Methylmercury-Mediated Down-Regulation of mtHSP70 and Phospholipase A_2 mRNA Expression in Human Neuroblastoma NB-1 Cells Identified by cDNA Macroarray Analysis

Rocky Pramanik,[a, b, 1] Masami Ishido,[a, b] and Manabu Kunimoto*[, a, b, 2]

[a]*Regional Environment Division, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba 305–8506, Japan and* [b]*CREST, Japan Science and Technology (JST) Cooperation, Kawaguchi, Saitama 332–0012, Japan*

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Methylmercury (MeHg) is a well-known environmental pollutant that causes severe neurological damage, especially in the developing nervous system. When human neuroblastoma NB-1 cells were treated with MeHg at sublethal concentrations, down-regulation of two apoptosis-associated genes, mtHSP70 (GRP75/ PBP74/ mtHSP75 /HSPA9B/mortalin) and phospholipase A_2 (PLA2), was identified using the cDNA macroarray technique. These observations were further confirmed by RT-PCR and Northern blotting experiments. Northern blotting data also demonstrated that only groups III and VI B of PLA2 were down-regulated, while group IIE remained unchanged. These results suggest that the mtHSP70 and PLA2 may be involved in the MeHg-mediated degeneration of neuronal cells.

Key words —— human neuroblastoma cell, methylmercury, gene expression, cDNA macroarray

INTRODUCTION

Methylmercury (MeHg) is known to provoke severe developmental neurological damage due to its disruptive effects on the mitosis of neuroblasts and neuronal migration, and induction of neuronal apoptosis.[1–4] However, the molecular mechanisms underlying these effects are not fully understood. Previously, we reported that MeHg inhibits the neurite outgrowth in NB-1 cells accompanied by selective down-regulation of 440 kDa ankyrin$_B$, a neuron-specific protein localized in axons.[5,6] To understand the molecular mechanisms of MeHg-mediated neurotoxicity, cDNA macroarray analysis was performed using NB-1 cells. The results showed that mtHSP70 (mtHSP75/grp75/mortalin/HSPA9B) and phospholipase A_2 mRNA expression were down-regulated in NB-1 cells treated with MeHg. These observations were further confirmed by RT-PCR and Northern blotting experiments.

MATERIALS AND METHODS

Materials —— Methylmercury chloride was from Tokyo Kasei (Tokyo, Japan) and [α-^{32}P]dCTP was purchased from Amersham (Amersham, U.K). The human neurobiology cDNA array membranes and PCR primers were from Clontech (MD, U.S.A.). All other tissue culture media, sera, and supplements were from Gibco BRL (CA, U.S.A.).

Cell Culture —— Human neuroblastoma NB-1 cells were cultured in 45% RPMI and 45% Eagle's minimum essential medium containing 10% fetal bovine serum and an antibiotic mix. Cells were subcultured once a week at a split ratio of 1 : 6.[5]

cDNA Macroarray —— Total RNA was prepared with Trizol reagent (Gibco BRL) as described in the user's manual. Macroarray analysis was performed as described previously.[7] Briefly, [α-^{32}P]dCTP-labeled ds-cDNA used to hybridize with a salmon sperm prehybridized neurobiology membrane (Atlas™ human neurobiology, Clontech) using the standard protocol. After washing, the image was analyzed with BAS2000 (Fuji Film, Tokyo, Japan) and relative radioactivity was calculated using Array

[1]Present address: Department of Radiation Oncology, Stritch School of Medicine, Loyola University Medical Center, Building 1 (114-B), Maywood, IL 60153, U.S.A.

[2]Present address: Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan

*To whom correspondence should be addressed: Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan. Tel.: +81-3-5791-6264; Fax: +81-3-3442-4146; E-mail: kunimotom@pharm.kitasato-u.ac.jp

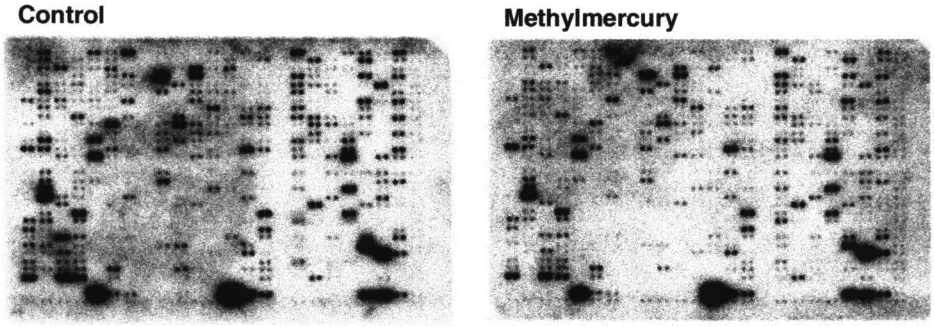

Fig. 1. cDNA Macroarray Analyses of Genes Expressed in NB-1 Cells

NB-1 cells precultured for 1 day were further cultured in the absence or presence of methylmercury chloride (MeHg) 1 μM for 2 days. Poly A$^+$ RNA isolated from the cells was reverse-transcribed to cDNA using gene-specific primers. cDNA array membranes (Atlas™ Human Neurobiology Membrane) were hybridized with [α-^{32}P]dCTP-labeled double-stranded cDNA. After washing, the radioactivity was detected using BAS2000 image analyzer (Fuji Film).

Gauge software (Fuji Film).

RT-PCR and Northern Blotting —— Total RNA was prepared from NB-1 cells using Trizol reagent (Gibco BRL) according to the manufacturer's instructions. PolyA$^+$ RNA was concentrated with biotin-labeled magnetic beads (Roche Diagonostics, Basel, Switzerland). Poly A$^+$ RNA was reverse-transcribed with oligo dT primer and Superscript II reverse transcriptase (Gibco BRL). One-fifth of the reverse-transcribed product was used as a PCR template with various sets of PCR primers for each target gene prepared according to the manufacturer's instructions (Clontech). A single discrete band was obtained for each set of primers. The bands from PCR products were further gel-purified and used as a probe for Northern blotting.

Total RNA from NB-1 cells was separated by electrophoresis in a 1% agarose gel containing 6.7% formaldehyde. RNA was transferred to a nylon membrane filter (Hybond-N$^+$, Amersham) with 10 × SSPE as the transfer solution as described previously.[8] The membrane was prehybridized at 68°C for 2–3 hr in Express Hybridization Solution (Clontech) containing 200 mg/ml of denatured salmon sperm DNA. Gel-purified cDNA fragments from PCR products were labeled with [α-^{32}P]dCTP and the random primer DNA labeling kit (Takara, Kyoto, Japan). The radioactive probe was added to the prehybridization solution and incubated for 12–16 hr at 68°C. The membrane was washed with 2 × SSPE/0.1% SDS and then with 0.1 × SSPE/0.1% SDS at 50°C. The radioactivity on the membrane was detected and quantified with BAS 2000.

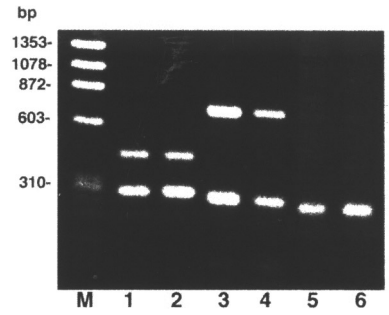

Fig. 2. RT-PCR Analyses of Selected Genes Expressed in NB-1 Cells

Poly A$^+$ RNA (1 μg) from control (lanes 1, 3 and 5) and MeHg-treated NB-1 cells (lanes 2, 4, and 6) were used for reverse-transcription reaction and one-fifth of the reverse-transcribed products were used for PCR reaction. In some PCR reactions, two sets of PCR primers were used at the same time and under the same conditions. Lanes 1 and 2, 40S ribosomal protein (upper bands) and glyceraldehyde-phosphate dehydrogenase (lower bands). Lanes 3 and 4, PLA2 (upper bands) and mtHSP70 (lower bands). Lanes 5 and 6, tyrosine hydroxylase.

RESULTS AND DISCUSSIONS

To examine the effects of MeHg on the gene expression pattern in cultured neuronal cells, cDNA macroarray analysis was performed using human neuroblastoma NB-1 cells. As shown in Fig. 1, the mRNA of many genes was down-regulated by treatment with MeHg at 1 μM. To confirm these observations, RT-PCR and Northern blotting analyses were performed. Only 4 of 12 genes selected from the macroarray experiment showed similar patterns in the RT-PCR and Northern blotting analyses. The RT-PCR results shown in Fig. 2 demonstrate that among the three housekeeping genes only 40S ribosomal protein (40S) (lanes 1 and 2, upper bands) remained unchanged, while phospholipase A_2 (PLA2) (lanes 3 and 4, upper bands) was down-regu-

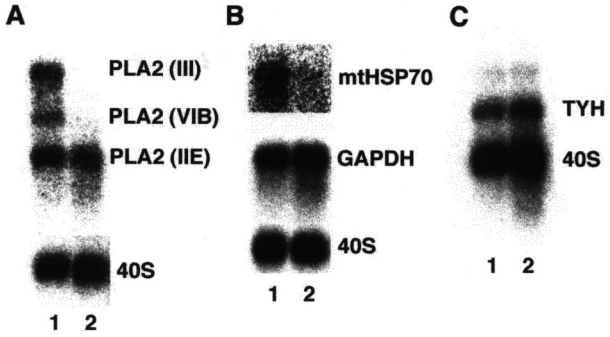

Fig. 3. Northern Blot Analyses of Selected Genes Expressed in NB-1 Cells

Twenty micrograms of total RNA from control (lanes 1) and MeHg-treated NB-1 cells (lanes 2) were subjected to Northern blot analysis as described in Materials and Methods. A) PLA2 (III), 4.4 kb (group III); PLA2 (VIB), 3.4 kb (group VIB); PLA2 (IIE), 1.5 kb (group IIE). B) mtHSP70, GAPDH, glyceraldehyde-phosphate dehydrogenase. C) TYH, tyrosine hydroxylase.

lated and glyceraldehyde-phosphate dehydrogenase (GAPDH) (lanes 1 and 2, lower bands) was increased by the MeHg treatment. Altered expression of two other genes occured, *i.e.*, down-regulation of mitochondrial HSP70 (mtHSP70) (lanes 3 and 4, lower bands) and up-regulation of tyrosine hydroxylase (TYH) (lanes 5 and 6). These RT-PCR results were further confirmed by the Northern blotting experiments (Fig. 3).

A large number of PLA2 have been identified within the central nervous system of rodents and humans.[9] Verity *et al.*[10] reported that MeHg stimulated PLA2 in cerebellar granule neuron cultures. It has been shown that cPLA2-deficient mice are resistant to neuronal injury compared with their wild-type littermate controls.[11] Therefore the decrease in PLA2 mRNA observed in our study raises the possibility that PLA2 could be involved in a defensive mechanism against MeHg-mediated cytotoxicity.[12] It is noteworthy that only two groups of PLA2, group III and group VIB, were down-regulated, while group IIE was not (Fig. 3A). Grouping of PLA2 mRNA was done based on the mRNA size from the gel.[9]

On the other hand, the decrease in mtHSP70 mRNA by MeHg treatment may explain the role of mtHSP70 in MeHg-mediated apoptosis. It has been shown that in the human breast adenocarcinoma cell line MCF-7 the elevated level of PBP74/mtHSP70 is associated with the resistance to ionizing radiation.[13] In addition, elevated levels of mortalin expression in human brain tumors have been reported.[14]

In addition, the up-regulation of TYH observed in our study was also consistent with the report of Rossi *et al.*,[15] who found that the increased levels of TYH by MeHg treatment are associated with altered locomotive activity in rats.

In the present study, three possible genes, PLA2, mtHSP70, and TYH, were identified and confirmed to be associated with the MeHg-mediated degeneration of neuronal cells. Further studies are needed to understand how each of these genes is involved in the process.

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REFERENCES

1) Graff, R. D. and Reuhl, K. R. (1996) Cytoskeleton toxicity of heavy metals. In *Toxicology of metals* (Chang, L.W., Ed.) Lewis Publishers, New York, pp. 639–657.
2) Kunimoto, M. and Suzuki, T. (1997) Migration of granule neurons in cerebellar organotypic cultures is impaired by methylmercury. *Neurosci. Lett.*, **226**, 183–186.
3) Kunimoto, M. (1994) Methylmercury induces apoptosis of rat cerebellar neurons in primary culture. *Biochem. Biophys. Res. Commun.*, **204**, 310–317.
4) Miura, K., Koide, N., Himeno, S., Nakagawa, I. and Imura, N. (1999) The involvement of microtubular disruption in methyl mercury induced apoptosis in neuronal and non neuronal cell lines. *Toxicol. Appl. Pharmacol.*, **160**, 279–288.
5) Kunimoto, M. and Suzuki, T. (1995) Selective down-regulation of 440 kDa ankyrin$_B$ associated with neurite retraction. *Neuroreport*, **6**, 2545–2548.
6) Kunimoto, M. (2000) A neuron-specific isoform of brain ankyrin, 440 kD ankyrin$_B$, as a useful tool in neurobiology and neurotoxicology. *J. Health Sci.*, **46**, 178–181.
7) Pramanik, R., Ishido, M. and Kunimoto, M. (2001) Effects of cadmium chloride on neurite outgrowth and gene expression in human NB-1 cells. *J. Health Sci.*, **47**, 478–482.
8) Pramanik, R., Ueno, A., Nishikawa, H., Nagata, T. and Inoue, H. (1996) Osteotropic factor-stimulated synthesis of thrombospondin in rat dental pulp cells. *FEBS Lett.*, **393**, 193–196.

9) Six, D. A. and Dennis, E. A. (2000) The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. *Biochim. Biophys. Acta*, **1488**, 1–19.
10) Verity, M. A., Sarafian, T., Pacifici, E. H. K. and Sevanian, A. (1994) Phopholipase A_2 stimulation by methyl mercury in neuron culture. *J. Neurochem.*, **62**, 705–714.
11) Sarafian, T. A., Bredesen, D. E. and Verity, M. A. (1996) Cellular resistance to methyl mercury. *Neurotoxicology*, **17**, 27–36.
12) Sapirstein, A. and Bonventre, J. V. (2000) Phospholipase A_2 in ischemic and toxic brain injury. *Neurochem. Res.*, **25**, 745–753.
13) Sadekova, S., Lehnert, S. and Chow, T. Y. (1997) Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: a possible role in induced radio-resistance. *Int. J. Radiat. Biol.*, **72**, 653–660.
14) Takano, S., Wadhwa, R., Yoshii, Y., Nose, T., Kaul, S. C. and Mitsui, Y. (1997) Elevated levels of mortalin expression in human brain tumors. *Exp. Cell Res.*, **237**, 38–45.
15) Rossi, A. D., Ahlbom, E., Ogren, S. O., Nicotera, P. and Ceccatelli, S. (1997) Prenatal exposure to methylmercury alters locomotive activity of male but not female rats. *Exp. Brain Res.*, **117**, 428–436.