Low linear energy transfer ionizing radiations, such as X-rays, generate reactive oxygen species (ROS), which cause biological damage. Our recent study demonstrated that high-dose X-ray irradiation induced the expression of antioxidant enzyme heme oxygenase-1 (HO-1) in human monocytic leukemia THP1 cells depending on the generation of secondary ROS, not primary ROS which are generated directly by X-irradiation. However, the source of generation of secondary ROS in X-irradiated THP1 cells remains unknown. To address this question, we investigated the kinetics of mitochondrial superoxide production in X-irradiated THP1 cells. Further, we investigated the types of ROS involved in the induction of HO-1 after X-irradiation. Mitochondrial superoxide generation in X-irradiated THP1 cells increased 3 h after X-irradiation, whereas secondary intracellular ROS generation was observed 24 h after irradiation. Treatment with polyethylene glycol catalase partly inhibited the upregulation of HO-1 by 5 Gy irradiation. We suggest that mitochondria are the source of the X-irradiation-induced secondary peak of intracellular ROS generation in THP1 cells and that hydrogen peroxide is partly involved in the upregulation of HO-1 after X-irradiation. Given that the kinetics of ROS and antioxidants are important factors for the cellular response in irradiated cells, these results will be helpful for treating radiation-induced injuries, such as acute radiation syndrome.

Key words: mitochondrial superoxide, hydrogen peroxide, heme oxygenase-

1. Introduction

Low linear energy transfer (LET) ionizing radiations, such as X-rays and γ-rays, generate reactive oxygen species (ROS), which cause biological damage and change cellular signaling depending on their concentration. Several enzymatic and nonenzymatic antioxidants play important roles in eliminating “intracellular” or “intercellular” ROS. Therefore, the kinetics of ROS and antioxidants in the cells after ionizing radiation is important for understanding cellular responses to ionizing radiation, such as cell death.

Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) belongs to the cap ‘n’ collar family of basic region leucine zipper transcription factors and mediates the coordinated transcription of several antioxidant genes such as heme oxygenase-1 (HO-1). HO-1 is an intracellular antioxidant enzyme that catalyzes the degradation of heme into biliverdin, ions, and carbon monoxide and confers protection against oxidative
stress. HO-1 also plays a crucial role not only as an antioxidant enzyme but also as an important modulator of DNA repair via generation of CO. As HO-1 regulates chemosensitivity and radiosensitivity in human non-small cell lung cancer cells, it is thought that HO-1 is involved in the cellular response to ionizing radiation.

We recently investigated the kinetics of intracellular ROS generation and the response of the Nrf2 system in human monocytic leukemia THP1 cells after X-irradiation, and we reported that X-irradiation directly generated primary ROS and generated secondary ROS after 24 h. We also reported that Nrf2 translocation into the nucleus was observed 6 h after 5 Gy irradiation, and this irradiation induced ROS-mediated HO-1 upregulation. However, the source of secondary ROS generation in 5 Gy-irradiated THP1 cells remains unknown. Recently, several studies suggested that mitochondria are responsible for secondary ROS generation after ionizing radiation. Therefore, in this study, we focused on the mitochondria and investigated the kinetics of mitochondrial superoxide production in THP1 cells. We also investigated the types of ROS (i.e. superoxide or hydrogen peroxide) involved in the induction of HO-1 after X-irradiation.

2. Materials and Methods

2.1. Reagents

The fluorescent probe ThiolTracker™ Violet and MitoSOX™ Red were purchased from Molecular Probes, Invitrogen Corporation (Eugene, Oregon, USA). Polyethylene glycol superoxide dismutase (PEG–SOD) and PEG–catalase (PEG–CAT) were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell culture

The human monocytic leukemia cell line THP1 was obtained from RIKEN Bio-Resource Center. THP1 cells were cultured in RPMI 1640 (Gibco® Invitrogen, Grand Island, NY, USA) supplemented with 1% penicillin–streptomycin (Gibco® Invitrogen) and 10% heat-inactivated fetal bovine serum (Bio Serum, Japan) at 37°C in a humidified atmosphere containing 5% CO₂.

2.3. In vitro irradiation of human monocytes

THP1 cells (1×10⁶ cells/ml) were exposed to X-rays (150 kVp, 20 mA, with 0.5 mm Al and 0.3 mm Cu filters) using an X-ray generator (MBR-1520R-3, Hitachi Medical Corporation, Japan) at a dose rate of 1.00–1.04 Gy/min. In some experiments, 100 U/ml PEG–CAT or 20 U/ml PEG–SOD was added to the culture medium 1 h after exposure to 5 Gy X-irradiation. Previous report by other group was referred to determine the concentration of PEG–CAT and PEG–SOD.

2.4. Measurement of mitochondrial superoxide

The fluorescent probe MitoSOX™ Red was used for the measurement of mitochondrial superoxide. Cells were incubated for 10 min at 37°C in the dark with 2.5 μM MitoSOX™ Red in Hanks’ Balanced Salt Solution (HBSS) containing Ca²⁺ and Mg²⁺. After incubation, the cells were washed with HBSS(+) and analyzed by flow cytometry (Cytomics FC500, Beckman Coulter).

2.5. Measurement of intracellular reduced thiols

The fluorescent probe ThiolTracker™ Violet, which is highly reactive to thiols and can be used as a convenient and effective indicator of intracellular glutathione (GSH), was used for the measurement of intracellular reduced thiols. Cells were incubated for 30 min at 37°C in the dark with 20 μM ThiolTracker™ Violet in phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺. After incubation, the cells were washed with PBS(+) and photographed using an Olympus IX71 (Tokyo, Japan) and DP2-BSW software (Olympus). The fluorescence intensity for ThiolTracker was analyzed by flow cytometry (Cell Lab Quanta™ Sc MPL, Beckman Coulter, Fullerton, CA, USA), equipped with a UV light source.

2.6. Intracellular HO-1 staining

The cells were fixed with Cytofix/Cytoperm™ solution (BD Biosciences, San Jose, CA, USA) for 20 min on ice. After washing with Perm/Wash™ solution (BD Biosciences), the cells were suspended in Perm/Wash™ solution and labeled with primary HO-1 antibodies (assay designs, Enzo Life Sciences, Inc., Farmingdale, NY, USA) for 40 min at room temperature. They were then washed with Perm/Wash™ solution and incubated with FITC-conjugated anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology Inc., CA, USA) for 30 min at room temperature in the dark. For the isotype control, the cells were stained with FITC-conjugated anti-mouse IgG antibodies only. After 30 min, the cells were washed with Perm/Wash™ solution and analyzed by flow cytometry (Cytomics FC500, Beckman Coulter).

2.7. Statistical analysis

Data are expressed as mean ± SD values, and P < 0.05 was considered to be statistically significant. The statistical analysis was performed using Microsoft Excel 2010 (Microsoft, USA) with the add-in software Statcel 3.

3. Results and discussion

3.1. X-irradiation increases mitochondrial superoxide generation

We have previously investigated the kinetics of intracellular ROS in X-irradiated THP1 cells using the 2',
7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent probe, which detects various types of ROS such as hydroxyl radical and hydrogen peroxide. As a result, although ROS directly generated by X-irradiation disappeared immediately after irradiation, 5 Gy X-irradiation induces a secondary peak of intracellular ROS generation 24 h after irradiation.10) With the aim of investigating the possibility that mitochondria are the source of the secondary peak of intracellular ROS generation after X-irradiation, the mitochondrial superoxide was measured using MitoSOX™ Red. The level of mitochondrial superoxide was higher in X-irradiated than in nonirradiated cells 3 h after irradiation (Fig. 1). Furthermore, the levels of mitochondrial superoxide in 5 Gy-irradiated cells remained higher 24–72 h after irradiation, whereas those in 1 Gy- and 2 Gy-irradiated cells were similar to those in nonirradiated cells. Therefore, it is possible that mitochondria are the source of the 5 Gy induced-second peak of intracellular ROS generation observed 24 h after 5 Gy irradiation10).

3.2 X-irradiation decreases intracellular reduced thiols

The redox status is an important indicator of cellular response to irradiation, such as cell death. Accordingly, we next investigated the cellular redox status using ThiolTracker™ Violet, which is a bright fluorescent probe that is highly reactive to thiols and can be used as a convenient and effective indicator of intracellular GSH and general redox. As shown in Fig. 2[A], reduced thiols were observed in both nonirradiated and X-irradiated cells. The fluorescence intensity for ThiolTracker™ Violet was quantified by flow cytometry. Although there was no difference in the level of reduced thiols (the fluorescence intensity for ThiolTracker/cell) between nonirradiated cells and X-irradiated cells (Fig. 2[B]), the fluorescence intensity for ThiolTracker/cell volume were lower in the 2 Gy- and 5 Gy-irradiated cells than in nonirradiated cells 6 h after irradiation (Fig. 2[C]). These results indicate that the cell volume of irradiated cells was larger than that of nonirradiated cells, which led to the change of intracellular redox status. Our previous report demonstrated that X-irradiation induced ROS-mediated HO-1 mRNA upregulation 6 h after irradiation even though the upregulation of intracellular ROS detected by H2DCFDA was not observed at this time. As it is reported that mitochondrial-derived ROS mediate HO-1 expression in sheared endothelial cells15), it may be that mitochondrial-derived superoxide generated after X-irradiation is responsible for the upregulation of HO-1 expression in the present study. Furthermore, we consider that although X-irradiation not only increases mitochondrial-derived superoxide but also affects the cellular redox status by changing cell volume within 6 h, more time is required until the ROS accumulated by those phenomena exceed the cellular antioxidant system. Therefore, there was a time lag between the upregulation of HO-1 mRNA and the change of redox status at 6 h after irradiation (Fig. 2[C]) and secondary peak of intracellular ROS generation occurred 24 h after irradiation10).

3.3 Hydrogen peroxide rather than superoxide is partly involved in the X-irradiation induced-HO-1 expression

We demonstrated the involvement of ROS in the upregulation of HO-1 expression by showing that the administration of N-acetyl-L-cysteine, a precursor of GSH, 1 h after X-irradiation inhibited this upregulation. To
investigate the types of ROS involved in the induction of HO-1 by X-irradiation, PEG–CAT or PEG–SOD were added to the culture medium 1 h after 5 Gy irradiation, and HO-1 protein expression was analyzed. CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen, whereas SOD catalyzes the dismutation of the superoxide anion into oxygen and hydrogen peroxide. The treatment with PEG–CAT or PEG–SOD did not affect the HO-1 expression of nonirradiated THP1 cells (Fig. 3). In line with our previous report\(^{10}\), HO-1 expression was higher in 5 Gy-irradiated cells than in nonirradiated cells. The treatment with PEG–CAT partly inhibited the upregulation of HO-1 by 5 Gy irradiation, although there was no statistical significant difference (Fig. 3).
In terms of PEG–SOD (20 U/ml), its effect was less than that of PEG–CAT. In our preliminary experiment, the suppressive effect of 100 U/ml PEG–SOD on the upregulation of HO-1 expression by 5 Gy-irradiation was less than that of 100 U/ml PEG–CAT (data not shown). We accordingly propose that hydrogen peroxide rather than superoxide was partly involved in the upregulation of HO-1 after X-irradiation. Furthermore, considering that, although superoxide reacts with glutathione, superoxide dismutase will always out-compete glutathione for reaction with the superoxide radical due to the difference of rate constant \(^{16}\), it is possible that the mitochondrial superoxide generated after irradiation are converted to H\(_2\)O\(_2\) by superoxide dismutase, and H\(_2\)O\(_2\) diffuse from mitochondria to the cytoplasm. Therefore, we consider that the H\(_2\)O\(_2\) is derived from mitochondria at least in part.

In conclusion, we suggest that mitochondria are the source of the 5 Gy induced-secondary peak of intracellular ROS generation in human monocytic THP1 cells. Furthermore, the present study demonstrated that hydrogen peroxide rather than superoxide is involved in the upregulation of HO-1 expression by X-irradiation. Hematopoietic disorder is one of acute radiation syndrome and results in infections due to low number of white blood cells. Given that the kinetics of ROS and antioxidants are important factors for the cellular response in irradiated cells, the present results will be helpful for not only cancer radiotherapy but also treatment for hematopoietic acute radiation syndrome.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


