Effects of Continuous Exposure of Mouse Primitive Neural Stem Cells to Methylmercury in Proliferation and Differentiation Stages

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Contents

1. Abstract

Methylmercury (MeHg) is a potent neurotoxin that causes Minamata disease and is particularly harmful during pregnancy, causing abnormal pregnancy or various adverse effects including congenital Minamata disease. Neural stem cells (NSCs) can proliferate and differentiate into neurons and glia, playing a key role in the formation of the CNS. Here, we examined the effects of continuous exposure of homogeneous embryonic stem cell-derived primitive NSCs to MeHg in the proliferation and differentiation stages. Cultured without MeHg in the proliferation stage, NSCs showed an exponential increase in the number of the cells up to day 4. However, continuous exposure of NSCs to MeHg induced apoptosis and caused a decrease in the number of NSCs in a dose- and time-dependent manner. Continuous exposure of NSCs to MeHg in the differentiation stage also caused a decrease in the number of NSCs but had no or little effect on differentiation from surviving NSCs into neurons and glia. The NSCs were about 20 times more susceptible to MeHg in the proliferation stage than the differentiation stage. These effects of continuous MeHg exposure on NSCs may be valuable in elucidating the mechanisms by which MeHg exposure during pregnancy causes congenital Minamata disease and reproductive problems. In particular, the present results suggests that MeHg even at a very low concentration may decrease the number of proliferating NSCs in the early stages of development of central nervous system (CNS) and cause shortage of NSCs required for normal development of CNS.

2. Keywords

Methylmercury

Neural stem cells

Developmental neurotoxicity

Congenital Minamata disease

Abnormal pregnancy

Embryonic stem cells

Proliferation

Apoptosis

3. Abbreviations

MeHg, methylmercury

NSC, neural stem cell

ESC, embryonic stem cell

NSS, neural stem sphere

FGF, fibroblast growth factor

PM, proliferation medium

ACM, astrocyte-conditioned medium

DM, differentiation medium

PBS, phosphate buffered saline

MAP2, microtubule-associated protein 2

GFAP, glial fibrillary acidic protein

DAPI, 4', 6-diamidine- 2'-phenylindole dihydrochloride

TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling

RT-PCR, real-time reverse transcription polymerase chain reaction

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

MBP, myelin basic protein

ACTB, β-actin

RPS29, ribosomal protein S29

RPL4, ribosomal protein L4

BSA, bovine serum albumin

4. Introduction

Methylmercury (MeHg) is a notorious neurotoxin that was shown to be responsible for Minamata disease. The initial case of Minamata disease was officially reported as a peculiar severe neurological disease of unknown etiology in May 1956 in Minamata City, Kumamoto Prefecture, Japan. At the end of the year, it was revealed that there had been 54 patients with similar symptoms since 1953 in the area around Minamata Bay, and that 17 of the patients had already died[1)-3)]. Typical symptoms of Minamata disease in children and adults were sensory disturbance, ataxia, muscle weakness, visual deficit, and auditory deficit[1)-5)]. Extensive studies performed mainly by researchers at Kumamoto University finally established that the disease was caused by the daily consumption of fish and shellfish that were heavily polluted by MeHg in waste water from a chemical factory[6)-8)]. In addition to children and adults, MeHg was shown to harm the embryonic/fetal nervous system through the mother's body and result in abnormal pregnancy defined as fetal death[9)] or congenital (or fetal) Minamata disease[10), 11)]. As of 2005, a total of 2265 people have been officially certified as Minamata disease patients in the Minamata area, and as many as 12300 people suffering from two or more symptoms of the Minamata disease acquired official support for medical expenses[3)]. In addition to the Minamata area, outbreaks of Minamata disease were also reported along the Agano River in Niigata prefecture, Japan, in the 1950s and 1960s[12)], where 690 people were officially certified[3)], and in Iraq in 1971 – 1972[13)].

Children with serious congenital Minamata disease showed symptoms similar to cerebral palsy together with symptoms characteristic of congenital Minamata disease, including

intelligence disturbance, cerebellar symptoms, and strabismus, although their mothers had moderate symptoms[10), 11)]. These results suggest that the nervous system is highly susceptible to MeHg in the developmental stage. During development of the nervous system, neural stem cells (NSCs) play a key role in proliferation and differentiation into neurons, astrocytes, and oligodendrocytes[14)]. Therefore, it is necessary to assess the influence of MeHg on NSCs to understand its effects on development of the fetal nervous system. NSCs, as well as neurons and glia[15)], have been reported to be induced to undergo apoptotic cell death by MeHg exposure using several types of NSCs, rat primary embryonic cortical NSCs and an NSC line originally derived from the neonatal mouse cerebellum (C17.2 cells)[16)], human NSC line derived from umbilical cord blood (HUCB-NSCs)[17)], and neural progenitor cells derived from the embryonic brain[18)]. However, NSCs were acutely exposed to MeHg in these previous studies, although fetuses in the uterus are generally exposed to MeHg chronically or continuously.

We reported previously that a large number of homogeneous NSCs can be directly produced via unidirectional neuronal differentiation from embryonic stem cells (ESCs) by the simple neural stem sphere (NSS) method[19)-21)]. ESC-derived NSCs can be stably expanded exponentially[22)] and differentiated into neurons and glia[19), 23), 24)]. The NSCs have been used successfully to study their responses to acute extrinsic stimuli, i.e., X-irradiation[25)] and heat shock[26)]. Here, we investigated the responses of NSCs to a not acute but continuous extrinsic stimulus, MeHg exposure, in the proliferation and differentiation stages. In addition, we focused on analyses of changes in cell number during MeHg exposure, because a sufficient

number of NSCs is necessary to generate appropriate numbers of neurons and glia required for development of a normal central nervous system (CNS). Consequently, we found that continuous MeHg exposure induces apoptotic cell death and decreases the number of NSCs in a time- and dose-dependent manner, and that NSCs are more susceptible to MeHg in the proliferation stage than in the differentiation stage.

5. Materials and methods

Preparation of NSCs and culture

Homogeneous NSCs were prepared from mouse ESCs by the NSS method, as described previously[19), 20)]. NSCs were plated onto dishes coated with Matrigel (BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced; Invitrogen, Carlsbad, CA) and allowed to proliferate exponentially in proliferation medium (PM) consisting of neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen) and 20 ng/ml fibroblast growth factor-2 (FGF-2) (R&D Systems, Minneapolis, MN). The medium was replaced every 2 days. To induce differentiation of NSCs into neurons and glia, the medium was switched from PM to differentiation medium (DM) consisting of neurobasal medium supplemented with 2% B-27 and 10% astrocyte-conditioned medium (ACM). The cells were cultured for up to 4 days without medium change.

Exposure of NSCs to MeHg

Proliferating NSCs were suspended in PM and plated at a density of 2×10^3 /cm^2 on

Matrigel-coated dishes. To evaluate the effects of exposure of NSCs to MeHg in the proliferation stage, the culture medium was replaced by PM containing various concentrations (0 – 1000 nM) MeHg (methylmercury chloride; Sigma-Aldrich, St. Louis, MO) on the next day after plating. The cells were cultured in media containing MeHg for 4 days, with culture medium changed on day 2. To investigate the effects of the exposure of NSCs to MeHg in the neural differentiation stage, proliferating NSCs in PM were plated as above. The PM was replaced by DM containing MeHg on the next day after plating. The cells were cultured for 4 days without medium change. The numbers of cells on dishes were determined every 24 hours using five or ten images acquired using an inverted phase-contrast microscope (Eclipse TE300; Nikon, Tokyo, Japan).

Immunofluorescence analysis

Cells plated and cultured on Matrigel-coated coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Immunocytochemistry was performed using standard protocols and antibodies as follows: Nestin (Rat-401, 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA); microtubule-associated protein 2 (MAP2) (1:200; Chemicon, Temecula, CA); glial fibrillary acidic protein (GFAP) (1:400; Chemicon); Alexa Fluor® 488- and 546-conjugated secondary antibodies (1:200; Molecular Probes, Eugene, OR). The nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). All fluorescence images were acquired using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

Cells on coverslips were fixed, and apoptotic cells were detected by TUNEL staining with an In situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Nuclei were counterstained with DAPI, and $TUNEL^+$ nuclei were assessed by fluorescence microscopy.

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Poly $(A)^+$ RNA was extracted from the cells cultured as above using Illustra™ QuickPrep Micro mRNA Purification Kits (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Each mRNA preparation was reverse transcribed into cDNA using random hexamer primers. Quantitative real-time RT-PCR was performed using a StepOnePlus™ Real Time PCR System (Applied Biosystems, Foster City, CA) and *Power* SYBR® GREEN PCR Master Mix (Applied Biosystems) with specific primer pairs. The primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Nestin, MAP2, GFAP, and myelin basic protein (MBP), which have been used previously[24], and the following sense and antisense primers were designed using Primer Express™ software (Version 2.0; Applied Biosystems): β-actin (ACTB), 5'-ATGGTGGGAATGGGTCAGAA-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'; ribosomal protein S29 (RPS29), 5'-TACTGGAGTCACCCACGGAAGT-3' and 5'-GGCACATGTTCAGCCCGTAT-3'; ribosomal protein L4 (RPL4), 5'-AAAGCTCCCATTCGACCAGAT-3' and

5'-TCTGATGACCTGCCAATTCACT-3'. The expression level of each gene was normalized relative to that of the housekeeping gene, RPS29.

6. Results

Dose- and time-dependent decreases in the number of NSCs in the proliferation stage associated with MeHg exposure

The effects of continuous MeHg exposure on the number of proliferating NSCs were evaluated by culturing NSCs in PM containing various concentrations of MeHg for 4 days. The morphologies of the cells exposed to MeHg were similar to those of control cells (Fig. 1A – C). Cultured without MeHg, NSCs showed an exponential increase in the number of the cells up to day 4, with a doubling time of about 24 hours (Fig. 1D). However, increases in the numbers of cells were inhibited by culture in the presence of MeHg in a dose-dependent manner (Fig. 1D). The numbers of cells cultured with more than 10 nM MeHg were significantly lower than those in the controls, and the number of cells cultured with 100 nM MeHg reached the maximum on day 2 of culture but did not increase thereafter (Fig. 1D). The majority of NSCs were lost during culture in the presence of > 300 nM MeHg, and the cell number dropped below the initial number on day 4 (Fig. 1D). The dose–response curve of each day in cell culture (i.e., days 1, 2, 3, and 4; Fig. 1E), indicated that the susceptibility of NSCs to MeHg increased in a time-dependent manner. Finally, the dose–response curve on day 4 showed a 50% inhibitory concentration (IC_{50} value) of about 20 nM MeHg. The results indicated that exposure of NSCs to MeHg in the proliferation stage decreases the number of

cells in a dose- and time-dependent manner.

Induction of apoptosis in proliferating NSCs by MeHg exposure

To determine whether the dose- and time-dependent decreases in number of proliferating NSCs by MeHg were due to apoptotic cell death, TUNEL staining was performed on the cells on day 1 after MeHg exposure. The ratio of $TUNEL^+$ to DAPI-stained (total) nuclei increased in a dose-dependent manner, with the percentage of apoptotic cells following incubation at 100 nM being 44.2%, which was 3.8-fold higher than that of the control cells (Fig. 2). These results indicated that MeHg at 100 nM induced severe apoptotic cell death in proliferating NSCs and caused the almost complete inhibition of increase in cell number of NSCs (Fig. 1D and E).

Effects of continuous exposure to MeHg on NSCs at the differentiation stage

To evaluate the effects of continuous exposure to MeHg in the differentiation stage, NSCs were induced to differentiate by changing the culture medium from PM to DM, ACM-supplemented medium, containing various concentrations of MeHg and cultured for 4 days. The control cells, which were cultured without MeHg, showed changes in morphology, and many neuron-like cells appeared (Fig. 3A), and the morphologies of the cells exposed to MeHg were similar to those of control cells (Fig. 3B, C). The control cells showed an exponential increase in cell number up to day 2, after which the increase gradually slowed and stopped by day 3 (Fig. 3D). The increase in cell number was inhibited by MeHg in a

dose-dependent manner (Fig. 3D). The numbers of cells cultured in the presence of > 300 nM MeHg were significantly lower than the controls from day 2 (Fig. 3D). The dose–response curves of cells cultured for a prolonged period indicated that the more prolonged exposure resulted in the more decrease in cell number (Fig. 3E), and the curve at day 4 showed an IC_{50} value of about 400 nM for MeHg. The value in the differentiation stage was about 20 times higher than that in the proliferation stage. These results suggest that the effects of MeHg on the number of NSCs in the differentiation stage are both dose- and time-dependent and NSCs in the differentiation stage were less susceptible to MeHg than those in the proliferation stage.

Effects of MeHg exposure on neural cell marker gene and protein expression in NSCs in the proliferation and differentiation stages

Real-time RT-PCR analysis was performed to investigate whether exposure of NSCs to MeHg in the proliferation and differentiation stages induces changes in cellular characteristics. The expression level of each target gene was normalized relative to that of *RPS29* mRNA in this experiment. In previous studies, we used the *GAPDH* gene as a housekeeping gene to normalize the expression level of the target genes[20), 22), 24)-26)]. However, because exposure to 100 nM MeHg upregulated *GAPDH* gene expression but not that of the *RPS29* gene as well as the other candidate housekeeping genes, *RPL4* and *ACTB* genes[26)] (Fig. 4A), we adopted the *RPS29* gene as a housekeeping gene in this analysis.

Quantitative gene expression analysis demonstrated high levels of expression of the *Nestin* gene, a marker of NSCs, in proliferating control cells cultured in PM without MeHg

for 4 days (Fig. 4B and C, left, white bar), whereas *MAP2* and *GFAP* genes, markers of neurons and astrocytes, respectively, were expressed at low levels (Fig. 4C, center and right, white bar, respectively), as reported previously[20), 22), 25), 26)]. The level of *Nestin* gene expression in the control cells was not significantly different from that in cells cultured for 4 days with higher concentrations of MeHg (100, 300, and 1000 nM) (Fig. 4B). Similarly, low levels of *MAP2* and *GFAP* genes expression were observed in cells exposed to high concentrations of MeHg (data not shown). In addition to gene expression analyses, immunofluorescence staining showed that almost all of the cells exposed to 100 nM MeHg for 4 days were positive for Nestin (Fig. 5A and B). These results indicated that the cells surviving after MeHg exposure in the proliferation stage retain the characteristics of NSCs represented by capacity for Nestin gene and protein expression.

Real-time RT-PCR analysis proved that NSCs in DM can be induced to undergo differentiation into neurons and astrocytes, as reported previously[25), 26)]. Induction of differentiation in the control cells reduced the level of *Nestin* gene expression slightly but not significantly (Fig. 4C, left, dark bar), and, in contrast, significantly upregulated the levels of *MAP2* and *GFAP* gene expression (Fig. 4C, center and right, dark bars). The expression levels of *MAP2* and *GFAP* gene in the cells exposed to MeHg were upregulated (data not shown) but were not different from those of the control cells (Fig. 4D, center and right, dark and black bars). Expression of the *MBP* gene, a marker of oligodendrocytes, was not detectable in this experiment (data not shown). Immunofluorescence staining confirmed that control NSCs differentiated into MAP2-positive neurons (Fig. 5C) and GFAP-positive astrocytes (Fig. 5E)

after culture for 5 days in DM, as reported previously[25), 26)]. Similarly, the NSCs exposed to MeHg differentiated into MAP2-positive neurons (Fig. 5D) and GFAP-positive astrocytes (Fig. 5F). These results suggest that differentiation from NSCs to neurons and astrocytes is not significantly affected by exposure to MeHg.

Proliferation and differentiation capacities of surviving NSCs after MeHg exposure

To investigate whether the cells surviving after MeHg exposure retain the characteristics of NSCs, such as capacity for self-renewal and differentiation, the cells were exposed to 100 nM MeHg for 4 days and surviving cells were subcultured in PM. First, the surviving cells increased exponentially in number in PM for 4 days similar to the control cells (Fig. 6). Immunofluorescence analysis indicated that almost all of the cells expressed Nestin protein after subculture of both surviving cells and control cells (Fig. 5H and G). These results indicated that the surviving cells after exposure to 100 nM MeHg for 4 days retained the capacities for proliferation and *Nestin* gene expression. Next, to examine whether the surviving cells after MeHg exposure retained neural differentiation capability, the cells were subcultured in PM for 1 day and subsequently in DM for 5 days to induce differentiation. Immunofluorescence analysis demonstrated that many MAP2-positive and GFAP-positive cells were present after subculture of the surviving cells and control cells (Fig. 5J and I, L and K). These results suggest that the surviving cells after MeHg exposure retained the characteristics of NSCs, i.e., capacities for proliferation and differentiation into neurons and glia.

7. Discussion

The present study clearly demonstrated that continuous exposure of NSCs to MeHg induces apoptosis in the proliferation stage and causes a decrease in the number of NSCs in a dose-dependent manner. Interestingly, the results presented here also indicated that the susceptibility of NSCs to MeHg in the proliferation stage increased during continuous MeHg exposure in a time-dependent manner, and became very high after exposure for 4 days (IC_{50}, 20 nM). On the other hand, previous studies have not indicated changes in the susceptibility of NSCs to MeHg during continuous exposure. For example, acute MeHg exposure for short periods (24 or 48 hours) has been reported to induce apoptotic cell death in various types of proliferating NSCs, including rat primary embryonic cortical culture[16], mouse NSC line (C17.2)[16], and human NSC line (HUCB-NSC)[17]. Although another study indicated that MeHg exposure for 6 days induced apoptosis in proliferating mouse neural progenitor cells derived from different regions of the embryonic brain (telencephalon and diencephalon), the authors have not reported the time-dependent changes in susceptibility to MeHg[18]. The IC_{50} values estimated from the results of previous reports[16-18], from 100 to 600 nM, are much higher than that of the present study after exposure for 4 days (IC_{50}, 20 nM). The biological half-life of MeHg has been reported to be considerably long, from 35 to 189 days, with an average of 72 days[28]. Therefore, embryos/fetuses *in utero* are generally thought to be exposed to MeHg continuously or chronically rather than acutely. The time-dependent increase in susceptibility of NSCs is thus very informative to estimate the neurotoxic effects of MeHg on nervous system development *in utero*.

It is speculated that apoptotic cell death is the main cause of the time-dependent increase in susceptibility to MeHg. Continuous MeHg exposure induces apoptosis in some NSCs. Therefore, the pool of proliferating NSCs decreases gradually during exposure, while all of the control cells continued to proliferate exponentially. In addition to apoptosis, other groups have shown that acute MeHg exposure inhibits proliferation of HUCB-NSCs[17] and primary fetal CNS cells in culture[29]. Further study remains to elucidate fully in which mechanism MeHg exposure inhibits proliferation of NSCs.

The susceptibility of NSCs to MeHg at the differentiation stage has been demonstrated to be lower than that at the proliferation stage, but it is comparable to or higher than the other types of neural cells, neurons and glia[15]. In addition, we showed that MeHg exposure decresed the number of cells but did not significantly affect the process of differentiation from the surviving NSCs to neurons and glia itself. The results regarding neuronal differentiation conflict with those obtained in rat primary cortical culture[16] but agree with those obtained in HUCB-NSCs[17]. With regard to differentiation into astrocytes, however, the present results were inconsistent with those obtained in HUCB-NSCs[17]. The reasons for these discrepancies are unclear, but may be due to the differences in origin of the NSCs. In fact, the susceptibilities of NSCs derived from the telencephalon and the diencephalon to MeHg were different[18]. In contrast, we employed ESC-derived primitive NSCs[19)-22)], which was in the default state[14].

From the present results, the effect of chronic exposure of embryos/fetuses to MeHg *in vivo* is presumed to be a decrease in the number of NSCs, and it likely explain CNS

maldevelopment and reproductive problems caused by prenatal MeHg exposure. Normal development of the CNS requires the concomitant and coordinated ontogeny of proliferation and differentiation to occur in a temporally and spatially controlled manner. Therefore, perturbations of the processes during development by MeHg exposure can result in disturbance of the structure and function of the CNS[30]. A number of *in vivo* studies in rodents and non-human primates have demonstrated the effects of MeHg exposure on neurodevelopment[31)-34)]. On the other hand, in humans, congenital Minamata disease was established to be caused by MeHg exposure of embryo/fetus during pregnancy[10), 11)]. Patients with this disease develop severe various neurological and mental symptoms, such as intelligence disturbance, primitive reflex, cerebellar symptoms, disturbance of body growth and nutrition, dysarthria, deformity of limbs, strabismus, etc.[10), 11)]. Pathological studies have demonstrated that cortical lesions of the brain are distributed more widely and more severely in congenital Minamata disease than in infantile and adult cases[35), 36)]. In addition, in many cases, hypoplasia and dysplasia of the nervous system were observed in congenital Minamata disease, and a specifically small brain was reported in two of three autopsy cases[35)]. Microcephaly accompanying congenital Minamata disease was indirectly suggested by the report that small heads were observed in 40% of children that were exposed to high levels of MeHg *in utero*[37)]. A sufficient number of NSCs is necessary for normal ontology of developmental processes in the CNS. Therefore, the decrease in NSC number by MeHg exposure may result in the pathogenesis of congenital Minamata disease, microcephaly, hypoplasia, and disturbance of CNS function. In addition, if the influence of MeHg on the

NSCs is not so severe, it may result in moderate symptoms depending on the severity. In the case of Iraqi infants that had been exposed to MeHg during pregnancy, a dose–response relationship was reported between the severity of symptoms and MeHg concentration in maternal hair[38]. In addition, an epidemiological study suggested a relationship between pre- or postnatal exposure to MeHg and psychiatric symptoms among the general population in Minamata[39]. Severe loss of NSCs during ontology may terminate development of the embryo/fetus and result in reproductive problems, such as miscarriage, abortion, and stillbirth. In fact, an epidemiological study indicated that the incidence rates of reproductive problems, defined as fetal death, stillbirth, and spontaneous abortion, were increased in two areas heavily contaminated with MeHg, around Minamata Bay in the period between 1956 and 1968, when the pollution became serious[9].

An epidemiological study on neurotoxicity suggested that MeHg exposure is more hazardous prenatally than postnatally[40]. MeHg in the environment is therefore a matter of great concern, and to protect human health and the environment from anthropogenic emissions and release of mercury and mercury compounds, the "Minamata Convention on Mercury" was agreed at the Intergovernmental Negotiating Committee in Geneva, Switzerland, in 2013. Epidemiological studies performed to date have mainly focused on assessment of the neurotoxic effects of MeHg on infants and children. However, as shown above, it will be necessary to assess the effects of MeHg on reproductive problems in addition to neurotoxic effects in future studies.

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9. Figures

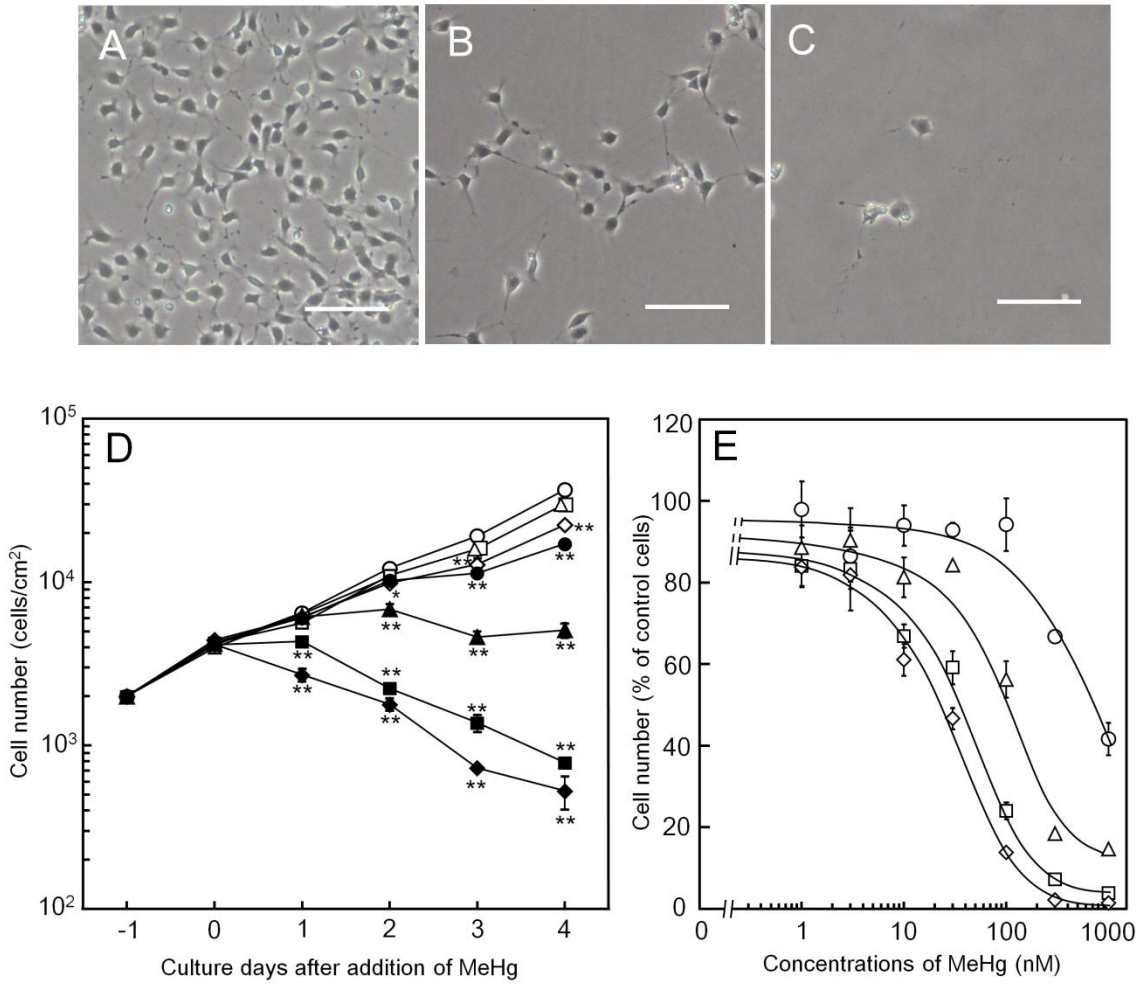

Figure 1. Changes in morphologies and cell numbers of NSCs after MeHg exposure in PM

(A – C) Phase-contrast micrographs of NSCs cultured for 4 days in PM with 0 nM MeHg (A), 100 nM MeHg (B), or 1000 nM MeHg (C). Scale bars: 100 μm. (D) Cell growth analysis of proliferating NSCs after MeHg exposure. NSCs were plated at a density of 2×10^3 /cm^2 on dishes and cultured for 1 day in PM and subsequently for 4 days in PM with 0 nM MeHg (control) (○), 1 nM MeHg (Δ), 3 nM MeHg (□), 10 nM MeHg (◊), 30 nM MeHg (●), 100 nM MeHg (▲), 300 nM MeHg (■), or 1000 nM MeHg (♦). The numbers of cells were counted every day. The values represent the means ± SEM (bars). **P* < 0.05, ***P* < 0.01 compared with control. (E) Dose–response curves on day 1 (○), day 2 (Δ), day 3 (□), and day 4 (◊). The cell numbers are shown as percentages of control cells and are expressed as means ± SEM (bars) of four determinations.

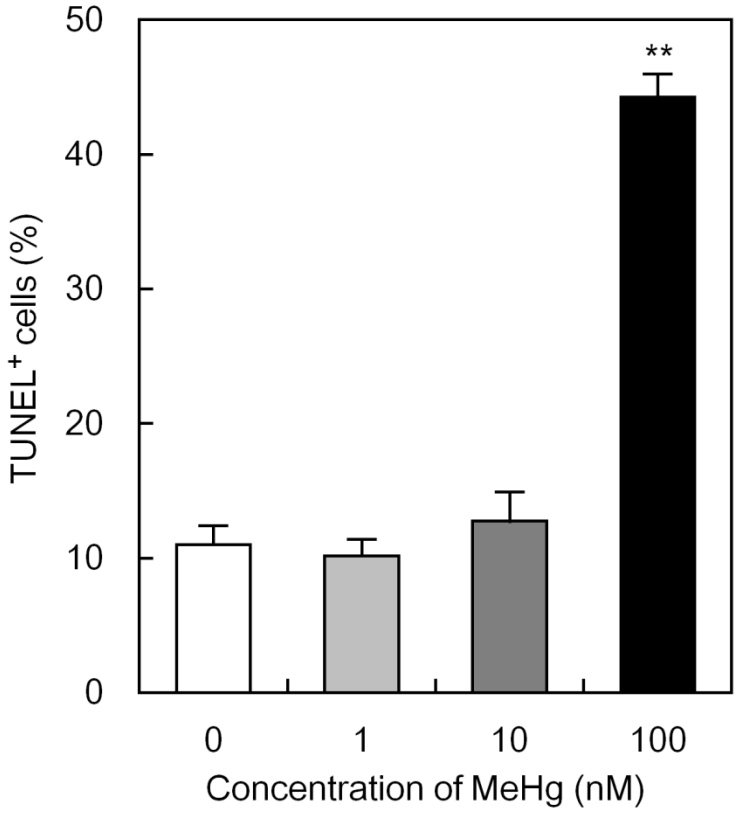

Figure 2. TUNEL detection of apoptotic cells after MeHg exposure in PM

TUNEL assay was performed on NSCs cultured for 1 day in PM with 0 nM MeHg (white bar), 1 nM MeHg (light gray bar), 10 nM MeHg (dark gray bar), or 100 nM MeHg (black bar). The $TUNEL^+$ cells are shown as percentages of the total cell number. All data are presented as the means ± SEM (bars) of three determinations. $^{**}P < 0.01$ compared with control.

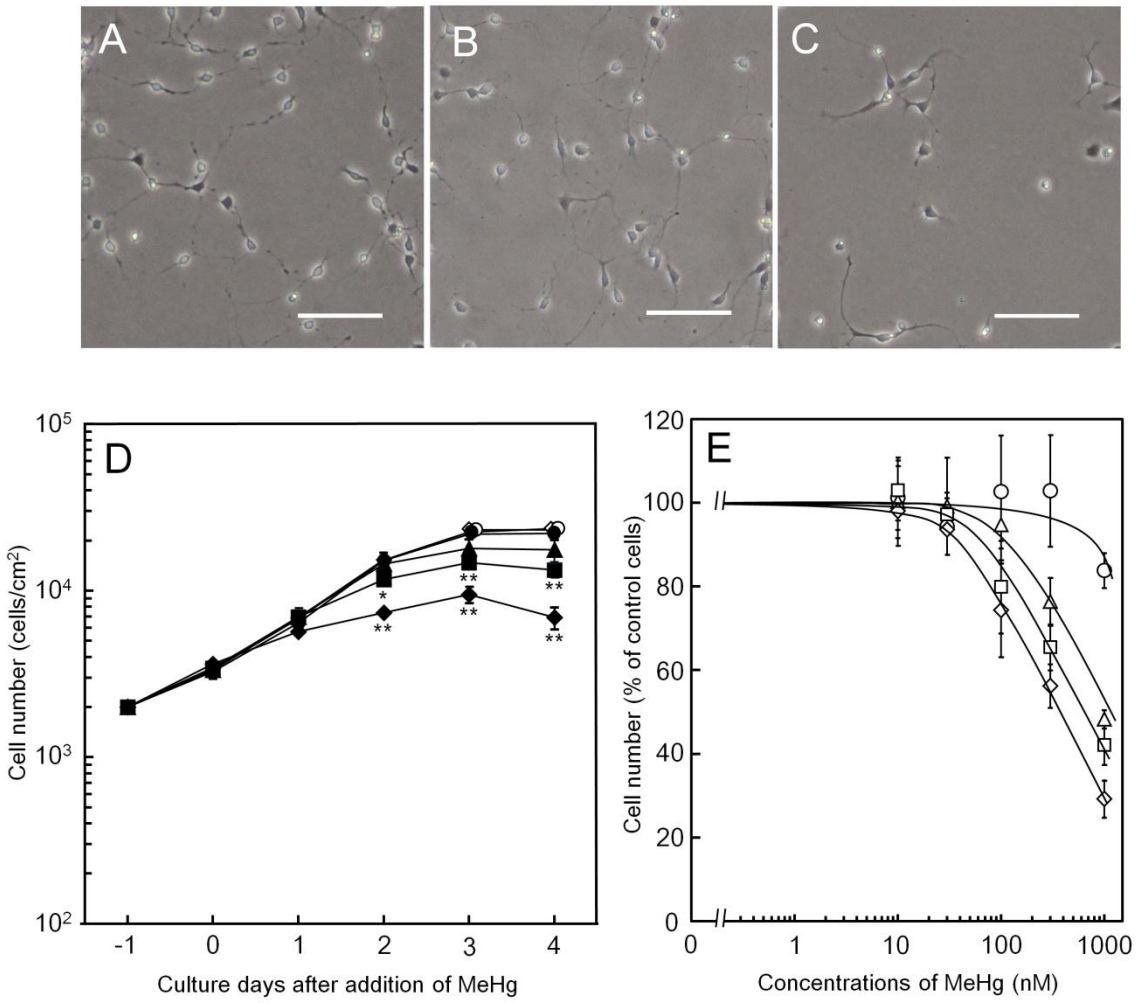

Figure 3. Changes in morphologies and cell number of NSCs after MeHg exposure in DM

(A – C) Phase-contrast micrographs of NSCs cultured for 4 days in DM with 0 nM MeHg (A), 100 nM MeHg (B), or 1000 nM MeHg (C). Scale bars: 100 μm. (D) Cell growth analysis of differentiating NSCs after MeHg exposure. NSCs were plated at a density of 2×10^3 /cm^2 on dishes and cultured for 1 day in PM and subsequently for 4 days in DM with 0 nM MeHg (control) (○), 10 nM MeHg (◊), 30 nM MeHg (●), 100 nM MeHg (▲), 300 nM MeHg (■), or 1000 nM MeHg (♦). The numbers of cells were counted every day. The values represent the means ± SEM (bars). **P* < 0.05, ***P* < 0.01 compared with control. (E) Dose–response curves on day 1 (○), day 2 (Δ), day 3 (□), and day 4 (◊). The cell numbers are shown as percentages of control cells and are expressed as means ± SEM (bars) of four determinations.

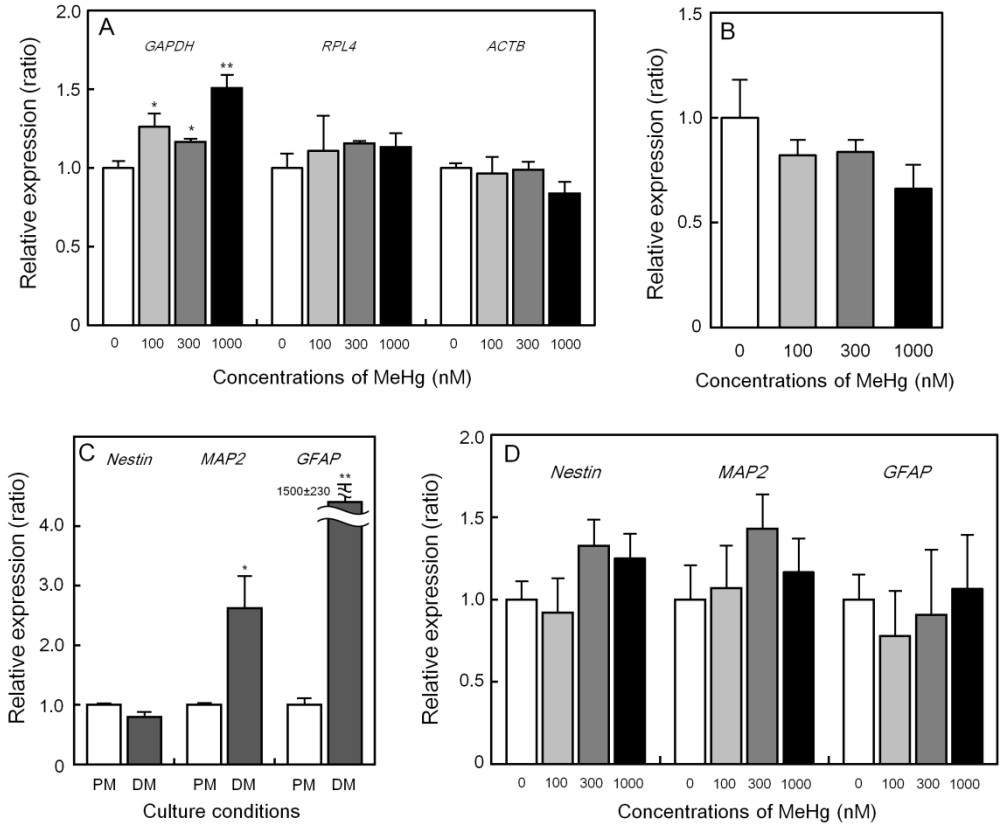

Figure 4. Gene expression analysis of NSCs in proliferation and differentiation stages after MeHg exposure

Gene expression levels were measured by quantitative real-time RT-PCR and normalized relative to that of *RPS29*. Data are presented as the means ± SEM (bars) of four determinations. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with control. (A) Determination of the expression levels of various housekeeping gene candidates, including *GAPDH*, *RPL*, and *ACTB*. NSCs were cultured for 4 days in PM with 0 nM MeHg (control) (white bars), 100 nM MeHg (light gray bars), 300 nM MeHg (dark gray bars), or 1000 nM MeHg (black bars). The ordinate represents the ratio of gene expression level to that of control. (B) Gene expression of *Nestin*, a neural stem cell marker, after MeHg exposure in the proliferation stage. NSCs were cultured for 4 days in PM with 0 nM MeHg (control) (white bars), 100 nM MeHg (light gray bars), 300 nM MeHg (dark gray bars), or 1000 nM MeHg (black bars). The ordinate represents the ratio of gene expression level to that of control. (C) Gene expression of neural cell markers after induction of neural differentiation. NSCs were cultured for 4 days in PM (control) (white bars) or DM (gray bars). The values of the gene expression levels of *Nestin* (left), *MAP2* (center), and *GFAP* (right) were standardized relative to those in PM. The means of gene expression level were 0.09 (*Nestin* in PM), 0.07 (*Nestin* in DM), 0.16 (*MAP2* in PM), 0.41 (*MAP2* in DM), 6×10^{-5} (*GFAP* in PM), and 0.10 (*GFAP* in DM). (D) Gene expression of neural cell markers after MeHg exposure in the differentiation stage. NSCs were cultured for 4 days in DM with 0 nM MeHg (control) (white bars), 100 nM MeHg (light gray bars), 300 nM MeHg (dark gray bars), or 1000 nM MeHg (black bars). The ordinate represents the ratio of gene expression level to that of control.

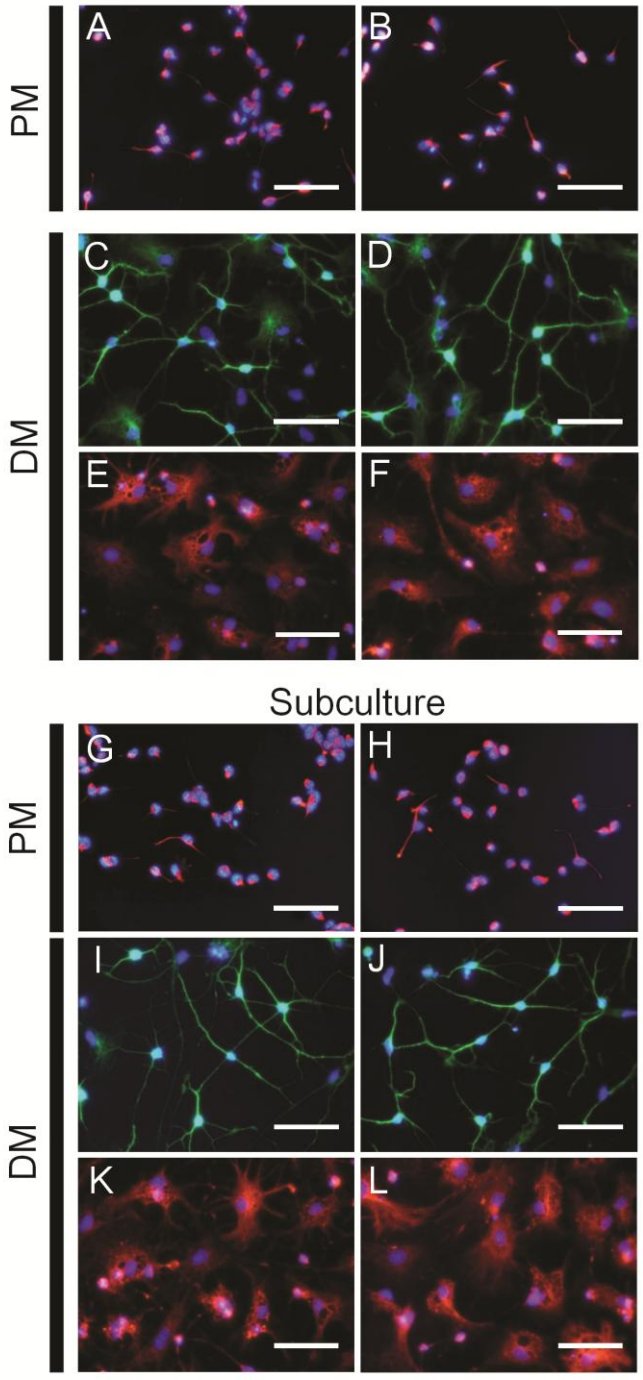

Figure 5. Immunofluorescence analysis of NSCs cultured in PM and DM, and surviving NSCs after MeHg exposure subcultured in PM and DM

(A – F) NSCs were cultured for 4 days in PM with 0 nM MeHg (control) (A) or 100 nM MeHg (B), or were cultured for 5 days in DM with 0 nM MeHg (control) (C and E) or 100 nM MeHg (D and F). (G – L) NSCs were cultured for 4 days in PM with 0 nM MeHg (control) (G, I and K) or 100 nM MeHg (H, J and L). Surviving cells after exposure were collected and subcultured for 4 days in PM (G and H) or for 1 day in PM and subsequently for 5 days in DM (I, J, K, and L). Fluorescence microscopy images of Nestin (red in A, B, G, and H), MAP2 (green in C, D, I, and J), and GFAP (red in E, F, K , and L) with DAPI counterstaining for nuclei (blue). Scale bars: 50 μm.

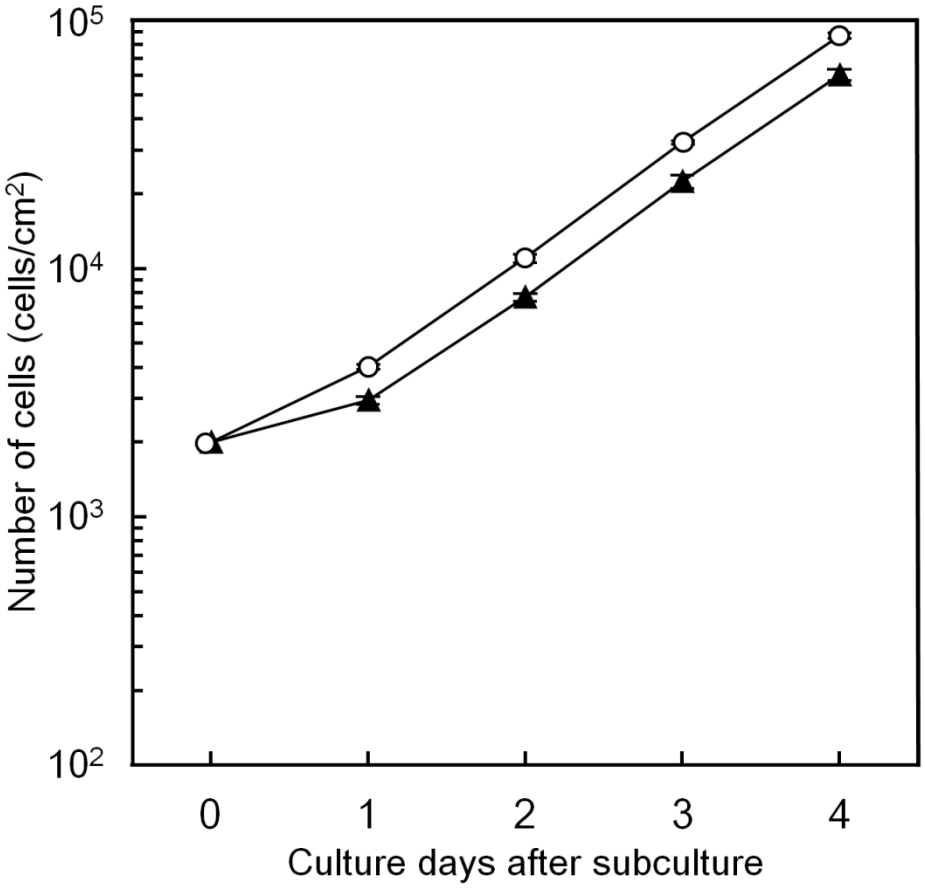

Figure 6. Cell growth analysis of subcultured NSCs after MeHg exposure in PM

After NSCs were cultured for 4 days in PM with 0 nM MeHg (control) (○) or 100 nM MeHg (▲), the surviving NSCs were collected. The cells were plated at a density of $2\times10^3/cm^2$ on dishes and subcultured for 4 days in PM. The numbers of cells were counted every day.

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11. Research achievements

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