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Effect of Whole Body X-irradiation on the Rat Glutathione-related Antioxidant System

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Ionizing radiation generates free radicals and ROS, and it has been reported that glutathione (GSH)-dependent antioxidant system is modulated in irradiated tissue and serum even after 24h of irradiation. However, dose-dependent effect on the system has not been clarified and there is no report on effect of irradiation to urinary GSH-dependent enzyme activities. Therefore, in present study, rats were exposed to a single X-irradiation at several doses; 1, 3, 5, and 7 Gy, and after 24h of irradiation, each liver, kidney, testis, and small intestine was exenterated and GSH content and activities of glutathione S-transferase (GST), glutathione reductase (GR), selenium-independent peroxidase and selenium-dependent peroxidase were assayed. Furthermore, those activities of serum and urine were assayed. In urine, effect of 2 and 4-Gy irradiation was also investigated. GR activity was up-regulated in liver and testis whereas GST activity was impaired in kidney. GSH content was increased in small intestine. However, these modulation were not in a dose-dependent manner. On the other hand, all of serum enzyme activities were unaffected, and most urinary enzyme activities were increased in a dose-dependent manner up to 2 Gy. Thus, it was demonstrated that X-ray modulated GSH-dependent antioxidant system of several tissues in dose-independent and tissue-specific manner, and it increased most urinary enzyme activities dose dependently. These results suggest that urinary GSH-related enzyme activities could be applicable for dose assessment in radiation emergency aid.

Key words: whole body X-irradiation, GSH-related antioxidant system, dose-dependent, tissue, serum, urine

1. Introduction

Ionizing radiation generates free radicals and reactive oxygen species (ROS), which cause cellular oxidative stress[1]. It has been suggested that cellular toxicity induced by ROS and oxidative stress leads to metabolic and morphological changes in animals and humans during flight as well as during radiotherapy

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and experimentation[2]. Cells are naturally equipped with several enzymatic and non-enzymatic antioxidant defense systems against oxidative stress[3]. The exposure of the human body to ionizing radiation depletes these endogenous antioxidants[4, 5] and ultimately leads to systemic disease. Thus, the diminution of cellular antioxidant capacity renders internal organs more susceptible to the effects of ROS[6].

GSH and GSH-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as ROS[7, 8]. GSH can either act as a substrate in the cytosolic GSH redox cycle or directly inactivate ROS such as superoxide and hydroxyl radicals[9]. GSTs are a family of drug-metabolizing dimeric proteins and are known to exist in the various organs of different species[10-13]. Most of them are localized in the cytosol and mammal GSTs are grouped to at least six classes, Alpha, Mu, Pi, Theta, Kappa, and Sigma[14-17]. GST class Alpha is mainly expressed in liver but is also present in kidney, testis, adrenal gland[9], and small intestine[18]. GST Mu class is also mainly expressed in liver. GST Pi class, GST P1-1 (rat GST-P and human GST-π), is mainly expressed in kidney but is also slightly present in lung, pancreas, placenta, testis, and small intestine[18-20].

It has been proposed that GST P1-1 detoxify alkylating agents by GSH conjugation and inactivate ROS that are known to be produced by oxidative stress conditions such as adriamycin and mitomycin C administration to drug resistant cells[21]. This form can also directly inactivate ROS with its free sulfhydryl groups. ROS are usually inactivated by enzymes such as superoxide dismutase (SOD), catalase (CAT), and selenium-dependent GSH peroxidase (Se-GSH-Px). Some ROS may generate lipid peroxides, and these can be reduced by selenium-independent GSH peroxidase (non-Se-GSH-Px) activity of Alpha[22] and Pi[21] class GSTs as well as Se-GSH-Px activity. Accordingly, the enzyme activity assayed with organic hydroperoxides such as cumene hydroperoxide (cumene-OOH) indicates total-GSH-Px activity. Additionally, it is known that several GST isozymes have high non-Se-GSH-Px activity towards thymine hyroperoxide[23]. In these reactions, GR contributes to the conversion of oxidized glutathione (GSSG) to GSH. Thus, GSH and GSH-related enzymes are one of the most important protective systems, and are thought to contribute to oxidative stress generated by radiation.

A previous report showed that whole body gamma-irradiation of 4.5 Gy decreased mouse liver GSH content and the activities of GST, SOD, and CAT within 24 h post-irradiation[24]. Other report demonstrated that GSH content of liver, lung, colon, and ileum was decreased 12 h post-8 Gy X-irradiation in rat[25]. Thus, ionizing radiation readily alters the GSH-related protective system of tissues. However, effect of radiation dose level to GSH content and GSH-related enzyme activities of each tissue is unknown. The present study was undertaken to elucidate the association between dose level of X-irradiation and GSH-related antioxidant enzyme activities of several tissues in whole-body X-irradiated rats. The effects of irradiation on GSH-related enzyme activities of serum and urine were also investigated.

2. Materials and Methods

2.1 Materials

Male Sprague-Dawley rats (six-week old) were obtained from CLEA Japan Inc.; 1-chloro-2,4-dinitrobenzene (CDNB), GSSG, 5,5′-dithiobis (2-nitrobenzoic acid), dithiothreitol (DTT), GR, and the reduced form of β-nicotinamide adenine dinucleotide phosphate (product of Oriental Yeast Co. Ltd.) were from Wako Pure Chemical Industries, Ltd., Osaka; GSH, hydrogen peroxide (H_2O_2, the product of Santoku Chemical Industries Co. Ltd.), and cumene-OOH were from Nacalai Tesque Inc., Kyoto. All other reagents were of special grade from Wako Pure Chemical Industries, Ltd.

2.1. Radiation exposure

Rats were acclimatized for one week before irradiation in a rearing cage and provided food and water *ad libitum*. They were then placed individually in an acrylic case with air holes, and exposed to a single dose of X-irradiation of up to 7 Gy using an X-irradiation apparatus (MBR-1520R-3, Hitachi Engineering and Services Co. Ltd., Ibaraki, Japan) under conditions of 150 kV and 20 mA with a 1.0-mm aluminum filter at a dose rate of 1.6 Gy/min. Six rats were used for the control and each irradiated group. For control rats, sham irradiation was performed by similar placement into the X-irradiator without exposure to introduce similar disquiet. Each rat was then immediately returned to the metabolic cage individually and provided food and water *ad libitum*. Urine of each rat was accumulated in a holder attached to the cage for 24 h after X-ray exposure.

2.2 Sample preparation

After 24 h of irradiation, each rat was etherized and blood was collected with a syringe from the abdominal aorta. Then, the liver, kidney, testis, and small intestine were resected. A 25-cm-length of the small intestine, including the duodenum, was used as a small intestine in this study. Each tissue was homogenized with 11 vol. of ice-cold 170 mM KCl, 4 mM EDTA, 5 mM DTT, and 45 mM Tris-HCl (pH 7.4). For homogenization, a Potter Elvehjem tissue grinder was used for liver, kidney, and testis samples, while a polytron homogenizer (Y stral GmbH D-7801, Dottingen, Germany) was used for small

Table 1. Changes in GSH content and GSH-related enzyme activities of liver, kidney, testis, and small intestine after 24 h of X-irradiation

Tissue	control	Dose (Gy)			
		1	3	5	7
Liver					
GSH	9.2 ± 0.86	9.2 ± 1.1	8.2 ± 1.6	10.5 ± 1.27	8.4 ± 1.6
GST	79.2 ± 13.4	74.2 ± 8.23	69.5 ± 6.82	84.0 ± 12.5	81.0 ± 10.2
Total-GSH-Px	43.1 ± 5.44	39.4 ± 1.52	47.4 ± 11.6	45.9 ± 17.1	43.5 ± 8.63
Se-GSH-Px	35.8 ± 3.60	34.0 ± 1.73	29.3 ± 10.4	35.5 ± 4.36	36.1 ± 5.26
GR	5.0 ± 0.33	6.4 ± 1.0 [a]	6.7 ± 1.0 [a]	6.1 ± 0.60 [a]	6.4 ± 1.0 [a]
Kidney					
GSH	4.0 ± 0.39	4.0 ± 0.44	4.4 ± 0.35	4.2 ± 0.45	4.0 ± 0.35
GST	21.6 ± 1.51	16.5 ± 1.06 [a]	12.9 ± 1.68 [a]	15.9 ± 2.23 [a]	15.7 ± 1.81 [a]
Total-GSH-Px	35.3 ± 3.54	33.0 ± 4.46	32.0 ± 4.37	33.1 ± 3.49	28.4 ± 2.18 [a]
Se-GSH-Px	26.0 ± 2.44	26.9 ± 3.74	25.2 ± 3.43	24.4 ± 4.63	19.6 ± 1.15 [a]
GR	11.3 ± 0.70	13.1 ± 1.32	12.1 ± 1.12	11.5 ± 0.685	11.5 ± 2.35
Testis					
GSH	4.9 ± 0.25	4.9 ± 0.52	4.6 ± 0.31	5.1 ± 0.15	5.0 ± 0.19
GST	42.4 ± 4.11	43.6 ± 2.01	43.0 ± 3.47	43.0 ± 6.99	36.6 ± 2.25 [a]
Total-GSH-Px	6.6 ± 1.10	5.7 ± 0.79	5.9 ± 0.70	5.6 ± 0.42	6.2 ± 0.42
Se-GSH-Px	1.8 ± 0.54	1.3 ± 0.28	1.5 ± 0.28	1.7 ± 0.29	2.0 ± 0.59
GR	0.62 ± 0.060	0.99 ± 0.12 [a]	0.81 ± 0.12 [a]	1.0 ± 0.12 [a]	0.79 ± 0.10 [a]
Small intestine					
GSH	3.8 ± 0.35	4.3 ± 0.30 [a]	4.5 ± 0.20 [a]	4.5 ± 0.37 [a]	3.6 ± 0.90
GST	16.6 ± 2.48	16.6 ± 1.88	15.5 ± 2.21	15.1 ± 0.260	11.2 ± 3.36 [a]
Total-GSH-Px	8.3 ± 0.52	9.3 ± 0.96	8.6 ± 0.71	9.7 ± 1.8	7.8 ± 1.2
Se-GSH-Px	7.7 ± 0.46	7.8 ± 0.97	7.0 ± 0.66	7.6 ± 0.79	6.2 ± 0.57 [a]
GR	8.0 ± 0.26	7.8 ± 0.59	7.9 ± 0.60	7.3 ± 0.85	6.6 ± 1.4

All values are expressed as mean ± SD of six rats. GSH content and each enzyme activity are described as μmol/g tissue and U/g tissue, respectively.
[a] Significant ($P < 0.05$) when compared with the control group.

intestine samples. The homogenate was centrifuged at 7,000 × *g* for 10 min and the supernatant was then centrifuged at 105,000 × *g* for 45 min. The cytosol fraction was subjected to enzyme assay. For quantitative determination of GSH, each tissue was homogenized with 11 vol. of ice-cold 5.5% trichloroacetic acid and the supernatant obtained after centrifugation at 7,000 × *g* for 10 min was subjected to assay. Collected blood and urine were centrifuged at 980 × *g* for 5 min and the supernatant was subjected to enzyme assay.

2.3 Methods for enzyme assays and other determination

GST activity was assayed with CDNB as a substrate, as described by Habig et al[26]. Se-GSH-Px and total-GSH-Px activities were determined with H_2O_2 and cumene-OOH, respectively, according to the method of Paglia and Valentine[27] as modified by Laurence and Burk[28]. GR activity was assayed with GSSG as a substrate according to the method of Carlberg and Mannervik[29]. GSH contents were determined as total non-protein sulfhydryl group using the method of Moron et al[30], with GSH as a standard. All enzyme activities were assayed at 25°C with a spectrophotometer (U-1100, Hitachi Engineering and Services Co. Ltd., Ibaraki, Japan). Urinary creatinine content was determined according to the method of Folin and Wu [31] to correct the urinary enzyme activities by concentration level of each urine.

2.4 Statistical analysis

All data were expressed as mean ± SD. Student's *t*-test or Mann–Whitney *U*-test was used for statistical analysis and $P < 0.05$ was accepted as significant (SPSS for Windows).

3. Results

3.1 Effect of X-irradiation on GSH content and GSH-related enzyme activities in liver, kidney, testis, and small intestine

Rats were exposed to a single dose of whole body X-irradiation of up to 7 Gy, and GSH content and activities of GST, Se-GSH-Px, total-GSH-Px, and GR were determined in liver, kidney, testis and small intestine samples exenterated after 24 h of irradiation. In liver, which exhibits the highest GSH redox activity along with kidney[32], GR activity was upregulated at any dose level whereas others were not affected by X-irradiation (Table 1). In kidney, only GST activity was impaired at any dosage whereas Se-GSH-Px, and total-GSH-Px activities were decreased at 7 Gy. Thus, GST has high susceptibility

Table 2. Changes in GSH-related enzyme activities of serum after 24 h of X-irradiation

Enzyme	control	Dose (Gy)			
		1	3	5	7
GST	35.9 ± 5.80	36.2 ± 4.70	33.6 ± 3.10	31.4 ± 4.90	32.9 ± 4.40
Total-GSH-Px	4.4 ± 0.92	4.9 ± 1.06	3.9 ± 0.94	3.9 ± 0.95	3.4 ± 0.87
Se-GSH-Px	4.5 ± 0.43	4.9 ± 0.98	4.2 ± 0.48	4.1 ± 0.51	4.1 ± 0.41
GR	27.6 ± 6.47	30.0 ± 6.14	32.9 ± 3.89	29.8 ± 2.66	29.7 ± 3.17

All values are expressed as mean ± SD of six rats. GST and GR activity were described as mU/ml. Both Px activities were described as U/ml.
[a] Significant ($P < 0.05$) when compared with the control group.

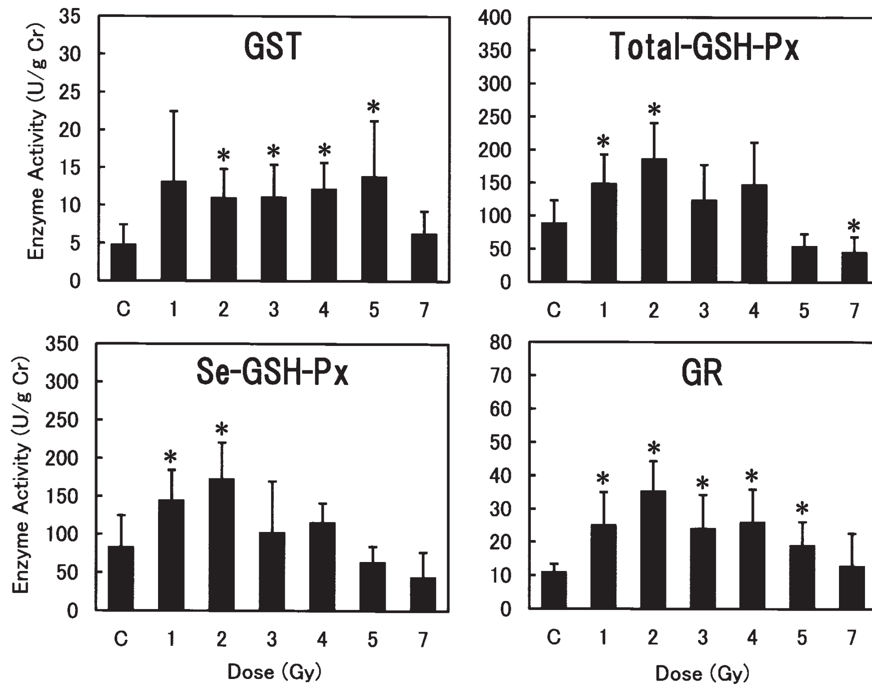

Fig. 1. Changes in GSH-related enzyme activities of urine after 24 h of X-irradiation.
Effect of X-ray irradiation on GST, total-GSH-Px, Se-GSH-Px, and GR activity was investigated. Value of each enzyme activity was described as unit per gram-creatinine (U/g Cr).
* Significant ($P < 0.05$) when compared with the control groups.

to radiation in kidney. In testis, GR activity was increased at any dosage as with liver whereas GST activity was decreased at 7 Gy. In small intestine, GSH content was increased up to 5 Gy whereas activities of GST and Se-GSH-Px were decreased at 7 Gy. It is noteworthy that the potentials of Se-GSH-Px, and total-GSH-Px activities of testis and small intestine are distinctly low and GR activity of testis is the lowest among the tissues examined.

3.2 Effect of X- irradiation on GSH related enzyme activities in serum and urine

In serum, any enzyme acativity did not vary by X-irradiation (Table 2). In contrast, urinary enzyme activities were affected by irradiation (Fig. 1). Specifically, GST activity uniformly increased at dosage between 2 and 5 Gy but it returned to the control level at 7 Gy. Whereas other activities were increased dose-dependently up to 2 Gy and fell gradually at higher doses, returning to control levels at 5 or 7 Gy. The variation pattern of each enzyme activity resembled a lognormal curve. The effects of irradiation to activities of Se-GSH-Px and total-GSH-Px were very similar.

4. Discussion

Ionizing radiation generates free radical and ROS, therefore GSH-dependent antioxidant system is easily modulated in radiated tissues. In fact, GSH-related redox system in several tissues and serum were affected by whole-body irradiation. However, dose-dependent effect on GSH-related enzyme activities have not been clarified. Furthermore, effect of ionizing radiation to urinary GSH-related enzyme activities has not been reported until now. Therefore, we investigated GSH-related enzyme activities

in several tissues, serum, and urine of rat exposed to several dose levels.

To date, several reports has shown that ionizing radiation alters tissue GSH content and GSH- related enzyme activities within 24h after radiation exposure, but the investigation time after irradiation is vary among individuals and the reason of an adoption of the time is generally unexplained. As to the regulation of transcriptional modulation of GSH-related enzymes by ionizing radiation, little information is available. Kim et al[33] demonstrated that mRNA levels of hepatic GST subunits Ya, Yb1, Yb2, Yc1, and Yc2 were increased by 2-4-fold at 15 to 24h after gamma-irradiation with 3 Gy, followed by return to the levels of untreated rats after 48h. It suggests that induction of each hepatic GST subunit by irradiation is most strongly enhanced at the period in transcription level. In terms of the points, we examined the effect of X-irradiation after 24h of whole body irradiation.

Chromosomal aberration analysis, which has been established as the gold-standard in radiation dosimetry, is used for evaluating radiation exposure[34]. However, it takes at least three days before the results are available. Recently, urine proteomic analysis for identify protein biomarkers using liquid chromatography, mass spectrometry and two-dimesional gel electrophoresis showed that significant changes in the urine proteome within 24h after total body X-irradiation[35]. Early changes of serum and urinary GSH-related enzyme activities post-irradiation may contribute to biodosimetry.

The experimental results suggested that the effect of X-irradiation on the GSH-related defense system was tissue specific and the irradiation-induced variation in these activities was not dose-dependent. The threshold dosage for GSH content and GSH-related enzyme activities was 1 or 7 Gy under the experimental condition of this study. It is undeniable that other detoxification enzymes also contribute to attenuate oxidative stress. Given that the tolerance of each tissue to X-radiation depends on its total antioxidant capacity, the effect of radiation on activities of other detoxification enzymes, in particular SOD and CAT, should be investigated.

ROS activate the expression of protective antioxidant genes including Se-GSH-Px, CAT, SOD, GR and GST[36], and also upregulate GSH synthesis via activation of γ-glutamilcysteine synthetase gene. It is known that activation of transcription factors AP-1 and NF-κB are involved in these upregulatory effects[36, 37]. Activation of these factors seem to be a reason of upregulation of GR activity and GSH content. However, it is suggested that higher ROS concentrations reversibly oxidize sulfhydryl groups of AP-1 and NF-κB accompanying by decrease of the transcription of submitted gene and attenuation of the cell protective system[37]. Therefore, it may involved in a decrease of enzyme activity at 7 Gy, the highest dose.

In liver, any enzyme activity was not decreased at any dose level. Its powerful antioxidant system may contribute to the resistibility for inactivation effect of high ROS concentration. GST P1-1, which has ROS-sensitive cysteine residues, is highly expressed in kidney. Hence, it is suggested that the inactivation of GST P1-1 may arise the sensitive decrease of kidney GST activity.

Among GSH-related enzyme activities, total-GSH-Px and Se-GSH-Px activities of testis and small intestine were markedly low level compared with those of liver and kidney. In addition, GR activity of testis was the lowest in tissues examined. We suggest that the low activities of both Px may account for the high radiosensitivity of testis and small intestine. We also speculate that the low potential of GR activity of testis leads to low GSH concentration earlier in this than in other tissues, owing to poor conversion activity of GSSG to GSH.

In general, radiation results in decreases of GSH content and GSH-related enzyme activities as described following reports. Sarkar et al[38] reported that diminution of liver and heart GSH contents occurred initially in rats exposed to 4 and 8 Gy of gamma-irradiation. Shingh et al[39] reported a decrease in GR activity of liver and spleen in rats exposed to gamma radiation of 4 Gy after 24 h of irradiation. In mouse, liver GSH content and activities of GST, SOD, and CAT were decreased after 24 h of whole-body gamma-irradiation of 4.5 Gy in radioprotective agent-free control. However, several reports demonstrated an inverse effect of radiation. Increases of GSH content in rat liver, kidney, and spleen were shown after 24 h of 800 R (approximately 7 Gy)[40]. In mouse, GSH contents in liver and heat were significantly increased, while those of kidney, lung, spleen, pancreas, brain, skeletal muscle, and bone marrow remained unchanged after 24 h of whole-body X-irradiation at 5 Gy[41]. Furthermore, an increase of rat spleen GR activity after irradiation was observed[38]. The reason for these inconsistent results is still unclear, but species and irradiation conditions (radiation quality and/or dose rate) may be related to the differences.

With respect to serum enzyme activity, previous investigation indicated that GR activity was unaffected by irradiation[39]. Our result that serum GSH-related enzyme activities including GR activity were unaffected by X-rays is consistent with the report. In contrast, urinary GSH-related enzyme activities were increased at 1 Gy, suggesting that it might be the threshold dose of these activities. As a whole, deviations of each urinary enzyme activity were larger than those of tissue and serum enzyme activity because of the extremely low activity in the absence of correction by urinary creatinine content. In this study, it was found that except for GST activity, each activity increased in a dose-dependent manner up to 2 Gy, suggesting its potential utility as a biomarker of

external radiation exposure. But no single enzyme activity appears to reveal whether the exposed dose level is less than or equal to 2 Gy. Recently, we found that rat urinary N-acetyl-beta-D-glucosaminidase (NAG) activity increased significantly at 3, 5, and 7 Gy compared with control before irradiation (data not shown). For future research, more investigation combinating other assay such as NAG activity measurement may clarify the suitability of GSH-related enzyme activities for dose assessment in radiation emergency aid after external ionizing radiation exposure.

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