Methylmercury induces hyaluronan synthesis in cultured human brain microvascular endothelial cells and pericytes via different mechanisms

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ABSTRACT — In a cerebrum damaged by methylmercury, where neuropathological lesions tend to localize along deep sulci and fissures, edematous changes in white matter have been proposed as the cause of such localization. Since hyaluronan has a high water-retention capability and can contribute to the progression of edematous changes, we hypothesize that methylmercury increases hyaluronan in brain microvascular cells. Our experimental results indicate that methylmercury induces the expression of hyaluronan in cultured human microvascular endothelial cells and pericytes through the induction of expressed UDP-glucose dehydrogenase and hyaluronan synthase 2, respectively. After exposure to methylmercury, hyaluronan largely accumulates in perivascular space, where it contributes to the progression of edematous changes.

Key words: Methylmercury, Endothelial cell, Pericyte, Hyaluronan

INTRODUCTION

Methylmercury, which is a recognized neurotoxic substance found in certain environmental pollutants, (Sanfeliu et al., 2003), has been the topic of considerable research in recent years. Methylmercury is a causal substance of Minamata disease. Histologically, cerebrum damage in Minamata disease patients is localized along the deep sulci and fissures in the cerebral cortex (Eto, 1997), but the mechanisms underlying this localization have yet to be completely understood. Experiments using common marmosets exposed to methylmercury indicated that the localized damage occurs after edematous changes in the white cortex of the cerebrum (Eto et al., 2001). This suggests that ischemia caused by cortex swelling may result in damage to neuronal cells along the deep sulci in the cerebrum.

In this letter, we report on our study into the mechanisms of methylmercury-induced edematous changes, which was conducted using a culture system of human brain microvascular endothelial cells and pericytes. We found that methylmercury induces the expression of VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and placenta growth factor (PIGF) in endothelial cells, as well as vascular endothelial growth factor-A (VEGF-A) in pericytes (Hirooka et al., 2013), in VEGF-related proteins. Since PIGF is specifically bound to VEGF-A, which is a decoy receptor for VEGF-A (Park et al., 1994), we postulate that methylmercury activates the VEGF-A-VEGFR-2 system between endothelial cells and pericytes in brain microvessels. This activation would increase the permeability sufficiently to cause swelling around the microvessels.

Hyaluronan is a type of glycosaminoglycan (GAG) chain that consists of a repeating unit of N-acetylglucosamine and glucuronic acid. Since the GAG has a high water-retention capability that can contribute to the progression of edematous changes, we hypothesize that methylmercury induces the expression of hyaluronan and promotes edematous changes in brain vascular tissue.

Hyaluronan is enzymatically synthesized by hyaluronan synthases (HASs) in what has come to be called the HAS family: HAS1, HAS2, and HAS3 (Weigel et al., 1997). The synthetic rate is regulated by these enzymes.
and UDP-glucose dehydrogenase (UGDH) (Clarkin et al., 2011), which converts UDP-glucose to UDP-glucuronic acid. In the present study, we investigated the effects of methylmercury on hyaluronan secretion and the expression of HASs and UGDH using cultured human brain microvascular endothelial cells and pericytes.

**MATERIALS AND METHODS**

**Materials**

Human brain microvascular endothelial cells and pericytes were purchased from DS Pharma Biomedical (Osaka, Japan), while HuMedia EG-2 and HuMedia SG-2, the growth medium for endothelial cells and pericytes, respectively, were obtained from Kurabo (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan), and the Bicinchoninic acid protein assay reagent kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). Antibodies for HAS1 (sc-23145), HAS2 (sc-66916), HAS3 (sc-66917), UGDH (sc-32354), and β-actin antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody (#7074) was purchased from Cell Signaling (Beverly, MA, USA), while horseradish peroxidase conjugated anti-goat IgG antibody (ab6885) was purchased from Abcam (Bristol, UK). The Hyaluronan Enzyme-linked Immunosorbent Assay Kit was purchased from Echelon Biosciences (Salt Lake City, UT, USA), while the ProteoExtract™ Native Membrane Protein Extraction Kit was obtained from Calbiochem (San Diego, CA, USA). The High-Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Assay were purchased from Applied Biosystems (Foster, CA, USA). Miscellaneous other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

**Cell culture and determination of hyaluronan**

Endothelial cells and pericytes were cultured separately at 37°C in 5% CO₂ in HuMedia EG-2 and HuMedia SG-2, respectively, in collagen-coated culture plates until they reached confluency. Sample plates with and without added methylmercury (1, 2, or 3 μM) were prepared in fresh HuMedia EG-2 (endothelial cells) or DMEM supplemented with 1% bovine serum albumin (pericytes) and treated for 24 hr. The conditioned medium was used for the determination of hyaluronan by ELISA. The cells were harvested by scraping the plates with a rubber policeman in calcium- and magnesium-phosphate-buffered saline and the cell homogenate was prepared by cell sonication. DNA content of the homogenate was analyzed by the fluorometric method (Setaro and Morley, 1976), and the accumulation of hyaluronan in the medium was expressed as ng/μg DNA.

**Real-time polymerase chain reaction analysis**

Total RNA was extracted from human brain microvascular endothelial cells and pericytes treated with methylmercury (1, 2, and 3 μM) for 12 hr. The complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit. Real-time polymerase chain reaction was performed using TaqMan Gene Expression Assay with 10 ng cDNA and 100 nM primers on a StepOnePlus RT-PCR system (Applied Biosystems). The thermal cycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The expression levels of HAS1, HAS2, HAS3, UGDH, and glyceraldehyde-3-phosphate dehydrogenase mRNA in each sample were then quantified by the comparative Ct method. The fold change for each gene was assessed after normalization of the intensity value to that of glyceraldehyde-3-phosphate dehydrogenase. The primer and probe sets for HAS1 (Hs00758053_m1), HAS2 (Hs00193436_m1), HAS3 (Hs00193436_m1), and UGDH (Hs00163365_m1) were obtained using the TaqMan Gene Expression Assay.

**Western blot analysis**

Human brain microvascular endothelial cells and pericytes were first treated with methylmercury at 3 μM for 24 hr, and then washed with ice-cold calcium- and magnesium phosphate-buffered saline containing 2 mM ethylene glycol tetraacetic acid. The membrane associated proteins and cytosolic proteins were extracted separately from the cells by using the ProteoExtract Native Membrane Protein Extraction Kit, and then concentrated via acetone precipitation. The precipitate was then suspended in 40 mM Tris-HCl buffer solution (pH 6.8) containing 10% glycerol and 2% sodium dodecyl sulfate, and then incubated for 5 min at 95°C. The protein content of the lysate was determined using a bicinchoninic acid protein assay reagent kit (Pierce). The cellular proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane (0.2 μm) at 2 mA/cm² for 1 hr. Next, the membranes were blocked with 5% skim milk in 20 mM Tris-HCl buffer solution containing 15 mM NaCl and 0.1% Tween 20 (pH 7.5), and then incubated with anti-HAS1 antibody (1:200), anti-HAS2 anti-
body (1:200), anti-HAS3 antibody (1:100), anti-UGDH antibody (1:200), 5'-NT antibody (1:200), and anti-β-actin antibody (1:1000) solutions at 4°C overnight. The protein bands were visualized via enhanced chemiluminescence using Chemi-Lumi One L and scanned by LAS3000 (Fujifilm, Tokyo, Japan).

Statistical analysis
The data were analyzed for statistical significance by analysis of variance (ANOVA) and Bonferroni’s multiple t-test, when possible. P values less than 0.05 were considered statistically significant.

RESULTS

Figure 1 shows the effects of methylmercury on the synthesis of hyaluronan in human brain microvascular endothelial cells. As can be seen in the figure, methylmercury significantly increased the secretion of hyaluronan into the culture medium from the cells in a dose-dependent manner (Fig. 1A). Although the expression of HAS1 mRNA was not detected, we detected HAS2 and HAS3 mRNAs; however, their expression was not changed by the methylmercury (Fig. 1B). At that time, we determined that the level of UGDH mRNA was significantly increased by methylmercury (Fig. 1B). Furthermore, while the expression of HAS2 and HAS3 proteins was unaffected by methylmercury (Fig. 1C), the expression of UGDH protein was found to have increased (Fig. 1D).

In human brain microvascular pericytes, methylmercury significantly increased the secretion of hyaluronan in a dose-dependent manner (Fig. 2A). More specifically, the levels of mRNAs for HAS2, HAS3, and UGDH

![Graphs and images](image�link)

Fig. 1. Effects of methylmercury on the secretion of hyaluronan and the expression of HAS2, HAS3, and UGDH in cultured human brain microvascular endothelial cells. [A] Secretion of hyaluronan in the conditioned medium of the cells treated with methylmercury (1, 2, or 3 μM) for 24 hr. Significantly different from the corresponding control, ** p < 0.01. [B] The expression of HAS2, HAS3, and UGDH mRNAs in the cells treated with methylmercury (1, 2, or 3 μM) for 12 hr. Significantly different from the corresponding control, * p < 0.05. [C] The expression of HAS2 and HAS3 proteins in the membrane fraction of the cells treated with methylmercury (3 μM) for 24 hr. 5'-Nucleotidase (5'-NT) was used as an internal standard. [D] The expression of UGDH protein in the soluble fraction of the cells treated with methylmercury (3 μM) for 24 hr. Actin was used as an internal standard.
were significantly elevated by the addition of methylmercury. The highest increase was observed in the HAS2 mRNA level (Fig. 2B). However, HAS1 mRNA was not detected. Western blot analysis showed that methylmercury significantly increased the expression of the HAS2 protein, but expression of HAS3 and UGDH proteins did not increase.

**DISCUSSION**

Because of its high water retention capability, hyaluronan can contribute to the progression of edematous changes in the extracellular matrix. In the present study, it was revealed that methylmercury increases the secretion of hyaluronan from human brain microvascular endothelial cells and pericytes. This suggests that methylmercury not only increases the permeability of brain microvessels via activation of the VEGF system between the endothelial cells and pericytes (Hirooka et al., 2013), but also forms a water-rich extracellular matrix by increasing hyaluronan from both endothelial cells and pericytes, thereby promoting edematous change progression around the microvessels. These events may contribute to the vasogenic edema in the brain after methylmercury exposure.

While the mechanisms underlying methylmercury-induced hyaluronan synthesis remain unclear, our results suggest that methylmercury promotes the synthesis of hyaluronan through the induction of UGDH and HAS2 expression in human brain microvascular endothelial cells and pericytes, respectively. In other words, the effect of methylmercury on the synthesis of hyaluronan is the same, but the mechanisms are different for endothelial cells and pericytes. Our previous studies indicated that both nonspecific and functional damage is caused in

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Fig. 2. Effects of methylmercury on the secretion of hyaluronan and the expression of HAS2, HAS3, and UGDH in cultured human brain microvascular pericytes. [A] Secretion of hyaluronan in the conditioned medium of the cells treated with methylmercury (1, 2, or 3 μM) for 24 hr. Significantly different from the corresponding control, * p < 0.05; ** p < 0.01. [B] The expression of HAS2, HAS3, and UGDH mRNAs in the cells treated with methylmercury (1, 2, or 3 μM) for 12 hr. Significantly different from the corresponding control, ** p < 0.01. [C] The expression of HAS2 and HAS3 proteins in the membrane fraction of the cells treated with methylmercury (3 μM) for 24 hr. 5'-Nuleotidase (5'-NT) was used internal standard. [D] The expression of UGDH protein in the soluble fraction of the cells treated with methylmercury (3 μM) for 24 hr. Actin was used as an internal standard.
pericytes, whereas functional abnormalities rather than nonspecific damage may occur to a greater extent in the endothelial cells of brain microvessels exposed to methylmercury (Hirooka et al., 2010a, 2010b). In addition, methylmercury exhibits toxicity in endothelial cells in a manner that is different from that observed in brain microvessel pericytes (Hirooka et al., 2010a). Thus, it is likely that the methylmercury target molecule(s) for endothelial cells differ from those that target pericytes. It is also possible that the response to the same target molecule(s) activated or inactivated by methylmercury will be different between these two cell types. However, these points have yet to be determined.

The present data suggests that change in extracellular matrix – such as an increase in hyaluronan – may be important for understanding the vasogenic edematous change induced by methylmercury. Additional studies should be performed to reveal functional damage to human brain microvascular endothelial cells and pericytes in order to clarify the mechanisms underlying the localization of damage along the deep sulci and fissures in the cerebral cortex of patients with Minamata disease.

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

REFERENCES


