Dimethylarsinic Acid Exposure Causes Accumulation of Hsp72 in Cell Nuclei and Suppresses Apoptosis in Human Alveolar Cultured (L-132) Cells

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We previously found that 72-kDa heat shock protein (Hsp72) was induced and accumulated in the nuclei, together with DNA damage, in human alveolar epithelial (L-132) cells by exposure to dimethylarsinic acid (DMAA), which is a main metabolite of inorganic arsenics in mammals. In the present study, the intracellular behavior of Hsp72 was investigated during the recovery from the DNA damage induced by exposure to DMAA. L-132 cells were exposed to 10 mM DMAA for 3 h, and then incubated in DMAA-free medium. The induction of Hsp72 by exposure to DMAA reached a peak at 6–9 h after removal of DMAA. However, the cell-nuclear distribution of Hsp72 was observed until 3 h after the start of DMAA-free incubation. We further investigated the appearance of apoptosis of L-132 cells after exposure to 10 mM DMAA for 3 h. Internucleosomal DNA fragmentation and morphological changes, as criteria for the evidence of apoptosis, were observed 6–22 h after the start of DMAA-free incubation. The appearance of apoptosis was followed by the release of Hsp72 from the cell nuclei. These results suggest a possibility that the cell-nuclear Hsp72 may suppress the appearance of apoptosis in DNA-damaged cells.

Key words dimethylated arsenic; dimethylarsinic acid; arsenite; Hsp72; apoptosis

Inorganic arsenics have been recognized as human carcinogens by epidemiological investigations, whereas numerous trials have failed to induce tumors in experimental animals.^{1,2)} To elucidate arsenic-induced genetic damage, we focused on dimethylarsenic produced in metabolic methylation which is generally believed to be the principal detoxication process for acute toxicity of inorganic arsenics.³⁾ We found that exposure to dimethylarsinic acid (DMAA), a major metabolite of inorganic arsenics in mammals, induced DNA damage such as DNA strand breaks, crosslinking with protein, and an increase in heterochromatin,^{4–7)} probably *via* the production of arsenic peroxy radical $[(CH_3)_2AsOO\cdot]$ in the further metabolic processing of DMAA.⁷⁾

Our *in vitro* experiment indicated the peculiar nuclear-distribution of 72-kDa stress protein (Hsp72) in human alveolar epithelial (L-132) cells by DMAA exposure.⁸⁾ We assumed that the Hsp72 played an important role in the recovery from the DNA damage induced by exposure to DMAA.⁸⁾ Recent reports showed that induction of Hsp72 was accompanied by DNA damage caused by DNA cross-linking agents⁹⁾ or UV irradiation¹⁰⁾ in cultured human cells, and further that Hsp72 induced by UV irradiation accumulated in the nuclei.¹⁰⁾ Welch and Suhan reported that the nuclear distribution of Hsp72 in mammalian cells induced by exposure to arsenite was observed during the recovery from the damage.¹¹⁾ A primary function of Hsp72 is thought to be to protect cells from damage due to stressors and allow them to recover and survive.¹²⁾ However, apoptosis may be a means of eliminating the cells which DNA damage has made irreparable.¹³⁾ Hsp72 is known to act to prevent apoptosis in stationary Ehrlich ascites carcinoma cells¹⁴⁾ and in primary cultured rat hepatocytes.¹⁵⁾

Here we describe the relationship between the nuclear distribution of Hsp72 and the occurrence of apoptosis in L-132 cells during the recovery from DNA damage induced by exposure to DMAA. We found that the release of Hsp72 from

the cell-nuclei led to the appearance of apoptosis.

MATERIALS AND METHODS

Cell Culture and Treatments L-132 cells, an established human alveolar cell line, (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were cultured in Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, U.S.A.) under a humidified atmosphere of 5% CO₂ at 37 °C. The cells were exposed to 10 mM DMAA (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) or to 42 °C for 3 h. Afterward, they were washed with PBS (phosphate-buffered saline) and were transferred to fresh Eagle's MEM, and then were incubated for the indicated time. For labeling proteins, the cells in methionine-free Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo) were incubated for 1 h with 370 kBq/ml of [³⁵S]methionine (NEN Research Products, Boston, MA, U.S.A.).

Analysis of Morphological Alterations by Light-microscopy DMAA-treated cells were washed twice with PBS and fixed in acetic acid-methanol (1:3) for 5 min. The fixed cells were spread on a slide glass and then were stained with 2% Giemsa's solution for 30 min. Morphological changes were detected by light-microscopy.

Agarose Gel Electrophoresis The cells (1.0 to 1.2×10^7) were collected by scraping, and then were centrifuged at 1500 rpm for 5 min. The cell-pellets were washed with ice-cold PBS. The cells were lysed in an ice-cold buffer (10 mM Tris-HCl (pH 7.4) containing 0.5% Triton X-100 and 1 mM EDTA) for 1 h. DNA was extracted with phenol and chloroform. The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, and then was dissolved in TE (10 mM Tris-HCl containing 1 mM EDTA) buffer (pH 8.0). Contaminated RNA was digested with

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RNase I (Sigma Chemical Co., St. Louis, MO, U.S.A.). Fragmented DNA was detected by 1.8% agarose gel electrophoresis, and was visualized with a UV illuminator following gel staining with ethidium bromide.

SDS-PAGE Cells were washed twice with ice-cold PBS, and then lysed in 62.5 mM Tris-HCl (pH 6.8) containing 2.5% [35 S]methionine SDS, 5% β -mercaptoethanol and 10% glycerol. The [35 S]methionine-labeled proteins were separated by 7.0% SDS-PAGE according to the method of Laemmli.¹⁶⁾ The gel treated with EN³HANCE (NEN Research Products) was autoradiographed at -80°C .

Immunochemical Detection of Hsp72 Hsp72 was analyzed by Western blot analysis as previously described.⁸⁾ Briefly, Hsp72 transferred onto nitrocellulose membrane (Hybond-C, Amersham, Buckinghamshire, England), was subjected to immunochemical detection as described in the manufacturer's instruction of blotting detection kit (Amersham, Buckinghamshire, England) using anti-72 kDa heat shock protein, clone C92F3A-5 (StressGen Biotechnologies Co., Victoria, Canada) as a first antibody.

Intracellular Distribution of Hsp72 Intracellular Hsp72 was detected as previously described.⁸⁾ Briefly, the cells on a slide glass were fixed and permeabilized by 2% paraformaldehyde and 0.2% NP-40, respectively. They were incubated with mouse anti-Hsp72 monoclonal antibody (StressGen Biotechnologies Co.) for 2 h at room temperature, and then were incubated with horseradish peroxidase-conjugated anti mouse IgG (H+L) (BioMakorTM, Rehovot, Israel) for 1 h at room temperature. After washing with PBS, the cells were incubated in a solution containing 0.3 mg/ml diaminobenzidine and 0.009% hydrogen peroxide for 15 min at room temperature. The air-dried cells were then observed by lightmicroscopy.

RESULTS

Hsp72 after Exposure of DMAA Induction of Hsp72: When L-132 cells were incubated in DMAA-free medium after exposure to 10 mM DMAA for 3 h, a significant increase in 72-kDa protein was observed 3 to 6 h after the start of the incubation (Fig. 1A), whereas, in the case of 42°C treatment, a condition that was generally utilized as a criterion for nuclear accumulation of Hsp72, the induction of 72-kDa pro-

tein was observed 0 to 3 h, after the treatment (Fig. 1A). A gradual increase in accumulation of Hsp72 was observed in the case of DMAA exposure till 9 h after the start of DMAA-free incubation, while it was not observed in the 42°C treatment (Fig. 1B).

Intracellular Distribution of Hsp72: Figure 2 shows the intracellular distribution of Hsp72 in L-132 cells after exposure of DMAA by immunomicroscopic analysis. We previously found that Hsp72 was accumulated in nucleoli and nucleoplasm of L-132 cells by exposure to DMAA.⁸⁾ Here, we observed that Hsp72 distributed in the cell nuclei immediately after 3 h exposure to DMAA. The cell-nuclear distribution continued for 3 h but not 6 h (Fig. 2), although the cellular induction of Hsp72 reached a peak at 6 h (Fig. 1).

Apoptosis in L-132 Cells by Exposure of DMAA Morphological Changes Induced by DMAA Exposure: The well-known markers of apoptosis are alterations in cell morphol-

Fig. 1. Induction of Hsp72 in L-132 Cells after Heat or DMAA Treatment

L-132 cells were heat (42°C)-treated for 3 h or exposed to 10 mM DMAA at 37°C for 3 h, and then incubated at 37°C in DMAA-free medium for the indicated time. (A) Proteins labeled with [35 S]-methionine for the final 1 h of incubation were analyzed by 7% SDS-PAGE. (B) Hsp72 was detected by immunoblotting using anti-Hsp72 monoclonal antibody.

Fig. 2. Nuclear Distribution of Hsp72 in L-132 Cells after DMAA Exposure

L-132 cells were exposed to 10 mM DMAA for 3 h, and then incubated in DMAA-free medium for the indicated time (C is control.). The immunostained cells were prepared as described in Materials and Methods.

Fig. 3. Morphological Alterations in L-132 Cells after DMAA Exposure

L-132 cells were exposed to 0 mM (A) or 10 mM (B) DMAA for 3 h, and then incubated in DMAA-free medium for 22 h. The open and closed arrows indicate the condensation and fragmentation, respectively, of cell nuclei.

Table 1. Cytotoxic Effects in L-132 Cells Observed after DMAA Exposure

Time after exposure (h)	Number of normal cells	Number of cells with morphological changes of nucleus (%)		Viability (%)	Detached cells (%)
		Condensation	Fragmentation		
Control	798	0	0		
0	1276	74 (5.48)	0	97.82	8.35
1	1350	59 (4.19)	0	97.59	7.68
3	1523	75 (4.69)	1 (0.06)	97.14	6.75
6	999	56 (5.22)	18 (1.68)	96.91	13.61
9	1027	67 (5.82)	57 (4.95)	95.02	12.55
22	903	95 (8.87)	73 (6.82)	87.50	27.15

L-132 cells were exposed to 10 mM DMAA for 3 h, and then incubated in DMAA-free medium for the indicated time. Cell viability was examined by dye-exclusion test using 0.5% trypan blue solution.

ogy, e.g., membrane blebbing, cellular shrinkage, nuclear and cytoplasmic condensation and margination of chromatin.¹⁷⁾ Figure 3 shows the nuclear condensation and fragmentation in L-132 cells exposed to 10 mM DMAA for 3 h and then incubated in DMAA-free medium for 22 h. Table 1 indicates cytotoxic effects of DMAA exposure on L-132 cells. The rate of nuclear condensation and fragmentation in these cells at 22 h after the removal of DMAA was 8.87% and 6.82%, respectively. Furthermore, the rate of detached cells and of nuclear fragmentation gradually increased from 6 h after the start of DMAA-free incubation.

Internucleosomal DNA Fragmentation Induced by DMAA Exposure: Detection of internucleosomal DNA fragmentation is frequently used as biochemical evidence of apoptosis. We observed that the DMAA-exposed (3 h) L-132 cells showed the fragmentation starting 6 h after shifting to DMAA-free medium (Fig. 4A), whereas no significant fragmentation was observed in the presence of 10 mM DMAA for any period tested (3 to 12 h) (Fig. 4B).

Fig. 4. Agarose Gel Electrophoresis of Internucleosomal DNA Fragmentation Induced after DMAA Exposure

L-132 cells were exposed to 10 mM DMAA for 3 h, and then incubated in DMAA-free medium for the indicated time (A), or exposed to 10 mM DMAA for the indicated time without post-incubation in DMAA-free medium (B). Lane M; DNA standard marker (λ -Hind III digests). Lane C; control.

DISCUSSION

We previously found that following exposure to DMAA L-132 cells showed a peculiar nuclear distribution of Hsp72 in a manner markedly different from that following heat treatment or arsenite exposure.⁸⁾ The present study indicated the difference between the mode of induction of Hsp72 by DMAA exposure and by heat treatment: exposure to DMAA caused a retarded induction of Hsp72 compared to that of heat treatment (Fig. 1). A noteworthy point on the Hsp72 in L-132 cells by exposure to 10 mM DMAA for 3 h is that it mainly localized in the nuclei at an early stage (<3 h) after the start of DMAA-free incubation (Fig. 3). Recent reports showed that Hsp72 was induced by DNA cross-linking agents⁹⁾ and UV irradiation¹⁰⁾ in cultured human cells. Our previous studies demonstrated that DNA damage in L-132 cells after exposure to 10 mM DMAA for 1 h was most remarkably observed 1–2 h after DMAA-free incubation, and subsequently the damage was gradually repaired.^{18,19)} Therefore, we assumed that Hsp72 would be accumulated in the nuclei of the cells, which received strong DNA damage at an early stage after DMAA exposure.

The relationship between DNA damage and the nuclear localization of Hsp72 remains obscure, although a recent study points out that migration of Hsp72 into nucleus was caused by increase of 8-hydroxydeoxyguanosine produced by exposure of the cultured cells to hydrogen peroxide.²⁰⁾ Our previous report²¹⁾ demonstrated that DNA damage was not fully repaired 24 h after exposure to 10 mM DMAA for 1 h, suggesting that DNA damage by exposure to DMAA was retained for a relatively long period. However, as shown in Fig. 1, a remarkable induction of Hsp72 was observed 3 h or longer after exposure to DMAA for 3 h, although Hsp72 accumulated in the nucleus decreased (Fig. 3). It seems that disappearance from cell-nuclei and retarded induction of Hsp72 by dimethylarsenics occur efficiently in terms of the recovery from its DNA damage.

One function of Hsp72 in carcinogenesis is that its increased amount in tumor cells caused a suppression of apoptosis.¹⁴⁾ An overexpression of Hsp72 makes mammalian tumor cells more malignant when transplanted, since they acquire resistance to apoptosis due to tumor necrosis factor- α .²²⁾ Furthermore, Hsp72 protects lymphocytes from radiation-induced apoptosis without changes of Bcl-2 expression.²³⁾ These facts suggest that Hsp72 may play an important role in the process of apoptosis suppression.

We investigated the relationship between the nuclear distribution of Hsp72 and the appearance of apoptosis in L-132 cells after exposure to DMAA. The post-incubation in DMAA-free medium caused the release of Hsp72 from the cell nuclei (Fig. 2) together with the cell apoptosis (Fig. 4 and Table 1). When 10 mM DMAA was successively present in the post-incubation medium, neither cell apoptosis nor release of Hsp72 from the nuclei was observed up to 12 h (Fig. 4). It seems likely that the cell apoptosis by exposure to DMAA appears during the recovery from the DNA damage following the release of Hsp72 from the cell-nuclei. Furthermore, our previous experiment showed that syntheses of total protein and RNA in L-132 cells exposed to 10 mM DMAA for 6 h remained approximately 45% and 52%, respectively, of control cells.²⁴⁾ These suggest that the suppression or

delay of apoptosis (Fig. 4) may be due to the action of Hsp72 itself in the cell-nuclei, rather than to the inhibition of protein and RNA synthesis.

The role of apoptosis in the development of malignant cells and its application to cancer therapy have recently been discussed.²⁵⁾ Among some agents which are carcinogenic but not genotoxic, nafenopin was reported to suppress apoptosis in a rat hepatoma cell line induced by TGF- β 1, suggesting that the suppression of apoptosis may play an important role in carcinogenicity.²⁶⁾ Furthermore, as pointed out in the transformation of colorectal epithelial cells to carcinoma cells, the inhibition of apoptosis in colorectal tumorigenesis may result in neoplastic progression.²⁷⁾ Thus, the suppression of apoptosis by cell-nuclear accumulation of Hsp72 may be important in clarifying the mechanism of arsenic carcinogenesis.

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