Original Article

Protective role of metallothionein in benzo[a]pyrene-induced DNA damage

Masaki Takaishi¹, Masumi Sawada², Akinori Shimada², Junko S.Suzuki³,
Masahiko Satoh^{1,4} and Hisamitsu Nagase¹

¹Laboratory of Hygienics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu City, Gifu 502-8585, Japan

²Department of Veterinary Pathology, Tottori University, 4-101 Koyama-minami, Tottori City, Tottori 680-8553, Japan

³Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

⁴Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan

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ABSTRACT — Metallothionein (MT) is known to reduce chemical carcinogenesis. Carcinogenesis induced by benzo[a]pyrene (B[a]P) which is an environmental chemical carcinogen is related to DNA adduct formation and oxidative damage through metabolic activation. Ten-week-old male MT-I/II null mice and wild-type mice were given a single injection of B[a]P (250 mg/kg, p.o.), and B[a]P-induced DNA damage was evaluated at 6-48 hr later. The frequencies of micronucleated reticulocytes (MNRET) in MT-I/II null mice were significantly increased compared with that of wild-type mice at 48 hr after B[a]P administration. At 48 hr after B[a]P administration, comet scores were significantly increased in MT-I/II null mice but not in wild-type mice. 8-Hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, was significantly increased in liver of MT-I/II null mice at 6 and 12 hr after B[a]P administration, although that of wild-type mice was only slightly changed. Because cytochrome P450 (CYP) plays a major role in the process of B[a]P metabolic activation, we attempted to reveal the effect of MT on metabolic activation of B[a]P. Although CYP1A activities were elevated in the livers of MT-I/II null mice and wild-type mice treated with B[a]P, it was not different between both strains of mice. In addition, MT levels in the livers of wild-type mice were significantly increased by the B[a]P treatment, whereas MT was not detected in livers of MT-I/II null mice with or without B[a]P treatment. These results demonstrate that MT acts as an endogenous defensive factor against B[a]P-induced DNA damage.

Key words: MT, B[a]P, Micronucleus test, Comet assay, 8-OHdG, EROD activity

INTRODUCTION

Benzo[a]pyrene (B[a]P) is one of the polycyclic aromatic hydrocarbons (PAH) referred to as a human carcinogen (IARC monographs, in preparation). B[a]P has been shown to induce gene mutations, chromosomal aberrations and other types of genotoxic effects in *in vitro* and *in vivo*. Most humans are more or less constantly exposed to B[a]P. As a product of incomplete combustion, B[a]P is present in tobacco smoke, diesel engine exhaust, urban air, various types of processed foods, coal-tar, creosote, asphalt, and various occupational settings such as coal gasification plants, coke ovens and iron foundries.

B[a]P is metabolized to 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) by cytochrome P450 (P450 or CYP). BPDE is the ultimate carcinogenic form of B[a]P and binds to the exocyclic nitrogen of deoxyguanosine in DNA. BPDE-DNA adducts in target tissues were closely related to development of cancer in animals and humans (Culp and Beland, 1994). In addition, oxidative DNA damage also plays an important role in the process of B[a]P carcinogenesis, because B[a]P produces quinone derivatives which easily generate reactive oxygen species (ROS) and oxidatively damage DNA (Penning *et al.*, 1996). As a result of hydroxyl radical interaction with DNA, many types of oxidized

Correspondence: Hisamitsu Nagase (E-mail: nagase@gifu-pu.ac.jp)

nucleosides have been reported (Poulsen *et al.*, 1998). 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is one of the oxidative DNA modifications and is extensively used for study of mutagenicity (Cheng *et al.*, 1992).

Metallothionein (MT) is a cysteine-rich low-molecular weight protein with a high affinity for metals such as zinc, copper, cadmium and mercury (Miles *et al.*, 2000). There are four isoforms of MT, namely MT-I, MT-II, MT-III and MT-IV. MT-I and MT-II are expressed in all tissues (Miles *et al.*, 2000). MT-III is expressed mainly in the brain and MT-IV in squamous epithelia. MT has been known to be involved in many physiological and pathophysiological processes such as intracellular storage, transport and metabolism of heavy metals, regulating essential trace metal homeostasis and playing a protective role in heavy metal toxicity (Miles *et al.*, 2000). MT also plays a protective role against toxicity of free radical-inducing agents such as paraquat, adriamycin and ionizing radiation (Satoh *et al.*, 1988, 1992; Sato and Bremner, 1993; Cai *et al.*, 1999). Detailed examinations of MT with free radicals have been extensively undertaken and have demonstrated that MT is a potent antioxidant to prevent various oxidative injuries from reactive oxygen and nitrogen species *in vitro* and *in vivo* (Sato and Bremner, 1993; Cai *et al.*, 1999, 2000). In addition, MT can reduce the adverse effects of the chemotherapeutic agents adriamycin and *cis*-diamminedichloroplatinum (II) (cisplatin) and other alkylating agents. (Satoh *et al.*, 1988; Naganuma *et al.*, 1987; Nakagawa *et al.*, 1995).

Pretreatment with MT-inducing metals such as zinc and bismuth can suppress chemical carcinogenesis caused by 7,12-dimethylbenz[*a*]anthracene (DMBA), 3-methyl cholanthrene, cisplatin and melphalan (Poswillo and Cohen, 1971; Duncan and Dreosti, 1975; Satoh *et al.*, 1993; Cherian *et al.*, 1994). Also the incidence of X-ray-induced thymic lymphomas was lowered by administration of bismuth nitrate as an MT inducer (Cai *et al.*, 1999). Recently, Cherian *et al.* (2003) have reported that high expression of MT in certain tumors may block apoptosis and may be related to tumor progression; a central role for p53 is to act as a tumor suppressor by inducing apoptosis.

Recently, MT-I/II null mice with both MT-I and MT-II null mutations have been developed and widely used in toxicological experiments (Michalska and Choo, 1993). Some researchers have reported that MT-I/II null mice were more susceptible than wild-type mice in chemical- and metal-induced carcinogenesis (Zhang *et al.*, 1998; Kondo *et al.*, 1999; Suzuki *et al.*, 2003; Waalkes *et al.*, 2004).

However, the involvement of MT in the genotoxicity

of B[*a*]P is not clear. MT-I/II null mice have been proven to be a good tool for elucidation of the normal functions of MT and the consequences of MT deficiency. In the present study, to elucidate the genotoxicity of B[*a*]P and the ability of MT to modify B[*a*]P toxicity, we investigated the susceptibility of MT-I/II null mice to B[*a*]P-induced DNA damage such as chromosomal aberrations, DNA strand breaks and oxidative DNA damage.

MATERIALS AND METHODS

Chemicals

B[*a*]P, nuclease P₁ and alkaline phosphatase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). A DNA Extractor WB Kit was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). An 8-OHdG ELISA kit (8-OHdG Check High sensitivity) and 8-OHdG antibody were purchased from Japan Institute for the Control of Aging (Shizuoka, Japan).

Animals

MT-I/II null mice were kindly provided by Dr. K.H.A. Choo (Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Australia) and were of a mixed genetic background of 129 Ola and C57BL/6 strains (Michalska and Choo, 1993). F1 hybrid mice were mated with C57BL/6 mice and their offspring were backcrossed to C57BL/6 for six generations. Both MT-I/II null mice and wild-type mice were generated by matings of heterozygous mice.

MT-I/II null mice and wild-type mice were routinely bred in the vivarium of the Gifu Pharmaceutical University. Both strains of mice were housed in cages in ventilated animal rooms with a controlled temperature of 24 ± 2°C, 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 of the Gifu Pharmaceutical University.

Treatments

Ten-week-old male MT-I/II null mice and wild-type mice were randomized into control and experimental groups (the groups of 0, 12, 24 and 48 hr after B[*a*]P administration on MT-I/II null mice consisted of 4 mice and the other group consisted of 5 mice). The groups of mice were given a single oral administration of B[*a*]P at a dose of 250 mg/kg. Peripheral blood was collected from the mouse tail vein, and then the liver and bone marrow were removed from each mouse under diethyl ether anesthesia at 0, 6, 12, 24 and 48 hr after the injection.

Another group of mice were orally administered a single dose of B[a]P at a dose of 30 to 750 mg/kg. Peripheral blood was collected from the mouse tail vein under diethyl ether anesthesia at 48 hr after injection.

Micronucleus test

The peripheral blood smeared on a slideglass (Matsunami Glass Industries, Osaka, Japan) was stained with acridine orange according to the method described by Hayashi *et al.* (1990). For each animal, 1,000 reticulocytes (RET) were scored for the micronucleus (MN) and the frequencies of micronucleated reticulocytes (MNRET) were expressed as percent of RET.

Single cell gel electrophoresis (comet) assay

The liver was gently homogenized according to the method described by Sasaki *et al.* (1997) with some modification. The slide preparation and electrophoresis were done according to the method described by Singh *et al.* (1988) with some modification.

Fifty nuclei were examined at 200 magnification using a fluorescence microscope equipped with a green filter. To quantify the average DNA strand breaks, all of them were classified into one of the following groups: only the head (normal; score = 0); tail length < head length (score = 1); tail length < 2 × head length (score = 2); tail length < 3 × head length (score = 3). Comet scores were the calculated sum of 50 nuclei.

Determination of 8-OHdG in DNA

The DNA was extracted from the liver with the DNA Extractor WB Kit according to the method of Kasai *et al.* (1987). DNA concentration was determined by the absorbance at 260 nm (A_{260} 1.0 = 50 µg DNA). The absorbance ratio (A_{260}/A_{280}) was between 1.80 and 1.85. The extracted DNA was digested with 6 units of nuclease P₁ and 200 mM sodium acetate (pH 4.8) at 37°C for 1 hr. The samples were incubated at 37°C for 1 hr after 2 units of alkaline phosphatase and 1 M Tris-HCl (pH 7.4) were added.

The amounts of 8-OHdG in the liver were measured by an ELISA kit according to the manufacturer's instructions. 8-OHdG amounts were presented as the 8-OHdG µg/g DNA ratio.

Immunohistochemical detection of 8-OHdG

The liver was fixed in 10% neutral buffered formalin solution and processed for paraffin embedding. Tissue 5-µm sections were prepared and placed on a slide glass (Matsunami Glass Industries, Osaka, Japan) and deparaffinized using xylene. Tissue sections were treated with 3% hydrogen peroxide for 15 min and washed with

0.01 M PBS. Mouse anti 8-OHdG antibody (1:400) was applied to each section at 4°C overnight.

Hepatic CYP1A enzymatic activity

7-Ethoxyresorufin-*O*-deethylation (EROD) activity in liver microsomes was determined by the method of Shimada *et al.* (1998). The EROD activity was expressed in nanomoles of resorufin formed per minutes per mg of proteins.

MT concentration measurement

MT (MT-I and MT-II isoforms) concentrations in the liver were measured by radioimmunoassay (Tohyama and Shaikh, 1981) as modified by Nishimura *et al.* (1990) using sheep anti-rat MT-I antiserum. Ten % liver homogenate was prepared in 10 mM Tris-HCl.

Statistical analysis

All values are expressed as the mean ± S.D.. Differences in the mean values were assessed by the ANOVA test followed by Newman-Keuls tests for *post hoc* comparison. Differences were considered statistically significant at $p < 0.05$.

RESULTS

MN test

The results of the MN test, which is an indicator of chromosomal aberration, are shown in Fig. 1. The frequencies of MNRET were significantly increased in the MT-I/II null mice at 48 hr after the B[a]P (250 mg/kg) administration (Fig. 1A). However, the frequencies of MNRET in wild-type mice were not increased by the B[a]P treatment.

Fig. 1B shows the effect of various doses of B[a]P on MN induction at 48 hr after administration. The frequencies of MNRET in both MT-I/II null mice and wild-type mice were increased by B[a]P treatment in a dose-dependent manner up to the dose of 250 mg/kg. At 125 and 250 mg/kg of B[a]P administration, the frequencies of MNRET were significantly increased in the MT-I/II null mice but not in the wild-type mice. At doses of 500 and 750 mg/kg B[a]P, the frequencies of MNRET in MT-I/II null mice were significantly increased compared with those in wild-type mice. As for the MT-I/II null mice treated with 750 mg/kg B[a]P, two out of five mice died within 48 hr after administration.

Comet assay

Comet scores, an indicator of DNA strand breaks, in the MT-I/II null mice were significantly increased at 48

Fig. 1. Frequencies of MNRET in peripheral blood of MT-I/II null mice and wild-type mice treated with B[a]P. (A) Times changed after B[a]P treatment. (B) Dose changed B[a]P treatment. Values are represented as mean $\pm$ S.D.. * Statistically significant differences from the corresponding control group ($p < 0.05$). # Statistically significant differences from the dose or times-matched MT-I/II null mouse group ($p < 0.05$). N.D.: not done.

hr after B[a]P administration (Fig. 2). In contrast, comet scores in the wild-type mice were not increased by B[a]P treatment.

Determination of 8-OHdG

The 8-OHdG levels, an indicator of oxidative DNA damage, in the livers of MT-I/II null mice, were signifi-

Protection from DNA damage by metallothionein

cantly increased compared with those in wild-type mice at 6 and 12 hr after B[a]P administration (Fig. 3). At 6 and 12 hr after B[a]P administration, 8-OHdG levels in

the livers of MT-I/II null mice were 4-fold and 2.5-fold higher than those in wild-type mice, respectively.

The 8-OHdG immunoreactivity in the livers of MT-

Fig. 2. Comet scores in the livers of MT-I/II null mice and wild-type mice treated with B[a]P. Values are represented as mean $\pm$ S.D.. * Statistically significant differences from the corresponding control group ($p < 0.05$).

Fig. 3. 8-OHdG levels in the livers of MT-I/II null mice and wild-type mice treated with B[a]P. Values are represented as mean $\pm$ S.D.. * Statistically significant differences from the corresponding control group ($p < 0.05$). # Statistically significant differences from the dose-matched MT-I/II null mouse group ($p < 0.05$).

I/II null mice and wild-type mice treated with B[a]P is shown in Fig. 4. Little 8-OHdG was detected in the livers of untreated wild-type mice and MT-I/II null mice (Figs. 4A and F). In wild-type mice, 8-OHdG in the liv-

er was slightly increased only at 12 hr after B[a]P administration (Fig. 4C). In contrast, 8-OHdG in the livers of MT-I/II null mice was apparently increased at 6 and 12 hr after administration (Figs. 4G and H).

Fig. 4. Immunohistochemical staining for 8-OHdG in livers obtained from B[a]P treated MT-I/II null mice and wild-type mice. (A) and (F), 0 hr after B[a]P administration. (B) and (G), 6 hr after B[a]P administration. (C) and (H), 12 hr after B[a]P administration. (D) and (I), 24 hr after B[a]P administration. (E) and (J), 48 hr after B[a]P administration. (A) to (E), wild-type mice. (F) to (J), MT-I/II null mice. 8-OHdG is indicated by white circles. Magnification; x 200.

Evaluation of hepatic CYP1A enzymatic activity

Hepatic EROD activities were significantly increased in both MT-I/II null mice and wild-type mice at 12, 24 and 48 hr after B[a]P administration (Fig. 5). However, the hepatic EROD activities of B[a]P-administered mice were not changed between the MT-I/II null mice and wild-type mice.

MT concentration in the liver

MT concentrations were determined in the livers of wild-type mice and MT-I/II null mice treated with B[a]P. The hepatic MT levels in the wild-type mice were signif-

icantly increased by B[a]P treatment. However, MT was not detected in the livers of MT-I/II null mice with or without B[a]P treatment (Table 1).

DISCUSSION

Because B[a]P is known to induce chromosomal aberration, many investigators have used the MN test for detecting B[a]P mutagenicity *in vivo* (Shimada *et al.*, 1992). In the present study, MT-I/II null mice are more susceptible to chromosomal aberration than wild-type mice at various doses of B[a]P. Itoh and Shimada (1996)

Fig. 5. EROD activities in the livers of MT-I/II null mice and wild-type mice treated with B[a]P. Values are represented as mean $\pm$ S.D.. * Statistically significant differences from the corresponding control group ($p < 0.05$).

Table 1. MT levels in the livers of MT-I/II null mice and wild-type mice treated with B[a]P

Times after B[a]P administration	MT concentrations ($\mu\text{g/g}$ tissue)	
	wild-type mice	MT-I/II null mice
0 hr	10.51 $\pm$ 4.21	N.D.
6 hr	41.67 $\pm$ 10.55*	N.D.
12 hr	40.09 $\pm$ 2.12*	N.D.
24 hr	29.98 $\pm$ 8.34*	N.D.
48 hr	11.92 $\pm$ 1.93	N.D.

Values are means $\pm$ S.D.. Statistically significant differences from control group.

Limit of detection for MT assay is 1.85 $\mu\text{g/g}$ tissue. N.D.: not detected.

* $p < 0.05$.

have reported that pretreatment with an MT inducer suppressed the MN induction by chromium compounds and selenium compounds. MN induced by anticancer drugs such as cisplatin, adriamycin, cyclophosphamide and L-phenylalanine mustard was also prevented by pretreatment with MT inducers (Nakagawa *et al.*, 1995). These results suggest that MT may play a protective role against chromosomal aberration induced by several compounds including B[a]P.

Comet assay is a very sensitive technique for detecting individual eukaryote cells of multiple classes of DNA damage and incomplete excision repair sites associated with the elimination of DNA damage (Fairbairn *et al.*, 1995). Sasaki *et al.* (1997) used the comet assay to detect DNA damage by various mutagens and found that hepatic DNA strand breaks were increased by oral administration of B[a]P. In DNA strand breaks caused by dimethylarsinic acid, MT-I/II null mice were more susceptible than wild-type mice (Jia *et al.*, 2004). The hepatic comet scores of MT-I/II null mice treated with B[a]P have a greater tendency to high score than wild-type mice in the present study.

The major metabolic active pathway of B[a]P proceeds through *trans*-dihydrodiol to *anti*-diol epoxide which alkylates the N^2 group of guanosine, and BPDE- N^2 -dG, which is the major adduct formed, is associated with B[a]P carcinogenesis (Culp and Beland, 1994). In addition, during metabolic processes, B[a]P also produces various quinone derivatives and ROS which are mutagens and carcinogens (Penning *et al.*, 1996). Formation of 8-OHdG has been widely used as a biomarker of oxidative DNA damage (Kasai *et al.*, 1987). 8-OHdG is thought to be a promutagenic lesion because this lesion induces G:C to T:A transversion unless repaired prior to replication (Cheng *et al.*, 1992). It has been suggested that organ-specific tumorigenesis may be related to formation of 8-OHdG in the target organs of animals treated with free radical generating carcinogens (Cho *et al.*, 1993). Oral administration of B[a]P to Sprague-Dawley rats increased hepatic 8-OHdG and decreased catalase and superoxide dismutase activities. (Kim and Lee, 1997). Our results showed that susceptibility to oxidative DNA damage induced by B[a]P was extremely increased in MT-I/II null mice.

In the present study we found that MT plays an important role in defense against B[a]P-caused genotoxicity such as chromosomal aberration, DNA strand breaks and oxidative DNA damage. By considering the peak times of B[a]P-induced DNA damage in three genotoxicity assays, the oxidative DNA damage occurred at first, then the DNA strand breaks, and finally, chromosomal aberration.

In addition, at the highest dose (750 mg/kg) of B[a]P, two out of five MT-I/II null mice died within 48 hr after B[a]P administration. Park *et al.* (2001) have reported that MT-I/II null mice were susceptible to cadmium and other metal-mediated lethality. Thus, MT may protect against the lethality of B[a]P.

B[a]P itself is chemically inert and requires metabolic activation by P450s and epoxide hydrolase in order to react with cellular DNA. B[a]P is first oxidized by P450s to form intermediate metabolites, B[a]P-7,8-epoxide, and these epoxides are hydrolyzed by epoxide hydrolase to form B[a]P-7,8-diol metabolites. B[a]P-7,8-diols are oxidized again by P450s to form highly reactive diolepoxides that cause initiation of cancers in several organs (Conney, 1982). B[a]P is also known to induce CYP1A1, CYP1A2 and CYP1B1 through an arylhydrocarbon receptor-dependent mechanism (Shimada *et al.*, 2002). Thus, we attempted to reveal whether MT affects the metabolic activation of B[a]P through P450s. There was no significant difference in hepatic CYP1A activities between MT-I/II null mice and wild-type mice with or without B[a]P treatment. The present results suggest that MT does not affect metabolic activation of B[a]P.

On the other hand, the synthesis of MT is induced by many metals, hormones, cytokines, stresses and chemicals (Miles *et al.*, 2000; Sato and Bremner, 1993; Cai *et al.*, 1999). In the present study, it was first found that B[a]P can induce MT in the liver. The hepatic MT levels in the wild-type mice were elevated at 6 hr and maintained until 24 hr after B[a]P administration. Therefore, B[a]P-caused genotoxicity such as oxidative DNA damage and DNA strand breaks in the liver may be prevented by not only endogenous MT but also B[a]P-induced MT.

Recently, MT-I/II null mice have been used to study the role of MT in the carcinogenesis of various mutagens. Zhang *et al.* (1998) have found that MT-I/II null mice were very sensitive to skin carcinogenesis caused by a single topical application of DMBA. Suzuki *et al.* (2003) have also demonstrated that MT-I/II null mice were sensitive to DMBA/12-*O*-tetradecanoylphorbol-13-acetate-induced two-stage skin carcinogenesis. In addition, Kondo *et al.* (1999) have reported that MT-I/II null mice had a high incidence of urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. These reports suggest that MT-I/II null mice are extraordinarily susceptible to chemical carcinogens. B[a]P can induce various tumors such as mammary, uterine, lung, skin, liver, stomach, esophagus and leukemia depending on routes of administration and animal species (IARC monographs, in preparation). The present study revealed that MT-I/II null mice have an increased susceptibility to DNA dam-

age caused by B[a]P oral administration. MT may prevent B[a]P-induced carcinogenesis because MT prevents B[a]P-induced DNA damage.

The mechanism of the protective role of MT in B[a]P-induced DNA damage is unknown. However, its mechanism is related to DNA adduct formation and oxidative DNA damage. MT is a potent antioxidant to various oxidative injuries from ROS *in vivo* (Satoh *et al.*, 1988, 1992; Sato and Bremner, 1993; Cai *et al.*, 1999). Thus, it is thought that prevention of B[a]P-induced DNA damage by MT may be due to scavenging of the ROS generated by B[a]P.

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