Cell Lines Derived from a Medaka Radiation-Sensitive Mutant have Defects in DNA Double-Strand Break Responses

Masayuki HIDAKA¹, Shoji ODA¹, Yoshikazu KUWAHARA², Manabu FUKUMOTO² and Hiroshi MITANI¹*

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It was reported that the radiation-sensitive Medaka mutant “ric1” has a defect in the repair of DNA double-strand breaks (DSBs) induced by γ-rays during early embryogenesis. To study the cellular response of a ric1 mutant to ionizing radiation (IR), we established the mutant embryonic cell lines RIC1-e9, RIC1-e42, RIC1-e43. Following exposure to γ-irradiation, the DSBs in wild-type cells were repaired within 1 h, while those in RIC1 cells were not rejoined even after 2 h. Cell death was induced in the wild-type cells with cell fragmentation, but only a small proportion of the RIC1 cells underwent cell death, and without cell fragmentation. Although both wild-type and RIC1 cells showed mitotic inhibition immediately after γ-irradiation, cell division was much slower to resume in the wild-type cells (20 h versus 12 h). In both wild-type and RIC1 cells, Ser139 phosphorylated H2AX (γH2AX) foci were formed after γ-irradiation, however, the γH2AX foci disappeared more quickly in the RIC1 cell lines. These results suggest that the instability of γH2AX foci in RIC1 cells cause an aberration of the DNA damage response. As RIC1 cultured cells showed similar defective DNA repair as ric1 embryos and RIC1 cells revealed defective cell death and cell cycle checkpoint, they are useful for investigating DNA damage responses in vitro.

INTRODUCTION

A wide range of genomic lesions from extracellular DNA-damaging agents such as ultraviolet radiation, ionizing radiation (IR), genotoxic chemicals, and reactive oxygen species can cause chromosomal changes, genetic mutations, and malignant transformations, all of which may lead to cancer or accelerate aging. DNA double-strand breaks (DSBs) are the most severe lesions and must be rapidly recognized and repaired in order for a cell to survive.1 DSBs activate the cell cycle checkpoint and are repaired by two main pathways: non-homologous end joining (NHEJ) and homologous recombination.2 If unrepaired or repaired incorrectly, DSBs can lead to apoptosis.3 It has been reported that in response to DSBs, the histone H2A variant, H2AX, is phosphorylated on Ser 139 (known as γH2AX)4 and is a key component in DNA repair.5–7 H2AX is also phosphorylated following the initiation of DNA fragmentation during apoptosis8–10 and can bind mediator of DNA damage checkpoint protein 1 (MDC1) which helps regulate the cellular response to DNA DSBs.11 The loss of MDC1 expression has been shown to impair the G2/M checkpoint.12 Although the molecular mechanisms of DNA repair,13–15 apoptosis,16–18 and the cell cycle checkpoint19–21 have been studied in detail, the specific regulatory mechanisms of the DNA damage response in vivo are still unknown.

Fish are useful models in radiation biology because they have a body plan that is morphologically and physiologically related to mammals. In particular, Medaka (Oryzias latipes) is a popular model organism as its eggs and embryos are transparent, making it easy to observe early developmental processes.22,23 Medaka also has the potential to be a useful in vivo model to investigate the DNA damage response, as demonstrated by the identification of a radiation-sensitive mutant, named ric (radiation induced curly tail malformation), during a genome-wide screening of N-ethyl-N-nitrosourea (ENU)-mutagenized Medaka investigating the in vivo mechanisms of genome stabilization.24 Homozygotes for the ric1 mutation, which is on one of three loci, show lower hatchability and higher malformation rates after γ-irradiation than the wild-type strain. Ric1 mutants in the pre-early gastrula stage also have defects in the early rejoining of DSBs induced by γ-rays.25 Although the ric1 mutant may be a useful model to investigate the response to DNA damage, it is nec-

*Corresponding author: Phone: +81-471-36-3670, Fax: +81-471-36-3669, E-mail: mitani@k.u-tokyo.ac.jp
1Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo; 2Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University.
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essary to first examine and understand the in vitro response to \(\gamma\)-rays, such as cell death and DNA damage checkpoint. In this study, mitotic inhibition, cell death, and formation of \(\gamma\)H2AX foci after ionizing irradiation were examined using three cultured cell lines derived from ric1-embryos.

**MATERIALS AND METHODS**

**Cell culture**
OLCAB-e3 and OLHdrR-e3 are fibroblast cell lines established from a single embryo of the wild-type strain.\(^{25}\) Primary culture of the cell lines established from a single embryo (RIC1-e9, RIC1-e42 and RIC1-e43) was performed as described by Komura *et al.*\(^ {26}\) Embryos from ric1 strains were maintained at 27°C for 5 days after fertilization. The embryos were sterilized for 10 s in Dakin’s solution (58 mM NaOCl, 6 mM HCl, and 93 mM NaHCO\(_3\)) washed three times in phosphate-buffered saline (PBS) and then transferred to L-15 medium. After removing the chorion and yolk sac, the embryos were minced with sterilized razors to obtain fibroblast-like cells, which were subcultured once a week in L-15 medium at 33°C for almost 2 years. Cells were cultured in L-15 medium (Irvine, CA, USA) supplemented with 20% fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan), 50 \(\mu\)g/ml streptomycin and 10 mM 2-[4-(-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.5) and incubated at 33°C.

**Irradiation**
Cells in culture dishes or glass bottom dishes were exposed to various doses of \(\gamma\)-rays from a \(^{137}\)Cs source at a dose rate of 10 Gy/min (Elan 3000; MDS Nordion, Ottawa, Canada).

**Neutral comet assay**
A neutral comet assay was performed as previously described with a few modifications.\(^{24,27}\) Briefly, cells were detached using 0.05% trypsin (Gibco–BRL, Grand Island, NY, USA) with 0.002% EDTA and suspended in L-15 medium. The cell suspension (2 \(\times\) 10\(^3\) /10 \(\mu\)l) was then mixed with 300 \(\mu\)l of 1% low-melting agarose in PBS and layered on top of a microscope slide coated with 1% agarose and spread evenly using a coverslip. After placing the slide on ice for 3 min to solidify the agarose, the coverslip was gently removed and the slide was immersed in a freshly prepared lysis solution (2% SDS and 30 mM EDTA 2Na) for 30 min in darkness. After washing with TBE, electrophoresis was performed at 20 V for 20 min. The DNA was subsequently stained with 100 ng/ml propidium iodide for 10 min in the dark. Sixty cells per slide were analyzed under a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) equipped with a TV camera (DP-70; Olympus, Tokyo, Japan), and the images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).\(^ {28}\) The assay was performed twice for each cell line and condition (i.e., irradiated versus non-irradiated). The tail moment (TM) was determined from the percentage of total fluorescence in the comet tail.

**Computerized video time-lapse microscopy**
In order to monitor cell death and the arrest of cell division, cells in 35 mm culture dishes were placed in an inverted microscope (IX-81; Olympus, Tokyo, Japan) equipped with a TV camera (Orca ER II; Hamamatsu Photonics, Hamamatsu, Japan) and the data were analyzed using IP Lab software (Scanalytics, Inc., Fairfax, VA, USA). Approximately 100 cells from each cell line were observed at 3 min intervals for 24 h. Each time-lapse analysis was repeated three times under the same condition in order to confirm the results.

**Immunocytochemistry**
One \(\times\) 10\(^5\) cells per dish were seeded in a 35 mm glass bottom dish (Matsunami Glass, Ind., Osaka, Japan) and incubated overnight at 33°C. At specific time points, the cells were irradiated and then incubated at 33°C for 15 min, 1 h, or 6 h. The irradiated cells were fixed by adding 0.5 ml per dish of 4% formaldehyde for 10 min at room temperature, washed three times with PBS, and then permeabilized with 0.5 ml per dish of 0.5% Triton X-100 (Wako) in PBS for 5 min at 4°C. The cells were incubated overnight with mouse monoclonal anti-phospho-H2AX (Ser139) antibody

![Fig. 1. DNA DSB repair in Medaka cells after 15 Gy \(\gamma\)-irradiations.](image-url)
(Upstate, Inc., Lake Placid, NY) at a 1:5,000 dilution in blocking buffer consisting of 0.5% Triton X-100 and 0.2% goat serum (100 μl per dish) at 4°C in a humidified box. After washing three times with PBS, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:5000 in 3% BSA (100 μl per dish) for 1 h at room temperature in the dark. The cells were then washed three times with PBS and counterstained with 20 μg/ml propidium iodide and RNase (200 μg/ml) for 5 min at room temperature. Stained cells were washed with PBS and then placed in 10% glycerol. The number of foci was counted for each time point under a fluorescence microscope (IX-81; Olympus, Tokyo, Japan) equipped with a TV camera (DP-70; Olympus,

Fig. 2. Morphologies and frequencies of cell death in cultured Medaka cells after 10 Gy γ-irradiation. (A) Two types of morphological change were observed during γ-irradiation-induced cell death: cell death with fragmentation (black arrow, apoptotic body) and cell death without cell fragmentation (white arrow, indicating necrosis). 10 Gy γ-irradiation induced cell death with fragmentation in wild-type cells within 1 h. In contrast, γ-irradiated RIC1 cells underwent cell death without cell fragmentation over a period of several hours. Scale is 25 μm (white bar). (B) A graph of cumulative cell death (percentage) over a period of 24 h following γ-irradiation. Each time point represents the accumulated percentage of cell death (mean ± SEM). The percentage of cell death in the non-irradiated controls was 5.0 ± 2.4% (OLCAB-e3), 0.9 ± 0.9% (OLHdrR-e3), 0.9 ± 0.9% (RIC1-e9), 5.4 ± 2.9% (RIC1-e42), 0.7 ± 0.7% (RIC1-e43).
Tokyo, Japan), and the images were processed using Adobe Photoshop. More than 40 cells were observed.

RESULTS

Rejoining of DNA DSBs after γ-irradiation

The ability of RIC1 cells to repair DSBs was examined using the neutral comet assay (Fig. 1). Immediately after 15 Gy γ-irradiation, the TMs of both wild-type and RIC1 cells were in an identical range. Although the TMs of the wild-type cells decreased significantly (P < 0.0005) 1 h after γ-irradiation, the TMs of the RIC1 cells had decreased only slightly in the same time period. However, after 4 h, the TMs of the RIC1 cells had also decreased significantly. The difference in the TMs between the RIC1 and wild-type cells at 4 h was smaller than those at 1 h and 2 h. These results indicate that cell lines derived from ric1 embryos have severe defects in the early rejoining of the DSBs induced by γ-irradiation and are also slower to repair damaged DNA.

Time-lapse analysis of cell death after γ-irradiation

OLCAB-e3 and OLHDrR-e3 cells began to shrink 4 h after γ-irradiation (10 Gy) and subsequently detached from the culture dish by membrane blebbing and fragmented into apoptotic bodies (Fig. 2A, upper panels), with the entire apoptotic process taking approximately 1 h. OLCAB-e3 and OLHDrR-e3 control cells that were not irradiated underwent cell death at very low levels. While γ-irradiation induced apoptosis in roughly 30% of the OLCAB-e3 and OLHDrR-e3 cells within 24 h after γ-irradiation (Fig. 2B), the highest rates were observed 4–12 h after γ-irradiation.

Although some of the RIC1 cells had detached from the dish 24 h after γ-irradiation, cell fragmentation was not observed. Instead, membrane blebbing was first observed followed by membrane breakage, which occurred over several hours as is typical of necrotic cells (Fig. 2A, lower panels). Approximately 10% of the γ-irradiated RIC1 cells underwent necrotic cell death (Fig. 2B).

Fig. 3. Cell division in cultured Medaka cells after 10 Gy γ-irradiation. Each time point represents the percentage of dividing cells after γ-irradiation. The number of dividing cells was determined by time-lapse microscopic observation. The average percentage of dividing cells for each non-irradiated control cell line was 12.4 ± 1.3% (OLCAB-e3), 12.1 ± 0.7% (OLHDrR-e3), 9.6 ± 1.8% (RIC1-e9), 11.1 ± 0.7% (RIC1-e42), and 12.6 ± 0.9% (RIC1-e43).

Fig. 4. Induction of γH2AX foci in cultured Medaka cells after 10 Gy γ-irradiation. (A) Within 15 min of γ-irradiation, γH2AX foci were observed in all of the cell lines, however, after approximately 1 h more than half of the foci had disappeared. After 6 h, most foci had disappeared in the RIC1 cells, while roughly 30% of the foci remained in the wild-type cells. (B) A graph representing the number of foci per nucleus at various time points after 10 Gy of γ-irradiation. For each cell line, foci from over 40 cells were used to calculate the average value.
Cell division arrest

As it is known that IR induces the G2/M checkpoint and mitotic inhibition, it was anticipated that in addition to apoptosis, the ric1 mutation would cause an aberrant induction of checkpoint. Microscopic observation of the morphological changes in dividing cells is a simple and reliable method for monitoring the arrest of cell division. Following γ-irradiation, all of the cell lines immediately stopped cell division (Fig. 3) and most of the OLCAB-e3, OLHdR-e3, and RIC1-e9 cells remained arrested for up to 16–20 h after γ-irradiation. In contrast, the RIC1-e42 and RIC1-e43 cells resumed cell division approximately 8–12 h after γ-irradiation. By 20–24 h after γ-irradiation, the rate of cell division of OLCAB-e3 and OLHdR-e3 cells had recovered to roughly 30% of that of the non-irradiated cells, while the RIC1-e9 cells had recovered to roughly 70% of the rate of the control cells.

γH2AX foci formation

We next examined the number of γH2AX foci induced by γ-irradiation (10 Gy) in each of the cell lines. In both the wild-type and RIC1 cells, H2AX was phosphorylated within 15 min of γ-irradiation (Fig. 4A, B), although the average number of γH2AX foci in the RIC1-e42 and RIC1-e43 cells was less than the wild-type cells. Although the comet assay revealed that most DSBs in the RIC1 cells were not rejoined within 1 h of γ-irradiation, more than half of the γH2AX foci disappeared in the same time-dependent manner as the wild-type cells (Fig. 4A, B, 10 Gy 15 min, 1 h). Several γH2AX foci were observed in the wild-type cells even 6 h after 10 Gy γ-irradiation, while almost no γH2AX foci were observed in the RIC1 cells at this time point. The RIC1 cells showed unstable γH2AX foci after 50 Gy (data not shown).

DISCUSSION

In this study, the following defects in the RIC1 cells were found: (1) a delay in the early repair of DSBs, (2) the cells undergo a lower frequency of death which occurs without cell fragmentation, (3) abnormal resumption of cell division (in two of three cell lines) and (4) unstable γH2AX foci. It has been reported that ric1 embryos have defective DNA repair, which was also observed in the cultured RIC1 cells. However, even though ric1 embryos show a high incidence of apoptosis, the number of foci remaining after 1 h of γ-irradiation and showed a later release from the arrest of cell division than the RIC1-e42 and RIC1-e43 cells, whereas defective DNA DSB repair and instability of γH2AX foci were common in all RIC1 cell lines. We therefore speculate that the number of foci remaining after 1 h relates to the duration of the delay in cell division. As the possibility exists that the RIC1 cell lines were derived from different organs, the observed differences may have been due to varying initial levels of γH2AX. Koike et al. has demonstrated tissue-specific DNA-PK-dependent H2AX phosphorylation, which may also help explain the difference in the number of γH2AX foci.

Cook et al. showed that when H2AX contains both phosphorylated Tyr142 and Ser139, it interacts with the pro-apoptotic factor JNK1 to induce cell death. We observed that γ-irradiation induced cell death in only 10% of the RIC1...
cells, whereas cell death was induced in 30% of the wild-type cells. These results indicate that the instability of γH2AX foci in RIC1 cells caused the impairment of JNK1 recruitment and the subsequent reduction in cell death. Lu et al. suggested that DNA fragmentation during apoptosis induced by UVA irradiation appeared to be dependent upon Ser139 phosphorylation given that caspase-3 activation occurred in UVA-irradiated H2AX+/− cells, whereas DNA fragmentation was not observed. In the present study, RIC1 cells also underwent cell death without cell fragmentation after γ-irradiation, which leads us to hypothesize that caspase-3 was activated, but cell fragmentation was not induced because of aberrant DNA fragmentation.

Our data suggest that the instability of H2AX Ser139 phosphorylation in RIC1 cells results in aberrant DNA repair and cell death, and implies the sustainability of Ser139 phosphorylation is necessary for both of these phenomena. Although we have demonstrated the ric1 gene regulates H2AX Ser139 phosphorylation, it remains unclear how it affects the balance between DNA repair and cell death, and if it also controls other protein modifications of H2AX, both of which are topics for future studies.

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