INTRODUCTION

Ionizing radiation induces various toxic effects such as bone marrow injury, intestinal lesions and carcinogenesis. In addition, although clinical radiotherapy shows high efficacy in cancer treatment, secondary carcinogenesis is a major problem in long-term radiotherapy. Radiation injury, including carcinogenesis, is caused by formation of intracellular oxygen radicals such as superoxide anions and hydroxyl radicals. Several studies have shown that metallothionein (MT) can efficiently eliminate reactive oxygen species and can play an effective role in protection against damage caused by oxidative stress (Sato and Bremner, 1993; Cai et al., 1999).

MT is a cysteine-rich low-molecular-weight protein with a high affinity for a wide range of metals such as cadmium and mercury. Expression of MT is induced by a number of stimuli, including exposure to various metals and many other factors such as glucocorticoïds, cytokines and ionizing radiation (Shiraishi et al., 1986; Koropatnick et al., 1989; Shibuya et al., 1995; Cai et al., 1999; Miles et al., 2000; Coyle et al., 2002). MT is thought to function in the homeostasis of essential metals such as zinc and copper, in the detoxification of heavy metals, mutagens and anticancer agents, in the resistance of tumor cells to anticancer agents and ionizing radiation, and in protection against chemical carcinogenesis (Naganuma et al., 1987; Lazo and Pitt, 1995; Shibuya et al., 1997; Role of metallothionein as a protective factor against radiation carcinogenesis

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ABSTRACT — In order to elucidate the involvement of metallothionein (MT) in radiation carcinogenesis, we examined the susceptibility of MT-I/II null mice to carcinogenesis and oxidative DNA damage resulting from X-irradiation. Eight-week-old female MT-I/II null mice and wild-type mice were exposed to whole-body X-irradiation at doses of 1.0, 1.5 or 2.0 Gy once a week for 6 weeks. Incidence of thymic lymphoma was determined at 24 weeks after the first exposure to X-irradiation. The frequency of thymic lymphomas induced by X-irradiation (at 1.5 and 2.0 Gy) was significantly higher in MT-I/II null mice than in wild-type mice. In addition, although the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were increased in the serum and urine of both strains of mice 24 hr after exposure to a single bout of whole body X-irradiation, these increases were significantly greater in the MT-I/II null mice than in the wild-type mice. Thus, the present results suggest that MT plays a protective role against carcinogenesis and oxidative DNA damage caused by X-irradiation.

Key words: Metallothionein, X-ray irradiation, Carcinogenesis, Oxidative DNA damage, Knockout mice

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Recent reports have shown that cellular damage caused by ionizing radiation was prevented by pretreatment with MT-inducing metals such as zinc and bismuth (Bakka et al., 1982; Matsubara, 1988; Renan and Dowman, 1989; Satoh et al., 1989; Miura et al., 1998; Cai et al., 1999). Our recent studies using MT-I and MT-II double knockout (MT-I/II null) mice have demonstrated that the protective effect of metal compounds on bone marrow injury caused by X-irradiation was due to the induction of MT (Shibuya et al., 2008). In addition, MT-I/II null mice were highly sensitive to bone marrow injury resulting from a low dose of X-irradiation (Shibuya et al., 2008). These findings suggest that MT plays a protective role against the acute toxic effect of ionizing radiation.

It has been previously reported that the incidence of thymic lymphoma caused by X-irradiation was prevented by pretreatment with bismuth, which is an MT-inducing metal (Kagimoto et al., 1991). However, the involvement of MT against radiation carcinogenesis is not clear because bismuth is not specific for MT induction. MT-I/II null mice are considered an effective model to evaluate the potential function of MT in radiation injury. In the present study, we examined the susceptibility of MT-I/II null mice to carcinogenesis and oxidative DNA damage caused by X-irradiation.

MATERIALS AND METHODS

Chemicals and radiation

10% neutral buffered formalin solution and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A LINCA ML-6MA (Mitsubishi Electric; Tokyo, Japan) was used as the X-ray source. The rate was 2.5 Gy/min.

Animals

MT-I/II null mice (Michalska and Choo, 1993) that possess a null mutation of the MT-I and MT-II genes were kindly provided by Dr. K.H.A. Choo (Murdoch Institute for Research into Birth Defects, Royal Children’s Hospital; Parkville, Australia), and maintained at the National Institute for Environmental Studies (NIES). MT-I/II null mice were originally developed on a mixed genetic background of OLA 129 and C57BL/6 strains. F1 hybrid mice were mated with C57BL/6J mice (CLEA Japan; Tokyo, Japan) and their offspring were backcrossed with C57BL/6J mice for six generations. MT-I/II null (MT-/-) mice and wild-type (MT +/-) mice were obtained by mating the heterozygous (MT +/-) mice. Both strains of mice were housed in cages in ventilated animal rooms at a controlled temperature of 23 ± 1°C, with a relative humidity of 55 ± 10% and a 12-hr light/dark cycle. The mice were maintained on standard laboratory chow and tap water ad libitum, and they received humane care throughout the experiment according to the guidelines established by the NIES.

Treatments and analyses

Eight-week-old female MT-I/II null mice and wild-type mice were randomly assigned to control and experimental groups, with 10-15 mice per group. Each experimental group was exposed to whole body X-irradiation at a dose of 1.0, 1.5 or 2.0 Gy once a week for 6 weeks. At 24 weeks after the first X-irradiation, the thymus was removed from each mouse following euthanasia by cervical dislocation. To evaluate the incidence of thymic lymphoma, thymus tissue was fixed in 10% neutral buffered formalin solution and embedded in paraffin. Deparaffinized tissue sections of 5 μm thickness were stained with hematoxylin-eosin.

In a separate experiment, eight-week-old males from both strains of mice (n = 4) were exposed to a single bout of whole body X-irradiation at a dose of 0.1, 0.5, 1.0 or 2.0 Gy. Urine was collected in a metabolic cage for 24 hr after X-irradiation, and blood was then collected from each mouse under ether anesthesia. The amounts of 8-hydroxy-2’-deoxyguanosine (8-OHdG) in the serum and urine samples were measured by an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistics

Differences in tumor incidence between MT-I/II null mice and wild-type mice were analyzed by χ² test. 8-OHdG levels are presented as the mean ± standard deviation (S.D.) for each experimental group (n = 4). Statistical analyses were performed using one-factor analyses of variance followed by Fisher’s protected least significant difference test for post-hoc comparisons. Differences between groups were considered significant at p < 0.05.

RESULTS AND DISCUSSION

Tumor incidence in the MT-I/II null mice and wild-type mice exposed to X-irradiation is shown in Table 1. X-irradiation at 1.5 and 2.0 Gy induced thymic lymphomas in 8 and 27% of wild-type mice respectively. In contrast, X-irradiation at both 1.5 and 2.0 Gy induced thymic lymphomas in 60% of MT-I/II null mice.

As an indicator of oxidative DNA damage, 8-OHdG levels were measured in the serum and urine of MT-I/II null mice.
null mice and wild-type mice after various doses of X-irradiation (Fig. 1). In wild-type mice, the amount of 8-OHdG in the serum was significantly increased in a dose-dependent manner at treatment levels above 1.0 Gy (Fig. 1A). In the MT-I/II null mice, 8-OHdG was significantly higher than that of wild-type mice at doses above 0.5 Gy (Fig. 1A). Additionally, X-irradiation significantly increased the amount of 8-OHdG in the urine of both strains of mice, and the elevation of 8-OHdG levels was significantly higher in MT-I/II null mice than in wild-type mice (Fig. 1B).

Several studies have reported that chemical- and metal-induced carcinogenesis was suppressed by pretreatment with MT-inducing metals such as zinc and bismuth (Poswillo and Cohen, 1971; Duncan and Droesti, 1975; Waalkes et al., 1989; Satoh et al., 1993). Moreover, MT-I/II null mice were extremely susceptible to carcinogenesis caused by 7,12-dimethylbenz[a]anthracene (DMBA) (Zhang et al., 1998; Suzuki et al., 2003), N-butyl-N-(4-hydroxybutyl)nitrosamine (Kondo et al., 1999), lead (Waalkes et al., 2004) and cisplatin (Waalkes et al., 2006). These studies suggest that MT acts as a protective factor against chemical carcinogenesis. Additionally, Kagimoto et al. (1991) demonstrated that pretreatment with MT-inducing metals suppressed the incidence of thymic lymphomas caused by X-irradiation. The present study clearly shows that MT-I/II null mice are hypersensitive to carcinogenesis resulting from X-irradiation (Table 1). Thus, MT plays an important role as an anticarcinogenic agent not only in chemical carcinogenesis but also in radiation carcinogenesis. In addition, our previous studies have demonstrated that MT-I/II null mice showed an increased sensitivity to clastogenicity of X-irradiation (Shibuya et al., 2008). In the present study, MT-I/II null mice were also highly sensitive to the oxidative DNA damage caused by X-irradiation (Fig. 1). These studies indicate that MT can prevent the genotoxicity that results from X-irradiation.

The mechanisms involved in protection against radiation carcinogenesis by MT remain unclear. However, MT is a strong reactive oxygen species scavenger (Sato and

### Table 1. Incidence of thymic lymphoma in MT-I/II null mice and wild-type mice after X-irradiation

<table>
<thead>
<tr>
<th>X-ray (Gy)</th>
<th>Wild-type mice</th>
<th>MT-I/II null mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/12 (0%)</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>1.0</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>1.5</td>
<td>1/12 (8%)</td>
<td>9/15 (60%)*</td>
</tr>
<tr>
<td>2.0</td>
<td>3/11 (27%)</td>
<td>9/15 (60%)*</td>
</tr>
</tbody>
</table>

* = significantly different from wild-type mice exposed to the same dose of X-ray (p < 0.05) as determined by χ² test. MT = metallothionein

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Fig. 1. 8-OHdG levels in (A) serum and (B) urine of MT-I/II null mice and wild-type mice are shown at 24 hr after X-irradiation by various doses. The values represent the mean ± S.D. for four mice per group. * = significant difference from the corresponding untreated group (p < 0.05). # = significant difference between wild-type and MT-I/II null mice within a particular irradiation dosage group (p < 0.05). MT = metallothionein.
Bremner, 1993; Cai et al., 1999). Moreover, in the present study, increased levels of oxidative DNA damage following X-irradiation were seen in mice that lacked MT-I/II. Thus, the antioxidant properties of MT have been proposed as the possible mechanism of defense against the toxic effects of ionizing radiation.

There is great variation in individual MT expression because human MT levels are altered under various conditions. In a study utilizing samples taken from Japanese cadavers of various ages, it was shown that MT levels increased in the kidney and liver in an age-dependent manner, whereas a small part of this population revealed low MT levels, irrespective of age (Yoshida et al., 1998). Moreover, it was recently demonstrated that polymorphisms in the human MT-IIA gene affected MT expression (Kita et al., 2006). Thus, individuals that express MT at low levels may exhibit enhanced susceptibility to radiation- and chemical-induced carcinogenesis.

In conclusion, we found that X-irradiation caused severe tumor incidence and oxidative DNA damage in MT-I/II null mice as compared to wild-type mice. These results suggest that MT is an important protective factor against the genotoxic and carcinogenic effects of X-irradiation. In addition, MT may be one of the factors responsible for sensitivity to radiation injury, including carcinogenesis.

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