Original Article

Resistance of human brain microvascular endothelial cells in culture to methylmercury: cell-density-dependent defense mechanisms

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ABSTRACT — Vascular toxicity is important for understanding the neurotoxicity of methylmercury, because microvessels strongly influence the construction of microenvironment around neurons. Previously, we found that low density-human brain microvascular pericytes are markedly susceptible to methylmercury cytotoxicity due to high expression levels of the L-type amino acid transporter 1 (LAT-1) that transports methylmercury into the cells. Although LAT-1 can be, in general, highly expressed in sparse cells that require amino acids for growth, we found that human brain microvascular endothelial cells, regardless of cell density, were resistant to methylmercury cytotoxicity. To investigate the mechanisms underlying this resistance, we exposed the endothelial cells at low and high cell densities to methylmercury and determined the extent of nonspecific cell damage, intracellular accumulation of methylmercury, expression of LAT-1 and LAT-2 mRNAs, and intracellular expression of reduced glutathione and metallothionein. These experiments indicate that sparse endothelial cells intracellularly accumulate more methylmercury via the highly expressed LAT-1, but are resistant to methylmercury cytotoxicity by higher expression of the protective sulfhydryl peptides, namely, reduced glutathione and metallothionein. It is suggested that both nonspecific and functional damage is caused in pericytes, whereas functional abnormalities rather than nonspecific damage may occur to a greater extent in the endothelial cells in the brain microvessels exposed to methylmercury. The previous and present data also suggest that methylmercury exhibits toxicity in endothelial cells in a manner different from that in pericytes in the brain microvessels.

Key words: Methylmercury, Endothelial cells, L-type large neutral amino acid transporter, Glutathione, Metallothionein

INTRODUCTION

Methylmercury is an environmental pollutant that causes severe neuropathy in the brains of humans and animals exposed to this pollutant (Sanfeliu *et al.*, 2003), as was observed in the case of patients with Minamata disease. Neuropathological lesions in the brain of patients

with Minamata disease are observed in specific areas, including the granular layers of the cerebellum and the calcarine, postcentral, precentral, and temporal transverse regions of the cerebral cortex (Eto, 1997). In common marmosets that were experimentally exposed to methylmercury, severe damage was observed in the occipital lobes with edematous changes in the white matter around the

deep sulci (Eto *et al.*, 2001), suggesting that the neuronal damage induced by methylmercury is a secondary manifestation of the edematous changes. However, the molecular and cellular mechanisms underlying the edematous changes are still unclear.

Since brain edema is defined as an abnormal accumulation of body fluid within the brain parenchyma due to the disruption of the blood-brain barrier or fluid accumulation in injured cells (Iencean, 2003; Marmarou, 2004), we hypothesized that methylmercury-induced neuropathy is induced by functional/nonspecific damage of the brain microvessels. The brain microvessels are composed of 2 different cell types—vascular endothelial cells and pericytes. The endothelial cells cover the luminal surface of the microvessels, and the pericytes wrap around and along the endothelial tube. Clarification of abnormalities in the brain microvascular endothelial cells and pericytes will contribute to the understanding of edematous lesions caused by methylmercury.

Previously, we studied the effects of methylmercury on human brain microvascular endothelial cells (HBMECs) and pericytes in a culture system. It was found that methylmercury retards the repair of wounded endothelial cell monolayers by inhibiting cell proliferation (Hirooka *et al.*, 2007), which is due to reduced expression of the fibroblast growth factor-2 (Hirooka *et al.*, 2009). On the other hand, pericytes at a high cell density are resistant, whereas those at a low cell density are susceptible to methylmercury cytotoxicity (Hirooka *et al.*, 2010). This cell-density-dependent susceptibility results from a higher expression and induction by methylmercury of the L-type amino acid transporter 1 (LAT-1) that transports methylmercury intracellularly (Simmons-Willis *et al.*, 2002) in the sparse pericytes (Hirooka *et al.*, 2010). The expression of LAT-1 appears to be higher in sparse cells, because such cells require higher amounts of amino acids for their growth. However, sparse and dense cultures of the endothelial cells were equally resistant to methylmercury cytotoxicity (Hirooka *et al.*, 2007).

In the present study, we investigated the susceptibility of cultured HBMECs to methylmercury-induced cytotoxicity. Unlike pericytes, the endothelial cells did not show cell-density-dependent susceptibility to methylmercury-induced cytotoxicity. With regard to response to methylmercury-induced cytotoxicity, the endothelial cells are resistant at low and high cell density, whereas pericytes are susceptible at low density but resistant at high cell density (Hirooka *et al.*, 2010). The present study was undertaken to investigate the mechanism underlying the resistance of sparse cultures of endothelial cells to methylmercury cytotoxicity.

MATERIALS AND METHODS

Materials

HBMECs were purchased from DS Pharma Biomedical (Osaka, Japan). HuMedia EG-2—a growth medium for endothelial cells—was purchased from Kurabo (Osaka, Japan); calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum, Invitrogen (Carlsbad, CA, USA); bovine serum albumin (BSA), Sigma-Aldrich Chemical (St. Louis, MO, USA); collagen-coated tissue culture dishes and plates, AGC Techno Glass (Chiba, Japan); methylmercury chloride, Tokyo Chemical Industry (Tokyo, Japan); CytoTox-ONE™ homogeneous membrane integrity assay—a lactate dehydrogenase kit, Promega (Madison, WI, USA); inorganic mercury standard solution for inductively coupled plasma mass spectrometry analysis, Perkin-Elmer (Waltham, MA, USA); iodoacetamide, Wako Pure Chemical Industries (Osaka, Japan); high-capacity cDNA reverse transcription kit, TaqMan gene expression master mix, and TaqMan primer and probe pairs for real-time reverse-transcription polymerase chain reaction, Applied Biosystems (Foster City, CA, USA); RNeasy lipid tissue mini kit, Qiagen (Tokyo, Japan); rabbit polyclonal anti gamma-glutamyl-cysteine synthetase light subunit (γ -GCS_L) antibody FL-274 and anti- γ -GCS heavy subunit (γ -GCS_H) antibody H-50, Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal mouse anti-horse metallothionein antibody (E9), Dako Cytomation (Carpinteria, CA, USA); horseradish peroxidase conjugated anti-rabbit IgG antibody and anti-mouse IgG antibody, Cell Signaling (Beverly, MA, USA); enhanced chemiluminescence western blotting detection reagents, Nacalai Tesque (Kyoto, Japan); Microcon centrifugal filter devices YM-3 (Microcon YM-3) and polyvinylidene difluoride membrane (pore size: 0.45 μ m), Millipore (Billerica, MA, USA); polyvinylidene difluoride membrane (pore size: 0.2 μ m), Atto (Tokyo, Japan); and L-cysteine, 2-mercaptoethanol, dithiothreitol, 25% glutaraldehyde, and other reagents, Nacalai Tesque (Kyoto, Japan).

Cell culture and cytotoxicity assay

HBMECs were cultured in 100-mm dishes at 37°C in HuMedia EG-2 in a humid atmosphere with 5% CO₂ until they reached confluency. They were then transferred into 24-well culture plates at a density of 5×10^3 cells/cm² and cultured for 24 hr in HuMedia EG-2 (sparse culture) or until they became confluent (dense culture). The medium was discarded, and the cells were washed twice with fresh HuMedia EG-2. Then, the cells were treated with meth-

ylmercury (1, 2, or 3 μM) at 37°C for 24 hr in 0.25 ml of fresh HuMedia EG-2. After treatment, the conditioned medium was harvested and used for the determination of lactate dehydrogenase activity—a marker of nonspecific cell damage. The cell layer was washed with CMF-PBS, fixed with methanol, and stained with Giemsa for morphological observation.

Accumulation of methylmercury

Sparse and dense cultures of endothelial cells in 6-well culture plates were incubated with methylmercury (1 or 2 μM) at 37°C for 24 hr in fresh HuMedia EG-2. Separately, the cultures were incubated at 4°C for 2 min in fresh HuMedia EG-2 to determine the nonspecific binding of methylmercury to the cell surface. After incubation, the conditioned medium was discarded, and the cells were washed with CMF-PBS containing 2 mM ethylene glycol tetraacetic acid. The cells were lysed in 6 ml of 60% nitric acid, and the lysate was digested by a microwave digester (Multiwave 3000; PerkinElmer). The digest was diluted to 18 ml with Milli Q water. Inorganic mercury in the digest was analyzed by inductively coupled plasma mass spectrometry (ELAN DRC II; PerkinElmer). The cell homogenate was prepared in CMF-PBS from sparse and dense cultures treated with methylmercury under the corresponding conditions and analyzed for DNA content by a fluorometric method (Kissane and Robins, 1958). The intracellular accumulation of methylmercury (pmol/ μg DNA) was estimated by dividing the intracellular methylmercury content (calculated by subtracting the methylmercury content bound to the cell surface from that accumulated in the cell layer) by the DNA content.

Real-time reverse-transcription polymerase chain reaction

Sparse and dense cultures of endothelial cells were treated with methylmercury (1, 2 or 3 μM) at 37°C for 12 hr in HuMedia EG-2. After treatment, total cellular RNA was extracted from the cells using the RNeasy lipid tissue mini kit. cDNA was synthesized from the mRNA by using the high-capacity cDNA reverse transcription kit. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed in triplicate using the TaqMan gene expression assay system with the 7500 real-time PCR system (Applied Biosystems), and the amplified DNA was analyzed by the comparative Ct method using glyceraldehyde-3-phosphate dehydrogenase as an endogenous control. The primer and probe sets for LAT-1 (Assay ID: Hs00185826_m1) and LAT-2 (Hs00794796_m1) were selected from TaqMan gene expression assay.

Interacellular content of reduced glutathione

Sparse and dense cultures of endothelial cells were incubated at 37°C for 24 hr in HuMedia EG-2 with or without methylmercury (1 and 2 μM). The conditioned medium was discarded, and the cell layer was washed with cold CMF-PBS containing 2 mM ethylene glycol tetraacetic acid and then suspended in 80 μl of 1 mM ethylenediaminetetraacetic acid disodium salt. The cell homogenate was prepared by sonication and centrifuged at $10,600 \times g$ for 10 min at 4°C. Aliquots of the supernatant were diluted 1:1 with 20 mM ammonium phosphate solution (pH 2.5) containing 1.8% acetonitrile and filtrated through Microcon YM-3. The filtrate (50 μl) was then injected into a high performance liquid chromatography system with a UV/VIS detector (LC-20A; Shimadzu, Kyoto, Japan) at 220 nm equipped with a C 18 ODS reversed-phase column (YMC-Pack-ODS-AM, 4.6 mm $\Phi \times$ 250 mm; YMC Co. Ltd., Kyoto, Japan). Elution was performed isocratically in a 20 mM ammonium phosphate solution (pH 2.5) containing 1.8% acetonitrile. The flow rate was 0.6 ml/min. A portion of the supernatant was assayed for protein content by a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL, USA) with BSA as the standard. The glutathione (GSH) content were expressed as nmol GSH/mg cellular protein.

Western blot analysis of γ -GCS and metallothionein-I/II

Sparse or dense cultures of endothelial cells were treated with methylmercury (2 μM) at 37°C for 24 hr in HuMedia EG-2. After treatment, the cell layers were washed with cold CMF-PBS containing 2 mM ethylene glycol tetraacetic acid. The cell lysate was prepared by adding 50 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol and 2% sodium dodecyl sulfate (SDS) to the cell layers and incubating at 95°C for 5 min. The protein content of the lysate was determined by the bicinchoninic acid protein assay reagent kit. To detect the γ -GCS protein, the protein (19 μg) was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (pore size: 0.45 μm). In the case of metallothionein-I/II, the protein (15 μg) was treated with 50 mM dithiothreitol and 5% 2-mercaptoethanol for 3 min at 95°C and reacted with 100 mM iodoacetamide for 30 min in the dark at room temperature. The protein was separated by 15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (pore size: 0.2 μm). The membrane was incubated for 1 hr in 2.5% glutaraldehyde; blocked for 1 hr in 20 mM Tris-HCl buffer containing 5% skim milk, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5); and incubat-

ed with rabbit polyclonal anti γ -GCS_L antibody (1:500), anti- γ -GCS_H antibody (1:250), or monoclonal mouse anti-horse metallothionein antibody (1:100) for 1 hr at room temperature. The blot was probed with horseradish peroxidase-conjugated secondary antibody, and the bands were visualized by an enhanced chemiluminescence procedure. The chemiluminescent intensity of the bands was quantified using the Image-J software.

Statistical analysis

Statistical significance was analyzed using analysis of variance and Bonferroni's multiple *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Fig. 1 shows the morphological observations of sparse and dense cultures of HBMECs after exposure to methylmercury for 24 hr. Previously, we reported that non-specific cell damage occurs in sparse cultures of human brain microvascular pericytes after the cells were exposed

to methylmercury at 1 μ M and more in a concentration-dependent manner, although dense cultures of the cell are resistant to methylmercury cytotoxicity (Hirooka *et al.*, 2009). In contrast, both sparse and dense cultures of endothelial cells were resistant to methylmercury cytotoxicity even at 3 μ M in the morphological examination (Fig. 1A). The leakage of lactate dehydrogenase from the cells was not increased by exposure to methylmercury (Fig. 1B), thus supporting the findings of morphological observation.

Fig. 2 shows the intracellular accumulation of methylmercury in the sparse and dense cultures of endothelial cells after exposure to methylmercury at 1 or 2 μ M for 24 hr; the accumulation in the sparse cells was significantly higher than that in the dense cells. The expression of LAT-1 mRNA was unchanged and that of LAT-2 mRNA was lowered after exposure to methylmercury in both sparse and dense endothelial cells (Fig. 3A). On the other hand, the expression of LAT-1 in the sparse cells was much higher than that in the dense cells, while that of LAT-2 in the sparse cells was significantly lower than that

Fig. 1. [A] Morphological observations of sparse and dense cultures of endothelial cells after exposure to methylmercury. Giemsa stain ($\times 100$). Scale bar = 250 μ m. [B] Lactate dehydrogenase leakage from the cells into the medium during exposure. Values are means $\pm$ S.E. for 4 samples. Sparse and dense cultures of HBMECs were incubated at 37°C for 24 hr in the presence of methylmercury (1, 2, and 3 μ M).

The susceptibility of vascular endothelial cells to methylmercury

Fig. 2. Intracellular accumulation of methylmercury in sparse and dense cultures of endothelial cells. Sparse and dense cultures of HBMECs were incubated at 37°C for 24 hr in the presence of methylmercury (1 and 2 μM). Values are means ± S.E. of 3 samples. **Significantly different from the corresponding dense culture, $P < 0.01$.

in the dense cells (Fig. 3B).

Fig. 4 shows the expression of sulfhydryl peptides (GSH, its rate-limiting enzyme γ -GCS, and metallothionein-I/II) that protect HBMECs from methylmercury cytotoxicity. γ -GCS is a heterodimeric enzyme composed of γ -GCS_H (heavy chain), which is a catalytic subunit with a M_r of 73 kDa (Huang *et al.*, 1993a) and γ -GCS_L (light chain), which is a regulatory subunit with a M_r of 27.7 kDa (Huang *et al.*, 1993b). We found that the GSH content in the sparse endothelial cells was markedly higher than that in the dense endothelial cells (Fig. 4A). Although the expression of γ -GCS_H in the sparse and dense endothelial cells was the same, that of γ -GCS_L was higher in the sparse cells (Fig. 4B). Furthermore, the expression of metallothionein-I/II in the sparse cells was also higher than that in the dense cells (Fig. 4C). However, the expression of the sulfhydryl peptides was not influenced by the exposure to methylmercury (Fig. 4B and C).

DISCUSSION

An understanding of the susceptibility of the component cells of microvessels, namely, endothelial cells and pericytes, to methylmercury is important, since vascular damage influences the development of brain lesions. Vas-

cular cells often respond to heavy metals in different manners depending on the cell type. For example, the proliferation of vascular endothelial cells is inhibited (Kaji *et al.*, 1995) but that of vascular smooth muscle cells is stimulated (Fujiwara *et al.*, 1995) by lead. We hypothesized that there are differences in the response to methylmercury between endothelial cells and pericytes. We have previously shown that pericytes at a high cell density are resistant but those at a low cell density are susceptible to methylmercury cytotoxicity (Hirooka *et al.*, 2010). In the present study, we demonstrated that cultured HBMECs at both low and high cell densities are resistant to methylmercury cytotoxicity at concentrations that cause nonspecific cell damage in sparse pericytes. The previous and present data suggest that methylmercury exhibits toxicity in endothelial cells in a manner different from that in pericytes in the brain microvessels. In the brain microvessels exposed to methylmercury, both nonspecific and functional damage may occur in pericytes, whereas functional abnormalities rather than nonspecific damage may affect endothelial cells to a greater extent.

Comparisons of the response characteristics of sparse and dense endothelial cells and sparse pericytes to methylmercury should be discussed. It was revealed that sparse endothelial cells had no susceptibility and exhibited higher accumulation of intracellular methylmercury following higher expression of LAT-1, higher content of intracellular GSH following higher expression of γ -GCS_L, and higher constitutive expression of metallothionein compared to dense endothelial cells. These results indicate that a higher accumulation of intracellular methylmercury due to a higher expression of LAT-1 occurs in sparse endothelial cells. However, the sparse endothelial cells are not as susceptible to methylmercury cytotoxicity as the dense endothelial cells because of a higher constitutive expression of the preventive sulfhydryl peptides, namely, GSH and metallothionein. In the case of sparse pericytes, methylmercury accumulates within the cells to a high level due to greater expression and induction of LAT-1; however, the expression levels of GSH and metallothionein were similar to that in dense pericytes, thus resulting in a higher susceptibility of sparse pericytes to methylmercury cytotoxicity (Hirooka *et al.*, 2010). Therefore, the most important difference between endothelial cells and pericytes at a low cell density appears to be the level of constitutive expression of intracellular GSH and metallothionein.

Methylmercury has a high affinity for the sulfhydryl group and is bound to the group of proteins, glutathione, and cysteine in the human body (Hughes, 1957). Since the conformational structure of the methylmercu-

[A]

[B]

Fig. 3. [A] Expression of LAT-1 and LAT-2 mRNAs in sparse and dense cultures of endothelial cells after exposure to methylmercury. Values are the percentage of the control $\pm$ S.D. of 3 samples. Significantly different from the corresponding control, * $P < 0.05$; ** $P < 0.01$. [B] Comparison of the expression levels of LAT-1 and LAT-2 mRNAs in sparse and dense cultures of endothelial cells. Values are the percentage of the expression in the dense endothelial cells $\pm$ S.D. of 3 samples. Significantly different from the corresponding dense cultures, * $P < 0.05$; ** $P < 0.01$. Sparse and dense cultures of HBMECs were incubated at 37°C for 12 hr in the presence of methylmercury (1, 2, and 3 μ M).

ry-cysteine complex is similar to that of the amino acid methionine, the complex is transported into cells by LAT (Hirayama, 1980). LAT has 2 isoforms—LAT-1 and LAT-2 (Pineda *et al.*, 1999; Yanagida *et al.*, 2001). It has been reported that the brain microvascular endothelial cells express both LAT-1 and LAT-2 *in vitro* (Kido *et al.*, 2001) and that both the LAT isoforms have the ability to transport methylmercury into the cells (Simmons-Willis *et al.*, 2002). We found that the expression of LAT-1 was higher and that of LAT-2 was lower in both the endothelial cells as well as pericytes (Hirooka *et al.*, 2010) when their cell density is low; at low cell density, intracellular methylmercury levels were increased, suggesting that LAT-1 is

responsible for the induction of methylmercury in both the endothelial cells and pericytes in the brain microvessels, although the LAT-1 mRNA expression is induced by methylmercury only in the pericytes.

The GSH and metallothionein have a protective effect against methylmercury cytotoxicity. GSH is involved in the excretion of methylmercury from astroglial cells, PC 12 cells, and brain capillary endothelial cells (Miura and Clarkson, 1993; Fujiyama *et al.*, 1994; Kerper *et al.*, 1996) and from the liver into the bile (Ballatori and Clarkson, 1983; Dutczak, and Ballatori, 1994). Both GSH (Hirrlinger *et al.*, 2000) and metallothionein (Sato and Kondoh, 2002) serve as intracellular antioxidants against

The susceptibility of vascular endothelial cells to methylmercury

Fig. 4. [A] Content of intracellular GSH in sparse and dense cultures of endothelial cells after exposure to methylmercury. Values are means $\pm$ S.E. of 3 samples. **Significantly different from the corresponding dense culture; $P < 0.01$. [B] Western blot analysis of γ -GCSH and γ -GCSL in sparse and dense cultures of endothelial cells after exposure to methylmercury. Values in the lower panels are means $\pm$ S.E. of 3 samples. [C] Western blot analysis of metallothionein-I/II in sparse and dense cultures of endothelial cells after exposure to methylmercury. Values in the lower panels are means $\pm$ S.E. of 3 samples. **Significantly different from the corresponding dense cultures, $P < 0.01$. Sparse and dense cultures of HBMECs were incubated at 37°C for 24 hr in the presence of methylmercury (2 μ M).

cytotoxic reactive oxygen species that is induced by methylmercury (Shanker *et al.*, 2004). In fact, the depletion of intracellular GSH by buthionine-*L*-sulfoxane enhances methylmercury cytotoxicity (Shanker *et al.*, 2004), whereas increased expression of metallothionein-I/II protects astrocytes from the toxicity (Aschner *et al.*, 1998; Yao *et al.* 2000) by scavenging reactive oxygen species (Sato and Kondo, 2002). These results suggest that the intracellular level of GSH and metallothionein is one of the definitive conditions in determining the susceptibility of cells to methylmercury cytotoxicity; this supports the hypothesis that the expression of high levels of intracellular sulfhydryl peptides in sparse endothelial cells involves a mechanism underlying more and similarly resistant than sparse pericytes and dense endothelial cells, respectively.

In summary, HBMECs, unlike pericytes, do not show cell-density-dependent susceptibility to methylmercury cytotoxicity *in vitro*; both pericytes and endothelial cells are resistant when the cell density is high, whereas only pericytes are susceptible at a low cell density. The present data revealed that the sparse endothelial cells accumu-

late methylmercury to a high level within the cells as do sparse pericytes, but the intracellular level of GSH and metallothionein are higher in the endothelial cells. Methylmercury exhibits toxicity in endothelial cells in a manner different from that in pericytes in the brain microvessels. Therefore, both nonspecific and functional damage may occur in pericytes, whereas functional abnormalities rather than nonspecific damage may have a greater effect on the endothelial cells of the brain microvessels exposed to methylmercury.

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