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Letter

Methylmercury causes neuronal cell death via M1-microglial activation in organotypic slices prepared from mouse cerebral cortex

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ABSTRACT — Methylmercury is an environmental pollutant that causes central nervous system injury. We reported that the expression of the inflammatory cytokines TNF-α and IL-1β was specifically induced in the brains of methylmercury-treated mice. In addition, we recently found that cytotoxic microglia (M1-microglia) may be involved in the induction of inflammatory cytokine expression by methylmercury in mouse cerebral slice cultures. In the current study, we investigated the involvement of M1-microglia in the neuronal cell death caused by methylmercury using mouse cerebral slice cultures. The results revealed that methylmercury activated steady state microglia (M0-microglia) to M1-microglia, but this activation was suppressed by pretreatment with minocycline, a microglial activation inhibitor. In addition, under the same conditions, minocycline suppressed neuronal cell death by methylmercury. These results suggest that methylmercury may induce neuronal cell death via activation to M1-microglia.

Key words: Methylmercury, Microglia, Neuronal cell death, Organotypic brain slice cultures

INTRODUCTION

Methylmercury is present in the environment and accumulates in fish and shellfish, causing central nervous system injury in humans who ingest them (Kazantzis, 2002). It was recently reported that the development of motor function and intelligence can be adversely affected in children born to women who ingested a large amount of fish and shellfish during pregnancy (Murata *et al*., 2011). We previously revealed that the expression of inflammatory cytokines TNF-α and IL-1β was specifically induced in the brains of mice treated with methylmercury (Iwai-Shimada *et al*., 2016, Takahashi *et al*., 2015). In addition, it has been suggested that the induction of inflammatory cytokines may be involved in central nervous system injury.

Microglia are considered to be the main cells involved in the induction of expression of inflammatory cytokines in the central nervous system. Steady-state microglia (M0-microglia) with elongated processes sense minor changes in the brain environment, and are activated to cytotoxic microglia (M1-microglia) to induce the expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Franco and Fernandez-Suarez, 2015). M1-microglia have been confirmed to increase at the onset of various neurodegenerative diseases (Tang and Le, 2016). Recently, Fujimura *et al*. and Shinoda *et al*. reported that M1-microglia were observed in the dorsal root ganglion of rats treated with methylmercury (Fujimura *et al*., 2019). However, the roles of M1-microglia in neuronal cell death in the central nervous system caused by methylmercury are poorly understood.

To investigate the involvement of M1-microglia in neuronal cell death caused by methylmercury, a com-

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bined culture system of neurons and microglia is required. Organotypic brain slices can be cultured while maintaining the cellular composition and structure of the brain, and has been used as a useful culture system for examining the intercellular communication networks in the brain. We recently improved the conventional preparation method for mouse brain slices, and produced mouse cerebral slice cultures in which most microglia exist in the mature state, as in the adult mouse brain (Hoshi *et al*., 2019). We also examined the effects of methylmercury, lead, tin and arsenic, which are known to exhibit neurotoxicity on M1-microglial activation using slice cultures, revealing that only methylmercury activated M0-microglia to M1-microglia (Hoshi *et al*., 2019). However, the effects of activation to M1-microglia by methylmercury on neuronal cell death is unknown. In the current study, we investigated the involvement of M1-microglial activation in methylmercury neurotoxicity using organotypic cerebral slice cultures.

MATERIALS AND METHODS

Animals

The present study was performed in accordance with the recommendations of Regulations for Animal Experiments and Related Activities at Tohoku University. All mice used in the study were C57BL/6 mice obtained from Japan SLC Inc. (Shizuoka, Japan). The mice were kept in plastic cages at 22 ± 2°C with a 12 hr light-dark cycle. Food (F-2, Oriental yeast, Tokyo, Japan) and filtrated tap water were provided *ad libitum*.

Organotypic cerebral slice cultures

We prepared organotypic cerebral slices as we recently reported (Hoshi *et al*., 2019). Briefly, the cerebral cortices prepared from P7 mice were dissected and placed in ice-cold Hank's balanced salt solution (HBSS) containing 6 mg/mL glucose and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for 5 min. The cerebral cortices were cut into 350 μm-thick sagittal slices using a Mcllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Cambridge, UK), and incubated on ice for 30 min in HBSS containing 6 mg/mL glucose and 15 mM HEPES. Two intact slices were placed on the culture plate insert (Koyama *et al*., 2007) with a hydrophilic polytetrafluoroethylene (PTEF) membrane (Millipore, MA, USA) of the six-well plate, and maintained in 5% CO_2 at 37°C. The incubation media were replaced after 24 hr.

Immunohistochemistry

Cultured slice tissues were immersed in ice cold phosphate-buffered saline (PBS) with the hydrophilic PTEF membrane for 1 min, and the slices were then fixed with 4% paraformaldehyde in PBS for 20 min at 22 ± 2°C. After washing with PBS for 10 min, the slices were incubated in permeabilized buffer (0.3% Triton X-100, 10% FBS in PBS) for 1 hr at 22 ± 2°C. The slices were then immersed in anti-Iba1 antibodies (Wako Pure Chemical, Osaka, Japan), anti-NeuN antibodies (Abcam, Cambridge, UK) diluted in PBS and incubated at 22 ± 2°C for 3 hr. After two washes with PBS for 5 min, the samples were incubated with fluorescent-conjugated antibody (Alexa Fluor Plus 555 and Alexa Fluor Plus 488, Thermo Fisher Scientific, MA, USA) diluted in PBS for 1 hr at 22 ± 2°C. Mounting was performed with VECTASHIELD mounting medium hard set with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., CA, USA). Confocal microscopy (FV1000, Olympus, Tokyo, Japan) was used for obtaining images.

Measurement of mRNA levels using real-time quantitative polymerase chain reaction

The slices were removed from the culture membrane using a spatula and collected in a 1.5 mL tube. The total RNA was isolated from the brain slices using Isogen II according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript® RT reagent kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (qPCR) was performed using KAPA SYBR (KAPA Biosystems, MA, USA) by Thermal Cycler Dice® (Takara Bio Inc.) with the following primers: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F:5'-AACTTTGGCATTGTGGAAGG-3', R:5'-ACACATTGGGGGTAGGAACA-3'.

CD11b, F:5'-CCAAGACGATCTCAGCATCA-3', R:5'-TTCTGGCTTGCTGAATCCTT-3'

CD16, F:5'-CCAAGACGATCTCAGCATCA-3', R:5'-TTCTGGCTTGCTGAATCCTT-3'.

CD32, F:5'- AATCCTGCCGTTCCTACTGATC -3', R:5'- GTGTCACCGTGTCTTCCTTGAG -3'.

GAPDH was used as an internal control, and the data are presented as GAPDH-corrected values.

Statistical analysis

Statistical significance was analyzed using one-way analysis of variance and Tukey's post hoc test.

RESULTS AND DISCUSSION

Minocycline, a tetracycline antibiotic, is often used as a compound that selectively inhibits activation of M0-microglia to M1-microglia (Kobayashi *et al*., 2013). M0-microglia are ramified with extended processes, and change to the spherical amoeboid-type when activated to M1-microglia (Cho and Choi, 2017). We recently found that microglia morphology could be changed from the ramified-type to the amoeboid-type by treatment with methylmercury in mouse cerebral slice cultures (Hoshi *et al*., 2019). Therefore, we first examined the effects of minocycline on the change in microglial morphology by methylmercury, and the pretreatment of 10 μM minocycline inhibited the reduction of the microglial processes by methylmercury (Fig. 1). Furthermore, under the conditions described above, the methylmercury-induced increase in expression of CD11b, CD16 and CD32, the M1-microglia specific cell surface antigens, was also suppressed by pretreatment with minocycline (Fig. 2). These results indicate that pretreatment with minocycline suppresses the activation to M1-microglia by methylmercury. We next performed immunostaining of mouse cerebral slices using antibodies against NeuN, a neuron specific marker. The results revealed that NeuN-positive cells were decreased by methylmercury (Figs. 3A, B). However, the decrease of neuronal cells caused by methylmercury was suppressed by pretreatment with minocycline. In addition, we found that clodronate-liposome, a microglia depleting agent, also suppressed methylmercury-caused neuronal cell death in the mouse cerebral slices (data not shown). These suggest that methylmercury may cause neural cell death by activating microglia from M0 to M1.

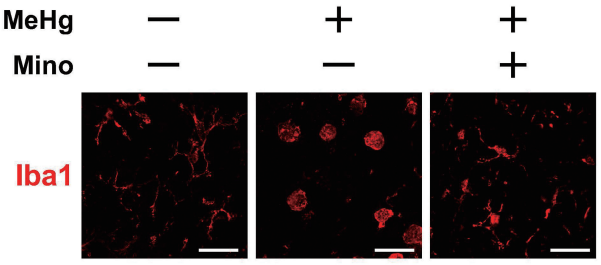

Fig. 1. Effects of minocycline on methylmercury-induced morphological changes of microglia in mouse cerebral slice cultures. Cerebral slices were pretreated with 10 μM minocycline (Mino) for 24 hr then exposed to 1 μM methylmercuric chloride (MeHg) for 6 hr, and immunostaining for Iba1 was performed. The scale bars indicate 50 μm.

Neuronal cell death is known to be caused by extracellularly releasing inflammatory cytokines and reactive oxygen species when M0-microglia are activated to M1-microglia. In recent years, it has also been reported that M1-microglia cause neuronal cell death by inducing cytotoxic astrocytes (A1 astrocytes) (Liddelow *et al*., 2017). Furthermore, microglia are known to be activated to M1-microglia by recognizing damage-associated molecular patterns released from damaged cells (Inoue, 2002). Our findings suggest that microglia may also be involved in increased adverse neurological effects due to methylmercury exposure. In future, it may be possible to elucidate the mechanisms underlying methylmercury toxicity through crosstalk between neurons and microglia by

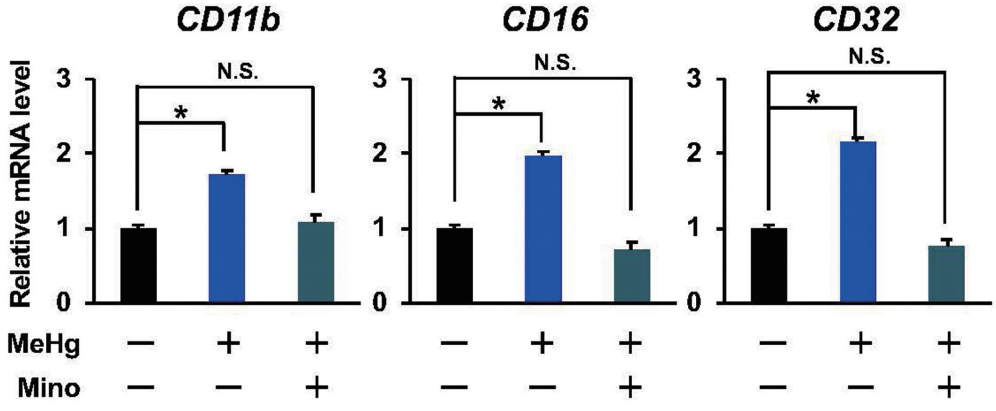

Fig. 2. Effects of minocycline on M1-microglial activation by methylmercury in mouse cerebral slice cultures. Cerebral slices were pretreated with 10 μM minocycline (Mino) for 24 hr and exposed to 1 μM methylmercuric chloride (MeHg) for 6 hr. The mRNA levels of CD11b, CD16 and CD32 were determined by real-time quantitative polymerase chain reaction (qPCR) (n = 3). The data are presented as the GAPDH-corrected value expressed as the mean ± standard deviation. Y-axis indicates the ratio with the non-treated slice (control) as 1. *P < 0.05, vs control. N.S. indicates P > 0.05 vs control.

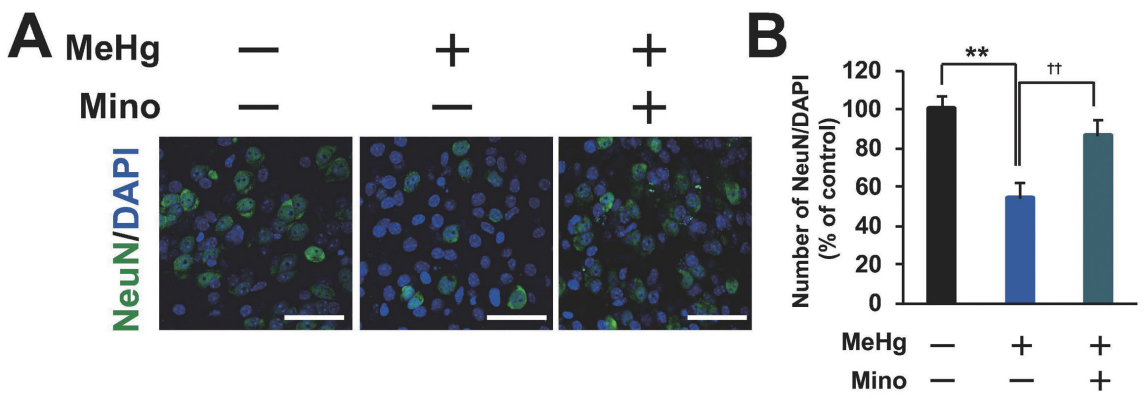

Fig. 3. Effects of minocycline on neuronal cell death caused by methylmercury in mouse cerebral slice cultures. (A) Cerebral slices were pretreated with 10 μM minocycline (Mino) for 24 hr and exposed to 1 μM methylmercuric chloride (MeHg) for 7 days. The slices were immunostained with NeuN antibodies (green) and nuclei were stained with DAPI (blue). The scale bars indicate 50 μm. (B) The number of NeuN positive cells were counted from 10 randomly obtained pictures, and the values shown were corrected by the number of DAPI-positive cells. The data are presented as the mean ± standard deviation, **P < 0.01 vs control. ††P < 0.01 vs MeHg treated group.

clarifying the mechanisms involved in neuronal cell death via M1-microglial activation caused by methylmercury.

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

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