Methylmercury Retards the Repair of Wounded Monolayer of Human Brain Microvascular Endothelial Cells by Inhibiting Their Proliferation without Nonspecific Cell Damage

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(Received April 25, 2007; Accepted May 7, 2007; Published online May 14, 2007)

Methylmercury (MeHg) is an environmental pollutant that causes severe neuropathy in the brain of exposed humans and animals. It is possible that MeHg induces functional damage of the brain microvessels and neuropathy occurs secondarily. Thus, the effects of MeHg on the maintenance of vascular endothelial cell monolayer were investigated using a culture system of human brain microvascular endothelial cells. MeHg did not damage the morphology of the monolayer; however, it retarded the repair of the wounded monolayer. The proliferation of endothelial cells was observed to be inhibited by MeHg when assessed by the cell number, [^{3}H]thymidine incorporation, and lactate dehydrogenase (LDH) leakage in sparsely growing cells. Cadmium also decreased the [^{3}H]thymidine incorporation but failed to decrease the cell number; inorganic mercury and lead did not exhibit any inhibitory effect under the same conditions. Considering these results together, it is suggested that MeHg exhibits toxicity in the brain microvessels when the endothelial monolayer is damaged. MeHg specifically inhibits the proliferation of endothelial cells during the repair process of the damaged monolayers. The present data support the hypothesis that the mechanism of MeHg-induced neuropathy in the brain includes changes in the microenvironment of the neurons caused by functional damage to the microvessels.

Key words —— methylmercury, endothelial cell, proliferation, brain, microvascular

INTRODUCTION

Methylmercury (MeHg) is well known as a potent neurotoxic compound that induces severe neuropathy in the brain of exposed humans and animals. The mechanisms by which MeHg induces neuropathy are incompletely understood. For example, there is a distinct difference in the distribution of pathological changes between the developing brain of the fetus and the adult brain after exposure to MeHg. In the adult brain, MeHg-induced damage is observed in specific areas such as the granular layer of the cerebellum and the calcarine region of the cerebrum, whereas the damage is widespread in the brain of the fetuses exposed to MeHg *in utero*.[1,2] However, the mechanism for this difference remains unclear. In addition, the most typical problem appears to be the mechanism by which MeHg exhibits the toxicity, particularly in the neurons. We hypothesize that the microenvironment of the neurons, which is formed by other cell types such as astroglial cells, pericytes, and endothelial cells, is altered by MeHg and that it contributes to the specificity of MeHg toxicity for the neurons.

There are several reports that support our hypothesis. For example, Eto *et al.*[3] demonstrated that acute MeHg exposure induces severe damage in the occipital lobes of the cerebrum of common marmosets. The concentration of MeHg was observed to be high in the edematous white matter of the cerebrum, suggesting that edema in the white matter is caused by MeHg, leading to secondary damage to the neurons. Thus, it is indicated that brain microvessels are a target for MeHg toxicity. In fact, on the basis of the observations of the devel-

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oping brains of chick embryos exposed to MeHg, Bertossi *et al.* suggested that the delayed maturation of the vessels with barrier properties is involved in the neurotoxic effects of the metal.[4)]

The brain microvessels mainly consist of endothelial capillaries covered by pericytes and outgrowths of astrocytes. Endothelial cells form tight junctions and regulate the selective transport of ions and nutrients such as amino acids and glucose, thus functioning as the blood-brain barrier.[5)] A large portion of previous studies conducted on MeHg neurotoxicity have focused on astrocytes and neurons.[2, 6, 7)] With regard to endothelial cells, the inhibitory effect of MeHg on the migration and tube formation of cultured human umbilical vein endothelial cells has been reported.[8)] However, little is known regarding the effects of MeHg on vascular endothelial cell functions.

The present study was undertaken to investigate the *in vitro* effects of MeHg on the maintenance of the monolayers of human brain microvascular endothelial cells. It was observed that MeHg does not damage the monolayers but retards the repair of wounded monolayers by inhibiting the proliferation of the cells.

MATERIALS AND METHODS

Materials —— Human brain microvascular endothelial cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). HuMedia EG-2, which is a growth medium for human endothelial cells, was purchased from Kurabo (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from MP Biomedicals, Inc. (Irvine, CA, U.S.A.). Collagen-coated tissue culture dishes and plates were obtained from Iwaki (Chiba, Japan). MeHg chloride was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). MeHg stock solution (10 mM) was prepared by dissolving MeHg chloride in CMF-PBS as a 1:1 equimolar complex with L-cysteine and stored at −80°C until use. [*Methyl*-^{3}H]Thymidine (9.25 MBq/mmol) was purchased from MP Biomedicals, Inc. (Irvine, CA, U.S.A.). CytoTox-ONE™ Homogeneous Membrane Integrity Assay—a lactate dehydrogenase (LDH) kit—was obtained from Promega (Madison, WI, U.S.A.). L-Cysteine and other reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Morphological Observation and Nonspecific Cell Damage —— Human brain microvascular endothelial cells were cultured at 37°C until confluent in HuMedia EG-2 in 100 mm dishes in a humid atmosphere with 5% CO_2. They were then transferred into 24-well culture plates at a density of 5×10^3 cells/cm^2 and cultured for 24 hr (sparse culture) or until confluent (dense culture) in HuMedia EG-2. The medium was then discarded, and the cell layer was washed twice with fresh HuMedia EG-2. The cell layer was then incubated at 37°C for 24 hr in 0.25 ml of fresh HuMedia EG-2 with or without MeHg (1, 2, or 3 μM), mercuric chloride (2 μM), cadmium dichloride (2 μM), or lead nitrate (2 μM). After incubation, the conditioned medium was harvested and the cell layer was washed with 0.25 ml CMF-PBS; the washing of the cell layer was combined with the corresponding conditioning of the medium and used for the LDH assay as a marker of nonspecific cell damage. The cell layer was fixed with methanol and stained with Giemsa for morphological observation.

Repair of Wounded Endothelial Monolayers —— Dense cultures of human brain microvascular endothelial cells were prepared in 60 mm dishes and the cell monolayer was wounded with a cell scraper. After wounding, the remaining monolayer was washed twice with fresh HuMedia EG-2 to remove the detached cells and incubated at 37°C for 24 hr in 1 ml of fresh HuMedia EG-2 with or without MeHg (1, 2, or 3 μM). The medium was then discarded, and the cells were washed twice with CMF-PBS; thereafter, the cells were stained with Giemsa and used for the morphological observation of the repair of the wounded area.

Cell Proliferation Assay —— The proliferation of human brain microvascular endothelial cells was evaluated by morphological observation as stated above, the number of cells, and [^{3}H]thymidine incorporation in the sparse cultures. Sparse cultures were prepared in 24-well plates and treated with or without MeHg (1, 2, or 3 μM), mercuric chloride (2 μM), cadmium dichloride (2 μM), or lead nitrate (2 μM) for 24 hr. The cells were then washed with CMF-PBS and incubated at room temperature for 4 min with 0.25 ml of CMF-PBS containing 0.25% trypsin and 0.02% EDTA. After incubation, 0.25 ml of CMF-PBS containing 10% FBS was added and the cell suspension was collected. The culture well was washed with 0.25 ml of CMF-PBS and the re-

sulting wash was mixed with the cell suspension. The cells were then collected by centrifuging at $150 \times g$ for 2 min and suspended in 0.25 ml of CMF-PBS. The number of viable cells was counted after adding 20 µl of 0.4% trypan blue in 0.85% saline by a particle counter (Sysmex CDA-500; Sysmex, Kobe, Japan). The sparse cultures were incubated separately in 6-well plates at 37°C for 24 hr in 1 ml of fresh HuMedia EG-2 with or without MeHg (1, 2, or 3 µM), mercuric chloride (2 µM), cadmium dichloride (2 µM), or lead nitrate (2 µM) and then labeled with [^{3}H]thymidine (10 kBq/ml) during the last 3 hr of incubation. The medium was then discarded and the cells were washed with CMF-PBS; the cells were then scraped off with a rubber policeman in the presence of 0.75 ml CMF-PBS. The culture was then washed with 0.75 ml CMF-PBS and the resulting wash was mixed with the cell suspension. The cell homogenate was prepared by sonication, and the incorporation of [^{3}H]thymidine into 5% trichloroacetic acid-insoluble fraction of the cell homogenate was determined by liquid scintillation counting. A portion of the cell homogenate was analyzed for DNA content by the fluorometric method.[9)]

Statistical Analysis —— Data were analyzed for statistical significance using Analysis of Variance (ANOVA) and Bonferroni's multiple t-test. $p < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the morphology of the dense culture of human brain microvascular endothelial cells after exposure to MeHg at 3 µM or lower for 24 hr. The monolayer was maintained even after the exposure to MeHg without any degenerative changes in the cells. Consistent with the morphological observations, MeHg did not induce increased LDH leakage from the cells. This indicates that MeHg is not cytotoxic to human brain microvascular endothelial cell monolayers.

In order to examine whether MeHg exhibits cytotoxicity during the repair of the damaged human brain microvascular endothelial monolayers, dense cell cultures were wounded and then exposed to MeHg at 3 µM or lower for 24 hr (Fig. 2). When the appearance of endothelial cells in the wounded

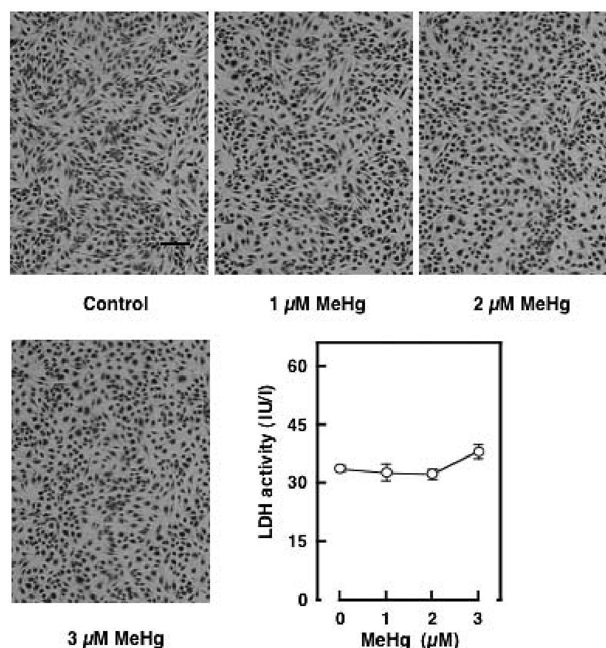

Fig. 1. Morphology of the Dense Cultures of Human Brain Microvascular Endothelial Cells After Exposure to MeHg (Original Magnification ×40) and LDH Leakage from the Cells During the Exposure

Dense cultures of human brain microvascular endothelial cells were incubated at 37°C for 24 hr. Scale bar = 250 µm. For LDH activity, values are means ± S.E. for 4 samples.

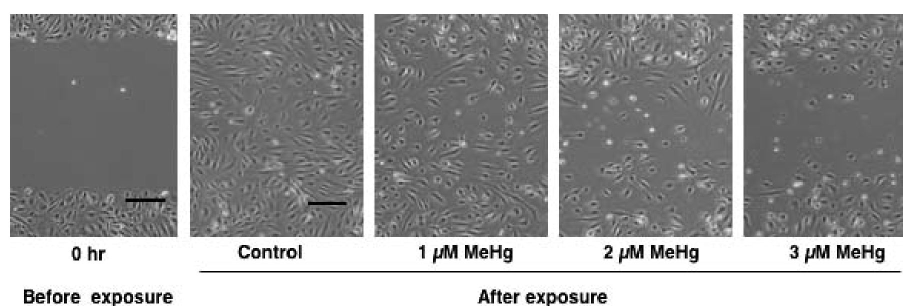

Fig. 2. Morphology of the Wounded Area of Human Brain Microvascular Endothelial Cell Monolayers After Exposure to MeHg (Original Magnification ×40)

Dense cultures of human brain microvascular endothelial cells were damaged and then incubated at 37°C for 24 hr in the presence of MeHg at 1, 2, or 3 µM. Scale bar = 250 µm.

area was regarded as repair of the damaged monolayer, MeHg was observed to inhibit the repair of the wounded endothelial cell monolayer in a concentration-dependent manner.

Since endothelial cell proliferation is crucial for the repair of damaged endothelial monolayers, the proliferative activity of the cells after exposure to MeHg was evaluated (Fig. 3). MeHg decreased the cell number without any degenerative changes in the cells in sparse cultures that were composed of proliferating cells. In fact, MeHg was observed to significantly decrease the number of cells when counted; further, the [^{3}H]thymidine incorporation also decreased. However, the LDH leakage from the cells remained unchanged following exposure to MeHg. These results suggest that MeHg inhibits the proliferation of human brain microvascular endothelial cells without nonspecific cell damage.

The proliferative activity of endothelial cells after exposure to inorganic mercury, cadmium, or lead was evaluated and compared to that after exposure to MeHg (Table 1). None of the metals were observed to induce any change in the LDH leakage, suggesting that the experiment was performed un-

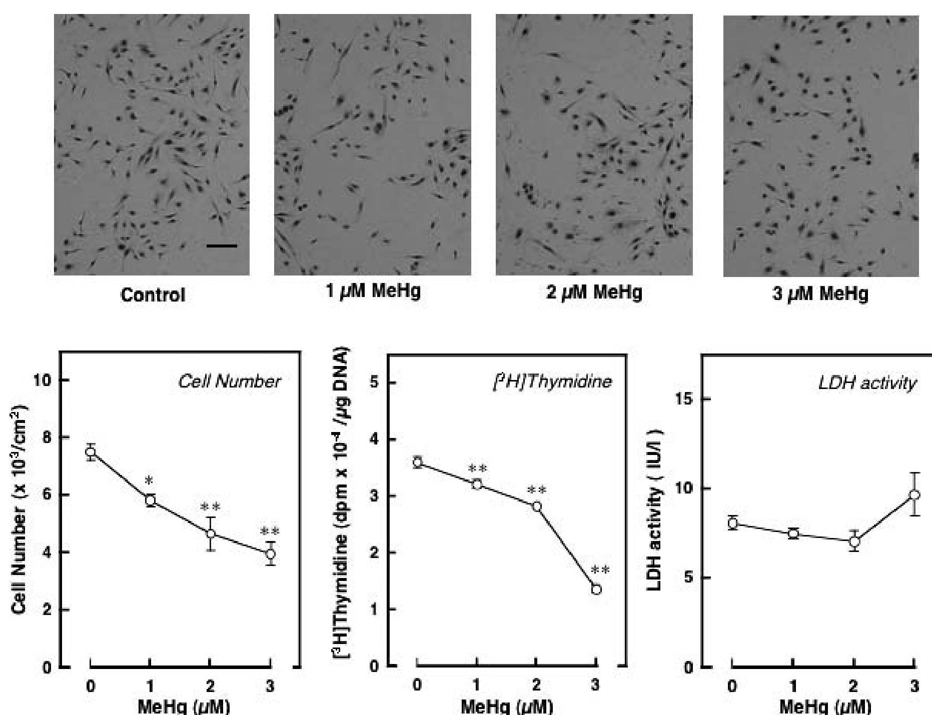

Fig. 3. Morphology of the Sparse Cultures of Human Brain Microvascular Endothelial Cells After Exposure to MeHg (Upper Panels; Original Magnification × 40); the Cell Number, [^{3}H]Thymidine Incorporation, and LDH Leakage from the Cells (Lower Panels)

Sparse cultures of human brain microvascular endothelial cells were incubated at 37°C for 24 hr in the presence of MeHg at 1, 2, or 3 μM and labeled with or without [^{3}H]thymidine during the last 3 hr of the incubation. Scale bar = 250 μm. For data in the lower panels, values are means ± S.E. for 4 samples.

Table 1. The Cell Number, Incorporation of [^{3}H]Thymidine into the Acid-Insoluble Fraction, and Leakage of LDH in Sparse Cultures of Human Brain Microvascular Endothelial Cells after Exposure to MeHg, $HgCl_2$, $CdCl_2$, or $Pb(NO_3)_2$

	Cell Number ($\times 10^3$ cells/cm^2)	[^{3}H]Thymidine (dpm $\times 10^{-4}$/μg DNA)	LDH activity (IU/l)
Control	7.49 ± 0.55	2.61 ± 0.08	3.28 ± 0.39
MeHg	5.14 ± 0.17**	1.93 ± 0.06**	3.21 ± 1.56
$HgCl_2$	7.13 ± 0.67	2.42 ± 0.06	3.92 ± 0.97
$CdCl_2$	7.07 ± 0.15	1.75 ± 0.10**	4.33 ± 0.55
$Pb(NO_3)_2$	6.93 ± 0.21	2.93 ± 0.09*	4.65 ± 1.53

Sparse cultures of human brain microvascular endothelial cells were incubated at 37°C for 24 hr in the presence to MeHg, $HgCl_2$, $CdCl_2$, or $Pb(NO_3)_2$ (2 μM each). Values are means ± S.E. of four samples. Significantly different from the corresponding control, $^*p < 0.05$, $^{**}p < 0.01$.

der nontoxic conditions. Although cadmium and MeHg significantly decreased the [^{3}H]thymidine incorporation, only MeHg significantly decreased the cell number, suggesting that the inhibitory effect of MeHg on human brain microvascular endothelial cells is stronger than that of cadmium. Lead significantly increased the [^{3}H]thymidine incorporation without changing the cell number, suggesting that the metal moderately stimulates the proliferation of the cells.

DISCUSSION

It has been suggested that the abnormal maturation of microvessels and damaged barrier function are important factors in the pathogenesis of brain disorders caused by neurotoxicants such as heavy metals.[10] Lead—a well-known neurotoxic heavy metal—induces cerebral edema and cerebellar hemorrhage via a dysfunction of the blood-brain barrier in the brain of exposed humans and animals,[10–13] suggesting that the abnormality of blood vessels may cause secondary degeneration of the neurons. Although a similar mechanism is possible in the pathogenesis of MeHg-induced neuropathy in the brain, the toxicity of MeHg to blood vessels has been unclear. In the present study, we investigated the effects of MeHg on the maintenance of human brain microvascular endothelial cell monolayers in cultures and found that MeHg is not toxic to the cells in a monolayer; however, it retards the repair of the wounded monolayer through the inhibition of cell proliferation without nonspecific cell damage. The present data partly support the hypothesis that the mechanism for MeHg-induced neuropathy in the brain includes changes in the microenvironment of the neurons caused by functional damage of the microvessels.

The repair of the damaged vascular endothelium is critical for the prevention of vascular disorders. Endothelial cell proliferation is an important event in the repair process, which is promoted by the endogenous fibroblast growth factor-2 (FGF-2) and the vascular endothelial growth factor (VEGF) through their specific receptors.[14, 15] We have previously reported that lead inhibits the proliferation of macrovascular endothelial cells[16] and retards the repair of wounded monolayers of the cells.[17, 18] This inhibition is caused by a weaker response of the cells to the endogenous FGF-2[19] through the inhibition of perlecan synthesis,[20] a heparan sulfate proteoglycan that promotes the binding of FGF-2 to its cell surface receptor.[21] Perlecan can potentiate the activity of VEGF165,[22] one of the alternative splicing isoforms of VEGF as well as that of FGF-2. In cultures, human brain microvascular endothelial cells synthesize perlecan as a predominant proteoglycan molecule[23] whose synthesis is induced by VEGF165.[24] Therefore, the expression of VEGF, FGF-2, their receptors, or perlecan may be suppressed by MeHg. However, the mechanism by which MeHg inhibits the proliferation of human brain microvascular endothelial cells is yet to be elucidated.

Previously, we have reported that cadmium inhibits the proliferation of endothelial cells derived from macrovessels by using the cells from the bovine aorta.[25] Since cadmium significantly decreased [^{3}H]thymidine incorporation in human brain microvascular endothelial cells, the metal may have reduced the proliferative activity of the cells, although the cell number did not change. It is suggested that the reduction in the proliferative activity may be a general response of endothelial cells to cadmium. In our previous study, we have demonstrated that lead inhibits the proliferation of bovine aortic endothelial cells;[16] however, in the present study, the metal significantly increased the [^{3}H]thymidine incorporation in human brain microvascular endothelial cells, suggesting a stimulation of cell proliferation. In microvessels, both endothelial cells[26] and pericytes[27] constitutively express VEGF. In contrast, in macrovessels, smooth muscle cells express VEGF,[28] whereas endothelial cells are deficient in this growth factor[26] and their proliferation depends on the endogenous FGF-2.[29] Thus, it is suggested that in macrovessels, the proliferation of vascular endothelial cells depends on FGF-2 while in microvessels, it depends on both FGF-2 and VEGF. This difference may induce the different responses of endothelial cells toward lead with regard to the proliferative activity. Of the heavy metals tested, only MeHg significantly decreased the cell number by inhibiting the [^{3}H]thymidine incorporation, suggesting that the metal strongly inhibits the proliferation of human brain microvascular endothelial cells. MeHg may be a heavy metal that exhibits toxicity to vascular endothelial cell proliferation in the brain microvessels.

In summary, it is suggested that MeHg is not cytotoxic to human brain microvascular endothelial cells but inhibits their proliferation, resulting in

a retardation of the repair of the wounded monolayers of the cells. MeHg may inhibit the angiogenic process or the repair of damaged microvascular endothelium by inhibiting endothelial cell proliferation in the fetal or adult brain. It is possible that the abnormal maturation of microvascular endothelium and its barrier properties contribute to the widespread neuronal damage in the brain of fetuses exposed to MeHg *in utero*. The present study supports the hypothesis that the microenvironment around the neurons, which is formed by other cell types such as endothelial cells, may be important in exhibiting MeHg toxicity specific to the neurons.

Acknowledgements This work was supported by "Academic Frontier" Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 2005–2009; a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. H.); a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. F.); a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (to C. Y.); and a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (to T. K.).

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