72-kDa Stress Protein (Hsp72) Induced by Administration of Dimethylarsinic Acid to Mice Accumulates in Alveolar Flat Cells of Lung, a Target Organ for Arsenic Carcinogenesis

Koichi Kato, a Kenzo Yamanaka, a Masayuki Nakano, b Akira Hasegawa, a and Shoji Okada c

Nihon University College of Pharmacy, a 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan, Chiba University Hospital School of Medicine, b 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8677, Japan, and University of Shizuoka School of Pharmaceutical Sciences, c 52-1 Yada, Shizuoka 422-8526, Japan.

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Our previous studies have demonstrated that the oral administration of dimethylarsinic acid (DMA), a main metabolite of inorganic arsenics in mammals, in mice causes DNA damage in the lung as well as the promotion and progression of lung- and skin-tumorigenesis. Moreover, we indicated that 72-kDa stress protein (Hsp72) was induced in cultured human pulmonary (L-132) cells by exposure to DMA and was accumulated specifically in the cell nuclei. The present in vivo study reveals the induction of Hsp72 by intraperitoneal administration of DMA to A/J mice used previously as an animal model of dimethylarsenic-induced lung tumorigenesis. The Hsp72 was observed in the lung, a target organ for arsenic carcinogenesis in human, and in the kidney as well, but not in the liver and spleen. By immunohistochemical analysis, the Hsp72 in lungs was exhibited to exist in the nuclei of alveolar flat cells, including capillary endothelial cells, which were previously found to increase the clumping of heterochromatin, an early morphological change in the developmental process of pulmonary carcinomas, after administration of DMA to mice. These in vivo observations suggest that the increase and accumulation of Hsp72 by administration of DMA to mice may occur specifically in target organs for arsenic carcinogenesis.

Key words dimethylarsinic acid; arsenite; Hsp72; immunohistochemistry

The International Agency for Research on Cancer (IARC) in 1980 and again in 1987 has concluded through epidemiological surveys that inorganic arsenics have human carcinogenicity, particularly for the lung and skin, while experimental evidence for their carcinogenicity is inadequate for evaluation. To investigate the carcinogenicity of arsenic compounds, we have focused on the dimethylarsonics produced in the metabolic processing of inorganic arsenics. Some reports indicated that in vivo and in vitro exposure to dimethylarsinic acid (DMA) induced lung-specific genetic damage such as DNA strand breaks, cross-linking between DNA and nuclear proteins, DNA modifications, and further, that the oral administration of DMA showed tumorigenic action including initiation, promotion and progression in mouse lung and skin, and in rat multiple-organs, e.g., urinary bladder, kidney, liver and thyroid gland, in vivo. We also assumed that free radical species, particularly the dimethylarsenic peroxy radical \( [(\text{CH}_3)_2\text{AsOO}^-]^{11} \) produced in the further metabolic processing of DMA was responsible for this genetic damage and tumorigenesis.

72-kDa heat shock protein (Hsp72) is one of the stress proteins induced by various oxidative stressors, including arsenite and other metals. The depressive roles of Hsp72 toward the stressors are generally thought to include protection and recovery of cell damage. Recent reports indicated that the induction of Hsp72 was observed in rat liver and kidney by the administration of cadmium chloride and mercuric chloride, respectively. The administration of a hepatocarcinogen, diethylnitrosamine, to rats caused an increase in the Hsp72 gene expression in liver. However, there are few data in vivo which showed the induction of Hsp72 by administration of arsenics, in spite of numerous data in vitro. We previously found that Hsp72 was induced and accumulated in the cell nuclei of human alveolar type II (L-132) cells DNA-damaged by exposure to DMA, and also found that the accumulation of Hsp72 in cell-nuclei would relate to the suppression of apoptosis. Referring to some reports indicating that the Hsp72 might be involved with the tumorigenic process through the function of apoptosis, we assume that the Hsp72 induced by dimethylarsenics may play an important role in DNA damage and tumorigenesis. In the present study, we examined whether Hsp72 was induced and accumulated in the lung, a target organ for tumorigenesis, by the administration of DMA to A/J mice. The Hsp72 was induced in lungs and existed in the nuclei of alveolar flat cells, including capillary endothelial cells in which the administration of DMA to mice increases in the clumping of heterochromatin, an early morphological change in the developmental process of pulmonary carcinomas.

MATERIALS AND METHODS

Animals Five-week-old male A/J mice were obtained from Japan SLC (Hamamatsu, Japan). Mice were acclimated in the animal facility for at least one week prior to use. This facility is environmentally controlled on a 12 h dark/light cycle at 23 °C and 55% humidity.

Chemicals DMA and As2O3 were purchased from Nacalai Tesque (Kyoto, Japan) and Merck (Darmstadt, Germany), respectively. The stock solution of DMA (60 mg/ml) was adjusted to pH 6.0 with NaOH, and that of arsenite (0.5 mg/ml) was prepared by dissolving As2O3 with minimal NaOH.

Western Blot Analysis of Hsp72 A/J mice intraperitoneally administered DMA (100-600 mg/kg) or arsenite (5 mg/kg) were sacrificed by exsanguination from the postcava under sodium pentobarbital anesthesia at the times indicated in the Figures and Table. Lung, kidney, liver and spleen in mice were excised and immediately placed in ice-cold 0.9% saline. The organs were homogenized in 5 vol. of 0.9%
saline, and centrifuged at 4°C for 30 min at 18800×g. The supernatant was solubilized with the lysis buffer (62.5 mM Tris–HCl buffer (pH 6.8) containing 2.5% SDS, 5% 2-mercaptoethanol and 10% glycerol). Total protein was analyzed according to the method of Bradford. The proteins (30 μg) were added in a gel lane of 7.0% SDS-PAGE according to the method of Laemmli. Immunoblotting analysis was performed by the method of Towbin. Briefly, the proteins were transferred onto a nitrocellulose membrane. After blocking non-specific binding with 3% bovine serum albumin (BSA), the membrane was incubated in the primary antibody, anti-Hsp72 monoclonal antibody clone C92FA-5 (StressGen Biotechnologies Corp., British Columbia, Canada), for 1 h at room temperature, and subsequently, in biotinylated anti-mouse IgG (Amersham International, PLC., Buckinghamshire, England) as a secondary antibody. Alkaline phosphatase-labeled streptavidin (Vector Lab., Inc., Burlingame, CA, U.S.A.) as a substrate (5-bromo-4-chloro-3-indolylphosphate and Nitroblue tetrazolium) development solution (KPL, Gaithersburg, MD, U.S.A.) was used to visualize Hsp72.

**Immunohistochemical Detection of Hsp72 in Lung**

Lungs were immersed in 10% buffered formalin overnight at 4°C. The specimens were embedded in paraffin wax. Sections (3 μm thick) were cut and then mounted on a 3-amino- propyltriethoxysilane-coated slide, and further dried overnight at room temperature. The sections were deparaffinized with xylene twice for 10 min, and hydrated with successive 95.5%, 90%, 80% and 70% (v/v) ethanol. Microwave treatment was applied for antigen retrieval; the sections in 10 mM sodium citrate buffer (pH 6.0) were boiled for 3 consecutive cycles for 5 min with a 1 min pause in between. Endogenous peroxidase activity in the sections was blocked by incubation with methanol containing hydrogen peroxide (0.3%) for 30 min. Nonspecific binding was blocked by incubation with phosphate-buffered saline (PBS) containing normal goat serum (10%) and Tween-20 (0.05%) overnight at 4°C. Anti-Hsp72 monoclonal antibody (mouse monoclonal IgG) as a primary antibody was diluted (1: 200) with PBS containing 1% BSA. The sections were incubated with the primary antibody solution at least 5 d at 4°C. The sections were washed with PBS containing Tween-20 (0.05%) and were incubated with goat anti-mouse IgG (Bethyl Lab., Inc., Montogomery, TX, U.S.A.) diluted (1: 200) with PBS containing 1% BSA. The sections were washed with PBS containing Tween-20 (0.05%) and incubated with monoclonal mouse PAP (ZYMED, San Francisco, CA, U.S.A.) and diluted (1: 200) with PBS containing 1% BSA. The sections were developed by immersion in PBS containing 0.3 mg/ml 3,3’-diaminobenzidine and 0.009% hydrogen peroxide for 1 h, and were then rinsed with running tap water. The sections were dehydrated through a graded ethanol series to absolute ethanol, clear in xylene, and mounted in Canada bal- sam.

**RESULTS AND DISCUSSION**

The Tissue-Specific Induction of Hsp72 in Mouse by DMA Administration

With rodents, little is known about the *in vivo* induction of Hsp72, in spite of numerous studies on its *in vitro* induction in cultured cells. We thus examined the induction of Hsp72 in various organs and tissues after exposure of A/J male mice to DMA. The Hsp72 was detected by an immunoblotting technique using mouse anti-Hsp72 monoclonal antibody. A remarkable induction of Hsp72 was observed in lung and kidney at 48 h after the administration of DMA at doses of 300 or 600 mg/kg (i.p.), but not in the liver and spleen (Fig. 1). Our previous studies revealed that the oral administration of DMA induced lung-specific DNA damage and the promotion and progression of lung and skin tumorigenesis in mice. Although our previous data indicated that DMA administration did not induce DNA damage in mouse kidney, a recent report described that the oral administration of DMA to rats showed nephrotoxicity. These suggest that Hsp72 is induced in the lung and kidney, possible target organs for genotoxicity and non-genotoxicity of dimethylarsenic, respectively. In addition, the Hsp72 in the lung and kidney was not induced by inorganic arsenics produced in the demethylation process of DMA in mice, but by dimethylarsenic itself, since the demethylation products of

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**Fig. 1. Organ-specific Induction of Hsp72 after the Administration of DMA**

A, DMA was injected (600 mg/kg bw, i.p.) into mice. Mice were sacrificed at indicated times after injection. Hsp72 was detected by Western blot analysis. B, DMA was injected (0–300 mg/kg bw, i.p.) into mice. Mice were sacrificed after 48 h. Hsp72 was detected by Western blot analysis. Sd: Hsp72 in cultured human alveolar cells exposed to DMA.
DMA have not been detected in mice.\textsuperscript{25)\textsuperscript{26)}

A report indicated an induction of Hsp72 in the kidney of female ND4 mice after intraperitoneal injection with arsenite.\textsuperscript{26)} As shown in Fig. 2, the induction of Hsp72 by the administration of arsenite (As$_4$O$_6$, 5 mg/kg, i.p.) was observed in all the organs (lung, kidney, liver and spleen), differently from that by the administration of DMA. Brown et al. demonstrated that the lung specific DNA damage was not induced by the administration of arsenite in rat.\textsuperscript{27)\textsuperscript{29)} Therefore, the non-specific induction of Hsp72 by the administration of arsenite may be ascribed to the formation of non-genotoxic damage, arising from an acute toxic mechanism such as combination with a variety of dithiol-containing molecules.

The Relationship between the Accumulation of Hsp72 and Induction of DNA Damage in Lung by DMA Administration in Mice

The distribution of Hsp72, which might be a protecting factor in the pulmonary tumorigenic process, was examined by immunohistochemical analysis. Hsp72 was observed in the nuclei of alveolar flat cells containing capillary endothelium after DMA administration (Fig. 3, Table 1). This may relate to our previous histopathological observation that the oral administration of DMA in mice induced a preferential increase in heterochromatin in the venular endothelium of the lung.\textsuperscript{30)} Although Hsp72 was also observed in the nuclei of pulmonary cells, in part of the venular endothelial cells, by the administration of arsenite (data not shown), we assumed that the induction and distribution of Hsp72 in the lung by dimethylarsenicals would be different in mechanism from those by arsenite, because the administration of arsenite in mice could not induce lung-specific DNA damage.\textsuperscript{27)\textsuperscript{29)} Some data represent the histopathological evaluations for lung cancer induced by the occupational exposure of arsenic in a copper smelter.\textsuperscript{28,29)} Our previous histopathological study demonstrated that oral administration of DMA in mice mainly induced peripheral papillary adenomas and adenocarcinomas.\textsuperscript{8)} The lung tumors are thought to be of Clara cells and alveolar type II cells.\textsuperscript{30)} The present finding, that Hsp72 induced by the administration of DMA in mice was accumulated in the nuclei in endothelial cells including alveolar wall capillaries (Fig. 3), may be consistent with those observations. As a possible role of Hsp72 in the lung tumorigenic processes, several studies indicated that Hsp72 would be complexed with the tumor suppressor gene products, e.g., p53,\textsuperscript{31)} and that it was concerned with the suppression of apoptic cell death.\textsuperscript{32,33)} The present study, indicating that Hsp72 induced by dimethylarsenicals, metabolites of inorganic arsenics, accumulated in the nuclei in the pulmonary cells in mice, may be helpful in understanding the mechanism of lung tumorigenesis by inorganic arsenics.

REFERENCES

1) International Agency for Research on Cancer (IARC), "IARC Mono-

Table 1. Cell-Nuclear Localization of Hsp72 in Alveolar Flat Cells of A/J Mice after DMA Administration

| Immunostained cell\textsuperscript{a1} count (per 0.3 mm\textsuperscript{2}) |
|-----------------|------------------|
| Control DMA\textsuperscript{a1} | 27±14.1 (n=6) |
| DMA\textsuperscript{a1} | 58±14.3 (n=5) |

Data represent the means±S.D.; significantly different from control levels (**p<0.05) by t-test. \textsuperscript{a1)} The immunostained cells were made up of alveolar flat cells containing capillary endothelium. \textsuperscript{a1)} 48 h after DMA administration (600 mg/kg).

Fig. 2. Hsp72 Induction in Various Organs after As$_4$O$_6$ Injection

A: As$_4$O$_6$ was injected (5 mg/kg, i.p.) into mice. C: Control received saline injection. Sd: Hsp72 in cultured human alveolar cells exposed to DMA. Mice were sacrificed after 24 h. Hsp72 was detected by Western blot analysis.

Fig. 3. Immunohistochemical Analysis of Hsp72 Localization in Alveolar Flat Cells

(A) Control. (B) Localization of Hsp72 at 48 h after injection of DMA (600 mg/kg, i.p.). (C) Hematoxylin-eosin stained lung after the administration of DMA. The arrows (in B, C) indicate the endothelial cells of alveolar wall capillaries.