Original Article

Characterization of antioxidant protection of cultured neural progenitor cells (NPC) against methylmercury (MeHg) toxicity

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(Received March 6, 2009; Accepted March 26, 2009)

ABSTRACT — Methylmercury (MeHg) is an environmental pollutant known to cause neurobehavioral defects and is especially toxic to the developing brain. With recent studies showing that fetal exposure to low-dose MeHg causes developmental abnormalities, it is therefore important to find ways to combat its effects as well as to clarify the mechanism(s) underlying MeHg toxicity. In the present study, the effects of MeHg on cultured neural progenitor cells (NPC) derived from mouse embryonic brain were investigated. We first confirmed the vulnerability of embryonic NPC to MeHg toxicity. NPC from the telencephalon were more sensitive to MeHg compared to those from the diencephalon. Buthionine sulfoximine (BSO) which is known to inhibit glutathione synthesis accelerated MeHg toxicity. Furthermore, antioxidants such as N-acetyl cysteine and α-tocopherol dramatically rescued the NPC from MeHg’s toxic effects. Interestingly, a 12 hr delay in the addition of either antioxidant was still able to prevent the cells from undergoing cell death. Although it is now difficult to avoid MeHg exposure from our environment and contaminated foods, taking anti-oxidants from foods or supplements may prevent or diminish the toxicological effects of MeHg.

Key words: Neural progenitor cell, Methylmercury, Oxidative stress, Antioxidant, Cell death

INTRODUCTION

Epidemiological surveys and clinical reports have steadily revealed that exposure during development to chemicals such as methylmercury (MeHg), polychlorinated biphenyls (PCBs), alcohol and tobacco smoke might be associated with lasting neurological effects such as cognitive deficits, and attention-deficit hyperactive disorder (ADHD) (Linnet et al., 2003; Moore et al., 2000; Strömland et al., 1994; Rogan et al., 1988; Thapar et al., 2003; Yolton et al., 2005). MeHg is now not only an issue due to outbreaks of poisoning in Japan (Minamata), Iraq and Peru, but also in people who eat fish on a regular basis (Davidson et al., 2004). Finding ways to avoid the toxic effects of low dose, chronic exposure to MeHg has become a worldwide health priority.

Pregnant women exposed to MeHg by virtue of the consumption of contaminated fish, for example, expose their fetus to the toxic effects of this compound. MeHg is known to preferentially accumulate in fetal tissues, including the brain (Sakamoto et al., 2002). MeHg-conjugated with L-cysteine shows molecular mimicry to L-methio...
nine, and consequently is able to rapidly cross the placenta via the neutral amino acid carrier system (Aschner, 2002; Kajiwara et al., 1996). Furthermore, as MeHg has a high affinity for fetal hemoglobin, levels of MeHg in fetal blood can be up to 25% higher than in the mother.

Minamata disease shows an age-dependent pattern of neuronal injury with marked anatomical selectivity of pathologic involvement. In adult Minamata disease, disorders of the occipital cortex and cerebellum are most prominent. In fetal Minamata disease, the entire cerebral and cerebellar cytoarchitecture is disrupted. Clinical studies suggested the selective sensitivity to MeHg of cortical or cerebellar immature progenitor cells (Amin-Zaki et al., 1976). It has been shown recently that neural progenitor cells (NPC) are more sensitive to MeHg than mature differentiated neural cells, and that MeHg induces apoptotic cell death at low doses (10^{-8}-10^{-6} M) via caspase3 and calpain activation (Tamm et al., 2006). With respect to its molecular mechanisms, many studies have revealed that MeHg toxicity is caused via oxidative stress in both neuronal and non-neuronal cells (Ercal et al., 2001; Kidd, 2005; Valko et al., 2005; Ahmad, 1995), thus raising the possibility that antioxidants inhibit MeHg toxicity.

In the present study, we first confirmed that 10^{-8}-10^{-6} M MeHg caused cell death to NPC (tNPC) isolated from the telencephalon. Notably, 10^{-7} M MeHg caused delayed cell death. Furthermore, we investigated the effects of the glutathione synthesis inhibitor, buthionine sulfoximine (BSO), on MeHg toxicity. We found that the reduction of this low molecular antioxidant accelerated cell death. These data encouraged us to characterize the protective effects of antioxidants. As antioxidants are consumed in the diet, we considered it important to investigate the protective effects of these compounds, as well as to consider the timing of their ingestion. We analyzed the protective effects of both pre- and delayed-delivery of antioxidants as a form of combating MeHg-induced cell death.

MATERIALS AND METHODS

Reagents

Dulbecco’s modified eagle medium (DMEM)/F12, insulin, apotransferrin, progesterone, putrescine, sodium selenite, poly-L-ornithine, fibronectin, DNase I, L-thyroxine and Hoechst 33342 were purchased from Sigma Co. (St. Louis, MO, USA). DMEM, Eagle’s Minimum Essential Medium (EMEM), RPMI 1640 medium, antioxidant antimycotic solution and fetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA). Human basic fibroblast growth factor (bFGF) and papain were purchased from R & D systems (Minneapolis, MN, USA) and Worthington Biochemical Corp (Lakewood, NJ, USA), respectively. BSO was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture

The culture of NPC was carried out as previously described (Watanabe et al., 2006). In brief, telencephalons or diencephalons were prepared from ICR mice embryos (E14.5) and gently dissociated by pipetting. The dissociated cells, which included NPC, were then seeded onto culture dishes pre-coated with 15 μg/ml poly-L-ornithine and 1 μg/ml fibronectin. The cells were cultured in DMEM/F12 medium containing 25 μg/ml insulin, 100 μg/ml human apotransferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 1% antibiotic antimycotic solution (Invitrogen) and 10 ng/ml recombinant human bFGF. The cells were passaged (5 x 10^5 cells/cm^2) after 4 days. One day after passage, the cells were exposed to 0.01-10 μM MeHg in the presence of bFGF for 2 days or 6 days. For long-term culture (6 days), the culture medium was changed and fresh MeHg was added every 2 days.

The serum-free cerebellar granule cell culture method used was a slightly modified version of the method of Fischer (Fischer, 1982) and Kimura-Kuroda et al. (2002). The cerebellum from newborn ICR mice (postnatal day 7) was freed from the meninges and cut sagittally into 2 pieces. The cerebella were then digested with 0.2 units/ml papain in PBS containing 0.2 mg/ml DL-cysteine, 0.2 mg/ml BSA, 5 mg/ml glucose and 0.02% DNase 1 for 25 min at 37°C. After incubation, the cerebella were gently dissociated using a fire-polished Pasteur pipette, and the cells then centrifuged and re-suspended in DMEM/F12 twice. The cell suspension was then passed through a mesh filter to remove large aggregates. Cells were re-suspended in serum-free DMEM/F12 medium containing 25 μg/ml insulin, 100 μg/ml human apotransferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 1 mg/ml fatty acid-free BSA, antioxidant antimycotic solution and 5 mM L-thyroxine. The cells were exposed to 0.01-1 μM MeHg for 2 days.

DMS114 human lung cancer cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum. PA1 human embryonic carcinoma cells and HT22 mouse hippocampal cells derived from the HT4 cell line were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum. IMR32 human neuroblastoma cells and neuro-2a mouse neuroblastoma cells were maintained in EMEM supplemented with 10% heat-inactivated fetal calf serum. All cells were seeded at a concentration of 4-5 x 10^4 cells/
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cm² and maintained in an atmosphere of 5% CO₂ at 37°C. One day after passage, the cells were exposed to 0.01-10 µM MeHg for 2 days.

Analysis of cell death

The number of live cells was determined using phase-contrast micrographs (Olympus BX50) based on cell morphology or with the use of a live-dead cell staining kit (BioVision Research Products, Mountain View, CA, USA). The live-dead cell staining was carried out as per the manufacturer’s instructions. Cell death was assessed by examination of nuclear morphology after staining with Hoechst 33342. Cells were stained with 10 µM Hoechst 33342 for 10 min at room temperature and examined under a fluorescence microscope (Olympus BX50). TUNEL staining was carried out with a specific kit (purchased from Takara Bio, Inc., Shiga, Japan) and performed as per the manufacturer’s instructions.

Statistical analysis

Experimental data represent the mean ± S.D. Statistical analyses were performed by using the Student’s t-test (Fig. 1), one-way ANOVA followed by a Bonferroni/Dunn multiple comparison test (Figs. 2, 3 and 5) and Turkey multiple comparison test (Fig. 4).

RESULTS

Cytotoxic effects of MeHg on neural progenitor cells, neural tumor cells and non-neural tumor cells

When exposed to MeHg, tNPC exhibited a shrunk appearance and few cells processes in phase-contrast micrographs (Fig. 1B). Shrunk and round-shaped cells were stained by propidium iodide (PI) (Figs. 1A and B), with these morphological changes considered to be indicative of cell death. Primary cultures of mouse tNPC, mouse NPC (dNPC) derived from diencephalons, and mouse cerebellar granule cells (CGC), were exposed to MeHg for 48 hr. CGC are known to be highly susceptible to MeHg toxicity (Kunimoto et al., 1992). Similarly to CGC, tNPC were not able to survive in the presence of 0.1 µM MeHg (Fig. 2A). On the other hand, as many dNPC did survive in the presence of 0.1 µM MeHg (Fig. 2A), these results show that tNPC are more sensitive to the effects of MeHg than are dNPC (Fig. 2B). As such, the severity of MeHg-induced cytotoxicity may depend on the origin of the cells examined.

The concentration dependency of the effects of MeHg on neural or non-neural tumor cell viability was also examined. tNPC showed the highest sensitivity to MeHg (Fig. 2C), with the 50% lethal concentration (LC₅₀) calculated for each cell type (Table 1) being three-fold lower for tNPC (0.056 µM) compared to dNPC (0.17 µM). Likewise, the LC₅₀ for tNPC was 3-40 times lower than that for neural tumor cells (0.19-2.3 µM), and more than 60 times lower than that for non-neural tumor cells (3.3-3.5 µM).

The extent of tNPC viablity was investigated (Fig. 2D) as a function of MeHg concentration in the range of 10 nM - 1 µM. Treatment with low concentrations of MeHg (10-25 nM) decreased the number of surviving tNPC after 6 days compared to control. Addition of more than 50 nM MeHg caused cell death to tNPC within 48 hr. Interestingly, the exposure of cells to 0.1 µM MeHg did not alter the number of viable cells in the first 24 hr, but did induce cell death thereafter. These results imply that there may be a time lag between exposure to MeHg and terminal MeHg-induced cell death.

Induction of cell death by MeHg

tNPC treated for 24 hr with 0.1 or 1 µM MeHg were stained with Hoechst 33342 to enable the nucleus to be visualized (Fig. 3A). Nuclear staining of tNPC exposed to MeHg showed chromatin condensation and nuclear fragmentation, in particular at 1 µM MeHg, and partially at 0.1 µM. Exposure of cells to MeHg also increased the number of TUNEL-positive tNPC (Fig. 3B).

Inhibition of glutathione synthesis promotes MeHg-induced cell death

tNPC treated for 24 hr with 0.1 µM MeHg did not show remarkable cell death until 24 hr (Fig. 2D). However, glutathione synthesis inhibitor, BSO, pretreated-tNPC caused cell death after 24 hr of culture with MeHg at 0.1 µM concentration (Fig. 4). BSO did not induce cell death without MeHg. Therefore, this data indicates that the glutathione plays an important role in resistance to MeHg toxicity.

Antioxidants rescue tNPC from MeHg-induced cell death

When used at a concentration of 0.1 µM, MeHg-induced cell death was delayed for as long as 24 hr (Fig. 2D). We investigated whether this time delay could be extended by exposing cells to antioxidants, given that previous reports have shown that MeHg induces cell death via the generation of reactive oxygen species (ROS) (Ally et al., 1984; Mori et al., 2007; Garg and Chang, 2006; Morken et al., 2005; Fonfria et al., 2002; Ahlbom et al., 2000; Atchison and Hare, 1994). Pre-treatment of tNPC with either of two antioxidants, N-acetyl cysteine (NAC)
MeHg induces morphological changes to, and cell death of, telencephalon-derived neural progenitor cells (tNPC). (A, B) tNPC were treated (B) with or (A) without 0.1 μM MeHg for 2 days and then stained using the live-dead staining kit. Dead cells were stained by PI, a red fluorescent dye that is cell impermeant in intact (live) cells, but can cross the disrupted membrane of dead cells. All cells stained with Live-Dye, a cell-permeable green fluorescent dye. Therefore, healthy cells show green fluorescence only (arrow head), while dead cells stained with both the cell-permeable Live-Dye and the cell impermeant PI, with the overlay of green and red giving the dead cells a yellow-red appearance (arrow). Cells stained by PI showed a shrunken morphology with few processes. Scale bars, 50 μm. (C) The numbers of live cells were counted on the basis of their morphology or using the live-death staining kit method. Each point represents the mean of six samples performed in two independent experiments. Vertical bars represent standard deviation of the mean. Statistical analyses were performed by Student's t-test. N.S. not significant.

Fig. 1
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Fig. 2. Effects of MeHg on primary cultured neural cells, neural tumor cells and non-neural tumor cells. (A) Phase-contrast micrographs of MeHg-treated tNPC, diencephalon-derived neural progenitor cells (dNPC) and cerebellar granular cells (CGC). tNPC, dNPC and CGC were incubated with or without MeHg (0.1 μM) for 2 days. Scale bar, 50 μm. (B, C) Effects of MeHg on cell viability. tNPC, dNPC and CGC in primary culture (B), and neuro-2a, IMR32, and HT22 cells (all neural tumor cells), and PA1 and DMS 114 cells (non-neural tumor cells) (C) were treated with various concentrations of MeHg for 2 days. The numbers of live cells were counted on the basis of their morphology. For tNPC and dNPC, each point represents the mean of 12 samples performed in 4 independent experiments. For the other samples, each point represents the mean of six samples performed in two independent experiments. (D) Effect of MeHg on tNPC viability. MeHg concentration-dependent cell death was analyzed by counting cell numbers over time. The numbers of live cells were counted on the basis of their morphology. Each point represents the mean of six samples performed in two independent experiments. Vertical bars represent standard deviation of the mean. (*) Statistical significance from tNPC is represented by asterisk(s) (*p < 0.05, **p < 0.005, ***p < 0.0005).
Fig. 3. Effects of MeHg on cell death in tNPC. (A, B) tNPC were treated with 0.1 μM or 1 μM MeHg for 24 hr and then stained with Hoechst 33342 (A) or TUNEL (B), as described in “Experimental Procedure”. The left column show a low magnification figures. Bar = 100 μm. The right column shows cells under high magnification. Bar = 20 μm.

Fig. 4. Cell death-promoting effect of BSO on MeHg toxicity. BSO (500 μM) was added 24 hr before the addition of 0.1 μM MeHg. After 24 hr of culture, the numbers of surviving cells were counted. The numbers of live cells were counted on the basis of their morphology. Each point represents the mean obtained from six distinct cultures. (*p < 0.05, **p < 0.005).
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Fig. 5. Cytoprotective action of antioxidants against MeHg-induced toxicity. NPC were cultured with 0.1 μM MeHg in the presence of NAC or α-tocopherol. (A) After 48 hr of culture, the number of surviving cells was counted. NAC (0.25-1 mM) and α-tocopherol (0.5-50 μM) were added 1 hr before the addition of 0.1 μM MeHg. (B) Antioxidants were added 1, 6, 12, 24 or 48 hr after the addition of 0.1 μM MeHg. (C) Following 1 hr treatment in the presence of 1 mM NAC, the culture medium was changed to normal medium (see top panel). (D) Cells were cultured with 0.1 μM MeHg for the indicated times and the culture medium then changed to normal medium. (E) Following treatment with 0.1 μM MeHg for the indicated times, the culture medium was changed to one containing 1mM NAC. (F) Cells were cultured in normal medium after the addition of both 1 mM NAC and 0.1 μM MeHg, as shown in panel. The numbers of live cells were counted on the basis of their morphology. Each point represents the mean obtained from six distinct cultures that were analyzed in two independent experiments. (*) Differs from control (***p < 0.05, ****p < 0.005, *****p < 0.0005). (†) Differs from the cells exposed to MeHg (0.1 μM) for 48 hr (†p < 0.05, ††p < 0.005, †††p < 0.0005).
or α-tocopherol, inhibited cell death in a dose-dependent manner (Fig. 5A). The addition of 1 mM NAC or 50 μM α-tocopherol strikingly rescued tNPC from cell death. Furthermore, even with a delay in delivery, these antioxidants were still able to reduce MeHg-induced toxicity (Fig. 5B). Even when exposed with a 12 hr delay following exposure of cells to 0.1 μM MeHg, cell death was significantly inhibited by the addition of NAC or α-tocopherol.

Pre-incubation of cells with NAC did not inhibit MeHg toxicity (Fig. 5C), indicating that the pre-treatment did not improve or strengthen the protection of tNPC against MeHg. tNPC cell death could be induced by just a 1 hr exposure to 0.1 μM MeHg (Fig. 5D), which suggests that although NPC can tolerate MeHg toxicity for as long as 12 hr, the process of MeHg-induced cell death begins soon after exposure to the compound. NAC (1 mM) treatment after the removal of MeHg from the culture medium prevented tNPC from undergoing cell death to the same extent as seen when the MeHg and NAC were co-administered (Figs. 5A and E). This data excludes the possibility that NAC’s effect occurs via an action on MeHg in the extracellular milieu. When NAC was administered simultaneously with MeHg, tNPC were able to survive even when they were transferred to a NAC- and MeHg-free culture medium (Fig. 5F). This data indicates that cells are able to survive once NAC counteracts the effects of MeHg toxicity.

**DISCUSSION**

In the present study, we confirmed that tNPC are highly sensitive to the toxic effects of MeHg. The exposure of cells to 0.1 μM MeHg caused delayed cell death. Reduction of endogenous glutathione accelerated this cell death. Under these experimental conditions, we have shown for the first time that antioxidants protect tNPC from the toxic effects of MeHg even when there is a time lag between exposure to MeHg and exposure to antioxidants.

Exposure of the fetus to MeHg results in defects of normal brain development, particularly in relation to the proliferation, migration and differentiation of NPC (Rice and Barone, 2000). Human neonates exposed to MeHg in utero show incomplete or abnormal migration of neurons to the cerebellar and cerebral cortices, and defective cortical organization of the cerebrum (Choi et al., 1978; Matsumoto et al., 1965). It has been shown recently that NPC are more sensitive to MeHg than mature differentiated neural cells in vitro (Tamm et al., 2006). In the present study, we used mouse NPC cultures from the telencephalon, diencephalon, and cerebellum, and neural and non-neural tumor cells to investigate the origin and specificity of MeHg toxicity in immature neural cells. Our results show that NPC, especially those derived from the telencephalon, are highly sensitive to MeHg, as are NPC from the cerebrum (Fig. 2). Consistent with the present study, bilateral cerebral atrophy and cerebellar hypoplasia are evident in fetal Minamata disease (Chang and Annau, 1984). Previously, our group reported that fetal neurogenesis is altered in a toxicologically heterogeneous manner upon exposure to 5-bromo-2-deoxyuridine (BrdU), a genotoxic reagent, with a higher sensitivity evident in the frontal cortex and cerebellum (Kuwagata et al., 2007; Ogawa et al., 2005). The present study also suggests that embryonic neurogenesis displays a heterogeneous sensitivity to toxic compound such as MeHg.

The mechanism of cell death might be characterized as being due to apoptosis. Exposure to 0.1 μM or 1 μM MeHg resulted in nuclear condensation and fragmentation, which are characteristic of cells undergoing apoptosis (Fig. 3A), while TUNEL-positive cells increased (up to 95% in tNPC) in response to a 24 hr exposure to 1 μM MeHg (Fig. 3B). As judged by Hoechist 33342 or TUNEL staining method, cell death of tNPC exposure to 0.1-1 μM MeHg may be apoptosis. Although species is different, similar intranuclear changes were observed in neural stem cell line C17.2 and primary cultures of cortical neural stem cells from E15 rat embryo exposed to low concentrations of MeHg (Tamm et al., 2006). These data suggest that cell death by low-dose MeHg in NPC derived from rat and mouse may be caused via apoptotic process.

Potential mechanisms involved in MeHg-induced toxicity have been described in many studies. MeHg has been reported to affect the structure of neural microtubules (Stummann et al., 2007; Daré et al., 2001; Castoldi

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**Table 1.** LC₅₀ values of methyl mercury for various cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>LC₅₀ (μM)</th>
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<tbody>
<tr>
<td>Neural cells in primary culture</td>
<td></td>
</tr>
<tr>
<td>tNPC</td>
<td>0.056</td>
</tr>
<tr>
<td>dNPC</td>
<td>0.17</td>
</tr>
<tr>
<td>CGC</td>
<td>0.02</td>
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<tr>
<td>Neural tumor cells</td>
<td></td>
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<tr>
<td>HT22</td>
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</tr>
<tr>
<td>IMR32</td>
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<tr>
<td>Neuro-2a</td>
<td>1.3</td>
</tr>
<tr>
<td>Non-neural tumor cells</td>
<td></td>
</tr>
<tr>
<td>DMS114</td>
<td>3.5</td>
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<tr>
<td>PAI</td>
<td>3.3</td>
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LC₅₀ values were determined from Figs. 2B and C. tNPC = telencephalon derived neural progenitor cells dNPC = diencephalon derived neural progenitor cells CGC = cerebellum granular cells

Values are the mean of two experiments performed in triplicate.
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et al., 2003; Hunter and Brown, 2000; Miura et al., 1999; Mottet et al., 1997), resulting in alteration of gene expression/protein regulation (Hwang and Nagamuna, 2006; Nagamuna et al., 2002; Diaz et al., 2004; Baraldi et al., 2002), and to impaired mitochondrial functions (Mori et al., 1984; Mori et al., 2007; Garg and Chang, 2006; Morken et al., 2005; Fonfría et al., 2002; Ahlbom et al., 2000; Atchison and Hare, 1994). In each report, ROS played a crucial role in mediating MeHg toxicity. MeHg has a high affinity for SH residues and inhibits the activities of peptide and enzyme, including glutathione and superoxide dismutase, which contain such residues (Kumagai et al., 1997; Gatti et al., 2004; Vicente et al., 2004; Nagamuna et al., 1998). Our data shows that glutathione synthesis inhibitor, BSO promotes MeHg toxicity while BSO itself does not cause cell death (Fig. 4). This data suggests that cell viability is compromised when antioxidant defenses are overwhelmed. Proliferating NPC are reported to exhibit a greater vulnerability to oxidative stress than differentiated neurons, despite their robust DNA repair and anti-oxidative responses (Sava et al., 2007). These studies suggest that antioxidants have a protective function. In the present study, antioxidants protected tNPC from cell death (Fig. 5A), while delayed-treatment with NAC or α-tocopherol rescued tNPC from MeHg-induced cell death (Fig. 5B). Interestingly, exposure to MeHg for only 1 hr was lethal to tNPC (Fig. 5C). However, a delay of 12 hr in addition of NAC was still able to prevent cell death (Figs. 5B and E). These data show that antioxidants are strong protective reagents against MeHg toxicity.

In epidemiological studies of the effects of MeHg exposure on health, umbilical cord mercury concentration was used as a biomarker of fetal exposure to MeHg. A range of studies in several countries have reported average MeHg concentrations in the cord blood; these include 0.38 μg/l and 4.9 μg/l in Canada (Morrisette et al., 2004; Butler Walker et al., 2006), 9.32 μg/l and 14 μg/l in Japan (Sakamoto et al., 2007; Tsuchiya et al., 1984) and 1.4 μg/l in Sweden (Vahter et al., 2000). In newborn rodents exposed to MeHg from their mothers, the brain: blood ratio of MeHg was reported to be 1-2: 10 (Newland and Reile, 1999; Sakamoto et al., 2002). From these reports, the concentration of MeHg in the infant rodent brain is estimated to be 0.04-2 μg/l (corresponding to 0.2-10 nM). These concentrations are within the range of levels that affected the survival of neural cells in the present study. When pregnant rats were exposed to 0.5 ppm MeHg in drinking water (this concentration corresponds to that found in highly contaminated fish), the whole-brain mercury concentration of their newborns was reported to be about 500 μg/l at birth (Newland and Reile, 1999). This dose equates to 2.3 μM, which is the same level as the concentrations examined here that induced immediate cell death (Fig. 2D). However, there were no statically significant effects of exposure on litter size or brain weight at birth in the report by Newland and Reile (1999). In consideration of our study, it is reasonable to speculate that antioxidants may protect the fetus from MeHg toxicity, thus making it important to investigate the role of antioxidants in this model.

The present study suggests that a daily diet including antioxidants, such as amino acids, vitamins, unsaturated fatty acids and flavonoids, might be useful in protecting fetal brains from MeHg toxicity. Further studies in vivo are required to confirm this possibility.

ACKNOWLEDGMENTS

We wish to express our thanks to Prof. E. DiCicco-Bloom for his many helpful suggestions throughout this work and to Ms. T. Saito for excellent technical work. This work was supported in part by a Research on Health Sciences focusing on Drug Innovation from The Japan Health Sciences Foundation (S.S).

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