Original Article

Methylmercury induces caspase-dependent apoptosis and autophagy in human neural stem cells

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ABSTRACT — Methylmercury (MeHg) is a well-known human neurotoxic agent whose exposure sources are mainly environmental and aquatic-derived food. MeHg is reported to induce central nervous system disability. However, the exact mechanism of MeHg-induced neurotoxicity is still unknown. In this study, to investigate which cell death signaling pathway is related with MeHg-induced cytotoxicity, the effects of MeHg on apoptosis and autophagy were evaluated in HB1.F3 human neural stem cells (NSCs). Human NSCs were treated with 1 μ M of MeHg for 48 hr and the effect of MeHg on cell signaling pathway was elucidated. MeHg inhibited Akt1/mTOR signaling that led to induction of caspase-dependent apoptosis and autophagy in the NSCs. Furthermore, retinoic acid (RA)-induced neuronal differentiation was inhibited by MeHg. Taken together, these results suggest that MeHg inhibits the differentiation of human NSCs by induction of caspase-dependent apoptosis and autophagy.

Key words: Methylmercury (MeHg), Human neural stem cell (NSC), Apoptosis, Autophagy

INTRODUCTION

Methylmercury (MeHg) is a well-known human neurotoxic agent. The routes of exposure are mostly environmental and dietary sources through aquatic system. The central nervous system (CNS) is the most sensitive target organ for MeHg, when exposures occur during the initial stages of brain development (Grandjean and Herz, 2011; Hassan *et al.*, 2012). Therefore, MeHg is able to induce CNS disability (Clarkson *et al.*, 2003) that leads to developmental deficits such as loss of intelligence quotient (IQ) points, reduction in language skills, impaired memory function and attention deficits in children exposed in-utero (Rice *et al.*, 2003). In earlier studies, MeHg was found to induce apoptosis in cerebellar neurons at low concentration while at higher concentration MeHg induced necrosis in neurons (Castoldi *et al.*, 2000; Daré *et al.*, 2000). In apoptotic cell death, cell shrinkage, plasma membrane

blebbing, activation of caspases, chromatin condensation and DNA fragmentation can be observed, whereas necrotic cell death can be characterized by cell and organelle swelling, loss of plasma membrane integrity and cell lysis (Orrenius *et al.*, 2003). MeHg can also activate p38 mitogen-activated protein kinase (MAPK) and c-Jun NH₂-terminal kinase (JNK) signaling cascades belonging to the stress-activated protein kinase (SAPK) family (Kim and Sharma, 2004) and oxidative stress-mediated extracellular signal-regulated kinase (ERK)1/2 (Lu *et al.*, 2011; Farina *et al.*, 2011). However, the exact mechanism(s) of MeHg-induced neurotoxicity still remains to be elucidated.

Apoptosis and autophagy are two closely related systems that induce cell death. Autophagy is a catabolic process involving the degradation of unmodified proteins and organelles through the lysosomal machinery. It is reported to be implicated in physiological cell death during development (Berry and Baehrecke, 2007). Autophagy is also

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positively regulated by phosphatase and tensin homolog (PTEN) and negatively regulated by oncogenic class I phosphoinositol-3-kinase (PI3K) signaling (Arico *et al.*, 2001; Furuta *et al.*, 2004). Autophagic cell death is morphologically characterized by an intact nucleus and the accumulation of cytoplasmic double-membrane autophagic vacuoles (Baehrecke, 2002; Reggiori and Klionsky, 2005). Autophagy-related gene (Atg) 6 (also known as beclin-1 and BECN1) is one of the essential components involved in autophagosome formation, and it displays increased expression during autophagy (Kondo *et al.*, 2005; Maiuri *et al.*, 2007). Microtubule-associated protein light chain 3 (LC3) is an autophagosome marker. Conversion of its cytosolic form, LC3-I, to its autophagic membrane form, LC3-II, is another key step in the induction of autophagy (Mizushima *et al.*, 2002; Kuma *et al.*, 2004). Therefore, an increase in the ratio of LC3-II to LC3-I coincides with autophagosome formation (Chang *et al.*, 2012).

In the present study, we investigated the effects of MeHg on human neural stem cells (NSCs) by using F3 immortalized human NSC line. We focused on the mechanisms of MeHg-induced cell death in human NSCs, as well as on the effects of non-cytotoxic levels of MeHg on differentiation of human NSCs.

MATERIALS AND METHODS

Chemical, reagents and antibodies

Methylmercury (II) chloride (MeHg) and retinoic acid (RA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and caspase-3/7 inhibitor I from CalBiochem (La Jolla, CA, USA). Anti-actin, anti-BECN1, and anti-poly ADP ribose polymerase (PARP) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nestin, and anti-glial fibrillary acidic protein (GFAP) were purchased from Millipore (Billerica, MA, USA) and MAP1LC3 (LC3) was purchased from Abgent (San Diego, CA, USA). Antibodies against mTOR, p53, cleaved-caspase 3, cleaved-caspase 7, and cleaved-PARP were purchased from Cell Signaling Technology (Beverly, MA, USA), and Akt1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from AbFrontier (Seoul, Korea).

Cell culture and MeHg treatment

HB1.F3 (F3) human NSCs have been described previously (Cho *et al.*, 2002). F3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4,500 mg/l) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium and

100 µg/ml streptomycin sulfate (GibcoBRL, Grand Island, NY, USA) in a humidified incubator in an atmosphere of 5% CO₂ at 37°C and subsequently cultured with or without MeHg. Dimethyl sulphoxide (DMSO) was used as a vehicle control.

Cell viability

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, 5×10^3 cells were placed into well of a 96-well plate and incubated with or without MeHg for 48 hr. After incubation, 10 µl of CCK-8 solution was added to each well to a final volume of 100 µl of culture medium containing viable cells and an additional incubation for 1 hr. Spectrophotometric absorbance at 450 nm was measured using a Bio-Rad Model 680 microplate reader (Bio-Rad).

Microscopy and photography

Cells were examined using a CK2 inverted microscope (Olympus, Tokyo, Japan) equipped with a model PC1015 digital camera (Canon, Tokyo, Japan).

Transmission electron microscopy (TEM)

F3 cells were fixed with a solution of 2.5% glutaraldehyde with 1% osmium tetroxide (OsO₄) buffer for 2 hr at 4°C and dehydrated with ethanol at 4°C. Then, cells were infiltrated in a 1:1 mixture of propylene oxide and Epon, and finally embedded in Epon by polymerization at 70°C for 24 hr. Ultrathin sections (40–70 nm) were cut and mounted on pioloform-coated copper grids. Sections were stained with lead citrate and uranyl acetate and viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

Western blot analysis

F3 cells were cultured with or without MeHg for 48 hr. Cells were lysed using Cell Culture Lysis 5 X Reagent (Promega, Madison, WI, USA) containing *Xpert* protease inhibitor cocktail solution (GenDEPOT, Barker, TX, USA), and transferred supernatant to a new tube after centrifugation at $10,000 \times g$ for 15 min at 4°C. After measuring the protein concentration of the lysates using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA), 25 µg of protein were separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered Saline containing tween-20 (TTBS) containing 5% skim milk for 1 hr at room temperature and immunoblotting was performed by incubation overnight at 4°C with the corresponding primary antibodies in 5% skim milk and then

with secondary antibodies conjugated to horseradish peroxidase for 1 hr at room temperature. After washing, the bands of interest were visualized using a model LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Quantification of Western blot analysis was conducted using the Multi Gauge version 3.0 program (Fujifilm).

4', 6-Diamidino-2-phenylindole (DAPI) staining and immunostaining

F3 cells (1×10^4) were seeded in eight-well chamber slides (BD BioCoat Poly-D-Lysin/Laminin; BD, Franklin Lakes, NJ, USA) and subsequently cultured with or without MeHg for 48 hr. Slides were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 10 min at 37°C. Slides were washed and fixed again with methanol and acetone in a volume ratio of 1:1. After being blocked with 3% bovine serum albumin (BSA) in TTBS for 1 hr, cells were incubated with a 1:200-1:500 dilution of primary antibody overnight at 4°C, washed, and incubated with a 1:200 dilution of Alexa Fluor 488-conjugated secondary antibody for 2 hr at room temperature. After washing, coverslips were mounted using FLUOROSHIELD™ mounting medium with DAPI (ImmunoBioScience, Mukilteo, WA, USA). The slides were visualized using a fluorescent microscope (Carl Zeiss, Jena, Germany).

Flow cytometry analysis

F3 cells (1×10^6) were seeded in T25 flask and subsequently cultured without or with MeHg (1 μ M). Forty-eight hours after MeHg treatment, cells were processed with an EzWay™ Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (KomaBiotec, Seoul, Korea) according to the manufacturer's instructions. Trypsinized cells (5×10^5) were washed with 0.5 ml cold PBS, and remove PBS by centrifugation at $1,000 \times g$ for 5 min at room temperature (RT). Washed cells were suspended with 0.5 ml cold $1 \times$ Binding buffer, and added 1.25 μ l of Annexin V-FITC and incubated for 15 min at RT in the dark. After removed the supernatant by centrifugation at $1,000 \times g$ for 5 min at RT, cells were washed with 0.5 ml cold $1 \times$ Binding buffer and added 10 μ l PI. The stained cells (1×10^4) with Annexin V-FITC and PI were analyzed by fluorescence-activated cell sorting (FACS) using a FACSCalibur apparatus (BD Bioscience, San Jose, CA, USA).

RESULTS

Effects of MeHg on human NSC proliferation and morphology

Human NSCs were treated with different concentrations (0.01, 0.05, 0.1, 0.5 and 1 μ M) of MeHg for 48 hr and the effect of MeHg on cell viability was measured. MeHg reduced viable cells in a dose-dependent manner. After 48 hr, treatment of cells with 1 μ M MeHg resulted in about 82% decrease in cell viability. However, at concentrations below 0.5 μ M, MeHg only marginally decreased cell viability compared to non-treated control cells (Fig. 1).

To determine the effect of MeHg on morphology of human NSCs, images of cells were taken by a digital camera at $100 \times$ magnification. As shown in Fig. 2A, cell shrinkage was evident in MeHg-treated cells compared with control or vehicle (DMSO)-treated cells. Because actin is involved in apoptotic cell death and cell shrinkage (Kayalar *et al.*, 1996; Pedersen *et al.*, 1999), the effect of MeHg on actin filaments in F3 cells was assessed by Western blot and immunofluorescence analyses. MeHg changed the pattern of actin filaments in F3 cells. However, the quantity of total actin remained unchanged (Figs. 2B and 2C). To determine whether neuronal differentiation of these cells by MeHg was affected, Western blot analysis of the stem cell marker Nestin was performed. The result showed that the level of Nestin remained unchanged in the cells following MeHg treatment (Fig. 2B).

Effect of MeHg on apoptosis in human NSCs

To determine the effect of MeHg on apoptosis and

Fig. 1. Effects of MeHg on cell viability of F3 human NSCs. F3 cells (5×10^3 cells/well) were treated with various concentrations (0-1 μ M) of MeHg for 48 hr. Cell viability was measured using a CCK-8 ($n = 10$). Values are the means $\pm$ S.E.M.

Fig. 2. Effects of MeHg on cell morphology of F3 human NSCs. (A) Morphological change of cells after treatment with MeHg. F3 cells were treated with various concentrations (0.1, 0.5 and 1 μM) of MeHg for 48 hr, and observed under 100 × magnification. (B) Western blot analysis of Nestin and Actin. Cells were treated with MeHg (0.1, 0.5 and 1 μM) for 48 hr and harvested, then cell lysates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (C) Immunofluorescence assay of Actin in F3 cells treated with MeHg. Cells were incubated for 48 hr, followed by fixing and immunostaining for Actin (green via Alexa Fluor 488) and nuclei (blue via DAPI). The scale bar represents 20 μm.

Fig. 3. Dose-dependent effects of MeHg on Akt1/mTOR expression. Western blot analysis of Akt1, mTOR, and p53. F3 cells were treated with various concentrations (0.1, 0.5 and 1 μM) of MeHg for 48 hr, and cell lysates were subjected to Western blot analysis. Blots were probed with antibodies as indicated.

autophagy in F3 NSCs, Western blot analysis for Akt1/mTOR, the upstream signal of apoptosis or autophagy, was performed. A dose-dependent decrease in Akt1 and mTOR expression levels was evident. In contrast p53 expression in MeHg-treated cells was increased (Fig. 3). Because p53 has a crucial role in cell cycle arrest and induction of apoptosis, the expression levels of apoptosis related proteins were investigated in MeHg-treated or control F3 cells. The expression levels of proteins involved in caspase-dependent apoptosis (c-caspase 3 and c-caspase 7) were increased by MeHg. As well, an increase in c-PARP protein level was evident in cells treated with MeHg (Fig. 4A). To confirm the induction of caspase-dependent apoptosis by MeHg, inhibitor assay was performed and apoptosis was clearly reduced (Fig. 4B). To further confirm the MeHg-mediated induction of apopto-

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Fig. 4. Effects of MeHg on apoptosis of F3 human NSCs. (A) Western blot analysis of apoptosis-related proteins. F3 cells were treated with 1 μ M MeHg for 48 hr, and cell lysates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (B) Inhibition of caspase-dependent apoptosis by caspase-3/7 inhibitor. F3 cells were pretreated with caspase-3/7 inhibitor I (180 nM) for 4 hr, and treated with 1 μ M MeHg for 48 hr, and then cell lysates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (C) Annexin V-FITC staining after MeHg treatment. F3 cells were treated with 1 μ M MeHg and cultures for 48 hr, and each group of cells stained with Annexin V-FITC and PI, then analyzed by flow cytometry. (D) Diagram of Annexin V-FITC staining. It represents the percentage of apoptosis of F3 cells.

sis, FACS analysis was performed. In F3 cells treated with MeHg, early apoptosis (Annexin V-positive and PI-negative) (Fig. 4C; lower right) and late apoptosis (Annexin V-positive and PI-positive) (Fig. 4C; upper right) were clearly increased compared with control or vehicle-treated cells (Fig. 4C). Fig. 4D is a diagram which represents the percentage of apoptosis of F3 cells. MeHg-treated cells have increased compared to non-treated group.

Effect of MeHg on autophagy in HB1.F3 human NSCs

To investigate the effect of MeHg on autophagy, Western blot analysis was carried out in F3 cells. MeHg increased the protein level of BECN1 and the ratio of

LC3-II to LC3-I at 48 hr (Fig. 5A). The increase in LC3-II protein level was further confirmed by immunofluorescence analysis. The number of LC3 dots was clearly increased in MeHg-treated cells (Fig. 5B). Furthermore, the effect of MeHg on autophagy was detected by transmission electron microscopy. Large numbers of autophagic vesicles (AVs) were found in MeHg-treated cells compared with control and vehicle-treated cells (Fig. 5C).

Effect of MeHg on neural differentiation in human NSCs

To induce the neural phenotype, F3 cells were cultured with RA in the presence or absence of MeHg for 48 hr. A morphological change was observed when cells treat-

Fig. 5. Effects of MeHg on autophagy of F3 human NSCs. (A) Western blot analysis of BECN1 and LC3. F3 cells were treated with 1 μ M MeHg for 48 hr, and cell lysates were subjected to Western blot analysis. Blots were probed with antibody of BECN1 or LC3. (B) Immunofluorescence of LC3 in MeHg-treated, vehicle-treated or control F3 cells. Cells were treated with 1 μ M MeHg for 48 hr, followed by fixing and immunostaining for LC3 (green via Alexa Fluor 488) and nuclei (blue via DAPI). The scale bar represents 10 μ m. (C) TEM analysis of MeHg-treated cells. F3 cells were treated with 1 μ M MeHg for 48 hr, fixed and analyzed via TEM. The scale bars represents 2 μ m. Nu denotes nucleus and * denotes autophagic vesicle.

ed with RA (Fig. 6A). To confirm the neuronal differentiation of these cells, Western blot analyses of the stem cell marker Nestin and the neural marker GFAP were performed after treatment of cells with MeHg. Differentiated cells induced by RA showed decreased expression level of Nestin compared with undifferentiated cells whereas, decreased expression level of Nestin was not observed in differentiated cells treated with MeHg. The opposite pattern was observed for GFAP expression. The expression level of GFAP in differentiated cells induced by RA was

increased compared with undifferentiated cells. However, MeHg-treated cells did not show such pattern of change compared to differentiated cells (Fig. 6B).

DISCUSSION

MeHg is a potent neurotoxicant that affects both the developing and mature central nervous system (Costa *et al.*, 2004; Atchison, 2005; Johansson *et al.*, 2007; do Nascimento *et al.*, 2008; Grandjean and Herz, 2011).

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Fig. 6. Effects of MeHg on differentiation of F3 human NSCs. (A) Morphological change of cells after differentiation. F3 cells were treated with 1 μ M RA in the presence or absence of 1 μ M MeHg and observed under 100 $\times$ magnification. (B) Western blot analysis of Nestin and GFAP. F3 cells were treated with 1 μ M MeHg for 48 hr, harvested, and the cell lysates were subjected to Western blot analysis. Blots were probed with antibody to Nestin or GFAP.

MeHg causes widespread and diffuse damage in infants, whereas in the adult brain MeHg causes focal damage. Exposure to MeHg is more dangerous to the fetus. Fetuses exposed in utero can be affected in the absence of maternal toxicity (Matsumoto *et al.*, 1965; Choi *et al.*, 1978; Takeuchi, 1982). Damage pattern of MeHg depends on the stage of development *when* the exposure occurs, as well as duration and concentration (Rodier, 1995). In the present study, we used human NSCs as a model system of fetal CNS to investigate neurotoxic effects of MeHg and mechanisms underlying these effects.

Exposure to MeHg can cause necrotic or apoptotic cell death in CNS neurons. Apoptotic cell death is triggered by receptor-mediated or mitochondria-mediated signaling

pathways that induce death-associated proteolytic and/or nucleolytic activities (Orrenius *et al.*, 2003). MeHg elicits mitochondrial-dependent apoptosis in developing rat hippocampal neurons and C17.2 mouse NSC line (Tamm *et al.*, 2006; Sokolowski *et al.*, 2011). However, several alternative cell death pathways including caspase-independent programmed cell death (PCD), necrosis-like PCD, and mitotic or autophagic cell death are present (Leist and Jäättelä, 2001; Jäättelä, 2002; Edinger and Thompson, 2004; Gozuacik and Kimchi, 2004; Levine and Yuan, 2005). Autophagy constitutes a stress adaptation that avoids cell death during nutrient starvation and which suppresses apoptosis (Maiuri *et al.*, 2007), or constitutes an alternative cell-death pathway (Edinger and

Thompson, 2004; Gozuacik and Kimchi, 2004). Autophagy and apoptosis are triggered by common upstream signals, sometimes resulting in combined autophagy and apoptosis, whereas cells switch between the two responses in a mutually exclusive manner (Baehrecke, 2005; Kroemer and Jäätelä, 2005). In a previous study, we demonstrated significantly increased autophagy in A549 lung cancer cells having stably elevated *BECN1* expression, while decreased levels of apoptosis-related proteins such as Bax and cleaved PARP were observed (Chang *et al.*, 2012). In the present study, we also examined the effect of MeHg on apoptosis and autophagy. Our results clearly showed induction of caspase-dependent apoptosis and autophagy in MeHg-treated human NSCs.

Differentiated cells have morphological features that correlate with their specialized functions in organs. These special features include membrane protrusions such as bristles, microvilli, stereocilia, filopodia, and lamellipodia, which are formed and maintained by the underlying actin cytoskeleton (Revenu *et al.*, 2004). Microvilli and microvilli-like structures are observed in a variety of polarized cells. Cell polarity is observed in most differentiated cells, even prokaryotes. Although observed in different forms in diverse cell types and species, polarity is defined by two fundamental properties, the asymmetric accumulation of mobile components between opposite poles of a cell and the orientated organization of inherently polar cytoskeletal filaments (particularly actin and microtubules) along the axis of polarity (Li and Gundersen, 2008). Neurons are highly polarized cells that might stimulate axon formation by localized actin instability (Bradke and Dotti, 1999). Studies of embryonic rodent hippocampal neurons have provided information on the factors involved in specifying neuronal cell polarity (Wiggin *et al.*, 2005; Arimura and Kaibuchi, 2007). Hippocampal neurons initially extend multiple morphologically indistinguishable and undifferentiated neurites. Symmetry of cytoskeletal filaments is broken when one of the neurites begins to grow rapidly and acquires axonal markers, such as dephosphorylated tau and microtubule-associated protein (MAP), whereas the other neurites remain unchanged. Over time, the short neurites begin to grow and differentiate into dendrites by acquiring dendritic markers such as MAP2 (Kosik and Finch, 1987). Our results suggest that structural change observed in actin filaments may result in inhibition of morphology and cellular differentiation.

In conclusion, MeHg inhibits the differentiation of F3 human NSCs through induction of caspase-dependent apoptosis and autophagy.

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