

Inhibitory Effects of Prior Low-dose X-ray Irradiation on Carbon Tetrachloride-induced Hepatopathy in Acatalasemic Mice

Kiyonori YAMAOKA^{1*}, Takahiro KATAOKA¹, Takaharu NOMURA², Takehito TAGUCHI¹, Da-Hong WANG³, Shuji MORI⁴, Katsumi HANAMOTO¹ and Shohei KIRA³

Acatalasemic mouse/Catalase/CCl₄/Hepatotoxicity/Low-dose X-irradiation/Antioxidant substances.

The catalase activities in blood and organs of the acatalasemic (C3H/AnLCS^bCs^b) mouse of C3H strain are lower than those of the normal (C3H/AnLCS^aCs^a) mouse. We examined the effects of prior low-dose (0.5 Gy) X-ray irradiation, which reduced the oxidative damage under carbon tetrachloride-induced hepatopathy in the acatalasemic or normal mice. The acatalasemic mice showed a significantly lower catalase activity and a significantly higher glutathione peroxidase activity compared with those in the normal mice. Moreover, low-dose irradiation increased the catalase activity in the acatalasemic mouse liver to a level similar to that of the normal mouse liver. Pathological examinations and analyses of blood glutamic oxaloacetic and glutamic pyruvic transaminase activity and lipid peroxide levels showed that carbon tetrachloride induced hepatopathy was inhibited by low-dose irradiation. These findings may indicate that the free radical reaction induced by the lack of catalase and the administration of carbon tetrachloride is more properly neutralized by high glutathione peroxidase activity and low-dose irradiation in the acatalasemic mouse liver.

INTRODUCTION

As the mutant, the acatalasemic (C3H/AnLCS^bCs^b) mouse has one-tenth to half lower catalase activities in blood and tissues than those activities in the normal (C3H/AnLCS^aCs^a) mouse, it provides a useful model to study the possible role of hydrogen peroxide (H₂O₂) of these mice in carcinogenesis.^{1,2)} For example, C3H mice spontaneously develop mammary tumors, though no mammary tumors are reported in the acatalasemic mouse.^{3,4)} It was also reported that the high-dose X-ray irradiation of hypocatalasemic mice readily induce signet ring cell carcinoma.⁵⁾

We have reported that unlike high-dose X-rays, the low-dose X-rays or low level radon inhalation reduced the lipid peroxide to a similar levels similar to those of juvenile animals, though radiation sensitivity varied depending on the age of the animals and the different organs and tissues. The superoxide dismutase (SOD) activity was also elevated, suggesting that low-dose radiation could activate the host defense system. It was also found that these changes continued for relatively longer periods after low-dose X-ray irradiation.^{6–8)}

Carbon tetrachloride (CCl₄) is frequently used as a chemical inducer of experimental liver cirrhosis. Transient hepatocellular damage such as degeneration and necrosis after the administration of CCl₄ is thought to be induced by trichloromethyl radical (•CCl₃). The radical induces an adverse reaction by forming radicals after its administration in the early stage between intracellular uptake and transformation into storage types. Thus many biological substances such as membrane lipids, proteins, nucleic acids, and microsome are injured by trichloromethyl radicals.⁹⁾ The mechanisms of CCl₄-induced hepatotoxicity is generally considered to result from the conversion of CCl₄ into •CCl₃ by the cytochrome P450 system in the endoplasmic reticulum of hepatocytes, in which the trichloromethyl radical and the highly reactive trichloromethyl peroxyradical are formed to initiate lipid peroxidation of the endoplasmic reticulum in the early stage of CCl₄-induced toxicity.^{9–13)} However, the mechanisms of how these initial events lead to the later cellular necrosis remain unclear. We previously found that CCl₄-induced hepatotoxicity was

*Corresponding author: Phone/Fax: +81-86-235-6852,

E-mail: yamaoka@md.okayama-u.ac.jp

¹Chair of Medical Radioscience, and Department of ³Public Health and ⁴Pharmacology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan; ²Low Dose Radiation Research Center, Central Research Institute of Electric Power Industry, 2-11-1 Iwado-kita, Komae-shi, Tokyo 201-8511, Japan.

Abbreviations: CCl₄, carbon tetrachloride; GOT, glutamic oxaloacetic transaminase; GPT, Glutamic pyruvic transaminase; SOD, superoxide dismutase; GPX, glutathione peroxidase; TBA, thiobarbituric acid; MDA, malondialdehyde; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; XOD, xanthine oxidase; NADPH, reduced nicotinamideadenine dinucleotide phosphate; GR, glutathione reductase; TCA, trichloroacetic acid; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); SEM, standard error of mean; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione.

enhanced in acatalasemic mice in comparison with the normal ones in the later phases of liver injury.¹⁴⁾ This might be due to the increased formation of hydroxyl radicals ($\bullet\text{OH}$) in the absence of catalase instead of superoxide anion.

Considering this background, we examined in the present study the histopathological changes of the liver after low-dose X-ray irradiation of hepatopathic mice treated with CCl_4 . We also investigated the various biochemical parameters, such as transaminase activities, lipid peroxidation levels, and endogenous antioxidants activities in these hepatopathic mice.

MATERIALS AND METHODS

Animals

Two strains of C3H mice originally provided by Feinstein *et al.*,¹⁵⁾ normal (C3H/AnLCs^aCs^a) and acatalasemic (C3H/AnLCs^bCs^b), six to seven weeks of age and 20–25 g of body weight were kept under an air-conditioned room (temperature 20°C and humidity 60%) at the Animal Center for Medical Research, Okayama University Medical School. They were fed on Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. The number of mice per experimental group is 6–12. We received an approval from the affiliated organs for animal experiments.

Each mouse was irradiated by a single dose of 0.5 Gy at a dose rate of 0.75 Gy/min of X-ray (maximum rated output voltage; 150 keV, ampere; 15 mA, filters; Al:Cu = 1.0:0.5), using an X-ray generator (Hitachi MBR-1505R2). The age-matched control mice were sham-irradiated. All the animals were killed by a dislocation of cramp.

Ten μl CCl_4 (20% in olive oil)/g weight was injected intraperitoneally to mice under light ether anesthesia 4 h after sham- or 0.5 Gy- irradiation. All animals were starved overnight after CCl_4 treatment. Each experimental group consisted of 6–12 mice. At 18 h following CCl_4 treatment, blood was collected from the heart after the dislocation of cramp. Serum was separated by centrifugation at 3,000 $\times g$ for 15 min for the assay of the activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Livers were quickly excised for the analyses of the activities of catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX), the total glutathione content, and the thiobarbituric acid reactive substances (TBARS) production, and parts of the livers were fixed in 10% neutral-buffered formalin for histological examinations.

Biochemical assays

Blood was collected by the above treatments, and serum was obtained by centrifugation at 3,000 $\times g$ for 15 min under 4°C. The activities of GOT and GPT were measured by the UV-rate method¹⁶⁾ using GOT and GPT-test kits (Wako Pure Chemical Industry, Co., Ltd.).

Lipid peroxide, malondialdehyde (MDA), was assessed by TBARS according to the method of Uchiyama and Mihara.¹⁷⁾

The liver was homogenized in 154 mM KCl on ice and refluxed for 60 min at 95°C in the presence of 0.3% (w/v) thiobarbituric acid (TBA) and 7.5% (v/v) acetic acid at pH 3.5. The optical density of colored product was read at 532 nm by using a spectrophotometer. 1,1,3,3-Tetraethoxypropane was used as the standard curve, and the results were expressed as n mol of malondialdehyde per mg of protein. The protein content was measured according to the method of Lowry *et al.*¹⁸⁾

SOD activity was determined by the spin-trapping method,¹⁹⁾ using a JES-TE100 electron spin resonance (ESR) device (JEOL). The measuring procedure of this ESR method was as follows: 15 ml of 450 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 85 μl of SOD extract specimen, and 50 μl of 2 mM hypoxanthine were placed in a test tube. To the mixture was added 50 μl of xanthine oxidase (XOD) (0.2 unit/ml) in the one. After agitation, the assay mixture was transferred into a special flat cell (product of JEOL).

The DMPO- O_2 -spin adduct was analyzed 45 s after the addition of XOD with the aid of an ESR spectrometer. Manganese oxide was used as an internal standard. The spin number was calculated by comparing the signal strength with that of 2,2,6,6-tetramethyl piperidine-*N*-oxide, the spin number of which is known.

Catalase activity was measured at 240 nm by a spectrophotometer in terms of H_2O_2 reduction rate at 37°C.²⁰⁾ The mixtures for assays consisted of 50 μl of 1 M Tris-HCl buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 900 μl of 10 mM H_2O_2 , 30 μl of deionized water, and 20 μl of the liver supernatant. The activity was calculated by using a molar extinction coefficient of $7.1 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$. In all assays, the assay mixtures were incubated at 37°C and then at 30°C for 5 min. The reactions were started by adding the liver supernatant prepared as described above.

GPX activity was assayed by coupling the reduction of tert-butyl hydroperoxide to the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase (GR).²¹⁾ The assay mixtures consisted of 100 μl of 1 M Tris-HCl containing 5 mM EDTA (pH 8.0), 20 μl of 0.1 M glutathione, 100 μl of GR solution (10 units/ml), 100 μl of 2 mM NADPH, 650 μl of distilled water, 10 μl of 7 mM tert-butyl hydroperoxide, and 10 μl of the liver supernatant. The oxidation of NADPH at 37°C was followed spectrophotometrically at 340 nm. One unit of activity is defined as the amount of GPX required to oxidize 1 μmol of NADPH per min.

Total glutathione content was measured by using a modified spectrophotometric technique.²²⁾ Briefly, 0.25 g of liver was suspended in 2.5 ml of 0.1 M phosphate buffer (pH 7.4), mixed with 1.25 ml of ice-cold 10% trichloroacetic acid (TCA) solution, then homogenized in a teflon-glass homogenizer. The homogenates were centrifuged at 14,000 $\times g$ for 15 min. Subsequently, 0.5 ml of the supernatant was treated with 3 ml of ice-cooled diethylether, and the diethylether layer was

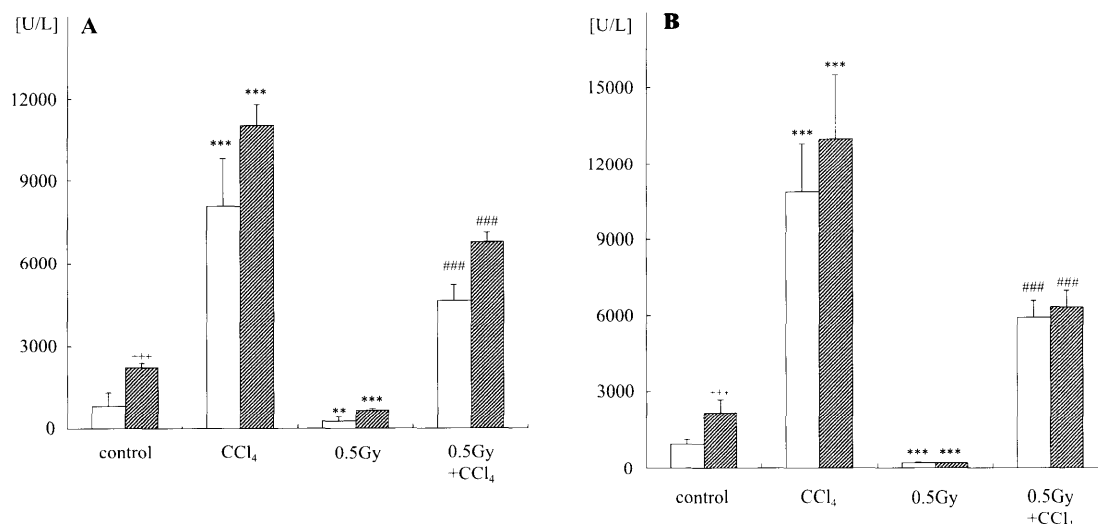


Fig. 1. Differences in the activities of GOT (A) and GPT (B) in serum between normal (open bars) and acatalasemic (hatched bars) mice, six to seven weeks of age, under non-treated control, CCl₄ administration, 0.5 Gy X-irradiation, or 0.5 Gy-irradiation prior to CCl₄-administration. The number of mice per experimental point is 6–12. +++ $p < 0.001$ by t -test, acatalasemic mice value vs. normal mice value under no treatment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ by t -test, each normal mice value or acatalasemic mice value under CCl₄ administration or 0.5 Gy X-irradiation vs. under no treatment, respectively. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ by t -test, each acatalasemic mice value or normal mice value under CCl₄ administration after 0.5 Gy X-irradiation vs. under CCl₄ administration. Each value indicates the mean \times SEM.

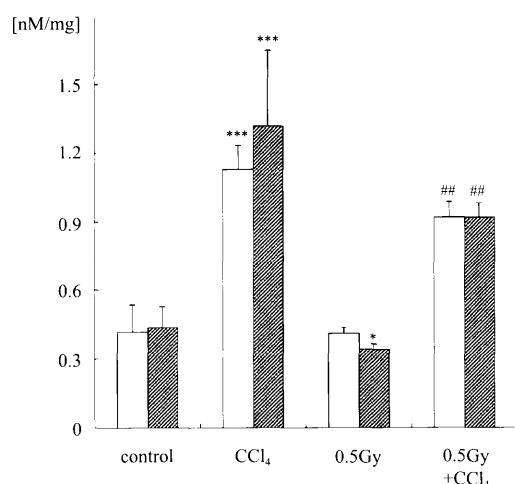


Fig. 2. Differences in liver lipid peroxide level between normal (open bars) and acatalasemic (hatched bars) mice under non treated control, CCl₄ administration, 0.5 Gy X-irradiation, or 0.5 Gy irradiation prior to CCl₄ administration. The number of mice for each experiment and significance are the same as in Fig. 1.

removed with a pipette. This procedure was repeated 5 times to remove excess TCA. The final supernatant was assayed for total glutathione content. The sample solution (25 μ l) was mixed with 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 733 μ l of 0.3 mM NADPH, and 10 μ l of GR (2 U/ μ l). The rate of change in absorbance was measured at 412 nm. Glutathione standards (1–20 μ g/ml) were analyzed in the same manner.

Histopathological examination

The left and central lobes of the liver were fixed in 10% formalin processed by graded ethanol and xylene and embedded in paraffin. Five μ m thick tissue sections were prepared and stained with hematoxylin-eosin (HE). The other parts were placed in a deep freezer at -80°C in assays as described below.

Small tissue specimens (4 \times 4 \times 2 mm) were collected from the liver, which showed grossly fatty degeneration. They were fixed by immersion for 12 h in 4% phosphate-buffered formalin at room temperature. After rinsing in phosphate-buffered saline for 2 h, the tissue was immersed into an octo-compound during a period of 2 h by the changing twice of 100% catalysed plastic containers. Two μ m thick sections were cut by a Sorvall microtome with glass knives, stretched on a water bath (23 $^{\circ}\text{C}$), put on a slide, and dried at room temperature. Lipids were demonstrated by Sudan Black B staining. For staining with Sudan Black B, the sections were briefly rinsed in 70% ethanol and stained in a filtered Sudan Black B solution containing 0.25% Sudan Black B in 70% ethanol. The sections were differentiated in 70% ethanol. Counterstain was performed by using 1% neutral red for 5 min, and the sections were mounted in glycerin-gelatine.²³⁾

Statistical analysis

The data values are presented as the mean \pm the standard error of mean (SEM). The statistical significance of differences was determined by Student's t -test for comparison between two groups or by two-way analysis of variance (ANOVA) and Dunnett's tests for multiple comparison.

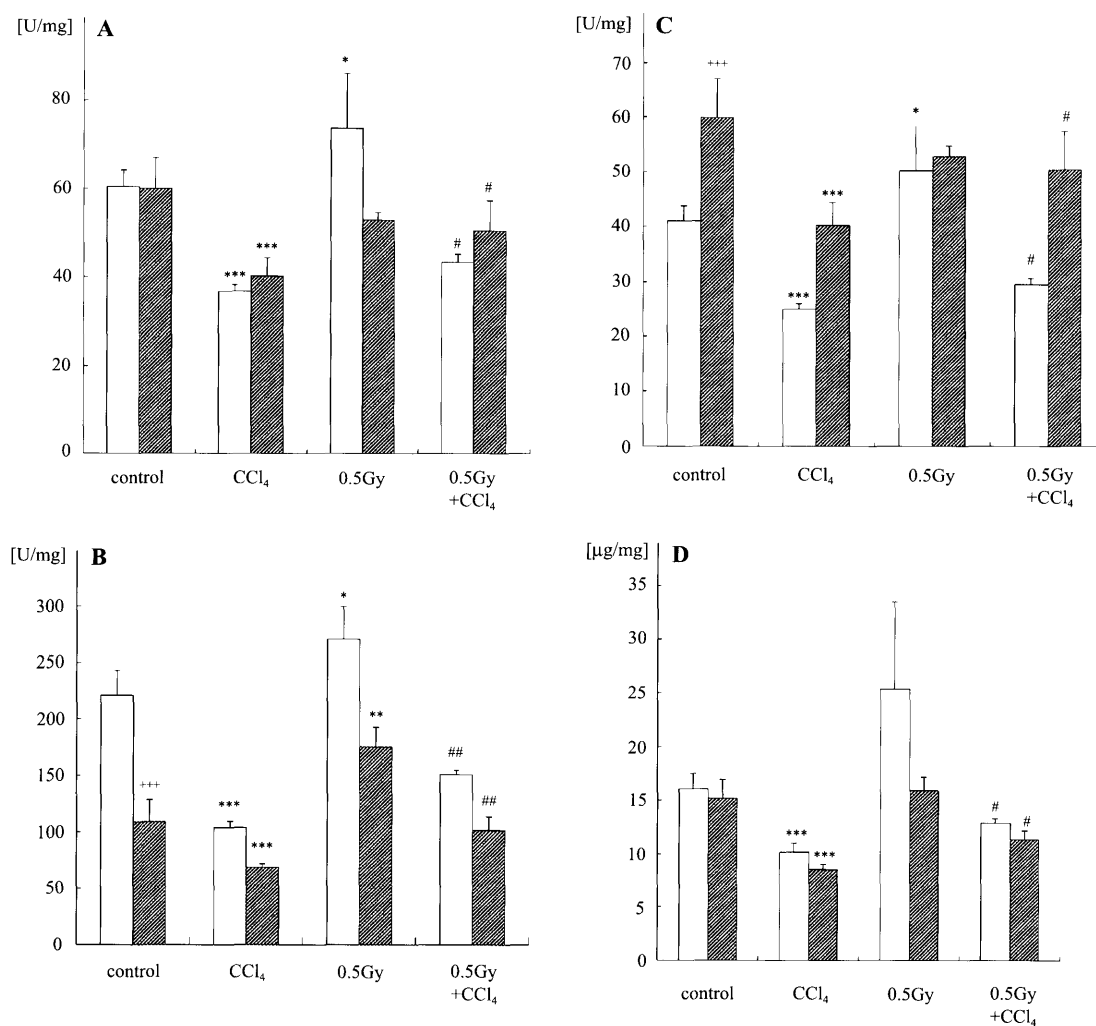


Fig. 3. Differences in activities of SOD (A), catalase (B), and GPX (C), and total glutathione content (D) in liver between normal (open bars) and acatalasemic (hatched bars) mice under non treated control, CCl₄ administration, 0.5 Gy X-irradiation, or 0.5 Gy-irradiation prior to CCl₄-administration. The number of mice for each experiment and significance are the same as in Fig. 1.

RESULTS

Differences between acatalasemic mouse and normal mouse

Under the conditions of non irradiation and no treatment with CCl₄, no significant differences were observed in the lipid peroxide level, the SOD activity, and total glutathione content between the acatalasemic mice and the normal mice, whereas the acatalasemic mice showed a significantly lower catalase activity and significantly higher GOT, GPT, and GPX activities than those of the normal mice (Figs. 1–3).

Changes caused by CCl₄ administration alone

There was a significant increase in the activities of GOT and GPT and the lipid peroxide level, and a significant decrease in the activities of SOD, catalase, and GPX and the total glu-

tathione content both in acatalasemic and normal mice after administration of CCl₄ (Figs. 1–3).

Changes caused by 0.5 Gy irradiation alone

There was a significant decrease in the activities of GOT and GPT and the lipid peroxide level, and a significant increase in the catalase activity in the acatalasemic mouse by 0.5 Gy irradiation. On the other hand, there was a significant decrease in the activities of GOT and GPT and a significant increase in the activities of SOD, catalase, and GPX in the normal mouse after 0.5 Gy irradiation. No significant changes were observed in the other experimental conditions (Figs. 1–3).

Changes caused by 0.5 Gy irradiation prior to CCl₄ administration

The activities of GOT and GPT and the lipid peroxide level

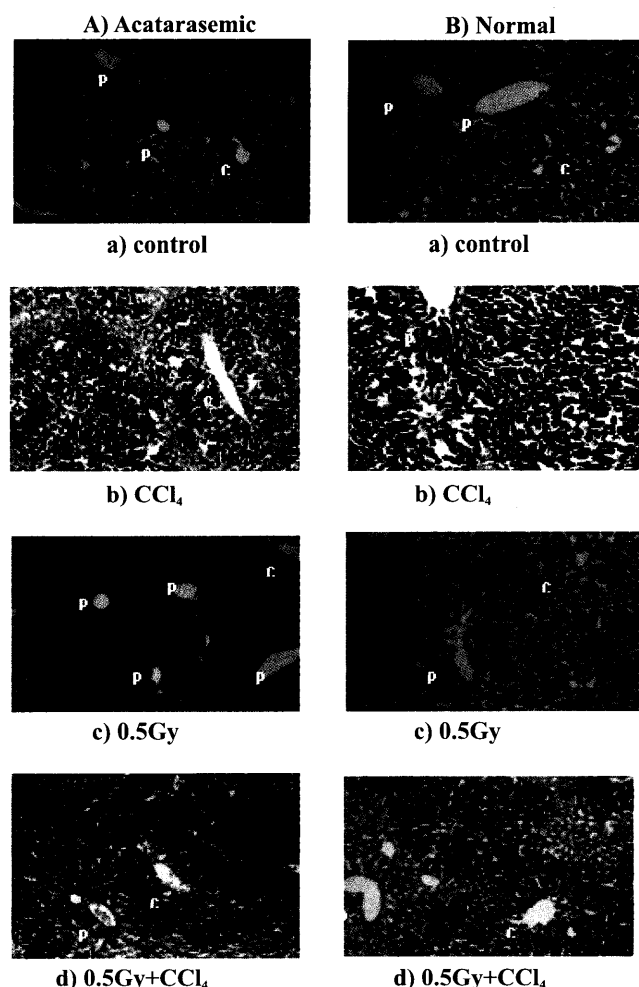


Fig. 4. Differences in liver histopathology between acatalasemic (A) and normal (B) mice under non-treated control (a), CCl₄ administration; (b), 0.5 Gy X-irradiation; (c) or 0.5 Gy irradiation prior to CCl₄ administration (d). All figures under lower magnification ($\times 100$) are stained with Sudan Black B (black-colored) for the detection of fatty degeneration surrounding the central vein (C) and the portal vein (P). The areas of cell necrosis surrounding the central vein in the CCl₄-administrated mice after 0.5 Gy X-irradiation were smaller in both mice in comparison with the CCl₄ administrated mice. No obvious difference was noted in the extent of the centrilobular hepatocyte damage by non treatment or 0.5 Gy X-irradiation in both mice.

in the group treated with CCl₄ significantly decreased in both acatalasemic and normal mice by 0.5 Gy irradiation prior to CCl₄ administration. In the same manner, the activities of SOD, catalase, and GPX and the total glutathione content in the group treated with CCl₄ significantly increased (Figs. 1–3).

Histological observations

Histological examinations of liver tissues revealed no significant changes before CCl₄ administration in acatalasemic

and normal mice from either the low-dose irradiated or the control sham-irradiated groups. At 18 h of CCl₄ treatment, liver tissues exhibited centrilobular necrosis of parenchymal hepatocytes associated with their hydropic (mitochondrial) swelling and fatty degeneration was more extensive in acatalasemic mouse liver than in the normal mice. However, in prior 0.5 Gy irradiated and CCl₄ treated groups, there was no obvious difference in the extent of the centrilobular necrosis between the acatalasemic and normal mice, reflecting the results of blood biochemistry. Moreover, in the result of the morphometric analysis using the Sudan Black B-stained sections, the fatty degeneration in livers of both normal and acatalasemic mice under 0.5 Gy irradiation prior to CCl₄ administration was reduced by 26–32%, compared with that under CCl₄ administration (Fig. 4).

DISCUSSION

The decreased catalase activities in acatalasemic mice might cause increased tissue or cellular levels of H₂O₂. As a consequence, the overproduction of OH radicals from the undecomposed excessive H₂O₂ in acatalasemic mice could induce more toxicity effect on the hepatocytes, leading to enhanced liver damage in acatalasemic mice. Catalase and GPX are the two most important enzymes in the regulation of intracellular H₂O₂ level in biological systems. The former is regarded as playing a major role in the break down for an excessive production of H₂O₂ in the liver.^{24,25)} In this study, the liver catalase activities in acatalasemic mice were significantly lower than in normal mice, but the liver GPX activities in acatalasemic mice were significantly higher than in normal mice. The present study suggests that no significant changes in the lipid peroxide level in the acatalasemic mouse liver under non irradiation conditions are due to the balance of low catalase activity and high GPX activity. Thus the high GPX activity neutralizes a free radical reaction induced by the lack of catalase.

It is also well known that the scavenging activity of SOD is a conversion of superoxide anion radical (O₂⁻) into H₂O₂. But under the administration of CCl₄, little O₂⁻ generates in the liver. It is certain that SOD activity did not elevate by inducing CCl₄. The decrease in SOD activity may be due to the damage of hepatocytes induced by the administration of CCl₄. However, this does not explain the significant difference between the irradiation group and the sham-irradiation group. In this study, we demonstrated that a pathological examination of the liver revealed fatty invasion induced by CCl₄ administration-related disorder. Glutathione directly reacts with reactive oxygen species (ROS), and GPX catalyzes the destruction of H₂O₂ and hydroperoxide.²⁶⁾ This catalysis generates GSSG, and finally GSH. However, GR catalyzes the regeneration of GSH from GSSG. Thus GR and GPX are both the enzymes in the glutathione-regenerating pathway, and the changes of both activities are in a similar fashion. The

total glutathione (reduced glutathione + oxidized glutathione; GSH + GSSG) content was increased by low-dose X-irradiation. However, the results of the behavior are clearly distinguishable between the activities of GR and GPX, the glutathione content. The CCl₄ hepatotoxicity is very likely caused by free radicals arising during the activation of CCl₄ by drug-metabolizing enzymes in the endoplasmic reticulum.⁹⁾ SH-donors, such as cysteamine and *N*-acetyl- cysteine, significantly lead to a suppression of CCl₄-induced acute hepatotoxicity.²⁷⁾ In the same manner, the radon and thermal therapy improved the pulmonary function of asthmatics patients by increasing the reduced activities of antioxidant enzymes.²⁸⁾

There was a significant increase in the transaminase activities and the lipid peroxide level in both livers from the normal and the acatalasemic mice after the administration of CCl₄. This might be indicated by the decrease in the activities of SOD, catalase, and GPX, all of which are the antioxidant enzymes detoxifying toxic O₂⁻ to H₂O₂ and H₂O₂ to H₂O, and the total glutathione content. On the other hand, we found that only the catalase activity in the acatalasemic mouse liver is more sensitive to radiation than that of the normal mouse liver. Moreover, a point mutation within the coding region, but no other major changes of the catalase gene, is responsible for the phenotype of an acatalasemic mouse, and transcriptional regulation is not different from normal counterparts because only enzymatic activities were measured in this study.²⁸⁻³⁰⁾

We found that a relatively low-dose (0.5 Gy) of irradiation, which is an adequate oxygen stress producer, increased the catalase activity in the acatalasemic mouse liver to the level similar to those in the normal mouse liver. We have reported that the enhancement in enzyme activities results from an induction of their synthesis shortly after low-dose irradiation.³¹⁻³²⁾ These findings also may indicate that the free radical reaction induced by the lack of catalase is neutralized by low-dose irradiation. These results suggest that activated functions of the living body by low-dose radiation may contribute to a suppression of aging and to the prevention or reduction of the ischemic brain and cardiac failure resulting from the cascade of cytokines and NO release.⁶⁻⁸⁾

On pathological examinations, there was a small area of fatty degeneration induced by 0.5 Gy irradiation prior to CCl₄ administration. With the decrease in GOT and GPT activities, fatty liver was almost improved 4 hr after irradiation. The lipid peroxide level was also significantly lower, in a similar fashion to GOT and GPT activities, suggesting that low-dose irradiation relieved functional disorder at least in the liver of mice with ROS-related diseases. This is because radiation can be considered to be a source of ROS. Yet it has recently been shown that low-dose irradiation (up to 0.5 Gy) induces various stimulating outcomes such as an increase in resistance to oxygen toxicity,³³⁾ enhancement of immune function³⁴⁾ and to the improvement of social behavior of mice.³⁵⁾ These effects

may be related to the induction of antioxidant enzymes and the degeneration of O₂⁻, H₂O₂, and •OH is inhibited by low-dose irradiation.

Little information has been available concerning the involvement of H₂O₂ or •OH in CCl₄-induced hepatotoxicity. However, the observed results in the present experiments clearly indicate that H₂O₂ or •OH are probably responsible for the enhanced CCl₄ toxicity in the livers of acatalasemic mice, and low-dose irradiation attenuating the hepatotoxicity of CCl₄ further substantiated that •OH radicals are the direct hepatotoxic agent in the later stages of CCl₄-induced liver injury.

We have previously conducted a study to examine changes in the activities of antioxidant enzymes, such as GPX and catalase in the brain, which is more sensitive to oxidative stress than other organ, at 3, 6, or 24 h following X-ray irradiation at doses of 0.5 or 5.0 Gy to the acatalasemic and the normal mice. The acatalasemic mouse brain was more damaged than the normal mouse brain by excessive oxygen stress, such as a high-dose (5.0 Gy) X-ray. On the other hand, we found that 5.0 Gy irradiation significantly decreased by 25–65% the activities of both GPX and catalase in the acatalasemic mice brains and that a relatively lower dose (0.5 Gy) irradiation specifically increased by 15–50% the activities of both GPX and catalase in the acatalasemic mice brains, making the activities closer to those in the normal mice brains. These findings may indicate that the free radical reaction induced by the lack of catalase is more properly neutralized by low-dose irradiation.³⁶⁾ In future clarification, this study is required to assess the difference of effects between high-dose and low-dose irradiations and the turnover rate of free radicals in acatalasemic mice livers in comparison with control mice livers.

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