

*Original Article*

## Methylmercury induces release of a cytotoxic factor from HEK293 cells into medium

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**ABSTRACT** — HEK293 cells were cultured in medium containing methylmercury (MeHg), followed by replacement with MeHg-free medium and further culturing. Thus, MeHg preconditioning medium (MeHg-PM) were obtained. Untreated HEK293 cells and C17.2 cells (mouse neural stem cells) were placed in the obtained MeHg-PM for culturing, which resulted in significantly inhibited cell growth. This cell growth inhibition was not affected by heating or proteinase K treatment, suggesting that neither proteins nor peptides caused the growth inhibition.

**Key words:** Methylmercury, Toxicity, HEK 293 cells, C17.2 cells

### INTRODUCTION

Methylmercury (MeHg) is a common environmental pollutant. In particular, this compound can accumulate in fish at relatively high concentrations. MeHg crosses the blood-brain barrier and accumulates in the brain, causing neuronal cell death and leading to central nervous system disorders (Harada, 1995; Simmons-Willis *et al.*, 2002). However, the underlying molecular mechanisms that result in toxicity are almost unknown. In recent years, some authors have reported that increased levels of extracellular glutamate, aspartic acid, and serotonin, among others, are involved in MeHg-induced neuronal cell death (Brookes and Kristt, 1989; Dave *et al.*, 1994; Allen *et al.*, 2001). However, little is known about extracellular factors involved in MeHg toxicity. Using cultured cell lines, this study examined the possible existence of a factor that is driven out of the cell by MeHg and subsequently shows cytostatic activity (cytotoxicity-inducing extracellular factor, CIEF).

### MATERIALS AND METHODS

#### Materials

Methylmercuric chloride (MeHgCl) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alamer blue was purchased from Invitrogen (Carlsbad, CA, USA). InartSep

C18 was obtained from GL Science (Tokyo, Japan). All other reagents used were of the highest grade available.

#### Cells and cell culture

Human embryonic kidney HEK293 cells, mouse neural stem C17.2 cells, mouse microglial BV2 cells and human cervical cancer HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 0.3% L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were maintained at 37°C in a humidified incubator under an atmosphere of CO<sub>2</sub> (5%) and room air (95%).

#### Preparation of MeHg preconditioning medium (MeHg-PM)

HEK293 cells were cultured in a 10 cm dish at a cell concentration of 6 × 10<sup>6</sup> cells/dish, and treated with MeHgCl for 2 hr. The cells were washed twice with PBS, transferred to fresh medium, and incubated for another 6 hr to release CIEF into the medium. The medium was collected and filtered using a syringe filter (0.2 µm pore size) (Corning, NY, USA) to remove cell debris. Collected medium was stored at -20°C before use.

#### Measurement of CIEF induced cell death

Cell viability was determined by alamer blue assay. C17.2 cells were seeded at a density of 5 × 10<sup>3</sup> cells/

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well/100  $\mu\text{L}$  of DMEM on a 96 well plate and incubated for 24 hr. Next, an aliquot of MeHg-PM (100  $\mu\text{L}$ ) was added and cells were incubated for another 48 hr. Subsequently, culture medium was replaced with Alamer blue solution (Invitrogen, CA, USA) and fluorescence was measured (Ex: 545 nm, Em: 590 nm) following a final 2 hr incubation.

### Measurement of mercury concentration

Mercury concentration (in MeHg-PM) was determined by atomic absorption spectrometry, using an RA-915+ Portable Mercury Vapor Analyzer (Twinsburg, OH, USA).

### Fractionation of MeHg-PM by ODS column

An aliquot of MeHg-PM (10 mL) was loaded onto a C18 (ODS) solid phase extraction column (GL Science, Tokyo, Japan), which was equilibrated using DDW. Next, the CIEF fraction was eluted using 20 mL of DDW, 10 mL of 20% methanol and 10 mL of 80% methanol. The collected fractions were freeze-dried, and the resi-

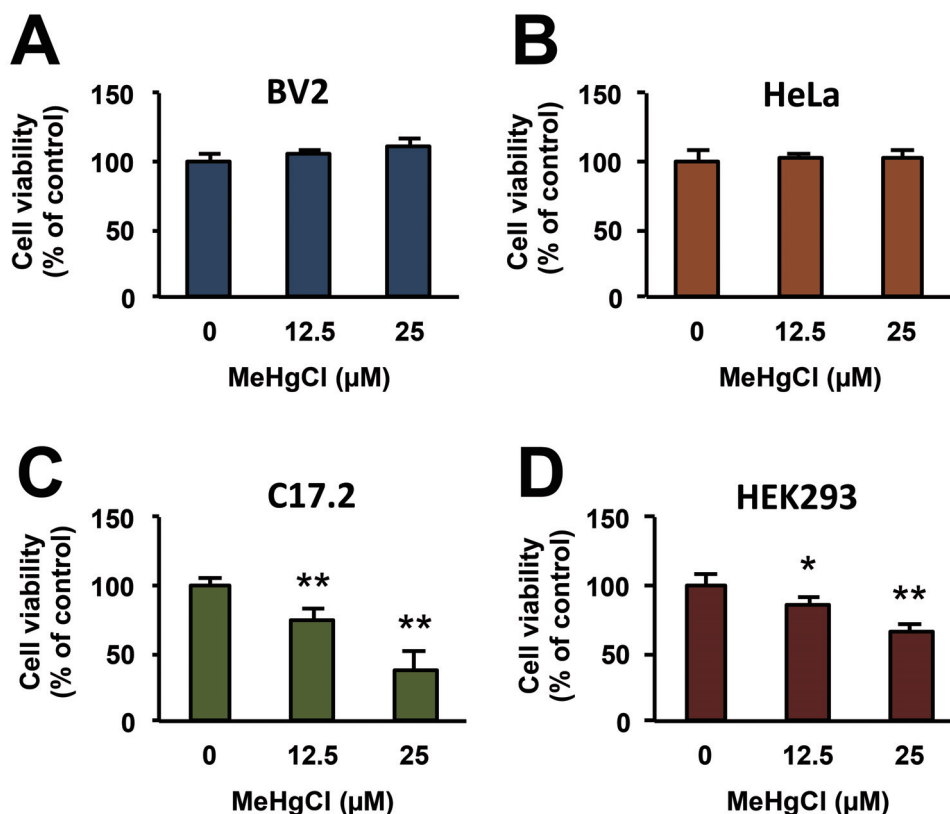
dues were re-dissolved into 10 mL of DMEM.

### Statistical analysis

Statistical significance was assessed by *t*-test. All *p* values are two-tailed.

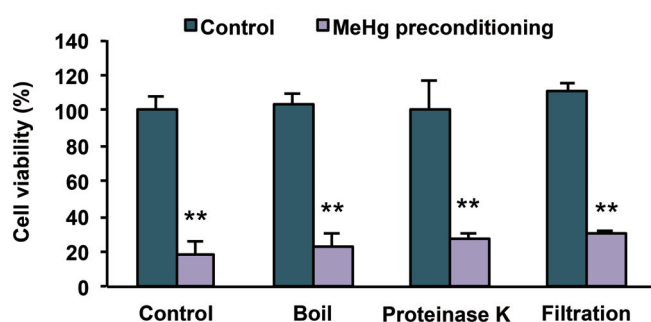
## RESULTS AND DISCUSSION

To confirm the existence of CIEF that is driven out of the cell by MeHg, we prepared MeHg preconditioning medium (MeHg-PM) according to the following protocol. HEK293 cells were cultured in medium containing MeHgCl for 2 hr. After washing with PBS, the cells were further cultured for 6 hr in fresh MeHg-free medium. The resulting culture medium was used as MeHg-PM. We placed HEK293 cells, C17.2 cells, BV2 cells and HeLa cells in the prepared MeHg-PM for culturing. The BV2 and HeLa cells showed almost normal growth, whereas the HEK293 and C17.2 cells showed significantly inhibited growth (Fig. 1). These results suggest that treating



**Fig. 1.** Release of CIEFs from the cells exposed to MeHg. HEK293 cells were treated with MeHgCl (0, 12.5 and 25  $\mu\text{M}$ ) for 2 hr, and the MeHg-PM was collected according to the protocol outlined in Materials and Methods. Indicated cell lines (BV2 (A), HeLa (B), C17.2 (C) and HEK293 (D)) were cultured in MeHg-PM and incubated for 48 hr. Cytotoxicity was subsequently determined by Alamer blue assay. Mean  $\pm$  S.D.,  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ .

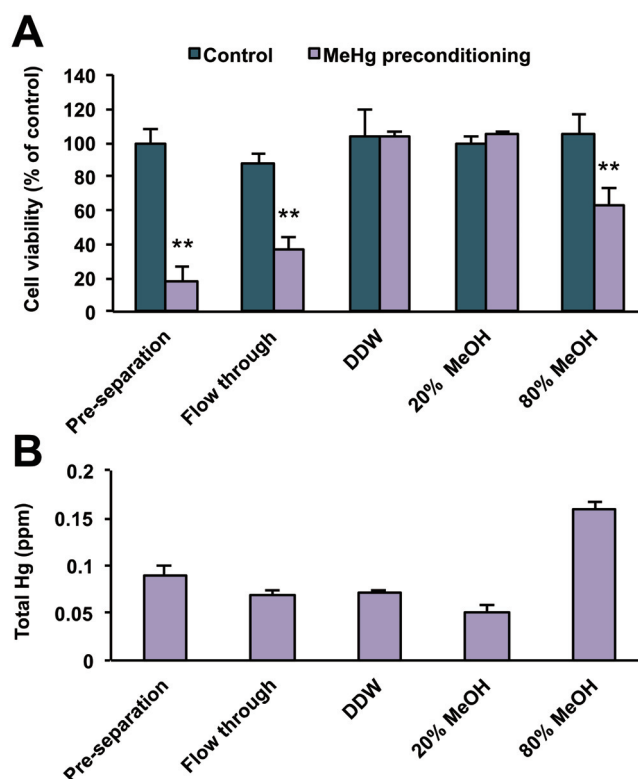
## Methylmercury induces release of a cytotoxic factor



**Fig. 2.** Cytotoxicity of MeHg-PM with proteins and peptides removed. HEK293 cells were treated with MeHgCl (25  $\mu$ M) for 2 hr, and MeHg-PM was collected according to protocol outlined in Materials and Methods. The MeHg-PM was incubated at 95°C for 15 min (Boil). Proteinase K (0.8 U/mL) was added to the MeHg-PM, followed by incubation at 37°C for 1 hr and a final incubation at 95°C for 15 min to terminate the reaction (Proteinase K). The MeHg-PM was filtered using an ultrafiltration membrane (molecular weight 3,000 cut off) (Filtration). C17.2 cells were cultured in the media for 48 hr, and the cytotoxicity was subsequently determined. Mean  $\pm$  S.D., n = 3; \*\*P < 0.01.

HEK293 cells with MeHg promotes the release of a CIEF from the cells, and this CIEF exerts a cytostatic effect on HEK293 and C17.2 cells. MeHg has been thought to show toxicity via inhibition of enzyme functions, for example. However, the present study shows that the toxicity of MeHg is based on a cytostatic factor that is driven out of the cell by MeHg, rather than the MeHg itself. This provides a new perspective for clarifying the toxicity mechanism of MeHg.

Next, we examined the properties of the CIEF that is driven out of the cell into culture medium by MeHg. The cytostatic effect of the MeHg-PM was not eliminated via heating or treatment with proteinase K (Fig. 2). This suggests that the CIEF contained in the MeHg-PM is neither protein nor peptide. In addition, the CIEF passed through a filter with a molecular weight cutoff of 3,000 Daltons (Fig. 2). These results suggest that the CIEF is a heat-stable, low molecular weight compound. When the MeHg-PM was fractionated on an ODS column, the CIEF did not bind to the column but appeared in the flow-through fraction (Fig. 3A). Meanwhile, a fraction eluted with 80% MeOH also showed cytostatic activity (Fig. 3A), which is probably attributable to a relatively high concentration of residual MeHg in the fraction (Fig. 3B). Further detailed studies successfully identifying the CIEF will represent



**Fig. 3.** Fractionation of MeHg-PM and the cytotoxicity of each fraction. MeHg-PM was loaded onto an ODS solid phase and extracted with DDW, 20% MeOH and 80% MeOH. Solvent of each fraction were removed and dissolved into DMEM. C17.2 cells were cultured in media for 48 hr, and the cytotoxicity was subsequently determined (A). The mercury concentration in each fraction was examined (B). Mean  $\pm$  S.D., n = 3; \*\*P < 0.01.

a breakthrough in clarifying the toxicity mechanism of MeHg.

#### ACKNOWLEDGMENTS

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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