Basic Study on Enhancement of Antioxidant Function by Low-dose Irradiation in Mouse Brain and Its Combined Effect with Ascorbic Acid

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We studied on the characteristics of changes in antioxidant function of mice brains at 4 h to 7 days after low-dose X-ray (0.1 to 2.0 Gy) irradiation, and the combination effect of this and administration of ascorbic acid (500 mg/kg body weight), an antioxidant enzyme. As a result, low-dose irradiation mostly showed decrease in lipid peroxide levels, increase in superoxide dismutase (SOD) activities and heat shock protein (HSP) levels. In addition, the combined use of 0.5 Gy irradiation with ascorbic acid administration showed a significant decrease in lipid peroxide level, suggesting the combined effect of antioxidant.

Key Words: low-dose X-ray, ascorbic acid, mouse brain, lipid peroxide level, superoxide dismutase (SOD) activity, heat shock protein (HSP) level

1. Introduction

It is widely accepted that exposure to radiation contributes to the development of cancer. In addition, an investigation involving atomic bomb survivors indicated that doses above 0.5 Gy are associated with an elevated risk of both stroke and heart disease.1) Since the 2011 nuclear accident in Fukushima, the effects of low-dose irradiation are at the forefront of the public’s attention. One of the main concerns is minimizing the impact of radiation exposure on health.

We have already reported that low-dose X-ray irradiation activates antioxidative functions in mouse organs.2) A report has suggested that the activation of antioxidative functions peaked at 4 h after X-ray irradiation.3) A possible mechanism of the activation is that reactive oxygen species (ROS) mediate the induction of antioxidative functions, such as Mn-superoxide dismutase (SOD) activity4) Interestingly, this activation induced by X-ray irradiation inhibits oxidative stress-induced damage of the liver5) and brain.6) For example, pretreatment with low-dose X-ray irradiation inhibits CCl4-induced liver damage due to activation of antioxidative functions in the liver.5) Another report has suggested that low-dose X-ray irradiation inhibits cold-induced brain injury in mouse.5) These damages are induced by free radicals or ROS. Therefore, the activation of antioxidative functions induced by low-dose X-ray irradiation plays an important role in reducing oxidative stress-induced damages.

Similarly, radon, a gas that emits α-rays, also activates antioxidative functions in mouse organs.2)
Radon inhalation inhibits ROS-induced transient global cerebral ischemic injury in gerbils due to activation of antioxidative functions in the brain. These facts are inconsistent with the findings of the aforementioned investigation involving atomic bomb survivors. Although both radiation and transient global cerebral ischemia induce ROS production, the effects are completely different because low-dose irradiation inhibits oxidative damages, while ischemia causes fatal damages. Since the atomic bomb survivors were exposed to high-dose radiation in a single event, it is conceivable that such an event may lead to stroke several decades later. Therefore, it is important to evaluate oxidative stress in the brain after X-ray irradiation.

Ascorbic acid reacts readily with superoxide anions and hydrogen peroxide. We reported that combined radon and antioxidant vitamin treatment could effectively inhibit alcohol-induced hepatopathy in mice due to activation of antioxidative function induced by radon inhalation. Therefore, combination of low-dose X-ray irradiation and antioxidant ascorbic acid administration may effectively inhibit oxidative damages in brain.

The purpose of this study was to evaluate activation of antioxidation by low-dose X-ray irradiation in the brain (Experiment 1) and to examine the combined effects of X-ray irradiation with ascorbic acid treatment (Experiment 2). To determine the oxidative stress, we examined the dose- and time-dependent changes in SOD activity and lipid peroxide (LPO) levels in the mouse brain following X-ray irradiation and that after X-ray irradiation and treatment with ascorbic acid.

2. Materials and methods

2.1 Animals

Male BALB/c (age, 8 wk; weight, approximately 23 g) and C57BL/6J mice (age, 8 wk; weight, approximately 23 g) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan), respectively. Ethical approval for all protocols and experiments was obtained from the animal experimentation committee of Okayama University.

2.2 X-ray irradiation

To examine the dose-dependent changes in antioxidative functions, we used BALB/c mice. Because they are sensitive to radiation compared with other strains, BALB/c mice received whole-body irradiation at doses of 0.1, 0.5, 1.0, or 2.0 Gy (tube voltage, 150 kV; tube current, 20 mA; filter, 0.5 mm Al and 0.2 mm Cu; distance between focus and target, 43.5 cm), delivered using an X-ray generator (MBR-1520R-3; Hitachi Power Solutions Co., Ltd., Ibaraki, Japan). The control mice were sham-exposed. The mice were euthanatized using CO2 4 h, 2 d, and 7 d after X-ray irradiation (Experiment 1).

2.3 Ascorbic acid administration

C57BL/6J mice were intraperitoneally injected with ascorbic acid at a concentration of 500 mg/kg body weight 2 h after X-ray irradiation. The mice were euthanatized using CO2 4 h after the administration (Experiment 2).

2.4 Biochemical assays

The LPO levels were assayed using a Bioxytech LPO-586 assay kit (OXIS Health Products, Inc.). Briefly, brains were homogenized in 10 mM PBS (pH 7.4) on ice. Prior to homogenization, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile was added per 1 mL of the buffer-tissue mixture. The resulting homogenate was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was used for assaying lipid peroxide levels. This assay is based on the measurement of malondialdehyde and 4-hydroxyalkenals, which react with a chromogenic reagent, the absorbance of which can be measured.
at 586 nm and is directly proportional to the LPO levels.

Brains were homogenized on ice in 10 mM phosphate-buffered saline (PBS, pH 7.4). The homogenates were centrifuged at 12,000 \( \times g \) for 45 min at 4°C, and the supernatants were used to assay SOD activity. The SOD activity in the brain was measured by the nitroblue tetrazolium (NBT) reduction method,\(^{11}\) using the Wako-SOD test (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan). Briefly, the extent of inhibition of the reduction of NBT was measured at 560 nm using a spectrophotometer (DS Pharma Biomedical, Suita, Osaka, Japan). One unit of enzyme activity was defined as 50% inhibition of NBT reduction.

Total glutathione (t-GSH) content was measured using the Bioxytech GSH-420TM assay kit (OXIS Health Products, Inc., Portland, OR, USA). Briefly, the brains were suspended in 10 mM PBS (pH 7.4), mixed with ice-cold 7.5% trichloroacetic acid solution, and homogenized. The homogenates were centrifuged at 3,000 \( \times g \) for 10 min. This assay is based on the formation of a chromophoric thione, the absorbance of which can be measured at 420 nm and is directly proportional to the t-GSH concentration.

Protein content in each sample was measured by the Bradford method, using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).\(^{12}\)

2.5 Determination of heat shock protein (HSP) 70 levels by western blotting

Western blot analyses with mouse monoclonal antibodies against HSP70 were performed using anti-HSP70 antibody (#4872; Cell Signaling Technology, Inc., Danvers, MA, USA). The bands were scanned and quantitated using the Chemi Doc XRS Plus System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer’s recommendations. Band intensities were normalized to \( \beta \)-actin levels to normalize for loading variations between lanes. Density of each band was assay using a software (Image Lab, Bio-Rad Laboratories).

2.6 Statistical analyses

The data are presented as the mean±standard error of the mean. Each experimental group consisted of samples from 4–6 animals. Statistically significant differences were determined using an unpaired \( t \)-test for comparisons between two groups and one-way or two-way analysis of variance (ANOVA) following Tukey’s test, for multiple comparisons where appropriate. \( P \)-values were considered statistically significant at \( P<0.05 \).

3. Results

3.1 Dose- and time-dependent changes in LPO levels in the BALB/c mouse brain after X-ray irradiation

First, we assayed the dose-dependent changes in LPO levels in the brain after X-ray irradiation because LPO is one of the indicators of oxidative stress. In addition, the LPO levels of 4 h after sham irradiation and 7 d after 0.1 Gy X-ray irradiated groups were significant higher than that of 2.0 Gy irradiated groups (Fig. 1A).

The LPO levels in the brain were significantly lower than the control levels 2 d after 0.1 Gy irradiation or 4 h and 7 d after 2.0 Gy irradiation (Fig. 1B).

3.2 Dose- and time-dependent changes in SOD activity in the BALB/c mouse brain after X-ray irradiation

An antioxidative enzyme, SOD reduces oxidative stress. We examined the dose-dependent changes in SOD activity in the BALB/c mouse brain after X-ray irradiation. The SOD activities of 2 d after 0.1, 0.5 or 1.0 Gy X-ray irradiated groups were significant lower than that of 2.0 Gy irradiated group (Fig. 2A).

The SOD activity was slightly higher than that
in the sham-irradiated controls 4 h, and 7 d after 0.1 or 0.5 Gy X-ray irradiation. The SOD activity was significantly decreased 2 d after 0.1 Gy irradiation. At 4 h after 1.0 Gy irradiation, the SOD activity was significantly higher than the control level. Similarly, at 4 h or 2 d after 2.0 Gy irradiation, the SOD activity was significantly higher than that of the control (Fig. 2B).

3.3 Changes in the HSP70 levels in the BALB/c mouse brain after X-ray irradiation
The HSP70 levels were increased at 4 h, and 2 d after 1.0 and 2.0 Gy irradiation, but the difference was not statistically significant (Fig. 3).
Changes in the SOD activity, t-GSH content, and LPO levels in the C57BL/6J mouse brain after treatment with X-ray irradiation, ascorbic acid.

The LPO levels of combination of 0.5 Gy irradiation with ascorbic acid administration were significantly lower than that of control mice, 0.5 Gy irradiated mice, or ascorbic acid administrated mice (Fig. 4).

4. Discussion

It is widely accepted that radiation induces ROS, such as hydroxyl radicals, which lead to oxidative stress and cause diseases. Although there are many reports on radiation-induced carcinogenesis, there are only a few reports on the non-cancerous brain diseases induced by low-dose irradiation. In this study, we focused on the brain because it is important to clarify the initial events in the brain after irradiation.
tion, specifically in the oxidative stress condition.

BALB/c mice, which are sensitive to radiation,\(^{10}\) are the best materials because it is expected that BALB/c mice show a clear biological response to radiation. However, C57BL/6J mice, which are resistant to radiation,\(^{10}\) may not show a clear response to radiation. Since one of the aims of Experiment 1 is to examine the response to radiation, we used BALB/c mice. Similar results have previously been reported, showing that continuous exposure to radon increases SOD activity, but it transiently returns to normal levels at approximately 2 d.\(^{13}\) Although SOD exists in multiple forms, including Mn-SOD, Cu/Zn-SOD, and extracellular SOD, which are located in the mitochondria, cytoplasm, or extracellular milieu, respectively, radon inhalation activates Mn-SOD in the mitochondria due to the induced oxidative stress.\(^{14}\) It has been reported that the production of hydroxyl and peroxynitrite radicals increase immediately after \(\gamma\)-ray irradiation and return to normal levels after 1 d, and that delayed production is observed over 7 d.\(^{15}\) Superoxide radicals are also produced, but the levels peak 3 d after \(\gamma\)-ray irradiation.\(^{15}\) The results may indicate that delayed production of ROS induced by X-ray irradiation contributes to the activation of
SOD in the brain. Although 0.5 Gy X-ray irradiation inhibits mild oxidative stress induced by ischemia-reperfusion injury in mice, severe oxidative stress was not inhibited. This is probably due to the fact that low-dose irradiation moderately activates antioxidative functions.

It has been reported that oxidative stress induces the expression of genes encoding HSP70. Therefore, the HPS70 levels after X-ray irradiation were examined in this study. Our results suggest that X-ray irradiation induces oxidative stress.

Our results may not indicate that low-dose X-ray irradiation activates antioxidative functions because we did not assay other antioxidative markers, such as levels of catalase and glutathione. To evaluate oxidative conditions in the brain after X-ray irradiation, we assayed the LPO levels. The results suggest that low-dose irradiation mostly showed decrease in lipid peroxide levels. In this study, there was a time lag between changes of SOD activity and LPO level. The possible reason may be that induction of Mn-SOD is mediated by ROS, while radicals can react with other molecules, such as lipids. These findings indicate that the response of LPO is much faster than that of SOD.

Excessive ROS is involved in the development of brain diseases, such as stroke, Parkinson's disease, and Alzheimer's disease. The mechanisms of stroke and heart disease among atomic bomb survivors have not been elucidated yet, but these diseases may be induced by oxidative stress. Therefore, we examined the combination effects of X-ray irradiation and ascorbic acid administration on oxidative stress in the brain. Treatment of ascorbic acid and $\gamma$-tocopherol also inhibited 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced brain damage. $\alpha$-tocopherol was found most effective in restoring inherent antioxidant system. Although ascorbic acid undergoes autoxidation in the presence of oxygen, it was prevented by GSH.

As ascorbic acid was oxidized to dehydroascorbic acid, this compound was rapidly converted back to ascorbic acid while GSH was converted to GSSG. In Experiment 2, we used C57BL/6J mice, which are resistant to radiation. The combination of ascorbic acid or $\alpha$-tocopherol and radon inhalation inhibited acute alcohol induced hepatopathy in ICR mice. In addition, activation of antioxidative functions by radon inhalation enhances the mitigation effects of pregabalin on chronic constriction injury-induced neuropathic pain in ICR mice. Examination of the effects of X-ray irradiation in radioresistance mice provides important insights for the future study, because combination of ascorbic acid with low-dose X-ray irradiation may enhance antioxidative functions in all strains of mice. The lethal dose (LD)$_{50}$ of C57BL/6 mice was about 26% higher than that of BALB/c mice. On the basis of our previous studies, the optimal radon concentration for activation of antioxidative functions in C57BL/6J mice will be about 25% higher than that of BALB/c mice. These results would suggest that combination of ascorbic acid with low-dose X-ray irradiation reduces oxidative stress in the brain. This may be helpful to inhibit brain diseases induced by oxidative stress. However, further studies are needed to understand the mechanism of the inhibition.

In conclusion, X-ray irradiation does not induce oxidative stress in the brain. In addition, the combination of 0.5-Gy irradiation with ascorbic acid administration reduced oxidative stress in the brain. Further studies are needed to clarify the effects of this combination for evaluating oxidative stress in the brain.

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要 旨

マウス脳における低線量照射による抗酸化機能の亢進と
アスコルビン酸投与併用による複合効果の基礎的検討

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本研究では、マウス脳における低線量 X 線（0.1〜2.0 Gy）照射の4時間〜7日後の抗酸化機能の
変化特性、および抗酸化物質であるアスコルビン酸（500 mg/kg 体重）の投与併用による複合効果
に関して検討した。その結果、低線量照射により概ね過酸化脂質の減少と SOD 活性・HSP 量の
増加が示された。また、0.5 Gy 照射とアスコルビン酸投与の併用により過酸化脂質の有意な減
少が示されたことから、抗酸化の複合効果も示唆できた。