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RESEARCH ARTICLE

Apoptotic cell death in erythrocytes of p53-deficient medaka (*Oryzias latipes*) after γ-irradiation

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ABSTRACT

Purpose: Previous studies have examined the effects of γ-irradiation (γ-IR) on wild-type and p53 mutant Medaka (*Oryzias latipes*) 24 hours after irradiation and in the present work, apoptosis and alterations in erythrocytes of 4, 8 and 24 h and 14 days after gamma-ray irradiation were reported as genotoxic biomarkers of γ-irradiation.

Materials and methods: Sexually mature wild-type, WT (Hd-rR) and p53(−/−) adult female medaka (*O. latipes*) were exposed to 4 Gy dose of γ-IR and sampling were collected after 4, 8 and 24 h and 14 days.

Results: Apoptosis and morphological alterations were observed from 4 h after irradiation and remarkably increased 8 h after irradiation in the wild-type. Apoptotic cell death has been observed 8 h after irradiation most prominently but subtle in p53 mutant medaka. All these phenotypes were recovered 14 days after irradiation in both strains. Although no micronuclei were seen in any group, nuclear abnormalities were observed in red blood cells. Both apoptosis and morphological alterations in erythrocytes were decreased after 24 and 14 days after γ-irradiation.

Conclusions: We conclude that apoptosis and malformations caused by 4 Gy γ-irradiation in the erythrocytes of medaka fish occurs from 4–24 h and the initial response until 8 h was p53-dependent.

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Introduction

Accidental nuclear radioisotope release into the ocean from nuclear power plants is of concern because of the serious ecological and health risks (Won and Lee 2014). Gamma ray radiation induces severe damage in diverse organisms at multiple levels from molecules to individuals (e.g. DNA, chromosomal aberrations, cells, proteins, growth and reproduction) (Sudprasert et al. 2006; Aquino 2012; Rhee et al. 2012; Sayed et al. 2014). In fish blood cells, Sayed et al. (2014) reported that gamma radiation induces chromosomal aberrations such as nuclear abnormalities.

Blood is an excellent indicator of the health of an organism, and hematological parameters are important for determining the effects of environmental stressors and toxic substances on fishes (Sayed et al. 2014). Recently, the interest of fish micronucleus (MN) assays for monitoring genotoxic effects after stressors has been increasing, especially in a number of teleostean fish (Al-Sabti and Metcalfe 1995; Takai et al. 2004a; Mekkawy et al. 2011; Sayed et al. 2014). The Japanese medaka (*Oryzias latipes*) is a unique vertebrate model for investigating the effects of irradiation (IR) (Yasuda et al. 2012).

Our previous study using medaka fish indicated the dangerous effects of 10 Gy γ-IR on blood cells of medaka using MN and morphological alterations as biomarkers (Sayed et al. 2014). It has been reported that the MN assay is a reliable test for detecting potential induces of DNA double-strand breaks (Takai et al. 2004a; Sayed et al. 2014). Several studies demonstrated that the metachromatic dye acridine orange (AO) is suitable for medaka gill blood cells (Takai et al. 2004b; 2008). The tumor protein p53 plays a critical role in determining whether DNA will be repaired or the damaged cell will undergo apoptosis. A mutant medaka strain has a p53 gene after X-rays (Takai et al. 2004a). Also, it has been demonstrated that AO distinguishes immature cells from mature cells and identifies micronuclei, although most of MN tests in fishes use erythrocytes as target cells for MN induction and Giemsa staining method for the MN detection (Takai et al. 2004a; 2008).

Because of the gender-independent response of medaka to spontaneous and X-rays-induced MN formation (Takai et al. 2004b), this study was conducted to elucidate the acute effects of γ-IR and its ability to induce apoptosis and morphological alterations in blood erythrocytes in adult female wild-type and p53-deficient medaka. Also, the present study addressed the issues of the using AO-staining for the peripheral erythrocytes MN assay and apoptosis detection after γ-IR.

Materials and methods

Fish

Sexually mature WT (Hd-rR) and p53(-/-) adult female medaka (*O. latipes*) were studied. The fish were kept at 26–28 °C

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under 14 h light: 10 h dark cycle. They were fed a powdered diet (Tetra-min, Tetra Werke Co., Mells, Germany) and brine shrimp (*Artemia franciscana*) three times a day.

γ-IR exposure

The WT and p53 (-/-) fish were divided into five groups, one control group and four groups exposed to γ-IR (six fish per group). The exposed fish were subjected to whole-body IR from ^{137}Cs γ-rays (Gammacell 3000 Elan, MDS Nordion, Ottawa, Canada) at a dose of 4 Gy (1 Gy/8 sec) at room temperature in a water-containing plastic tube without anesthesia. The fish were dissected 4, 8, 24 h and 14 days after γ-IR.

Apoptosis detection

Apoptotic erythrocytes were detected using acridine orange (AO) stain (Cat. No. A1031, Life Technologies, Carlsbad, CA). The modified protocol according to Sayed et al. (2015) was used to detect the apoptosis in red blood cells (RBC), after preparation of blood smears on clean glass slides, the slides were washed in 1× PBS (pH = 7.2). AO buffer (17 µg/l AO in 1× PBS buffer) was added to the slides for 30 min in the dark. Decolorization process was achieved by washing the slides every 30 min with 1× PBS four times. Fixation was in paraformaldehyde 4% for 5 min. Cell observation was made under an Olympus fluorescence microscope (PX50) provided with a digital Olympus color video camera (DP70).

Micronucleus test and erythrocytes alterations

Blood smears were obtained by caudal incision and blood was collected onto clean grease-free microscope slides 4, 8, 24 h and 14 days after IR. The smears (seven slides from each fish) were dried, fixed in absolute methanol for 10 min, and stained with Giemsa stain as reported previously (Tavares-Dias and Moraes 2003). Slides were selected on the basis of staining quality, coded, randomized and scored blindly. In each group 10,000 cells (minimum of 1000 per slide) were examined using the method of (Al-Sabti and Metcalfe 1995) under a 40× objective and 10× eyepiece to identify micronucleated and morphologically altered erythrocytes in separate studies. The established criteria for identifying micronuclei (Schmidt 1975) were followed strictly to ensure authentic scoring.

Statistical analysis

The means, standard divisions and ranges were estimated. One-way analysis of variance was used to analyze the data using SPSS software (SPSS 1998) at the 0.00001 significance level. Tukey's HSD test was used for multiple comparisons and to verify the frequency of apoptotic and altered erythrocyte.

Ethics statement

All experiments were performed in accordance with the Japanese laws and university guidelines for the care of experimental animals according to The University of Tokyo Animal Experiment Enforcement Rule.

Results

Apoptosis detection

γ-IR (4 Gy) of WT (Hd-rR) medaka. Apoptosis in red blood cells 4 h, 8 h, 24 h and 14 days after γ-IR of adult female WT was recorded. Compared to the unirradiated control, 4.67 ± 0.56% (Figure 1(a) and Table 1), there was a significant increase in numbers of AO-positive apoptosis in blood smear as 6.17 ± 0.48 and 10.67 ± 0.88%, 4 h and 8 h after irradiation, respectively (Table 1; Figure 1(c,e)). Twenty-four h and 14 days after γ-IR, although the apoptosis was still observed, percentages had decreased to 5.67 ± 0.56 and 4.17 ± 0.4%, respectively (Table 1; Figure 1(g,i)).

γ-IR (4 Gy) of p53- mutant medaka. Apoptosis in red blood cells of p53 (−/−) was also recorded 4 h, 8 h, 24 h and 14 days after γ-IR. Compared to the unirradiated p53(−/−) control as 3.5 ± 0.22%, numbers of AO-positive apoptosis in blood smear was 3.83 ± 0.31% 4 h after γ-IR, suggesting there was no increase in 4 h (Figure 1(b,d); Table 1). However, there was a significant increase in apoptosis as 7.00 ± 1.18 and 5.33 ± 0.33% after 8 and 24 h, respectively (Table 1; Figure 1(f,h)). Fourteen days after γ-IR, although the apoptosis was still observed, percentages had decreased to 3 ± 0.26% (Table 1; Figure 1(j)).

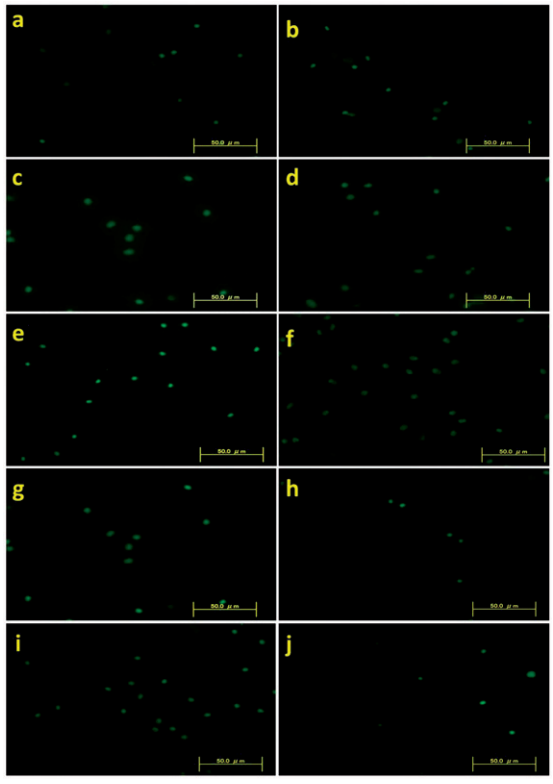

Figure 1. The apoptotic cells of sexually mature, (a) WT control; (b) p53 control; (c) WT 4 h after γ-IR; (d) p53 4 h after γ-IR; (e) WT 8 h after γ-IR; (f) p53 8 h after γ-IR; (g) WT 24 h after γ-IR; (h) p53 24 h after γ-IR (i) WT 14 days after γ-IR and (j) p53 14 days after γ-IR in light green color under the fluorescence microscope stained with acridine orange.

Table 1. Percentage of apoptotic erythrocytes as mean ± SD (range) per 100 cells after γ-irradiation in adult female medaka *Oryzias latipes* ($n = 6$).

	Dose of irradiation	0	4 Gy	4 Gy	4 Gy	4 Gy
	Time after irradiation	–	4 h	8 h	24 h	14 days
Wild-type fish	Apoptotic cells	4.67 ± 0.56^{bc} (3–7)	6.17 ± 0.48^{b} (5–8)	10.67 ± 0.88^{a} (8–13)	5.67 ± 0.56^{a} (4–7)	4.17 ± 0.4^{c} (3–5)
	Dose of irradiation	0	4 Gy	4 Gy	4 Gy	4 Gy
	Time after irradiation	–	4 h	8 h	24 h	14 days
p53 −/− mutant fish	Apoptotic cells	3.5 ± 0.22^{b} (3–4)	3.83 ± 0.31^{b} (3–5)	7 ± 1.18^{a} (3–11)	5.33 ± 0.33^{bc} (4–6)	3 ± 0.26^{b} (2–4)

Different letters ^{a,b,c,d} between conditions indicate statistical significance, $p < .00001$.

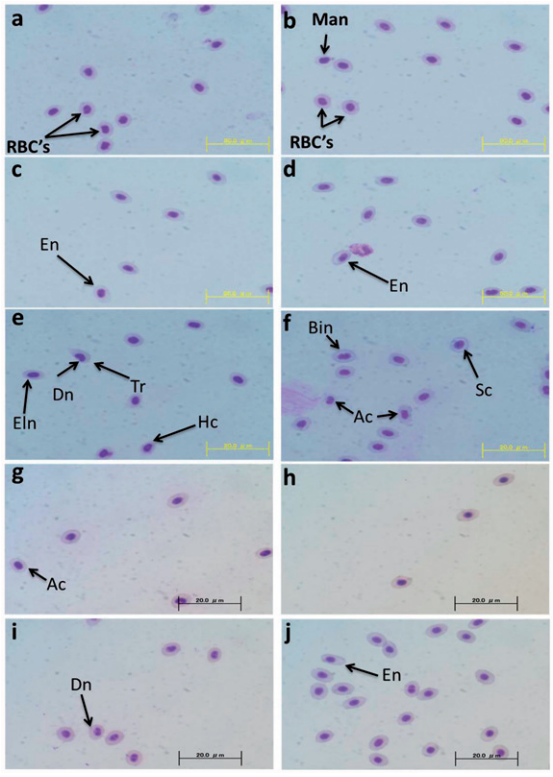

Figure 2. Giemsa staining of blood film of sexually mature, (a) WT control; (b) p53 control; (c) WT 4 h after γ-IR; (d) p53 4 h after γ-IR; (e) WT 8 h after γ-IR; (f) p53 8 h after γ-IR; (g) WT 24 h after γ-IR; (h) p53 24 h after γ-IR (i) WT 14 days after γ-IR; (j) p53 14 days after γ-IR. RBC: red blood cells; Man: Macronucleus; Bin: bilobed nucleus; Eln: elongated nucleus; En: eccentric nucleus; Sc: swelled cells; Ac: acanthocytes; Dn: deformed nucleus; Hc: Hemolyzed cels; Sc: swelled cells; Tr: teardrop-like cell. Scale bar =20 μm.

Erythrocytes alterations, micronuclei and nuclear abnormalities

Phenotypic description of erythrocytes after γ-IR. Morphological changes in red blood cells and some pathological cells were observed in all groups of adult female medaka with different levels and degree. The typical alterations in the appearance of red blood cells from fish irradiated with 4 Gy are shown in (Figure 2); these changes included acanthocytes and some red blood cells exhibited an irregular cell surface with small projections and appeared as teardrop-like cells with pointed spines. Other morphological changes in red blood cells from fish irradiated with 4 Gy as hemolyzed cells (Lysis of cell membrane of some of red blood cells), and swollen red blood cells were observed in fish irradiated.

The fish showed signs of recovery 24 h and 14 days after γ-IR. The frequency of morphological alterations had declined (Figure 2i and j). This finding suggests that 24 h and 14 days allowed complete repair of the damage after 4 Gy γ-IR.

Micronuclei were not observed in control or in fish irradiated with 4 Gy; however, other nuclear anomalies were seen, as macronucleus (the nucleus is large than normal one), eccentric nucleus (in which the nucleus located at only one side of the cytoplasm not as central position), elongated nucleus (rod-like structure), and deformed nucleus, and biolobed nucleus (biolobed shape but not dividing). Those nuclear abnormalities and more were recorded before as described as (Sayed et al. 2014).

γ-IR (4 Gy) of WT (Hd-rR) medaka. The normal structure of the of red blood cells of WT and p53(−/−) medaka (*O. latipes*) was described before by Sayed et al. (2014). Table 2 summarizes the frequency of red blood cell abnormalities. The percentages of altered erythrocytes increased significantly 4 and 8 h after γ-IR in both strains as 2.67 ± 0.21 and 6.00 ± 0.58%, respectively, compared to the unirradiated control as 2.17 ± 0.31; 24 h and 14 days after γ-IR, the percentages were decreased to 3.5 ± 0.34 and 2.67 ± 0.21%. At 8 h after γ-IR erythrocytes alterations represented the highest level in the WT.

γ-IR (4 Gy) of p53-mutant medaka. The blood samples showed very little and a small number of morphological alterations in erythrocytes under microscope in p53(−/−) medaka (*O. latipes*) described before (Sayed et al. 2014) (Table 2).

The percentages of altered erythrocytes increased significantly 8 and 24 h after γ-IR in $p53^{-/-}$ as 4.00 ± 0.26 and 3.5 ± 0.22% compared to the unirradiated control and 4 h after γ-IR as 1.33 ± 0.21 and 1.5 ± 0.22%, respectively. Twenty-four h and 14 days after γ-IR, the percentages had decreased. The percentages of erythrocyte deformations decreased significantly 14 days after irradiation as 1.5 ± 0.22 in $p53^{-/-}$.

Discussion and conclusion

In this study, we verified the morphological alteration and the apoptotic cell death of red blood cells 4, 8 and 24 h and 14 days after the gamma-ray irradiation using a wild-type and p53 mutant medaka. As a result, AO-positive apoptosis and morphological alterations were observed from 4 h after the irradiation and remarkably increased 8 h after the irradiation in the wild-type. On the other hand, in p53 mutant medaka, apoptotic cell death has been observed 8 h after the irradiation most prominently but subtle compared to the wild type, confirming the resistance of p53 mutant to apoptosis and morphological alterations after gamma-ray irradiation. All these phenotypes were recovered 14 days after the irradiation in both strains.

The authors have previously reported for morphology of the blood cells 24 h after γ-ray irradiation with 2 or 10 Gy in

Table 2. Percentage of altered erythrocytes as mean ± SD (range) per 100 cells γ-irradiation in adult female medaka *Oryzias latipes* ($n = 6$).

	Dose of irradiation	0	4 Gy	4 Gy	4 Gy	4 Gy
	Time after irradiation	–	4 h	8 h	24 h	14 days
Wild-type fish	Altered cells	2.17 ± 0.31^{c} (1–3)	2.67 ± 0.21^{bc} (2–3)	6 ± 0.58^{a} (4–8)	3.5 ± 0.34^{a} (3–5)	2.67 ± 0.21^{bc} (2–3)
	Dose of irradiation	0	4 Gy	4 Gy	4 Gy	4 Gy
	Time after irradiation	–	4 h	8 h	24 h	14 days
p53 −/− mutant fish	Altered cells	1.33 ± 0.21^{b} (1–2)	1.5 ± 0.22^{b} (1–2)	4 ± 0.26^{a} (3–5)	3.5 ± 0.22^{b} (3–4)	1.5 ± 0.22^{b} (1–2)

Different letters ^{a,b,c,d} between conditions indicate statistical significance, $p < .00001$.

medaka (Sayed et al. 2014). To take these results into consideration, while the number of deformed red blood cells was significantly increased 24 h after 10 Gy of gamma-ray iradiation, a significant but without dose-dependent increase was observed in 2 and 4 Gy irradiation in wild-type. Furthermore, only acanthocyte was observed in 2 and 4 Gy irradiation but ameoboid and sickle cell was not appeared which was observed in the blood cells of 10 Gy irradiation. From the above, it is difficult to evaluate the morphological and apoptotic effect of red blood cells according to the dose dependency in the 2–4 Gy of gamma radiation in wild-type medaka. Moreover, the detection threshold of phenotype in 24 h after gamma irradiation of medaka erythrocytes may be inferred about 4 Gy. Moreover, in the present study, it was confirmed that gamma-ray irradiation did not induce micronuclei in erythrocyte in fish. Although there is a report that X-rays induce micronuclei in gill cells (Takai et al. 2004a), radiation response of erythrocyte is considered different from that of the tissue cells.

In the previous study, both 2 and 10 Gy gamma-ray irradiation induced deformation of the red blood cells at 24 h and the phenotype was recovered 14 days after the irradiation in p53 mutant medaka. These phenomena were directly confirmed from the results in this study after 4 Gy gamma-ray irradiation. However, the phenotype in 2 and 4 Gy irradiated blood cells showed no dose dependency. Therefore, 4 Gy may be the lowest dose to detect the morphological changes in the p53-dependent red blood cell responses using our method technique.

Prior studies have evaluated the effect of acute irradiation at 24 h after the irradiation and that was the earliest timepoint. In this study we verified the effect of the shorter duration, 4 and 8 h after irradiation and found the most remarkable phenotype at 8 h after irradiation. This result suggested that the effect on erythrocyte caused by acute gamma-ray irradiation appeared earlier than considered so far in testis or gills (Kuwahara et al. 2002; 2003; Takai et al. 2004b; Yasuda et al. 2012). In the midbrain of medaka embryos apoptosis occurs from 6 h and lasts up to 30 h with the highest apoptosis at 12 h after gamma-ray irradiation (Yasuda et al. 2015). The apoptosis and erythrocyte deformation observed in this study between 4 and 8 h after irradiation was p53-dependent, and p53-independent apoptosis was induced after 8–24 h. This time lag pattern between p53-dependent and independent response was similar to the initial response observed in the brain of medaka embryos after gamma-ray irradiation.

Responses of red blood cells after gamma-ray irradiation found in this study were similar to that of UVA irradiation (Sayed et al. 2016). Since the red blood cells of the fish are nucleated, it is considered that the nuclear DNA damage by UVA and ionizing radiation may induce apoptotic cell death in erythrocytes. Tumor suppressor p53 is a transcription factor, and p53-dependent apoptosis and erythrocyte morphological change found in this study is expected due to the gene expression. In the future, understanding the molecular mechanism of the radiation responses in red blood cells and of p53 involvement will be revealed by the detailed analysis using p53 mutant.

In conclusion, we conducted an experiment to irradiate the 4 Gy of γ-ray to medaka and revealed that radiation response of red blood cells occurs from 4–24 h, and the initial response until 8 h was p53-dependent.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are response for the content and writing of the paper.

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