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Apoptosis and morphological alterations after UVA irradiation in red blood cells of p53 deficient Japanese medaka (*Oryzias latipes*)

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ABSTRACT

Morphological alterations in red blood cells were described as hematological bioindicators of UVA exposure to investigate the sensitivity to UVA in wild type Japanese medaka (*Oryzias latipes*) and a p53 deficient mutant. The fewer abnormal red blood cells were observed in the p53 mutant fish under the control conditions. After exposure to different doses of UVA radiation (15 min, 30 min and 60 min/day for 3 days), cellular and nuclear alterations in red blood cells were analyzed in the UVA exposed fish compared with non-exposed controls and those alterations included acanthocytes, cell membrane lysis, swollen cells, teardrop-like cell, hemolyzed cells and sickle cells. Those alterations were increased after the UVA exposure both in wild type and the p53 deficient fish. Moreover, apoptosis analyzed by acridine orange assay showed increased number of apoptosis in red blood cells at the higher UVA exposure dose. No micronuclei but nuclear abnormalities as eccentric nucleus, nuclear budding, deformed nucleus, and bilobed nucleus were observed in each group. These results suggested that UVA exposure induced both p53 dependent and independent apoptosis and morphological alterations in red blood cells but less sensitive to UVA than Wild type in medaka fish.

1. Introduction

Ultraviolet (UV) radiation represents one of the most important factors to impact the Earth's ecosystems and all solar UV radiation (A and B) is known to enhance phototoxic damage in the presence of various pollutants [6,12]. Ultraviolet-A (UVA) radiation (320–400 nm) is scattered rapidly in water with biologically useful amounts to at least 100 m deep in clear aquatic environments [23]. UVA radiations are slightly affected by ozone level according to World Health Organization [35] with intensity more constant than UV-B during the day and throughout the year [21,27]. UV radiation (UVR) is naturally a stressor to most forms of life. Recent changes in UVR exposure at both global and local level regarding the potentially damaging effects of this stressor have been studied [14,35]. Many studies on the negative impact of UVA radiation on the level of cells, tissues and molecules have been reported [5,8]. Many other harmful UVA induced effects are recorded including mortality and alteration in embryonic stages, metabolic rate and growing fish, oxidative DNA damage and immunological characteristics [5,14,16]. UVA has longer wavelength and contains less energy per photon than UV-B and UV-C, it causes indirect DNA damage by promoting the generation of reactive oxygen species (ROS). However, UVA irradiation has been shown to have synergistic detrimental effects when combined with exogenous photosensitizers and to increase ROS mediated DNA damage [7,33].

Several methods for the assessment of UV damage have been described including physiological, morphological, biochemical and histopathological studies in vertebrates [14,16,20–23]. Many studies have reported that blood cell indicates, as reflected in changes in their morphology and distribution in the blood, are good indicators of the systemic response to external stress [2,9,11,18,19,23,31,34].

The Japanese medaka (*Oryzias latipes*), native to East Asian freshwater systems and a small aquarium fish, is used widely in ecotoxicological studies [10,17,36] and is a unique vertebrate model for investigating the effects of gamma rays irradiation [25,39,40]. Their whole genome has been sequenced and several genetically modified medaka using targeting mutation or random mutagenesis including TILLING system has been produced in decades [13,37]. In addition to the general negative impacts, the UVR destroys the fish immune system including blood cells [22,23].

To our knowledge, the current study is the first to record the dangerous effects of UVA radiation on medaka red blood cells. Accordingly, the

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present study aimed to elucidate the negative impacts for the first time of UVA exposure (366 nm) for 15, 30, and 60 min/day for 3 days and its ability to induce apoptosis and morphological alterations in red blood cells in wild type and the p53 deficient medaka.

2. Materials and Methods

2.1. Fish

Adult wild type (WT; Hd-rR) and homogenic p53 deficient adult female Japanese medaka (*O. latipes*) were originally made by TILLING mutation [38]. Fish were reared at 20–21 °C under 14 h light: 10 h dark cycle, fed by live brine shrimp (*Artemia franciscana*) and/or the powdered diet (Tetra-fin, Spectrum Brands Japan Inc., Tokyo, Japan) three times a day.

2.2. Experimental Design

The UVA doses were selected according to Mekkawy et al. [16]. The fish in each group were treated as follows at the same time:

Group I: The fish were kept for three days under the same laboratory conditions as the fish in the other groups and are to be considered as control fish.

Group II: The fish were exposed to UVA of 15 min/day for three days.

Group III: The fish were exposed to UVA of 30 min/day for three days.

Group IV: The fish were exposed to UVA of 60 min/day for three days.

2.3. UVA Exposure

The fish were exposed to UVA using a UV Lamp of 366 nm (ULTRA-VIOLET Products, Inc., UVL-56, San Gabriel, CA, USA). Experimental containers with the fish were fitted from above with the UV lamp at 8 cm above the bottom of plastic containers [16]. At this level, the intensity of this lamp was 2450 μW/cm^2 as measured using a UV meter and three replicates for each group (three samples from each replicate) were prepared to be examined.

2.4. Micronucleus Test and Red Blood Cells Alterations

Blood samples collected by caudal incision of the fish were smeared onto clean glass slides 24 h and 14 days after UVA exposure. The smears (seven slides from each fish) were dried, fixed in absolute methanol for 10 min, and Giemsa stained [32]. Slides were selected on the basis of staining quality and randomized to be scored blindly. In each group, 10,000 cells (minimum of 1000 cells per slide) were examined under 40× objective to identify micronucleated and morphologically altered red blood cells according to the method by Al-Sabti and Metcalfe [1] The established criteria for identifying micronuclei [26] were followed strictly to ensure authentic scoring. Morphological alterations of red blood cells such as acanthocytes, teardrop-like cells, sickle cells, swollen cells, crenated cells and alterations of nuclear morphology were scored.

2.5. Apoptosis Detection

Apoptotic red blood cells were detected by acridine orange (AO) (Cat. No. A1031, Life Technologies, 5791 Van Allen Way Carlsbad, CA 92008, USA) staining. The modified protocol reported by Sayed [24] was used to detect the apoptosis in red blood cells: Blood smears were prepared on clean glass slides, washed in 1× PBS (pH = 7.2), and AO staining solution (17 μg/l AO in 1× PBS) was put on the slides for 30 min in the dark. Then, the slides were washed every 30 min with 1× PBS for 4 times to decolorize and fixed in 4% paraformaldehyde in 1× PBS for 5 min. Finally, apoptotic red blood cells were scored under a fluorescence microscope (BX50, Olympus) equipped with a digital still camera (DP70, Olympus).

2.6. Statistical Analysis

One-way analysis of variance was conducted to analyze the data using SPSS software [29] at 0.0001 significance level. Tukey's HSD test was used for multiple comparisons and verification of frequency of erythrocyte alterations. Dunnett's t-test was used to compare the three UVA treated groups with the control group.

2.7. 2.7. Ethics Statement

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

3. Results

3.1. Apoptosis in Red Blood Cells of WT and p53 Deficient Mutant Medaka

Fig. 1a, e, and 2a, e show the blood smears from normal wild type (WT; Hd-rR) and p53 deficient medaka (*O. latipes*), representing the normal structure of red blood cells in control. Even without exposure to UVA, some apoptotic red blood cells (7.00% ± 1.26 and 3.67% ± 0.33) were present in WT and p53 deficient fish, respectively, and fewer p53 deficient red blood cells were apoptotic than WT ($p < 0.0001$) (Table 1 and Fig.5).

3.2. Apoptosis in Red Blood Cells after UVA Exposure of WT Medaka

In WT medaka, the percentages of apoptotic red blood cells 24 h and 14 days after UVA exposure of 15 min/day for three days were 13.0 ± 1.41% and 4.83 ± 0.40%, respectively, indicating erythrocyte apoptosis significantly increases 24 h after the UVA exposure and returns to the control level 14 days after the exposure (Fig 1b, f, Table 1 and Fig 5). Apoptotic red blood cells also increased 24 h after UVA exposure of 30 min/day for three days (19.67 ± 1.74%) and decreased to the control level (6.67 ± 0.42%) (Figs. 1c, g and Table 1). In contrast, exposure of WT fish to UVA of 60 min/day for three days induced much more apoptosis of red blood cells: the percentages of apoptosis in red blood cells were 29.7 ± 2.30% 24 h after the exposure. Unlike red blood cells exposed to 15 and 30 min/day for three days, the apoptotic red blood cells did not decreased to the control level even 14 days after UVA radiation (10.2 ± 0.48%, Fig. 1d, h and Table 1).

3.3. Apoptosis in Red Blood Cells after UVA Exposure of p53 Deficient Medaka

In p53 deficient medaka, apoptosis in red blood cells significantly increased (4.83 ± 0.31%) 24 h after UVA exposure of 15 min/day for three days as shown in Fig. 2b and Table 1, Fig 5). Fourteen days after UVA exposure, percentage of apoptotic red blood cells was 4.33 ± 0.33, which was slightly higher than the control level (Fig. 2f, Table 1 and Fig 5). The percentages of apoptotic red blood cells were 8.17 ± 0.87% and 4.67 ± 0.33% 24 h and 14 days after UVA exposure of 30 min/day for three days, respectively (Fig. 2c and g, Table 1 and Fig 5), showing that apoptosis in p53 deficient red blood cells increased depending on the radiation dose and that the increase of apoptotic red blood cells was returned to the control level in 14 days after the UVA radiation. UVA exposure of 60 min/day for three days exhibited higher level of apoptosis and the percentages of apoptosis in red blood cells 24 h and 14 days after the exposure were 16.7 ± 1.20% and 6.00 ± 0.58%, respectively (Fig. 2d and h, Table 1 and Fig 5). When p53 deficient medaka were exposed with UVA of 60 min/day for three days, the