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Induction of Rhodanese, a Detoxification Enzyme, in Livers from Mice after Long-term Irradiation with Low-dose-rate Gamma-rays

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Low-dose-rate radiation/Long-term-irradiation/Rhodanese/Antioxidants/Superoxide dismutase.

The health effects of low-dose radiation exposure are of public concern. Although molecular events in the cellular response to high-dose-rate radiation exposure have been fully investigated, effects of long-term exposure to extremely low-dose-rate radiation remain unclear. Protein expression was analyzed by two-dimensional electrophoresis in livers from mice irradiated for 485 days (22 hr/day) at low-dose-rates of 0.032 μGy/min, 0.65 μGy/min and 13 μGy/min (total doses of 21 mGy, 420 mGy and 8000 mGy, respectively). One of the proteins that showed marked changes in expression was identified as rhodanese (thiosulfate sulfurtransferase). Rhodanese expression was increased after irradiation at 0.65 μGy/min and 13 μGy/min, while its expression was not changed at 0.032 μGy/min. Rhodanese is a detoxification enzyme, probably related to the regulation of antioxidative function. However, antioxidative proteins, such as superoxide dismutase (SOD)1 (also known as Cu,Zn-SOD) and SOD2 (also known as Mn-SOD), which can be induced by high-dose-rate radiation, were not induced at any low-dose-rates tested. These findings indicate that rhodanese is a novel protein induced by low-dose-rate radiation, and further analysis could provide insight into the effects of extremely low-dose-rate radiation exposure.

INTRODUCTION

The biological effects of low-dose-rate radiation exposure are of public concern as well as scientific importance.[1–3] Epidemiological studies in the high-background radiation areas have been performed to assess health effects of chronic exposure to low-dose-rate radiations in humans.[4] In these studies, the confounding factors such as lifestyles of residents living in the high-background radiation areas have made it difficult to reveal a small variation caused by low-dose-rate irradiation. Then, animal experiments under the fully controlled condition are needed for evaluating the effects of long-term irradiation at low-dose-rates.

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Tanaka *et al.* studied effects on lifespan of continuous exposure to low-dose-rate gamma-rays (16 μGy/min, 0.83 μGy/min and 0.038 μGy/min) for approximately 400 days using about 4,000 specific pathogen-free (SPF) B6C3F1 mice.[5] The significant life-shortening was observed in both male and female mice irradiated with 16 μGy/min, and in female mice irradiated with 0.83 μGy/min as compared to the lifespan of the non-exposed control mice. Furthermore, they demonstrated that the observed life-shortening was due to early death from a variety of neoplasms, not from increased incidence of certain neoplasms.[6] Molecular analysis for these events has not been studied in mice exposed to long-term, low-dose-rate radiation. It is possible to detect the effects of low-dose or low-dose-rate radiation at the molecular level using RNA-based analysis.[7–18] Based on the current information, many of gene expressions are changed by high-dose and/or low-dose radiation exposure.[7–19] Protein expression can be also a specific marker for evaluating radiation effects. While proteome analysis in protein expression with regard to the effects of high-dose radiation has been performed,[20–22] few studies are there on the low-dose radiation effects.[23–25] Proteome analysis is thus anticipated to accumulate more precise information about the effects of low-dose or low-dose-rate radiation. Detection of

changes in protein expression in mice exposed to long-term, low-dose-rate radiation would reveal what kinds of proteins are involved in the biological effects.

Here, we analyzed changes in protein expression after long-term, low-dose-rate gamma-ray irradiation, using two-dimensional electrophoresis (2DE), a common method for proteomic analysis. We identified rhodanese as a new clue for evaluating biological effects after long-term, low-dose-rate irradiation. We also compared the expression of rhodanese with those of other antioxidative enzymes.

MATERIALS AND METHODS

Mice and Irradiation

Male SPF C57BL/6J mice, 7 to 8 weeks of age, were obtained from the animal breeding facility (CLEA Japan, Tokyo) and irradiated under the same conditions as described previously.[5,6] Briefly, continuous irradiation was carried out with ^{137}Cs gamma-rays for 485 days (22 hr/day) at dose rates of 0.032 μGy/min, 0.65 μGy/min and 13 μGy/min (total doses of 21 mGy, 420 mGy and 8000 mGy, respectively). Dosimetry and animal cares under a barrier-condition were carried out as those described previously.[5,6] We examined total of 12 mice (3 per each dose-rate and 3 for the non-exposed control) out of 14 mice autopsied, in which the gross appearance was normal, without organ hypertrophy, neoplasia, and/or hair loss, and the livers were perfused with cold-PBS, stored at –80°C until use.

All experiments were conducted in accordance with the relevant legal regulations of Japan, and with the Guidelines for Animal Experiments of the Institute for Environmental Sciences.

Reagents

Antibodies against rhodanese, superoxide dismutase (SOD)1, SOD2 and heat shock protein 60 (HSP60), as well as the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP), were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Sample Preparation

Liver tissues were homogenized in 5 volumes of lysis solution (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10 and 1% DTT). Lysates were centrifuged (20,000 × g) at 20°C for 30 min. The supernatants were used for two dimensional electrophoresis (2DE) and Western blot analysis.

Two-dimensional electrophoresis (2DE)

Isoelectric focusing (IEF) was performed using Immobiline dry strip pH 3–10 (Amersham Biosciences) and Ampholine pH 3.5–10. Solubilized protein (100 μg) was loaded onto a strip. SDS-PAGE was performed using 9–18% gradient gels. Protein spots were detected by SYPRO Ruby staining (Molecular Probes, Inc. OR). For 2DE protein spot analysis including MS (Mass Spectrometry)/MS and MASCOT search analysis, we used the technical services of ProPhoenix Co., Ltd. (Hiroshima, Japan).

Western Blot Analysis

Protein concentration was determined by Bradford's method.[26] Equivalent amounts (33 μg/lane) of protein were subjected to SDS-polyacrylamide gel electrophoresis (7.5% or 12 % gels), as described elsewhere.[27] Separated proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with PBS-Tween (0.05%) solution containing 5% non-fat dry milk at 4°C overnight, followed by immunoblotting with antibodies against rhodanese, SOD1, SOD2 and HSP60 for 2 h at room temperature. After four times washes with PBS-Tween solution, immune complexes were detected with appropriate secondary antibodies and ECL reagents. Signal bands were developed and densitometry analysis was performed using a LAS 1000 and FLA-5100 analysis system (Fuji Film, Japan).

Statistical Analysis

Statistical analysis was performed using Student's *t* test, and P value is indicated in the figure legend.

RESULTS AND DISCUSSION

Based on the data from 2DE, expression of liver proteins in the control mice were evaluated and compared with that in mice irradiated at three different dose rates of 0.032 μGy/min, 0.65 μGy/min and 13 μGy/min (total doses of 21 mGy, 420 mGy and 8000 mGy, respectively). Several different spots were detected in samples from irradiated mice as compared to the control images (Fig. 1A). The remarkably increased proteins were targeted in mice irradiated at two different low-dose-rates of 0.65 μGy/min and 13 μGy/min, and four candidates were selected. Three of these appeared to be serum albumin precursor and hemoglobin chains by Prophoenix database search analysis (ProPhoenix Co. Ltd., Hiroshima). The expressions of these three proteins were observed at all dose-rates, but appeared not to be increased in a dose-rate-dependent manner. In addition, these changes were considered to be due to the blood remaining in the livers even though they were perfused. Thus, they were excluded as non-targeted increased proteins (NIPs) (Fig. 1B). As a result, a single protein of 32 kDa, which was increased at two dose-rates of 0.65 μGy/min and 13 μGy/min, was selected as a candidate for identification. By the Mascot search engine (http://www.matrixscience.com), the Mascot search score (the Mowse score) was 119 and 14% of the sequence was matched, concluding that the protein was identified as rhodanese (thiosulfate sulfurtransferase) (Table 1). Rhodanese expression was confirmed by Western blot analysis (Fig. 2). In consistent with the 2DE, irradiation at

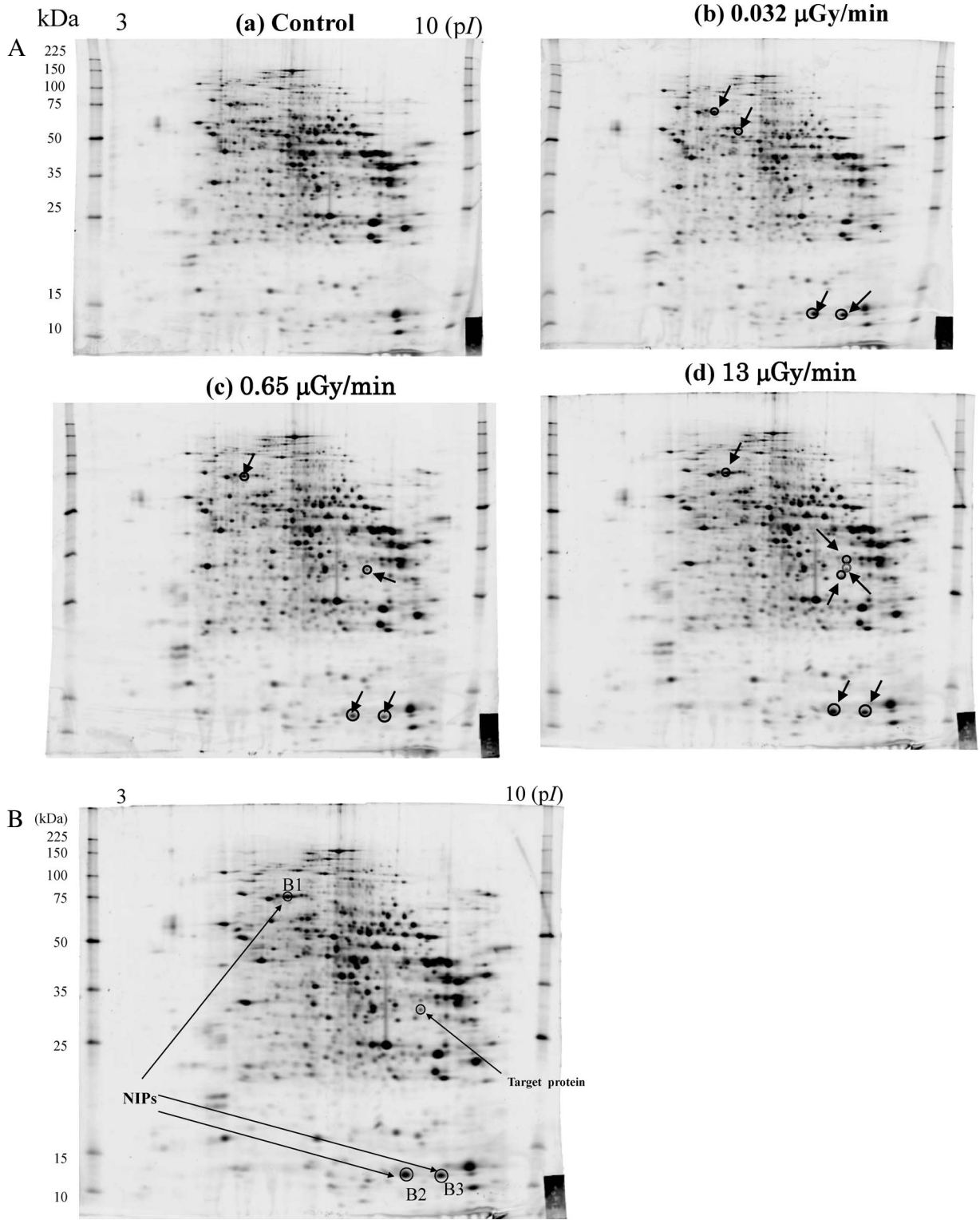

Fig. 1. Two-dimensional electrophoresis of liver proteins from mice after long-term irradiation with low-dose-rate gamma-rays. A: 2DE images. The image (a) is a control image and the image (b),(c) and (d) are for liver proteins after irradiation at dose rates of 0.032 μGy/min, 0.65 μGy/min and 13 μGy/min, respectively. 2DE was performed and analyzed using the sample from one of three mice in each group. Spots indicated by circles and arrows were observed as increased when compared to the control. B: Enlarged 2DE-image (the dose rate, 13 μGy/min). Non-targeted increased proteins (NIPs) are likely to be serum albumin precursor (B1), and hemoglobin chains(B2, B3). The target protein was analyzed by MASCOT search.

Table 1. Identification of a protein induced by long-term irradiation with low-dose-rate gamma-rays.

Protein name	NCBI Accesion No.	Molecular Weight [Da] /pI calculated	Sequence Coverage [a)]	Mowse Score [b)]
Thiosulfate sulfurtransferase, mitochondrial	6678449	33673 / 7.71	14%	119

[a, b)]Values of a sequence coverage and score were determined according to the Mascot search engine.

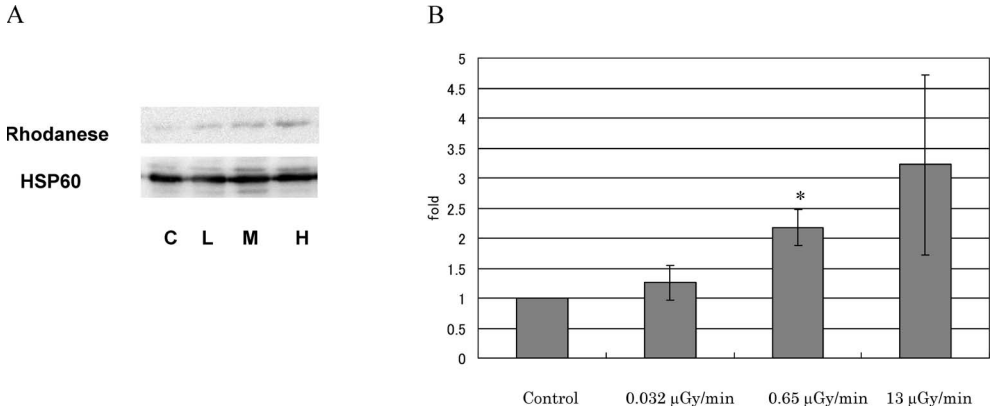

Fig. 2. Rhodanese expression in livers from mice after long-term irradiation with low-dose-rate gamma-rays. A: Western blot analysis of rhodanese. Image is representative of three independent mice. C, L, M and H refer to data from control(C) mice and mice irradiated with dose rates of 0.032 μGy/min(L), 0.65 μGy/min(M) and 13 μGy/min(H), respectively. HSP60 was used as a loading control for mitochondrial proteins. B: Densitometry analysis of Western blot signals. Each point is the mean of three independent experiments from three mice, and error bars are S.E.M.(n = 3). * $P < 0.05$ compared to the control case.

a low-dose-rate of 0.032 μGy/min did not induce the expression but did the expression at 0.65 μGy/min and at 13 μGy/min (Fig. 2B).

Rhodanese is a detoxification enzyme found in both prokaryotes and eukaryotes of mice, rats and humans. In mammals, it is present in mitochondria and plays a role in detoxification of cyanide and H_2S.[28–32)] However, its cellular function is not much understood. Rhodanese activity is also considered to be related to the regulation of sulfane sulfur metabolism. Sulfane sulfur is a highly reactive sulfur atom, covalently bound to another sulfur atom and has antioxidant action.[33)] Thus, the increased amounts of rhodanese can also be related to antioxidant activity. This notion is consistent with the recent observation that the effects of low-dose irradiation can be related to the increased activity of antioxidants.[34)] The expression of antioxidative enzymes, such as SOD1 (Cu,Zn-SOD) and SOD2 (Mn-SOD), is known to be induced by high-dose-rate irradiation.[35,36)] SOD1 was previously demonstrated to be induced by low-dose (0.2 Gy) irradiation at a dose rate of 3.81 Gy/min;[35)] however, its expression level was not changed (Fig. 3). SOD2, which functions in mitochondria, is induced by fractionated irradiation(2 Gy × 30) at a dose rate of 0.46 Gy/min, leading to radioresistance.[36)] Levels of SOD2 expression did not change in the liver of mice after long-term irradiation at low-dose-rates (Fig. 3).

Fig. 3. Superoxide dismutase expression in livers from mice after long-term irradiation with low-dose-rate gamma-rays. A: Western blot analysis of superoxide dismutase 1(SOD1). B: Western blot analysis of superoxide dismutase 2(SOD2). Image is representative of three independent mice. C, L, M and H refer to data from control(C) mice and mice irradiated with dose rates of 0.032 μGy/min(L), 0.65 μGy/min(M) and 13 μGy/min(H), respectively. HSP60 was used as a loading control.

Rhodanese induction by irradiation may lead to increases in compounds with sulfane sulfur, resulting in the induction of antioxidants. Radiation induces reactive oxygen species (ROS), and oxidative stress induces antioxidants. Indeed, compounds with sulfane sulfur, such as allyl disulfide, were reported to prevent radiation-induced damage.[30,37)] Moreover, the possibility that rhodanese plays a role in inhibition of cell proliferation via sulfane sulfur cannot be ruled out.[30)] It should be noted that the activity of rhodanese is low in

malignant hepatomas and the liver of tumor-bearing mice, and the amount of sulfane sulfur is also low in the liver of tumor-bearing mice.[33,38] These findings suggest that control of sulfane sulfur levels by rhodanese could be associated with tumorigenesis. Rhodanese induction by long-term, low-dose-rate irradiation might function as a defense system against tumor induction. Because Tanaka *et al.* demonstrated that irradiation at 0.83 and 16 μGy/min shortened the longevity of mice,[5,6] abilities of rhodanese including antioxidative or other functions against tumorigenesis may not have been sufficient to repair the radiation effects that result in the development of neoplasms and the life-shortening.

In mouse liver, acute high-dose irradiation induces redox signaling proteins[21] and fractionated irradiation induces proteins such as PKCμ and Rab2.[24] Toxicity markers after treatment with cisplatin, a DNA-damaging agent, have been detected in rat liver and human cell lines such as HepG2 cells.[39] However, rhodanese is not included among radiation (or cisplatin)-induced proteins previously reported. These results suggest that the effects of long-term gamma-irradiation at low-dose-rates, as shown in this study, differ from those of acute or fractionated irradiation. Indeed, although comparing the effect of the low-dose-rate, long-term radiation with that of high-dose-rate, short-term radiation is important, it is difficult to perform the comparative study in this experiment. Because the result might be dependent on when mice are irradiated acutely in the experimental period of 485 days. Nevertheless, considering rhodanese induction at 8 Gy (a total dose) in our study, the finding that rhodanese is not included in altered proteins in the livers of mice irradiated with 10 Gy using high-dose-rate irradiation (0.25 Gy/min)[21,22] is consistent with the notion that rhodanese is induced specifically in low-dose-rate irradiation.

Rhodanese could be a new clue for evaluating effects of long-term radiation exposure at low-dose-rates. In the livers from mice irradiated continuously at low-dose-rates, the mRNA expression of rhodanese is not induced (T. Ono *et al.* unpublished data). The relationship between RNA and protein expressions has been analyzed in mouse tissues, and it was reported that RNA expression is incompletely corresponded to protein expression in mitochondrial proteins.[40] The induction of rhodanese, a mitochondrial protein in this study, could be post-transcriptionally regulated.

This study provides the first evidence that a detoxification enzyme, rhodanese, is related to the effects of long-term radiation exposure at low-dose-rates. This protein might be thus a key to clarify low-dose radiation effects and novel defense systems.

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