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Possible involvement of cathepsin B released by microglia in methylmercury-induced cerebellar pathological changes in the adult rat

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ABSTRACT

There is increasing evidence that cathepsin B (CB), a lysosomal cysteine protease, is one of the toxic molecules that are secreted by activated microglia. We herein provide evidence that CB released by activated microglia may play a role in the methylmercury (MeHg)-induced pathological changes observed in the cerebellum of the adult rat. Pathological changes tended to progress slowly after treatment with MeHg (5 mg/kg) for 12 consecutive days. At 5 days after the final treatment of MeHg, there was a mild pyknotic change of the granule cells, whereas a marked accumulation of activated microglia was observed in the granule cell layer of the lingual and central lobe. At 8 days after the final treatment, intense pyknotic changes of the granule cells and the accumulation of activated microglia were observed throughout the cerebellar vermis. CB first significantly increased at 3 days after the final treatment of MeHg as the mature form. CB mainly increased in activated microglia which accumulated in the granule cell layer. The coadministration of CA074, an irreversible CB inhibitor, with MeHg significantly reduced the severity of pyknotic changes of the granule cells. Furthermore, primary cultured microglia secreted the mature CB in the culture medium following cellular activation. These observations strongly suggest that CB secreted by activated microglia is thus closely associated with the MeHg-induced severe pyknotic changes of the cerebellar granule cells. The treatment of CA074 could be a potentially effective therapeutic intervention to prevent the pathological changes in the cerebellum caused by ingestion of MeHg-contaminated food.

An intoxication of methylmercury (MeHg), also known as Minamata disease, commonly results from the ingestion of MeHg-contaminated food. MeHg is easily absorbed from the intestine and therefore if transported to the central nervous system (CNS) through the blood–brain barrier. Among CNS neurons, the granular cells in the cerebellum are especially vulnerable to MeHg intoxication. Exposure to MeHg during development results in an impaired migration of the granule cells and impaired synaptogenesis, thus leading to a disordering of the cerebellar architecture [7]. On the other hand, exposure to MeHg during adulthood can also result in loss of the granule cells from the internal granule cell layer, while Purkinje cells remain intact [13,24]. MeHg has also been reported to affect functions of astrocytes [1,2] and microglia [9,17]. The degeneration of the cerebellar granule cells by MeHg involves an apoptotic process based on the ultrastructural features and intranucleosomal DNA fragmentation [13]. In the cultured cerebellar granule cells, MeHg induced both apoptosis and necrosis in a concentration-dependent manner [5,11]. However, little is known about the precise mechanism underlying the pyknotic changes of the cerebellar granule cells after chronic treatment with MeHg.

There is substantial evidence that cathepsin B (CB, EC 3.4.22.1), a typical cysteine lysosomal protease, is involved in the apoptotic process. CB has been implicated in the activation of the proinflammatory caspase 11 and 1 [21,23]. Furthermore, CB can cleave the Bcl-2 family member Bid [22], which may lead to cytochrome *c* release from the mitochondria, thus subsequently causing caspase activation. In addition to the functions of intracellular proteolysis, there is accumulating evidence that CB released from microglia may play a crucial pathological role in the CNS [3,14,15]. Ryan et al. [18] used an immortalized murine microglial cell line, BV-2 cells, to demonstrate that CB was secreted in a heavy-chain form, in addition to the proform, upon stimulation with LPS. It has recently been demonstrated that CB secreted from microglia is a major causative factor of microglia-induced neuronal apoptosis [10]. More recently, Gan et al. [8] conducted functional genomic studies to identify CB as one of the genes transcriptionally induced by amyloid-β

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(Aβ) peptides in BV-2 cells. They have also further shown that an inhibition of CB in BV-2 cells using either small interference RNA-mediated gene silencing or a specific inhibitor for CB, CA074, leads to a decrease in the neurotoxic effects caused by Aβ-activated BV-2 cells. These observations strongly suggest that CB is involved in neuronal apoptosis through different two pathways: intracellular proteolysis after leakage from the lysosome and extracellular proteolysis after secreted from activated microglia.

Here, we demonstrate for the first time that CB was intensely expressed in activated microglia in advance of severe pyknotic changes of the cerebellar granule cells following chronic treatment with MeHg. The coadministration of CA074 significantly inhibited the severity of pyknotic changes of the cerebellar granule cells. Our results strongly suggest that CB secreted by activated microglia is therefore closely associated with the progression of the pathological changes induced by chronic treatment with MeHg.

All experimental procedures of this study were approved by Animal Care and Use Committee, Kyushu University. Adult Wistar rats (8 weeks) supplied by CLEA Japan were maintained on a 12 h light/12 h dark cycle at 23 °C. According to the method described previously [12,19,26], MeHg (98% purity, Tokyo Kasei Kogyo, Japan) and L-cysteine (Sigma, St Louis, MO) were dissolved at a molecular ratio of 1:1 with 10% condensed milk. The animals were divided into three groups ($n = 5$ for each group) and then were treated with L-cysteine for 12 consecutive days, MeHg solution (5 mg/kg) for 6 consecutive days, and MeHg solution (5 mg/kg) for 12 consecutive days. MeHg solution was orally administered with a stainless catheter. In some animals ($n = 6$), CA074 (*N*-(L-3-*trans*-propyl-carbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline) (Peptide Institute, Osaka, Japan), an irreversible CB inhibitor, was coadministered (3 mg/kg, i.p.) with MeHg for 12 consecutive days.

For histological and immunohistochemical analyses, the cerebellum was prepared from animals of each group ($n = 5$–8): vehicle, on the day of treatment with MeHg for 6 consecutive days (6d), on the day after treatment with MeHg for 12 consecutive days (12d), 3 days after the final treatment with MeHg for 12 consecutive days (12 + 3d), 5 days after the final treatment with MeHg for 12 consecutive days (12 + 5d), 8 days after treatment with MeHg for 12 consecutive days (12 + 8d). Each animal was anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and then killed by intracardiac perfusion via the heart with isotonic saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). After post-fixing in the same fixative for 24 h, serial saggital sections (30 μm) were then prepared by a cryostat and then stained with hematoxylin and eosin (H&E). The severity of pyknotic changes in the cerebellar granule cells was graded arbitrarily by 0 (no change), 1 (mild), 2 (moderate), 3 (severe), and 4 (very severe). The data are expressed as the means ± S.E.M. and the statistical analysis was performed using Mann–Whitney *U*-test. For immunohistochemistry, the sections were incubated with 10% normal goat or horse serum for 4 h at room temperature. They were incubated with anti-CB IgG (1:500, Upstate, Lake Placid, NY, USA) in phosphate buffered saline (PBS) with 0.1% Tritin-X-100 and 1% bovine serum albumin (BSA) for 3 days at 4 °C. After washing with PBS, the sections were incubated with biotinylated anti-rabbit IgG overnight at 4 °C. After washing with PBS, the sections were treated with 0.5% streptavidin-Alexa 488 (Molecular Probes, Eugene, OR, USA) for 2 h at 24 °C. For double fluorescent staining, the sections were stained with the following combinations of antibodies for 3 days at 4 °C: mouse monoclonal anti-CD11b IgG (OX42) (1:200, Serotec) and rabbit polyclonal anti-CB IgG (1:200, Upstate), or mouse monoclonal anti-GFAP IgG (1:400, Sigma) and rabbit polyclonal anti-CB IgG (1:200, Upstate). After washing with PBS, the sections were incubated with a mixture of 0.5% Alexa488 anti-rabbit IgG and Cy3 anti-mouse IgG (Amersham). After washing with PBS, the sections were mounted in the anti-fading medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and then were examined with a confocal laser-scanning microscope (CLSM) (LSM510MET, Carl Zeiss, Jena, Germany).

For immunoblot analyses, the cerebellum was prepared from animals of each group (vehicle, 6d, 12d, 12 + 3d, 12 + 5d, 12 + 8d, $n = 3$, each) who were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and then were killed by intracardiac perfusion with isotonic saline. The soluble fractions obtained from the cerebellum homogenates by differential centrifugation as described below were electrophoresed in SDS-polyacrylamide gels. The proteins on SDS gels were transferred electrophoretically to nitrocellulose membranes and then they were incubated at 4 °C overnight under gentle agitation with anti-CB IgG (1:500, Upstate). After washing, the membranes were incubated with 0.5% horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG (Amersham). Subsequently, the membrane bound HRP-labeled antibodies were detected by the enhanced chemiluminescence detection system (ECL kit, Amersham) with image analyzer FLA3000 (Fuji Photo Film, Tokyo, Japan) and the protein bands were densitometrically analyzed. The data are expressed as the means ± S.E.M. and the statistical analysis was performed using Student's *t*-test. The supernatants of primary cultured microglia [20] were also subjected to immunoblot analysis using anti-CB IgG at 48 h after treatment with 10 nM chromogranin A (CGA; Peptide Institute, Osaka, Japan).

As reported previously [12], neuronal pyknotic changes were observed in the cerebellar granule cell layer and occipital cortex, but not in the hippocampus or the brain stem after chronic treatment with MeHg in adult rats. In the present study, the cerebellum was focused to examine a possible involvement of CB in MeHg-induced neurotoxicity in the mature brain, because pyknotic changes of the cerebellar granule cells were most severely and constantly observed after chronic treatment with MeHg in adult rats. The severity of neuronal damage and changes in the immunoreactivity for CB were assessed in the cerebellum after chronic treatment with MeHg. In the cerebellum of vehicle-treated rats, Purkinje cells showed intense granular immunoreactivity for CB (Fig. 1A and E). On the other hand, the cerebellar granule cells and glial cells in the granule cell layer showed sparse granular immunoreactivity for CB as reported previously [16]. After treatment with MeHg (5 mg/kg) for 12 consecutive days, rats showed severe contracture of hind legs and tail-election (data not shown). At this stage, neither neuronal degeneration (Fig. 1B) nor change in the immunoreactivity for CB (Fig. 1F) was found. At 5 days after the final treatment, a mild degeneration as evidenced by pyknotic changes was detected in the cerebellar granule cells but not in Purkinje cells (Fig. 1C). At this stage, however, there was intense CB immunoreactivity in glial cells with activated morphology accumulated in the granular cell layer (Fig. 1G). At 8 days after the final treatment, cerebellar granular cells showed severe pyknotic changes (Fig. 1D). At this stage, Purkinje cells also showed slight morphological changes. In the granular cell layer, glial cells that expressed intense immunoreactivity for CB still accumulated in the granule cell layer (Fig. 1H). Double staining immunohistochemistry was conducted to identify glial cells that showed intense immunoreactivity for CB. The CB-immunostained cells (green) were found to correspond closely with OX42-positive microglia (red) (Fig. 1I) but not with GFAP-immunostained astrocytes (red) (Fig. 1J). It was also noted that GFAP immunoreactivity around CB-immunostained microglia was markedly reduced.

To address possible pathological roles of CB, we examined the effects of CA074 on the MeHg-induced pyknotic changes of the cerebellar granule cells. The coadministration of CA074 with MeHg markedly reduced the MeHg-induced pyknotic changes of the cerebellar granule cells (Fig. 1K) and the mean pathological grade on 8