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Prevention of Adverse Effects of γ-Ray Irradiation after Metallothionein Induction by Bismuth Subnitrate in Mice

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Abstract—*The effect of preinduction of metallonthionein (MT) by bismuth subnitrate (BSN) on the adverse effects and antitumor activity of γ-ray irradiation was investigated in mice. Preinduction of MT by oral administration of BSN significantly reduced the lethal effects and bone marrow injury caused by total body irradiation with γ-rays. A significant increase in the MT concentration in bone marrow was observed in mice treated with BSN. In tumor-bearing mice, pretreatment with BSN did not compromise the antitumor activity of γ-ray irradiation although bone marrow injury was remarkably suppressed. These results suggest that BSN pretreatment is an effective method for protection against side-effects in radiotherapy.*

INTRODUCTION

RADIOTHERAPY is widely used in clinical cancer treatment because of its high efficacy in reducing tumor cells. However, γ-irradiation shows some serious side-effects such as bone marrow injury [1] and intestinal lesions [2, 3], which limit the dose and irradiation frequency. Cell killing by irradiation is known to be caused by unrepaired or misrepaired DNA double strand breakages induced through intracellular radical formation [4].

Recently, Bakka *et al.* [5] found reduced sensitivity to radiation in Cd-resistant cells, which are rich in metallonthionein (MT), compared with Cd-sensitive cells. In addition, Matsubara *et al.* [6] reported that the mortality rate in irradiated mice was minimized by preadministration of MT inducing metals such as zinc, cadmium and manganese. MT is a cysteine rich protein of low molecular weight [7] which has a high affinity for metals such as Cd, Zn, Cu and Hg [8], and is induced by various metals and other xenobiotics [8–14]. Although the physiological roles of MT have still not been completely clarified, it is known that MT detoxifies some harmful heavy metals [15–18] and plays a role in the maintenance of zinc homeostasis [19]. Furthermore, it has recently been reported that MT appears to act as a radical scavenger, at least *in vitro* [20, 21].

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We have previously reported that the pretreatment of mice with bismuth subnitrate (BSN: antidiarrheic) to induce MT in tissues prevented the side effects of *cis*-platinum and Adriamycin® without affecting their antitumor activities [22–24]. Since radiotherapy is often used clinically in combination with anticancer drugs, we attempted to examine the effect of BSN preadministration on both the adverse and antitumor effects of γ-irradiation in mice.

MATERIALS AND METHODS

Animals

Male ICR mice (5 weeks old) were purchased from Charles River Japan, Inc., Atsugi, Japan. C57BL/6, DBA/2 and B6D2F$_1$ male mice (8 weeks old) were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The animals were given free access to food and tap water.

Chemicals

BSN was purchased from Iwaki Co., Ltd., Tokyo, Japan. $^{203}HgCl_2$ (2.4 mCi/mg) was purchased from New England Nuclear, Boston, Mass., U.S.A. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan.

Tumor cells

P388 leukemia and colon adenocarcinoma 38 cells were kindly supplied by Dr. T. Tsuruo, Japanese Foundation for Cancer Research, Tokyo,

Japan. P388 leukemia cells were passaged by i.p. transplantation in male DBA/2 mice. Colon adenocarcinoma 38 cells were maintained by s.c. transplantation in the backs of male C57BL/6 mice. The viability of tumor cells was tested by trypan blue exclusion.

Treatment by drugs and irradiation

Male ICR mice were pretreated orally with BSN once a day for 5 consecutive days. Total body irradiation with γ-rays (^{60}Co, 6–9 Gy) was performed 24 h after the last BSN administration. The mice were sacrificed 10 days after irradiation. The number of leukocytes in the blood was counted using a Coulter counter as an indicator of bone marrow lesions. Lipid peroxidation in the bone marrow cells was determined by quantitating thiobarbiturate-reactive substances (TBA-RS) by the method of Ohkawa *et al* [25]. The level of TBA-RS was expressed as nmol malondialdehyde (MDA)/mg protein. The MT levels in bone marrow cells were determined by our modification [22] of the ^{203}Hg-binding assay [26, 27]. The protein levels in bone marrow cells were determined by Lowry *et al.*'s method [28].

Evaluation of antitumor activity

Mice implanted with P388 leukemia or colon adenocarcinoma 38 cells were used for evaluation of the influence of pretreatment with BSN on the antitumor effect of irradiation. P388 leukemia cells (1×10^6 cells/mouse) were inoculated i.p. into B6D2F$_1$ mice on day 0. The mice were orally administered BSN once a day from days 1 to 5, and irradiated with γ-rays (^{60}Co, 6 Gy/mouse) on day 6. The antitumor effect was evaluated from the change in survival times of the mice. Colon adenocarcinoma 38 cells (2×10^6 cells/mouse) were inoculated s.c. in the right legs of B6D2F$_1$ mice on day 0. BSN was administered orally once a day from days 1 to 5. On day 6 the mice were fixed on a board in a corrected position so that only their right legs were in the field of irradiation by γ-rays (^{60}Co, 6 Gy/leg). The antitumor effect was evaluated by tumor weight on day 16 (10 days after γ-irradiation), and the number of leukocytes in the blood was counted on day 16.

Statistical calculations

Student's *t*-test was used for statistical analysis of data for the different groups of animals and experiments.

RESULTS

The effect of pretreatment with various doses of BSN on the survival rate of mice irradiated with a lethal dose of γ-rays is shown in Table 1. In the group without BSN pretreatment, all mice died within 30 days after irradiation (Table 1). However, pretreatment with more than 50 mg/kg of BSN led to a dose-dependent increase in the survival rate (Table 1). At a dose of 200 mg/kg, no mice died during the 30 day observation period (Table 1).

Table 1. Effect of pretreatment with BSN on lethal effects of γ-ray (^{60}Co) irradiation in mice

Pretreatment with BSN(mg/kg/day)	^{60}Co irradiation (Gy/mouse)	Survival* rate (%)
0	0	100
0	9	0
25	9	0
50	9	43
100	9	57
150	9	86
200	9	100

Mice ($n = 7$) were pretreated p.o. with BSN once a day for 5 days, and irradiated with γ-rays (^{60}Co) 24 h after the last administration of BSN.
*Determined 30 days after γ-ray (^{60}Co) irradiation.

The effect of pretreatment with 200 mg/kg of BSN, which completely suppressed the lethal effect of γ-ray irradiation as indicated in Table 1, on the number of total leukocytes as an index of bone marrow toxicity was examined (Fig. 1). The number of total leukocytes in mice irradiated by γ-rays without BSN pretreatment markedly decreased in a dose-dependent manner. However, the decrease was significantly suppressed by pretreatment with BSN (Fig. 1). The effect of pretreatment with BSN on lipid peroxidation in bone marrow cells was also examined (Fig. 2). Although a dose-dependent increase in the level of MDA was observed in mice irradiated with γ-rays, the increase was significantly reduced by pretreatment with BSN (Fig. 2). At 24 h after the last administration of BSN, the time of γ-ray irradiation, the concentration of MT in bone marrow cells of BSN-treated mice was two-fold higher than that in those of untreated mice (Fig. 3).

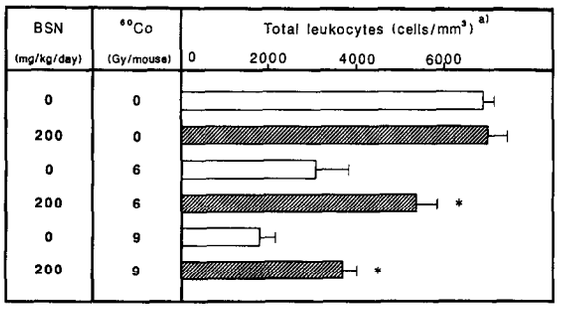

*Fig. 1. Effect of pretreatment with BSN on the number of total leukocytes reduced by γ-ray irradiation. Mice (ICR) were administered BSN (200 mg/kg/day) once a day for 5 days and irradiated with γ-rays 24 h after the last administration of BSN. The results are indicated as the means ± S.D. for five mice. BSN-untreated mice, □; BSN-treated mice, ▨. (a) Determined 10 days after γ-ray irradiation. *Significantly different from BSN-untreated mice ($P < 0.001$).*

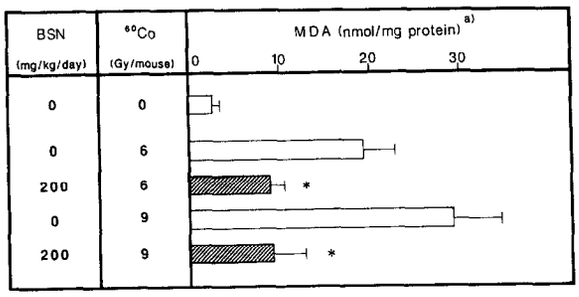

*Fig. 2. Effect of BSN pretreatment on lipid peroxidation in bone marrow cells caused by γ-ray irradiation. Mice (ICR) were treated as described in the legend to Fig. 1. The results are indicated as the means ± S.D. for five mice. BSN-untreated mice, □; BSN-treated mice, ▨. (a) Determined 10 days after γ-ray irradiation. *Significantly different from BSN-untreated mice ($P < 0.001$).*

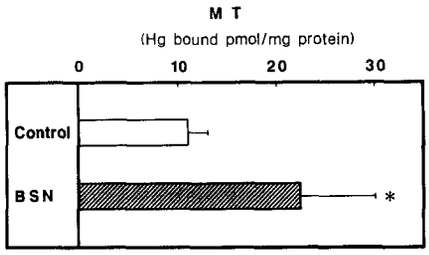

*Fig. 3. Concentration of MT in bone marrow cells of mice treated with BSN. Mice (ICR) were administered BSN (200 mg/kg/day) once a day for 5 days. MT was determined 24 h after the last administration of BSN. The values are the means ± S.D. for five mice. *Significantly different from the control ($P < 0.001$).*

Figure 4 shows the effect of BSN pretreatment on the antitumor activity of total body γ-irradiation indicated by prolongation of the survival time in mice inoculated i.p. with P388 leukemia cells. The mean of survival time of the control mice was 11.0 ± 1.8 days (Fig. 4). It was prolonged to 22.3 ± 2.0 days by a single γ-ray irradiation of 6 Gy (Fig. 4). Pretreatment with BSN had no modifying effect on the survival time of mice irradiated with γ-rays (Fig. 4). In another set of experiments, the effect of BSN pretreatment on the tumor-reducing activity of γ-irradiation was examined using mice inoculated s.c. with colon adenocarcinoma 38 cells. Tumor weight was significantly reduced by the γ-irradiation (Table 2). As in the case of P388 leukemia cells (Fig. 4), the antitumor activity of γ-rays was not affected by BSN pretreatment (Table 2). The remarkable decrease in the number of total leukocytes caused by γ-ray irradiation observed in the mice without BSN pretreatment was significantly improved by BSN preadministration (Table 2).

DISCUSSION

Radiotherapy is widely used alone or in combination with chemotherapy because of its high efficacy against solid tumors. However, radiotherapy

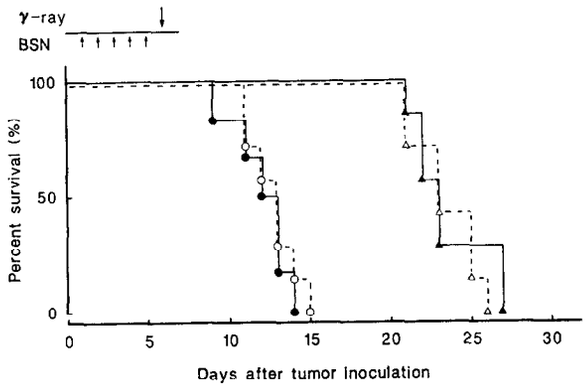

Fig. 4. Effect of pretreatment with BSN on the antitumor activity of γ-ray irradiation in mice inoculated i.p. with P388 leukemia cells. Mice ($B6D2F_1$, $n = 7$) were inoculated i.p. with P388 leukemia cells on day 0. BSN was administered orally to mice once a day from day 1 to 5 and the mice were irradiated on day 6. Untreated ●—●; BSN alone, ○---○; γ-ray (^{60}Co, 6 Gy) alone, ▲—▲, BSN-pretreatment + γ-ray (^{60}Co, 6 Gy), △---△.

can cause severe side-effects, such as bone marrow lesions and intestinal injury, and these adverse effects prevent the proper utilization of radiotherapy in clinical practice.

Radiation damage can be ascribed to free radicals induced by γ-ray irradiation [4]. In fact, lipid peroxidation in the bone marrow cells was enhanced by the irradiation (Fig. 2) in the present experiment. However, the extent of lipid peroxidation in the bone marrow caused by irradiation was significantly reduced by BSN treatment prior to irradiation (Fig. 2). Also, the decrease in the number of leukocytes, an indication of bone marrow injury, was much less with BSN pretreatment (Fig. 1). Finally, the MT level in the bone marrow cells was increased by the BSN preadministration (Fig. 3). Thus, the BSN-induced reduction of the lethal effects and bone marrow lesions of γ-irradiation can be explained at least partly by MT-induction in the bone marrow, a target tissue of the irradiation.

Matsubara *et al.* reported that protection against lethal effects of radiation was provided by pretreatment of mice with Zn, Cd or Mn, which might induce MT [6]. Bakka *et al.* indicated that cadmium-resistant cultured cells with a high MT content were substantially resistant to cytotoxicity of radiation [5]. In addition, it has recently been reported that MT might play a role in scavenging free radicals [20, 21]. We have previously demonstrated that the induction of MT in the hearts of mice by appropriate heavy metals prevents the lethal and cardiac toxicities of Adriamycin® which is considered to generate active oxygen [29]. These facts appear to support the assumption that the protective effect of BSN pretreatment against radiation damage observed in the present study is attributable to the increased level of MT which scavenges free radicals induced

Table 2. Effect of pretreatment with BSN on antitumor activity and bone marrow injury of γ-ray irradiation in mice inoculated s.c. with colon adenocarcinoma 38 cells

Dose			
^{60}Co (Gy/mouse)	BSN (mg/kg/day)	Tumor weight* (g)	Total leukocytes* (cells/mm^3)
0	0	0.199 ± 0.110	7060 ± 598
0	200 × 5	0.202 ± 0.048	6840 ± 493
6	0	0.018 ± 0.010	2940 ± 541
6	200 × 5	0.023 ± 0.009	5060 ± 581†

Mice ($B6D2F_1$) were inoculated s.c. with colon adenocarcinoma 38 cells on day 0. BSN was administered orally once a day from day 1 to 5, and γ-rays were irradiated on day 6. The values are the means ± S.D. for five mice.

*Determined 10 days after γ-ray irradiation.

†Significantly different from BSN-untreated mice ($P < 0.001$).

by γ-irradiation and protects the bone marrow in particular from radiation injury.

As indicated in Fig. 4 and Table 2, BSN pretreatment did not affect the antitumor activity of the radiation. We have previously reported selective protection by BSN pretreatment against the side-effects of *cis*-platinum and Adriamycin® [22–24] These favorable effects of BSN, specific for the side-effect of anticancer drugs and radiation, can be explained by the fact that BSN induces MT in some normal tissues of mice, but not in the tumor tissues so far tested [23].

In conclusion, the experimental results obtained in the present study clearly demonstrated that oral administration of BSN markedly reduced the lethal effects and bone marrow damage of γ-ray irradiation without compromising its tumor-reducing effect. In clinical practice, radiotherapy is often carried out with chemotherapy. Since BSN has long been used clinically as an antidiarrheic and has recently been reported to be an effective agent for preventing the side-effects of anticancer drugs such as *cis*-platinum and Adriamycin®, BSN pretreatment appears to be one of the most promising tools not only in radiotherapy but also in combined radiotherapy and chemotherapy for neoplasms.

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REFERENCES

1. Henschke UK, Morton JL. Mortality of rhesus monkeys after single total body irradiation. *Am J Roentgenol Radiat Ther* 1957, **77**, 899–909.
2. Willoughby DA. Pharmacological aspects of the vascular permeability changes in the rat's intestine following abdominal radiation. *Br J Radiol* 1960, **33**, 515–519.
3. Paterson E. The mechanism of death following whole body radiation. *Br J Radiol* 1957, **30**, 577–581.
4. Chapman JD, Gillespie CJ. Radiation-induced events and their time scale in mammalian cells. *Adv Radiat Biol* 1981, **9**, 143–198.
5. Bakka A, Johnsen AS, Endresen L, Rugstad HE. Radioresistance in cells with high content of metallothionein. *Experientia* 1982, **38**, 381–383.
6. Matsubara J, Tajima Y, Karasawa M. Promotion of radioresistance by metallothionein induction prior to irradiation. *Environ Res* 1987, **43**, 66–74.
7. Margoshes M, Vallee BL. A cadmium protein from equine kidney cortex. *J Am Chem Soc* 1957, **79**, 4813–4814.
8. Webb M. Metalothionein. In: Webb M, ed. *The Chemistry, Biochemistry and Biology of Cadmium*. Amsterdam, Elsevier/North-Holland Biomedical Press, 1979, 195–266.
9. Suzuki Y, Yoshikawa H, Induction of hepatic zinc-binding proteins of rats by various metals. *Ind Health* 1976, **14**, 25–31.
10. Eaton DL, Stacey NH, Wong KL, Klaassen CD. Dose–response effects of various metal ions on rat liver metallothionein, glutathione, heme oxygenase, and cytochrome P-450. *Toxicol Appl Pharmacol* 1980, **55**, 393–402.
11. Onosaka S, Yoshiya S, Min KS, Fukuhara T, Tanaka K. The induced synthesis of metallothionein in various tissues of rat after injection of various metals. *Eisei Kagaku* 1983, **29** 221–225.
12. Etzel KR, Shapiro SG, Cousins RJ. Regulation of liver metallothionein and plasma zinc by the glucocorticoid dexamethasone. *Biochem Biophys Res Commun* 1979, **89**, 1120–1126.
13. Karin M, Herschman HR. Dexamethasone stimulation of metallothionein synthesis in HeLa cell cultures. *Science* 1979, **204**, 176–177.

14. Oh SH, Deagen JT, Whanger PD, Weswig PH. Biological function of metallothionein. V. Its induction in rats by various stresses. *Am J Physiol* 1978, **234**, E282–E285.
15. Nordberg GF. Effect of acute and chronic cadmium exposure of the testicles of mice. *Environ Physiol* 1971, **1**, 171–187.
16. Webb M. Protection by zinc against cadmium toxicity. *Biochem Pharmacol* 1972, **21**, 2767–2771.
17. Yoshikawa H. Tolerance to acute metal toxicity in mice having received a daily injection of a low dose. *Ind Health* 1974, **12**, 175–177.
18. Leber PA, Miya TS. A mechanism for cadmium- and zinc-induced tolerance to cadmium toxicity: involvement of metallothionein. *Toxicol Appl Pharmacol* 1976, **37**, 403–414.
19. Cousins RJ. Regulatory aspects of zinc metabolism in liver and intestine. *Nutr Rev* 1979, **37**, 97–103.
20. Shiraishi N. Utsumi K, Morimoto S *et al.* Inhibition of nitroblue tetrazolium reduction by metallothionein. *Physiol Chem Phys* 1982, **14**, 533–537.
21. Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydrogen radicals. *Biochim Biophys Acta* 1985, **827**, 36–44.
22. Naganuma A, Satoh M, Imura N. Prevention of lethal and renal toxicity of *cis*-diammine-dichloroplatinum(II) by induction of metallothionein synthesis without compromising its antitumor activity in mice. *Cancer Res* 1987, **47**, 983–987.
23. Naganuma A, Satoh M, Imura N. Specific reduction of toxic side-effects of Adriamycin® by induction of metallothionein in mice. *Jpn J Cancer Res* 1988, **79**, 406–411.
24. Satoh M, Naganuma A, Imura N. Metallothionein induction prevents toxic side-effects of cisplatin and Adriamycin® used in combination. *Cancer Chemother Pharmacol* 1988, **21**, 176–178.
25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979, **95**, 351–358.
26. Piotrowski JK, Bolanowska W, Sapata A. Evaluation of metallothionein content in animal tissues. *Acta Biochim Pol* 1973, **20**, 207–215.
27. Kotsonis FN, Klaassen CD. Comparison of methods for estimating hepatic metallothionein in rats. *Toxicol Appl Pharmacol* 1977, **42**, 583–588.
28. Lowry OH, Rosebrough NJ, Farr AL, Randall RL. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
29. Satoh M, Naganuma A, Imura N. Involvement of cardiac metallothionein in prevention of Adriamycin® induced lipid peroxidation in the heart. *Toxicology* 1988, **53**, 231–237.