol into the nucleus to mediate the bystander response. Our results suggest two possible mechanisms that induce chromatin damage in bystander cells arising from the interaction of unknown factor(s) secreted from α -particle-irradiated cells: (1) Long-lived unknown modulating factors may interact with the cell surface and thereby induce some signals to transfer to a nearby cell or move to a nearby cell nucleus, inducing genomic instability at the chromatin level. (2) As a result of direct effects of long-lived unknown modulating factors, secondary radicals, such as $\text{OH}\cdot$, $\text{H}\cdot$ and e_{aq}^- , could be produced in the medium around the cells and attack bystander cells indirectly.

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