

Long-term persistence of X-ray-Induced Genomic Instability in Quiescent Normal Human
Diploid Cells

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Abstract

Ionizing radiation can induce genomic instability in the progeny of irradiated cells, as was demonstrated in various experimental systems. Most *in vitro* studies have utilized replicating cells, but it is not clear whether radiation-induced genomic instability persists in quiescent cells. Here we show the induction of X-ray-induced genomic instability in normal human diploid cells irradiated and maintained in a quiescent state for up to 24 months while cells were subcultured approximately once every two to three months. Every 12 months, a fraction of the irradiated cell population was stimulated to divide by culturing at a low density, and we found that these cells showed increased frequencies of phosphorylated ATM foci, decreased colony-forming ability, and increased frequency of chromosomal aberrations. No significant increases in ROS levels were detected in long-term cultured cells. These results suggest that there are ROS-independent mechanism(s) induced by radiation, which can generate persistent delayed effects in quiescent cells, and could ultimately contribute to carcinogenesis.

Introduction

Accumulating evidence suggests that ionizing radiation can cause various delayed effects in cells that have not directly absorb radiation energy (1-5). These effects, observed in non-irradiated cells, are now collectively described as non-targeted effects, which include radiation-induced genomic instability. Genomic instability is manifested in the progeny of surviving cells and is measured as the expression of various delayed effects such as delayed reproductive death or lethal mutation, delayed chromosomal instability, and delayed mutagenesis (6-9). Since radiation-induced genomic instability leads to the accumulation of gene mutations and chromosomal rearrangements, it is thought to play a pivotal role in radiation-induced carcinogenesis (10-13).

Recent advances in stem cell biology suggest the possible involvement of tissue stem cells in the development of cancer (14-17). Stem cells are able to proliferate both asymmetrically and symmetrically, and until they are stimulated to divide, some stem populations undergo quiescence in contact with a stem cell niche (18-22). Such quiescence in niche has been hypothesized to account for why cancer stem cells are refractory to chemotherapy and radiotherapy (23, 24). In contrast to the proliferating tissue stem cells, whose surviving progenies manifest radiation-induced genomic instability during the successive cell divisions, survived quiescent stem cells remain residing in the radiation-exposed tissues until they face circumstances that trigger their proliferation and increase the risk of manifesting genomic instability. Since genetic changes leading to

carcinoma are thought to accumulate in non-hematopoietic stem cells, and most of these cells remain in a quiescent state for the better part of their life span, it is highly relevant to examine the persistence of radiation-induced genomic instability in cells maintained in a quiescent state (17). As reported recently, sustained excess relative risk of solid cancers demonstrated in Atomic bomb survivors has suggested that radiation-exposed tissue stem cells residing in a niche may undergo proliferation after a long period of quiescence (25, 26). Thus, our present study was designed to determine whether or not irradiated cells that have remained in a quiescent state for long time are indeed capable of inducing delayed phenotypes after they are forced to divide. Current studies are also indispensable for the better understanding of the late effects of radiation, because non-cancerous late effects are also known to be stemmed from delayed dysfunction in stem cells of various adult tissues.

In the present study, normal human diploid cells were maintained in a confluent (quiescent) state for up to 24 months after irradiation. We found that those cells stimulated to divide after the confluence showed the delayed induction of DNA double strand breaks, as well as various delayed phenotypes, including delayed reproductive death and delayed chromosome instability, thereby indicating the persistence of radiation-induced genomic instability. Interestingly, no significant increase in ROS levels were detected in long-term cultured cells, which implicated ROS-independent mechanism(s) capable of contributing to the succession and perpetuation of the initial insults of the genome caused by ionizing radiation.

Materials and Methods

Cell culture and irradiation

Normal human diploid fibroblast-like cells were cultured in MEM supplemented with 10% fetal bovine serum (TRACE Bioscience PTY Ltd., Australia)(27). Exponentially growing cells were irradiated with an X-ray generator at 150 kVp and 5 mA with a 0.1-mm copper (SOFTEX M-150WE, Softex, Osaka). Cells remaining in a confluent state were irradiated at a dose rate with 0.44 Gy/min. After irradiation with 4 Gy, the cells were cultured in T75 flasks (75 cm²) for up to 24 months, during which time the medium was changed every 2 to 3 days. At subculture, cell numbers were determined using a cell counter (Microcell Counter, Sysmex Co. Ltd., Tokyo). The population doubling numbers (PDNs) were calculated as follows: $PDNs = \ln(N_1/N_0)/\ln 2$, where N_1 and N_0 are the cell number at the end of each passage and the number of cells inoculated, respectively.

Analysis of delayed effects

The procedure used for the analysis of delayed effects is summarized in Figure 1. Twelve and 24 months after irradiation, cells were collected by trypsinization and a portion of both the control and X-irradiated cells was kept on ice to examine ROS levels. Another portion of cells was grown in T75 flasks at a low density (1×10^6 cells/flaks), and these cells were cultured for an additional 3 and 7 days in order to examine any delayed DNA damage, delayed chromosomal instability, and to determine levels of ROS. Delayed induction of DNA double

strand breaks was determined by examining phosphorylated ATM foci. After 3 and 7 days in culture, the cells were replated onto 22 x 22 mm cover slips for immunofluorescence study using monoclonal antibody against phosphorylated ATM protein. At the same time, another portion of the proliferating cells was plated onto 100-mm dishes at a clonal density (10^2 cells per 100 mm-dish) to determine delayed reproductive death and giant cell formation in the colonies. After two weeks incubation, twenty formed colonies were randomly isolated from both populations, and the clonal cells were directly replated onto coverslips. Twenty-four hours after isolation, the cells were stained with monoclonal antibody against phosphorylated ATM protein.

Detection of delayed DNA damage

Delayed induction of DNA double strand breaks was determined by phosphorylated ATM foci as described previously (28). Cells cultured on coverslips were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100, and were washed extensively with phosphate-buffered saline (PBS). The primary antibodies, anti-phosphorylated ATM monoclonal antibody (Clone 10H11.E12, Rockland, Gilbertsville, PA) was diluted in 100 μ l of TBS-DT (20 mM Tris-HCl, 137 mM NaCl, pH7.6, containing 50 mg/ml skim milk and 0.1% Tween-20), and the antibody was applied on the coverslips. The samples were incubated for 2 hours in a humidified CO₂ incubator at 37°C. The primary antibody was washed with PBS, and Alexa488-labelled anti-mouse and anti-rabbit IgG antibodies (Molecular Probes,

Inc., OR) were added. The coverslips were incubated for 1 hour in a humidified CO₂ incubator at 37°C, washed with PBS and counterstained with 0.1 mg/ml of DAPI. The samples were examined with a F300B fluorescence microscope (Leica, Tokyo). Digital images were captured and the images were analyzed by FW4000 software (Leica). The formation of phosphorylated ATM foci was determined in 10³ cells for each group.

Analysis of delayed reproductive death and giant cell formation

Cells were trypsinized and counted using a cell counter (Microcell Counter, Sysmex Co. Ltd., Tokyo). Aliquots of 10² cells were plated onto 100-mm dishes and incubated for 14 days before they were fixed with ethanol and then stained with 3% Giemsa. Colonies of more than 50 cells were counted. The cells, which occupied an area in the colony several times greater than the rest of the cells, were considered to be giant cells, as described previously (29).

Analysis of senescence-like growth arrest

Senescence-like growth arrest was examined by senescence-associated β -galactosidase (SA- β -gal) staining. Cells were washed briefly in PBS and fixed with 2% formaldehyde containing 0.2% glutaraldehyde for 5 min at room temperature. Then, the cells were washed extensively in PBS and incubated in SA- β -gal staining solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside

(X-gal), as described previously (30).

Analysis of delayed chromosomal instability

Exponentially growing cells were treated with 0.033 $\mu\text{g/ml}$ Colcemid (GIBCO, Grand Island, NY) for 1 hour, and mitotic cells were collected. The mitotic cells were treated with 0.075 M potassium chloride for 20 min, fixed in ice-cold Carnoy's fixative (methanol:acetic acid, 3:1) for 30 min, and spread on slide glasses using an air-drying method. After these cells were stained with 3% Giemsa, chromosome aberrations were classified as previously described (31). Three independent experiments were performed, and more than 400 metaphases were counted for each sample.

Determination of oxidative stress in long-term culture

Oxidative stress was evaluated using DCFH fluorescence (32). A part of the confluent cell cultures and cells cultured for 3 and 7 days at a low density were kept on ice, washed once with PBS, and then treated with 1 μM DCFH-DA (Molecular Probes) for 30 minutes. The cells were washed with PBS, and fluorescence intensity was measured using a fluorescence spectrophotometer F2000 (Hitachi, Tokyo, Japan). The excitation and emission wavelengths were 503 nm and 524 nm, respectively.

Statistical analysis

The data were analyzed statistically using Wilcoxon test.

Results

Rare cell division in a confluent culture

In order to maintain the cells at a confluent state, control cells (1×10^7) were subcultured in T75 flasks (Figure 2). These cells underwent only one to two cell doublings within one passage. However, in X-irradiated population, some fractions of cells were expected to lose their proliferative potential, as the clonogenic surviving fraction of 4 Gy of X-rays was approximately 0.05. While no significant cell loss by apoptosis was observed, some giant cells caused by X-ray-induced senescence-like growth arrest were observed, as they were positive for SA- β -gal staining. Thus, such giant cells might be gradually eliminated from a population, most of the irradiated population contained cells that had lost proliferative potential. Therefore, the population doubling numbers (PDNs) at early passages (Figure 3) might have been underestimated. Fresh medium was supplied every 3 or 4 days, and the cells were subcultured every two (first 12 months) or three (12 to 24 months) months. The cultures were trypsinized, and 1×10^7 cells were reseeded to maintain confluent cultures. As shown in Figure 3, the total PDN of the control and 4 Gy-irradiated cells at 24 months was about 18.

Delayed induction of DNA damage

Twelve and twenty-four months after irradiation, cells were collected by trypsinization, and a portion of both the control and X-irradiated cells were cultured at low density for 3 and 7 days to analyze delayed induction of DNA damage. In addition, colonies formed by the control and irradiated populations were independently isolated, and clonal cells were cultured to analyze the induction of delayed DNA damage. The cells were fixed and stained with an antibody recognizing phosphorylated ATM, i.e., the active form of ATM protein (Figure 4). Because phosphorylated ATM forms discrete foci at sites of DNA double strand breaks, we determined the number of foci in order to estimate the delayed induction of DNA double-strand breaks in 10^3 cells. While phosphorylated ATM foci were rarely detected in the control cells (0.013 foci/cell), the number of such foci was significantly higher ($p < 0.05$) in cells derived from the exposed confluent cultures (Table 1). The frequency of phosphorylated ATM foci did not significantly differ between cells cultured for 3 days and 7 days, indicating a similar probability of genomic instability in proliferating cells, irrespective of the number of days in culture. Because it is possible that observed DNA damage was due to the initial damage that had occurred when cells were irradiated, the frequency of phosphorylated ATM foci was also determined in cells clonally expanded after long-term quiescence. As shown in Table 2, no increased in the number of phosphorylated ATM foci was observed in any of the clones derived from irradiated population, as radiation-induced genomic instability is expressed randomly among the progenies of surviving cells. However, a higher number of

clones with an increased frequency of foci was observed in irradiated clones as opposed to the non-irradiated clones. This finding clearly indicated that delayed DNA damage did occur when quiescent cells were forced to proliferate.

Delayed induction of reproductive cell death, giant cells and chromosomal instability

Delayed reproductive cell death, as determined by decreased colony-forming ability, was also apparent in cells from the exposed cultures (Table 3). In addition, the delayed induction of giant cells was more frequent in colonies formed by irradiated cells (Table 4). As shown in Table 5, the cells subcultured for 3 and 7 days also showed delayed induction of chromosomal aberrations. The frequency of every type of aberration was higher in the exposed cells than in the non-irradiated cells. The induction of chromatid-type aberrations as well as unstable chromosomal aberrations such as dicentric and ring chromosomes, indicate that delayed chromosome rearrangements occurred only after the confluent cells were forced to proliferate. In addition, the appearance of such non-clonal and multiple aberrations demonstrates that delayed chromosome instability was induced in those cells derived from exposed cultures that had been maintained in a quiescent state for a long period of time after irradiation.

Determination of oxidative stress in long-term culture

Levels of oxidative stress were evaluated using DCFH fluorescence (Figure 5). While X-irradiation immediately and directly induced a significant increase in fluorescence intensity, there were no significant increases in ROS levels in the exposed cells after they had been maintained in a confluent state for a long period of time. It is possible that ROS levels increased in cells seeded at a low density. Therefore, cells cultured for 3 and 7 days at a low density were also examined; once again, no significant increases in ROS levels were observed in these latter two groups.

Discussion

It is well established that ionizing radiation can induce genomic instability in the progeny of irradiated cells. However, most studies conducted thus far have utilized replicating cells. Here, we demonstrated persist genomic instability for up to 24 months in quiescent normal human diploid cells. It was of interest that the incidence of delayed phenotypes was almost the same in cells cultured for 12 and 24 months, indicating that radiation-induced genomic instability was comparably maintained in quiescent cultures. These results are in disagreement with those showing that radiation induced genomic instability gradually disappeared in normal proliferating human cells (33). Notably, gradual disappearance of genomic instability was not observed in p53-defective cells. For example, unstable clones

isolated from Chinese hamster cells retained their genomic instability for over 45 population doublings (34). Furthermore, we previously observed that the delayed activation of a DNA damage checkpoint in the progeny of p53-defective human tumor cells surviving radiation exposure (35). Thus, it can be concluded that in proliferating normal cells, in which DNA damage response is intact, initially unstable cells are gradually eliminated by delayed reproductive cell death or by apoptosis. The absence of growth-related cell death in quiescent cells could maintain a state of genomic instability for a long period of time after irradiation.

It should be mentioned that radiation-induced genomic instability has not been commonly observed in the previous studies using normal human cells. For example, Dugan and Bedford reported finding no evidence for the induction of such instability, although they observed a senescence-related chromosomal instability in the progeny of both irradiated and unirradiated cells (36). Such senescence-related changes were also observed in the current study. For example, as shown in Table 4, the frequency of dicentric chromosomes was higher in cells cultured for 24 months, which might be due to shortened telomeres. The reason for this discrepancy remains unknown; here, we attempt to use cells from early passages in order to avoid the possibility that senescence-related changes mask persistent radiation effects. In the present study, we confirmed the significant induction of genomic instability only in the progeny of irradiated cells. Therefore, these results indicate that the observed genomic instability in quiescent cells is indeed dependent on irradiation.

Recently, several studies have proposed the importance of tissue stem cells as targets

for carcinogenesis (14-17). In contrast to the somatic cells that divide continuously, tissue stem cells stay quiescent in a stem cell niche (18-21). Although we did not use stem cells in this study, radiation-induced genomic instability is likely to persist over the lifetime of quiescent cells such as those found in niche. Such persistent inheritance of radiation-induced genomic instability in stem cells was well-described in mice (10), and has been suggested in exposed humans (25, 26, 37, 38). Conditions enforcing cell division (e.g., tissue damage caused by radiation exposure) could provide opportunities for quiescent stem cells to manifest radiation-induced genomic instability.

The mechanism(s) underlying the perpetuation of genomic instability in quiescent cells remains to be determined. To date, elevated ROS levels have been considered as a cause of persisted instability (39-44). Recent *in vivo* studies have suggested that inflammatory-type tissue response provides microenvironment that leads to persistent genomic instability (42). Moreover, it has been shown that dysfunctional mitochondria are involved in the persistence of radiation-induced genomic instability (39-44). Thus, it is reasonable to consider that persistent oxidative stress is among the mechanisms associated with radiation-induced genomic instability. We, therefore, examined ROS levels using DCFH fluorescence in irradiated quiescent cells. Although direct ROS production was observed immediately after irradiation, no increases were observed in ROS levels of quiescent cells cultured for a long period of time after irradiation. We also examined ROS levels in cells forced to proliferate for 3 and 7 days at a low cell density. However, neither of these groups showed increased levels

of ROS. Thus, our results clearly indicated that ROS-independent mechanism can contribute to the perpetuation of radiation-induced genomic instability. As we reported previously, X-ray-induced large deletions potentially cause unstable chromosome regions (PUCRs), which could be transmitted through generations (9, 45). These deletions are not DNA breaks by themselves, but abnormal structures in higher-order chromatin created through mis-rejoining of DNA double strand breaks. Such structural radiation signature may mediate perpetuation of genomic instability in quiescent cells.

In summary, the present study demonstrated that X-irradiation induces genomic instability in normal quiescent human diploid cells, and this instability persists for up to 24 months after irradiation. These findings indicated that ROS-independent mechanisms "memorize" initial DNA damage, and are somehow associated with radiation-induced genomic instability. As no delayed phenotypes accumulate in the quiescent cells observed here, such a DNA damage memory is most likely to induce delayed DNA breakage, which result in the induction of delayed phenotypes, only after quiescent cells are exposed to a condition that initiate cell proliferation.

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Figure legends

Figure 1. Experimental procedure for the analysis of delayed effects.

Figure 2. Morphology of normal human diploid cells cultured in different conditions.

(A) Confluent 4 Gy-irradiated cells cultured for 12 months, (B) A portion of 4 Gy-irradiated cells cultured at confluence for 12 months, reseeded at a low cell density.

Figure 3. Population doubling numbers of control and 4 Gy-irradiated cells cultured at confluence.

Control (open circles) and 4 Gy-irradiated (closed circles) cells maintained at confluence were subcultured every two or three months, and the population doubling numbers were calculated as described in Materials and Methods.

Figure 4. Phosphorylated ATM foci in cells derived from confluent cultures maintained for 12 months.

A portion of cells from control (A) and 4Gy-irradiated (B) confluent cultures was grown at a low cells density. Cells were stained with anti-phosphorylated ATM antibody as described in Materials and Methods. Phosphorylation of ATM is detected with Alexa-488-labelled secondary antibody (green), and chromosomal DNA is counterstained with DAPI (blue). Triangles indicate the foci-positive nuclei.

Figure 5. Oxidative levels determined by DCFH fluorescent assay.

Portions of both control (open bars) and 4 Gy-irradiated (closed bars) confluent cultures maintained for 12 and 24 months were treated with 1 μM DCFH-DA for 30 minutes. In addition, cells cultured for 3 days (3D) and 7 days (7D) at a low density were treated with 1 μM DCFH-DA for 30 minutes. Fluorescence intensity was determined by a fluorescence spectrophotometer as described in Materials and Methods.

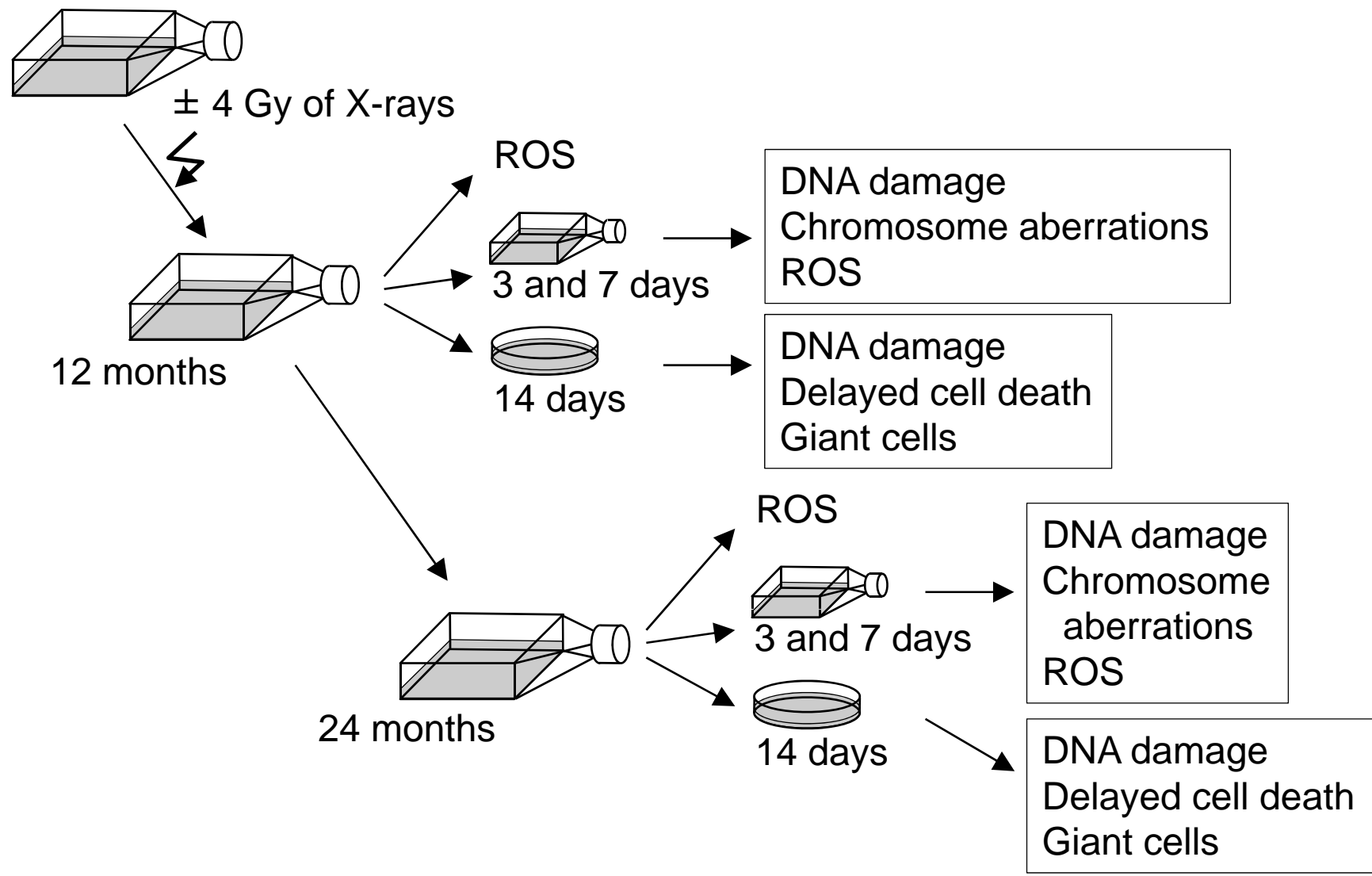


Figure 1 Suzuki et al

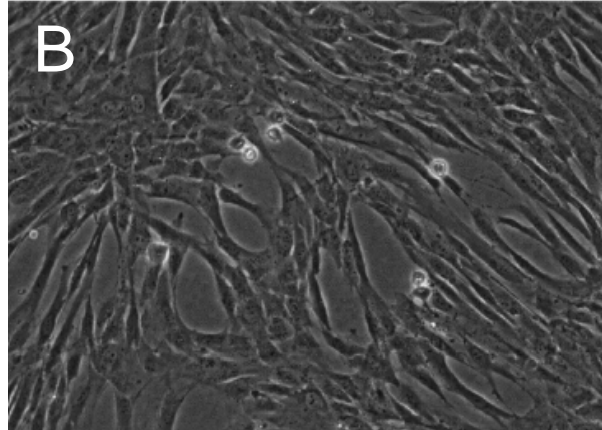
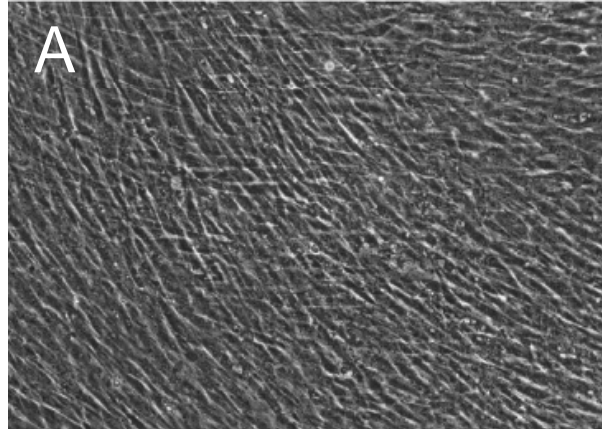


Figure 2 Suzuki et al

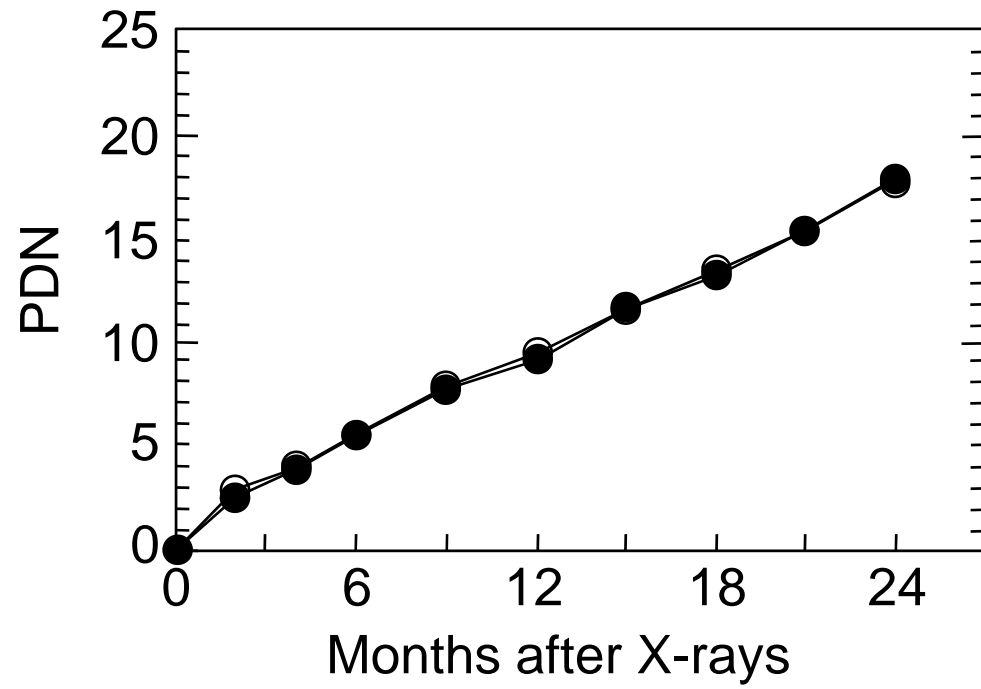


Figure 3 Suzuki et al

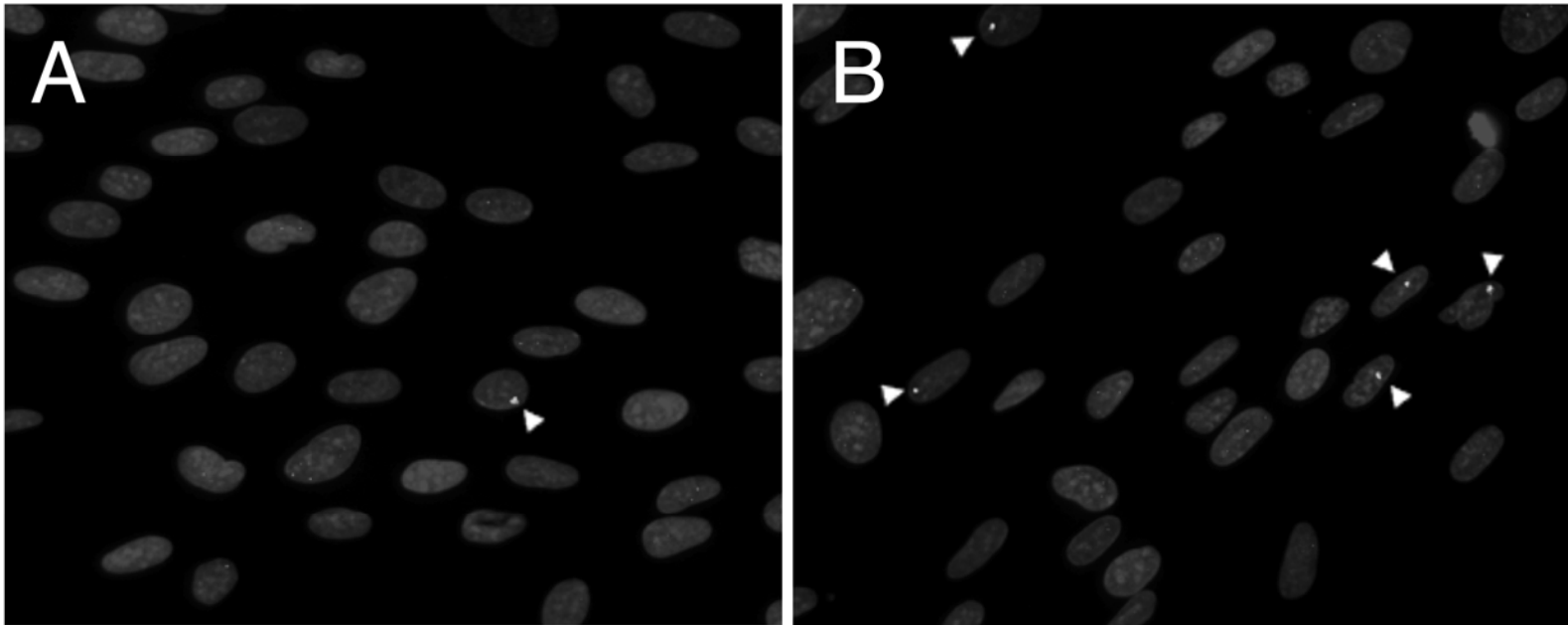


Figure 4 Suzuki et al

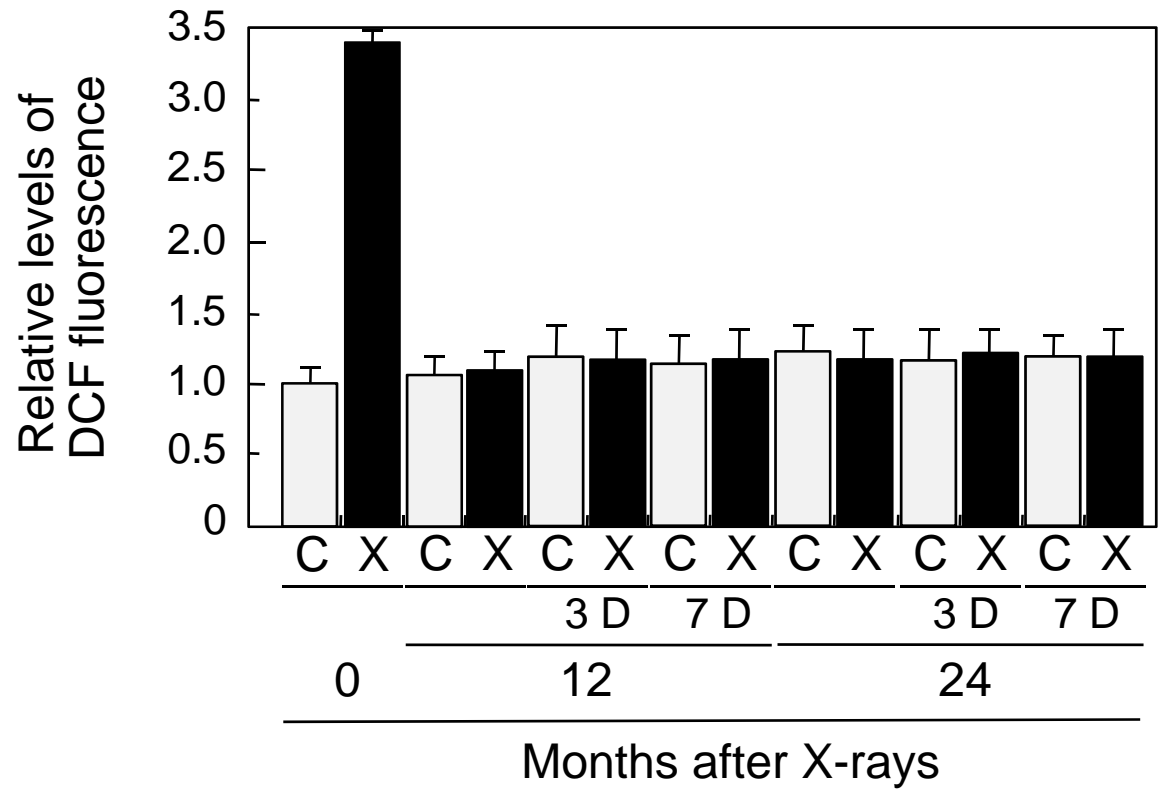


Figure 5 Suzuki et al

Table 1 Delayed induction of phospho-ATM foci

Cells	Total no. cells counted	% cells with foci	No. foci per cell ($\times 10^{-2}$)
12 months after X-rays			
- 3 days			
0 Gy	1000	1.3 ± 0.6	1.3 ± 0.6
4 Gy	1000	5.0 ± 0.2	7.0 ± 2.0] $p < 0.01$
- 7 days			
0 Gy	1000	1.5 ± 0.7	1.5 ± 0.7
4 Gy	1000	5.3 ± 0.3	7.5 ± 2.2] $p < 0.01$
24 months after X-rays			
- 3 days			
0 Gy	1000	1.7 ± 0.6	2.7 ± 1.2
4 Gy	1000	7.0 ± 0.2	9.3 ± 3.5] $p < 0.05$
- 7 days			
0 Gy	1000	1.6 ± 0.6	2.8 ± 1.4
4 Gy	1000	6.9 ± 0.5	9.5 ± 3.7] $p < 0.05$

Table 2 Delayed induction of phospho-ATM foci in isolated colonies

Cells	Total no. cells counted	% cells with foci	No. foci/cell (x10 ⁻²)	Cells	Total no. cells counted	% cells with foci	No. foci/cell (x10 ⁻²)
12 months after X-rays							
C1	1000	1.3	1.3	X1	1000	4.3	7.3
C2	1000	1.2	1.2	X2	1000	1.0	1.3
C3	1000	1.0	1.3	X3	1000	1.3	1.9
C4	1000	1.1	1.5	X4	1000	1.2	1.7
C5	1000	1.2	1.3	X5	1000	1.1	1.6
C6	1000	1.3	1.3	X6	1000	4.5	6.3
C7	1000	1.1	1.1	X7	1000	7.3	11.9
C8	1000	1.0	1.5	X8	1000	1.1	1.3
C9	1000	1.0	1.8	X9	1000	1.2	3.3
C10	1000	1.3	1.9	X10	1000	1.3	4.1
C11	1000	1.5	1.5	X11	1000	6.3	9.2
C12	1000	1.9	1.9	X12	1000	3.1	4.8
C13	1000	1.1	1.4	X13	1000	3.3	6.9
C14	1000	1.0	1.3	X14	1000	2.3	3.1
C15	1000	1.1	1.3	X15	1000	1.2	1.5
C16	1000	1.1	1.4	X16	1000	9.3	14.5
C17	1000	1.3	1.7	X17	1000	1.0	1.3
C18	1000	1.7	2.1	X18	1000	1.1	1.1
C19	1000	1.5	1.8	X19	1000	3.3	4.3
C20	1000	1.7	1.9	X20	1000	1.9	2.0
24 months after X-rays							
C1	1000	1.1	1.3	X1	1000	5.3	11.3
C2	1000	1.3	1.7	X2	1000	4.2	6.1
C3	1000	1.5	1.9	X3	1000	3.3	5.0
C4	1000	1.7	2.1	X4	1000	2.9	4.3
C5	1000	1.2	1.8	X5	1000	1.5	1.9
C6	1000	1.3	1.5	X6	1000	1.3	3.3
C7	1000	1.3	1.8	X7	1000	1.1	1.7
C8	1000	1.0	1.9	X8	1000	1.0	2.8
C9	1000	1.1	2.0	X9	1000	7.3	13.3
C10	1000	1.0	1.3	X10	1000	7.2	8.3
C11	1000	1.2	1.8	X11	1000	9.3	11.0
C12	1000	1.4	1.9	X12	1000	4.0	5.3
C13	1000	1.3	1.5	X13	1000	1.1	1.8
C14	1000	1.7	2.6	X14	1000	3.7	5.3
C15	1000	1.2	2.7	X15	1000	2.3	3.3
C16	1000	1.1	1.7	X16	1000	1.0	2.0
C17	1000	1.3	1.5	X17	1000	4.7	7.7
C18	1000	1.0	1.8	X18	1000	5.3	10.5
C19	1000	1.1	1.8	X19	1000	7.1	6.7
C20	1000	1.2	1.7	X20	1000	1.8	3.1

Table 3 Delayed reproductive death

Cells	Cloning efficiencies (%)
12 months after X-rays	
0 Gy	32.7 ± 1.5
4 Gy	17.0 ± 2.0] $p < 0.01$
24 months after X-rays	
0 Gy	10.7 ± 0.6
4 Gy	4.0 ± 1.0] $p < 0.01$

Table 4 Delayed induction of giant cells

Cells	No. colonies with giant cells / No. colonies counted (%)
12 months after X-rays	
0 Gy	9 / 264 (0.034)
4 Gy	44 / 228 (0.193)] $p < 0.01$
24 months after X-rays	
0 Gy	13 / 320 (0.041)
4 Gy	19 / 220 (0.086)] $p < 0.01$

Table 5 Induction of delayed chromosomal instability

Cells	No. cells counted	No. metaphases with aberrations (%)	No. aberrations				
			Dic (with Frag)	Gaps	Breaks	Fragments	Ring
12 months after X-rays							
- 3 days							
0 Gy	418	7 (1.7)	0	3	2	2	0
4 Gy	402	44 (10.9)	12 (12)	20	12	15	0
] p<0.01				
- 7 days							
0 Gy	513	7 (1.4)	0	2	3	2	0
4 Gy	472	52 (11.0)	10 (10)	25	13	4	0
] p<0.01				
24 months after X-rays							
- 3 days							
0 Gy	500	17 (3.4)	3 (1)	7	7	3	0
4 Gy	652	86 (13.2)	34 (13)	34	22	20	9
] p<0.01				
- 7 days							
0 Gy	515	19 (3.7)	3 (3)	8	7	4	0
4 Gy	635	81 (12.8)	27 (19)	27	15	7	5
] p<0.01				