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# METHYLMERCURY INDUCES APOPTOSIS OF RAT CEREBELLAR NEURONS IN PRIMARY CULTURE

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Cerebellar neurons in primary culture were exposed to methylmercury, a well-established neurotoxicant known as a cause of Minamata disease, at 0.1 - 1.0 μM for up to 72 hours and compared with the neurons undergoing apoptosis induced by withdrawing $K^+$ from the medium. Cerebellar neurons treated with methylmercury at up to 0.3 μM showed morphological changes characteristic to apoptosis, depending on methylmercury dose; formation of apoptotic vesicles, disappearance of neurites and condensation of nuclear chromatin. In addition, soluble DNA prepared from the methylmercury-treated cells exhibited the typical DNA fragmentation pattern similar to that in cells undergoing apoptosis induced by $K^+$ withdrawal. At higher concentration of methylmercury, however, a non-apoptotic pathway of cell death started to predominate over the apoptotic pathway. These results indicate that the death of cerebellar granule neurons induced by methylmercury is, at least at lower doses, apoptotic . 

Since the outbreak of Minamata disease (neurological syndrome caused by methylmercury poisoning) in Japan in the early 1950's, methylmercury has been recognized as an extremely hazardous environmental pollutant (1). Numerous biochemical, physiological and morphological investigations on the neurotoxic effects of methylmercury have demonstrated that the damage is remarkably selective, being limited to specific focal areas such as granule cells of the cerebellum and the neurons in the interstices of the visual cortex (1 - 3). However, little is known about the mechanism for neuronal cell death caused by methylmercury.

Several lines of evidence suggest that cell death occurs by one of two general pathways, necrosis or apoptosis (4, 5). Necrosis is associated with non-physiological conditions that disrupt cellular homeostasis. By contrast,

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apoptosis is a type of regulated cellular self-destruction that functions in the normal control of development and tissue homeostasis and may be regulated by various factors throughout life. Cells undergoing apoptosis exhibits nuclear chromatin condensation and apoptotic vesicle formation. A biochemical hallmark of cells undergoing apoptosis is internucleosomal cleavage of DNA into oligonucleosome-length fragments (6). While several events are known to initiate apoptosis in non-neuronal cells, only a few specific insults have been associated with apoptosis of cultured neurons (7 - 9). Recently, rat cerebellar granule neurons in primary culture have been shown to undergo apoptosis by lowering $K^+$ concentration in the serum-free medium from 25 mM to 5 mM (10). To examine the possible involvement of apoptosis in the methylmercury-induced neuronal death, biochemical and cytological alterations associated with methylmercury-induced death of cerebellar neurons were compared with those of the apoptosis induced by $K^+$ withdrawal. Results obtained indicate that methylmercury-induced death of cerebellar neurons in primary culture is apoptotic and that this process may play a role in the neuronal death associated with Minamata disease.

## Materials and Methods

### *Materials*

All tissue culture media and supplements were from GIBCO. RNase A and Hoechst 33258 (bisbenzimide) were from Boehringer Mannheim. 1-β-D-arabinofuranosylcytosine, actinomycin D and poly(L-lysine) were from Sigma. Methylmercury chloride was from Nacalai Tesque Inc. (Kyoto, Japan).

### *Primary culture of rat cerebellar neurons*

Cultures enriched in granule neurons were obtained from dissociated cerebella of 8-day-old Wistar rats (CLEA Japan, Co., Tokyo Japan) as described by Gallo *et al.* (11). Cells were plated in basal Eagle's medium (BME) supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin on poly(L-lysine)-coated Corning dishes (35mm). 1-β-D-Arabinofuranosylcytosine (10 μM) was added to the culture medium 24 hours after plating.

### *Treatment of cultures*

To induce apoptosis of granule neurons, culture medium was replaced with serum-free BME medium containing 5 mM KCl and other supplements 7 days after plating as described by D'Mello *et al.* (10). In case of methylmercury treatment, ethanol solution of methylmercury chloride was added to the culture 6 to 8 days after plating to make final concentration indicated elsewhere. Ethanol concentration in the medium was no more than 0.001% (v/v), which exerts no adverse effects on the survival of neurons.

### *Cell viability analysis*

Cell viability was assessed by crystal violet staining (12, 13). Cells were fixed by adding 1/10 volume of 11 % glutaraldehyde for 15 min and stained with 0.1 % crystal violet in 0.2 M 4-morpholinr-ethanesulphonic acid, pH 6.8, for 20 min at room temperature. The dye incorporated into the cells was eluted into 10 % acetic acid and the absorbance at 590 nm, which is proportional to the number of viable cells, was measured spectrophotometrically.

***Nuclear condensation analysis***

Cells grown on chamber slides (LabTek, Nunc) coated with poly(L-lysine) were fixed with 4 % paraformaldehyde for 15 min at room temperature and washed 3 times with phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.5 % Triton X-100 in PBS and incubated with 5 μM bisbenzimide in PBS for 1 min at room temperature. After washing with PBS, stained nuclei were observed under fluorescent microscope.

***DNA fragmentation analysis***

Fragmentation of DNA was analyzed as described by Hockenberry *et al.*(14). After digesting with RNase A (100 μg/ml) at 37°C for 60 min, the soluble DNA prepared from 5 x $10^6$ cells was subjected to electrophoresis in a 1.2 % agarose gel and visualized by ethidium bromide staining.

## Results and Discussion

It is well established that high level of $K^+$ ( 25 mM) in culture medium ensure proper development and prolong survival of cerebellar neurons in primary culture, most of which are granule neurons (11). Upon withdrawal of $K^+$ from the medium by switching to one contains low but more physiological $K^+$ concentration (5 mM), differentiated granule neurons start to degenerate and die *via* an apoptotic pathway (10) (Fig. 1 B and Fig. 2). Viability of the neurons undergoing apoptosis was slightly higher than that reported previously (10), which is probably because of the difference in the assay systems; viability estimated by crystal violet staining is reflecting the amount of total protein of cells attached to substrata (12). Methylmercury at submicromolar concentrations induced degeneration and death of the differentiated granule neurons even in the presence of high $K^+$ (Fig. 1 C and Fig. 2). To examine the possible involvement of apoptosis in the methylmercury-induced death of granule neurons, these cells were compared with those undergoing apoptosis by $K^+$ withdrawal on the basis of indicators of apoptosis such as chromatin condensation, apoptotic vesicle formation and DNA fragmentation.

The morphological changes associated with the death of methylmercury-treated granule neurons (at 0.3 μM for 72 hr) are quite similar to those undergoing apoptosis induced by $K^+$ withdrawal; shrunken cell bodies with condensed nuclei, fragmented and less dense neurites, and cellular fragmentation were clearly evident (Fig. 1 B & C). The condensation of nuclear

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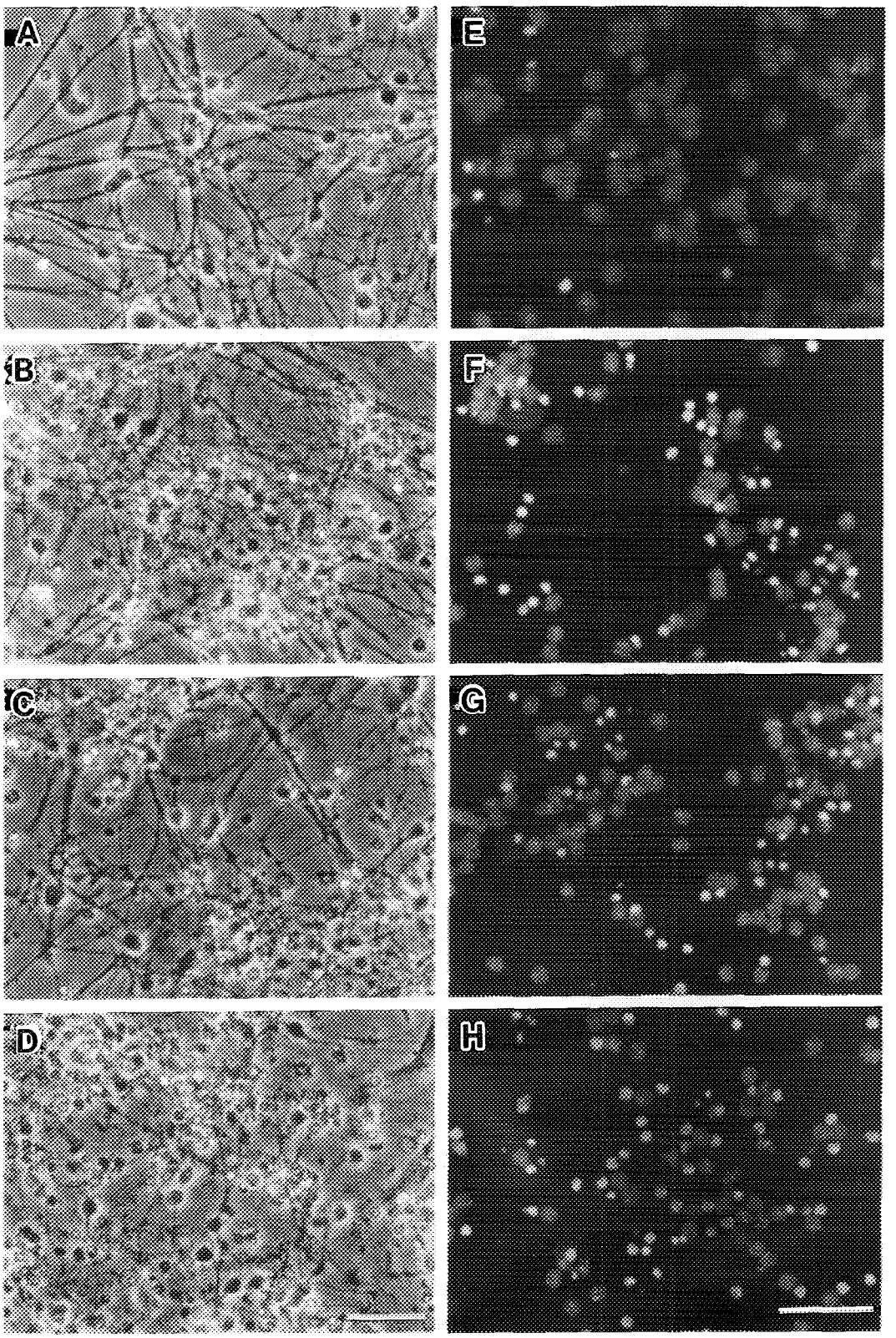
Figure 1. Morphological changes of granule neurons after the treatments with methylmercury or $K^+$ withdrawal. Cerebellar granule neurons were allowed to mature for 6 - 7 days after plating in the medium containing 10 % fetal calf serum and 25 mM KCl. The neurons were then treated with methylmercury at 0 (A, E), 0.3 (C, G) or 1.0 μM (D, H) in the same medium for 72 hours or switched to serum-free medium containing 5 mM KCl and cultured for 48 hours (B, F). Phase-contrast micrographs of unfixed cells (A - D) and fluorescent micrographs of the fixed cells after staining with bisbenzimide (E - H) were shown. Scale bar, 40 μm.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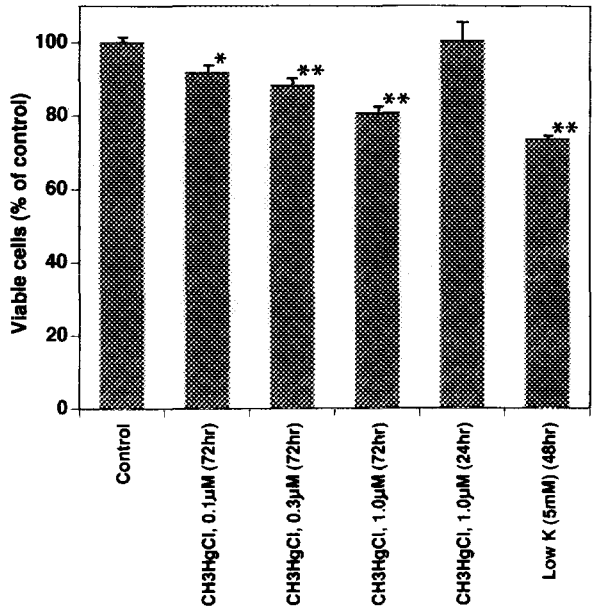


Figure 2. Survival of cerebellar neurons after the treatments with methylmercury or $K^+$ withdrawal. Neurons were treated with methylmercury or $K^+$ withdrawal as in Figure 1. Survival of these cells were estimated by crystal violet staining as described in Materials and Methods. Each bar represents mean $\pm$ SD of 3 culture wells. Statistically significant differences from control were estimated by the Student t test: * $p<0.01$, ** $p<0.001$.

chromatin was verified by the staining with bisbenzimide. Shrunken or fragmented nuclei of granule neurons treated with methylmercury were brightly stained with the dye (Fig. 3 B & D) just like those of the cells undergoing apoptosis by $K^+$ withdrawal (Fig. 3 A & C). A significantly larger proportion of methylmercury-treated cells possessed nuclei with condensed chromatin than control cells (Fig. 1 E & G). At higher methylmercury concentration (at 1.0 μM for 72 hr), however, morphology of the nuclei was slightly different from those of cells undergoing apoptosis: condensed nuclei were larger in size and less bright (Fig. 1 F & H), suggesting that at this concentration of methylmercury another pathway of cell death started to predominate over the apoptotic pathway.

To further confirm that the death induced by methylmercury was apoptotic, the occurrence of DNA cleavage into oligonucleosomal-sized fragments, a hallmark of apoptosis, was demonstrated in the methylmercury-treated granule neurons (Fig. 4). In the granule neurons treated with methylmercury at 0.1- 0.3 μM for 72 hr , DNA fragmentation was observed in a dose-dependent manner. At 1 μM, however, DNA fragmentation was less evident, suggesting that non-apoptotic (possibly necrotic) cell death was taking place. This is consistent with

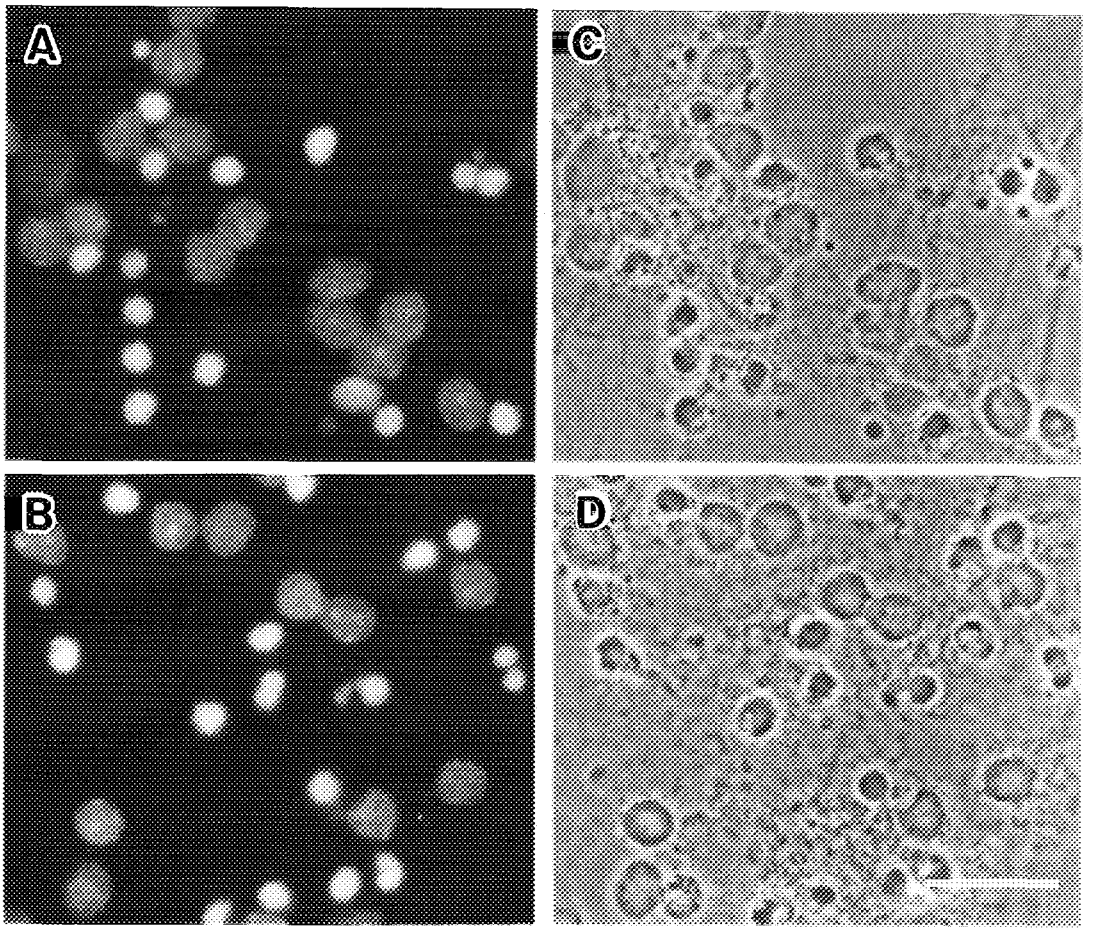


Figure 3. Condensation of nuclear chromatin in cerebellar neurons after the treatments with methylmercury or $K^+$ withdrawal. Neurons cultured as in Figure 1 were treated with methylmercury at 0.3 μM for 72 hours (B, D) or switched to serum-free medium containing 5 mM KCl and cultured for 48 hours (A, C). Fluorescent micrographs of the fixed cells after staining with bisbenzimide (A, B) and corresponding phase-contrast micrographs (C, D) were shown. Scale bar, 20 μm.

the appearance of differently condensed nuclei at this concentration (Fig. 1 H). It is noteworthy that neither DNA fragmentation nor cell death was observed after 24-hr treatment with methylmercury, even at 1.0 μM.

Actinomycin D, a transcriptional inhibitor, has been shown to inhibit neuronal death induced by $K^+$ withdrawal, while it is toxic to cells in long term exposure (i.e., more than 48 hr) (10). When actinomycin D (1 μg/ml) was added to the culture of granule neurons being treated with 0.3 μM methylmercury, DNA fragmentation and chromatin condensation were significantly inhibited, whereas viability was not rescued (data not shown). It is likely that cell death

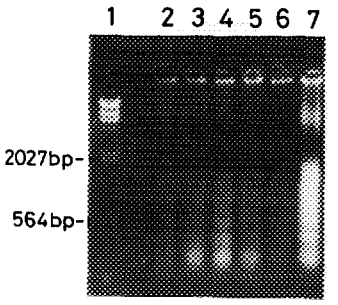


Figure 4. DNA fragmentation analysis of cerebellar neurons after the treatments with methylmercury or $K^+$ withdrawal. Soluble DNA was extracted from neurons treated with methylmercury at 0, 0.1, 0.3 or 1.0 μM (lanes 2, 3, 4 and 5, respectively) for 72 hours or at 1.0 μM for 24 hours (lane 6) or cultured in the serum-free medium containing 5 mM KCl for 48 hours (lane 7). DNA from equal number of plated cells ($6 \times 10^6$) was loaded in each lane. Lane 1 shows DNA molecular size marker.

observed in the presence of actinomycin D was mainly from the toxic effect of this drug.

An excitatory amino acid, glutamate, is also known to induce death of cerebellar granule neurons, but no DNA fragmentation associated with the death has been observed, suggesting that death of granule neurons is not always accompanied by DNA fragmentation and the death induced by glutamate occurs *via* necrosis (10, 15). Loo *et al.* reported that β-amyloid peptide induces apoptosis in cultured central nervous system neurons (16), while Behl *et al.* claimed that the death is rather necrotic than apoptotic (17). Methylmercury has been regarded as an inducer of necrotic death of neurons by disrupting cellular homeostasis through denaturation of cellular components including proteins and lipids (1, 18, 19). This may be valid at least at higher concentrations (i.e., >1 μM) in an *in vitro* system. However, at lower doses, which are more relevant for understanding the mechanism of methylmercury intoxication, the death of neuronal cells is rather apoptotic than necrotic as demonstrated above.

Recently, Nakamura *et al.* have shown that DNA fragmentation *in situ*, visualized by labeling the free 3'-OH end of chromosomal DNA with biotinylated -dUTP introduced by terminal deoxynucleotidyl transferase, occurred selectively in the granular layer of cerebellum in methylmercury-intoxicated rats (20). Their observation strongly suggests that methylmercury selectively induces apoptotic death of granule neurons *in vivo*, which is consistent with the *in vitro* results of this report.

Death of granule neurons induced by methylmercury is thus apoptotic, at least at lower doses. This process may play an important role in the neuronal death associated with Minamata disease.

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