1
2
3 Effects of subcytotoxic cadmium on morphology of glial fibrillary
4
5
6 acidic protein network in astrocytes derived from murine neural
7
8
9 stem/progenitor cells
10
11
12
13
14
15
16
17
18
19
20
21

22 Hideki Mori^{*}, Go Sasaki, Mayu Nishikawa, and Masayuki Hara
23
24
25
26
27
28
29
30
31

32 Department of Biological Science, Graduate School of Science, Osaka Prefecture
33
34
35 University, Gakuen-cho 1-2, Naka-ku, Sakai, Osaka 599-8570, Japan
36
37
38
39
40
41
42
43
44
45
46

47 ^{*}Corresponding author: E-mail address: morihide@b.s.osakafu-u.ac.jp (H.Mori).
48
49
50

51 Tel. and Fax: +81-72-254-8342
52
53
54
55
56
57
58
59
60

Abstract

The susceptibility of mouse neural stem/progenitor cells (NSPCs) to heavy-metal cytotoxicity was assessed by measuring cell viability following exposure to heavy metal chlorides (ZnCl_2 , CdCl_2 , CuCl_2 , and HgCl_2 , respectively). We determined half-maximal inhibitory concentration (IC_{50}) values, subcytotoxic doses, capacity for neural differentiation, and morphological features of glial fibrillary acidic protein (GFAP) network at the subcytotoxic doses of heavy metal ions. Experiments were performed using two protocols for the exposure at subcytotoxic doses of heavy metal ions; these protocols included simultaneous exposure with the induction of NSPC differentiation and sequential exposure after the induction for 1 week. Exposure to HgCl_2 using both protocols reduced the ratio of neuronal NSPC differentiation. Although sequential exposure to CdCl_2 reduced the size of GFAP network, simultaneous exposure did not induce any change. In conclusion, image analyses of the cytoskeletal morphology of NSPCs as a novel tool for assessing neurodevelopmental cytotoxicity enabled us to obtain new information about the localization of cytoskeletal proteins.

Keywords: neural stem cell, developmental neurotoxicity, cadmium chloride, glial fibrillary acidic protein, morphology

1. Introduction

Neural stem/progenitor cells (NSPCs) possess the capability for self-renewal and multipotency that can give rise to neuronal and glial cells, which constitute the central nervous system (CNS) (Gage, 2000; Reynolds et al., 1992). The development of a culture technique that maintains the self-renewal capacity and multipotency *in vitro* expanded the potential of NSPCs to provide an alternative to animal experiments and for development as promising treatments for intractable diseases and injuries of the CNS (Bal-Price et al., 2010; Breier et al., 2010; Mori and Hara, 2013).

Some studies innovated comprehensive gene expression elucidation using microarray and cell population analyses by flow cytometry in order to establish the feasibility of applying embryonic and neural stem cells in developmental neurotoxicity and cytotoxicity assays (Baek et al., 2012; Bal-Price et al., 2010; Breier et al., 2010; Theunissen et al., 2012). The flow cytometric and gene expression analyses using microarrays provided a huge data set detailing protein expression and related genes rapidly and broadly. However, no information about the cytoskeletal morphology and intracellular localization of proteins was obtainable with these approaches. Therefore, there are still concerns that important symptoms for the evaluation of neurotoxicity could be missed or neglected in some cases. Microscopic imaging of cells provides

1
2
3 information on cellular characteristics, which is related to the cell types and states, as
4
5
6 well insight into the localization of intracellular and membrane proteins. Recently,
7
8
9 automated methods for the analysis of cell morphology based on microscopic images
10
11
12 have been developed to evaluate the quality of cultured stem cells (Matsuoka et al.,
13
14
15 2013; Mori et al., 2007). The application of image-based analysis on cultured stem cells
16
17
18 can provide a rapid and highly sensitive assay system for the assessment of
19
20
21 neurodevelopmental toxicity.
22
23
24

25 Heavy metals enter the body from the atmosphere through respiration, oral ingestion of
26
27
28 food and drinks in daily life, and from implanted orthopedic and dental materials
29
30
31 (Grandjean and Landrigan, 2006). Among the heavy metals, copper and zinc (Cu^{2+} and
32
33
34 Zn^{2+} , respectively) are essential elements for the body. On the other hand, mercury and
35
36
37 cadmium (Hg^{2+} and Cd^{2+}) are toxic non-essential elements, which can be deleteriously
38
39
40 incorporated in the body. However, the precise mechanisms underlying the toxicity of
41
42
43 heavy metals are yet to be completely elucidated.
44
45
46

47 In this study, we evaluated the developmental neurotoxicity of four heavy metal ions in
48
49
50 NSPCs, using cell viability assay and immunocytochemical analysis. Zn^{2+} as an
51
52
53 essential element as well as Cu^{2+} , Cd^{2+} , and Hg^{2+} , the neighboring metals in the
54
55
56 elemental periodic table were used.
57
58
59
60
61
62
63
64
65

2. Materials and methods

2.1 Cell culture

NSPCs were obtained from the cerebra of embryonic day 14 (E14) ICR mice (Japan SLC, Hamamatsu, Japan) using the neurosphere culture technique (Mori et al., 2013).

The protocols for the care and use of animals in this study complied with the relevant laws and institutional guidelines for animal welfare at the Osaka Prefecture University.

NSPCs were cultured on polystyrene dishes in neurosphere-culture medium (DMEM/F12, Sigma-Aldrich, St. Louis, MO, USA) supplemented with antibiotic-antimycotic mixed solution (Nacalai Tesque, Kyoto, Japan), 2% (v/v) B27 supplement (Life Technologies, Grand Island, NY, USA), recombinant human epidermal growth factor (EGF, 20 ng/mL, PeproTech, Rocky Hill, NJ, USA), and recombinant human basic fibroblast growth factor (b-FGF, 20 ng/mL, PeproTech) at 37 °C and in 5% (v/v) CO₂ atmosphere. The cells were suspended at a density of 200,000 cells/mL in neurosphere culture medium, which was replaced every few days with fresh medium, and passaged using 0.05% (v/v) trypsin solution once a week.

2.2 Induction of neural differentiation

To induce neural differentiation, neurospheres formed by the NSPCs after 3 days of

1
2
3 culturing were further cultured on coverslips (Matsunami, Kishiwada, Japan) coated
4
5
6 with poly-L-ornithine (Sigma-Aldrich) in the differentiation medium containing
7
8
9 DMEM/F12 supplemented with antibiotic-antimycotic mixed solution, 2% (v/v) B27
10
11
12 supplement, and 1% (v/v) fetal bovine serum (FBS, Sigma-Aldrich).
13
14
15

16 To investigate the effect of heavy metals on neural differentiation, NSPCs were tested
17
18
19 using two protocols with different exposure times to heavy metals. One protocol
20
21
22 involved simultaneous heavy metal exposure and induction of neural differentiation
23
24
25 (Simultaneous exposure, Fig. 1) while the other protocol exposed cells to heavy metals
26
27
28 7 days after induction of neural differentiation (Sequential exposure, Fig. 1).
29
30
31
32
33
34

35 *2.3 Cell viability testing*

36
37

38 NSPCs were seeded on 96-well plates at 50,000 cells/mL (100 μ L/well), and incubated
39
40
41 at 37 °C and 5% (v/v) CO₂ atmosphere for 3 days. PC12 cells were seeded at 20,000
42
43
44 cells/mL. The metal chlorides (ZnCl₂, CdCl₂, CuCl₂, and HgCl₂, Nacalai Tesque, Kyoto,
45
46
47 Japan) were prepared as stock solution dissolved in distilled water at 25 times the final
48
49
50 concentration, and were added to the culture medium after sterilization by filtration. An
51
52
53 equal volume of distilled water was given as a control sample. Cells were exposed to
54
55
56 culture medium containing ZnCl₂, CdCl₂, CuCl₂, and HgCl₂ at concentration ranges of
57
58
59
60
61
62
63
64
65

0.01–1, 0.005–1, 0.005–0.5, and 0.002–0.1 mM, respectively. After 24-h heavy metal exposure, cell survival was measured using a cell counting kit (07553-15, Nacalai Tesque) according to the manufacturer's instructions.

2.4 Immunocytochemistry

The differentiated NSPC samples were placed on coverslips, fixed in 4% (w/v) paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 30 min on ice and then washed with PBS. After the samples were permeabilized with 0.3% (v/v) Triton X-100 (Nacalai Tesque) for 5 min and washed with PBS, they were incubated for 1 h at room temperature in PBS containing 10% (v/v) goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.01% (v/v) Triton X-100. Next, the samples were incubated with anti-tubulin β III mouse monoclonal antibodies (1:500, Covance, Princeton, NJ, USA) and anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibodies (1:100, Sigma-Aldrich) in PBS containing 10% (v/v) goat serum at 4 °C overnight. After washing in PBS, the samples were incubated with anti-mouse IgG AlexaFluor488 conjugated (1:500) and anti-rabbit IgG AlexaFluor568 conjugated (1:500; Life Technologies) secondary antibodies and 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Mashikimachi, Japan) in

PBS containing 10% (v/v) goat serum for 1 h at room temperature. For the double staining of F-actin and GFAP, anti-rabbit IgG secondary antibodies conjugated with FITC (1:500, Life Technologies) against GFAP polyclonal antibodies were used with rhodamine-phalloidin (cytoskeleton, Denver, CO, USA). After washing thrice with PBS, the samples were mounted on glass slides, air-dried, and observed using fluorescence microscopy (Optiphot2, Nikon, Tokyo, Japan). The images were captured using a digital camera (DS-5Mc, Nikon).

2.5 Statistical analysis

All experiments were performed in triplicate, and representative data are shown. Statistical analysis was performed using computer software Excel 2010 (Microsoft, Redmond, WA, USA). Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was assessed using the Bonferroni-Holm test with $p < 0.05$ considered significant.

3. Results

3.1 Viability of NSPCs and PC12 cells after exposure to heavy metal ions

The sensitivity of NSPCs to heavy metals was evaluated by performing a cell viability

assay using water-soluble tetrazolium (WST) solution (cell counting kit). The viability of PC12 cells was assessed using the same assay to enable a comparison between NSPCs and an example of neuronal cells. Fig. 2 shows the survival ratios of NSPCs and PC12 cells after exposure to heavy metals. The number of viable cells decreased concentration-dependently following treatment with the four metal ions.

The viability-concentration graph showed a more gradual decrease in cell viability following incubation with Cu^{2+} and Cd^{2+} ions, than it did with Zn^{2+} and Hg^{2+} ions, and the half-maximal inhibitory concentration (IC_{50}) values were calculated for each heavy metal (Table 1).

The order of the IC_{50} values in NSPCs was $\text{Zn}^{2+} \ll \text{Hg}^{2+} \leq \text{Cu}^{2+} < \text{Cd}^{2+}$. Furthermore, the sensitivities of cells to individual heavy metal ions differed between the two cell types (Zn^{2+} and Cd^{2+} , $\text{PC12} < \text{NSPC}$; Cu^{2+} , $\text{PC12} \approx \text{NSPC}$; and Hg^{2+} , $\text{PC12} > \text{NSPC}$).

3.2 Neural differentiation of NSPCs after exposure to heavy metals

The concentrations of the metal ions used to evaluate their toxicity against neuronal and glial differentiation of NSPCs were chosen based on the results of the viability assays described above. The subcytotoxic concentrations previously determined to result in cell survival rates above 80% were used in the differentiation assay (Table 2).

1
2
3 In order to determine the differentiation stage that was susceptible to heavy-metal
4
5
6 toxicity, two experimental protocols with different metal ion exposure times were used
7
8
9 (Fig. 1). “The protocol that involved simultaneous heavy metal exposure and cell
10
11
12 differentiation (Fig. 1, simultaneous exposure)” was used to investigate the influence of
13
14
15 heavy metals on the early stage of NSPC differentiation. “The protocol involving
16
17
18 sequential heavy metal exposure after cell differentiation for 7 days (Fig. 1, sequential
19
20
21 exposure)” was used to investigate the influence of heavy metals on the late stage of
22
23
24 NSPC differentiation, and the susceptibility of cells after neuronal or glial
25
26
27 differentiation. The ratio of neuronal/glial cells differentiated from NSPCs was
28
29
30 measured using cell counting after immunostaining with lineage markers. Distribution
31
32
33 of neuronal and glial lineages (β III tubulin- and GFAP-positive cells, respectively) is
34
35
36 shown in Table 2. Exposure to Zn^{2+} , Cd^{2+} , and Cu^{2+} did not show different effects on
37
38
39 cell differentiation. However, Hg^{2+} exposure elicited a significant decrease in the
40
41
42 number of cells in the neuronal lineage.
43
44
45
46
47
48
49
50

51 *3.3 Cytoskeletal morphology of cells following heavy metal exposure*

52
53

54 Representative fluorescence microscopy images are shown in Fig. 3A. All samples
55
56
57 exhibited numerous star-shaped astrocytes differentiated from NSPCs. The cells adhered
58
59
60

1
2
3 to the glass slide and then spread out. We found that the area of astrocytes
4
5
6 immunostained with anti-GFAP antibodies was smaller in the cell samples prepared
7
8
9 after sequential exposure to Zn^{2+} and Cd^{2+} and differentiation than in the control
10
11
12 samples incubated without metal ions. However, we did not find this in the cell samples
13
14
15 that were simultaneously exposed and differentiated. The GFAP network of astrocytes
16
17
18 sequentially exposed to Zn^{2+} and Cd^{2+} and cell differentiation presented with a notably
19
20
21 shrunken appearance. Figure 3B and C show the immunostained area of GFAP network
22
23
24 of astrocytes calculated using an image analysis program (ImageJ, NIH,
25
26
27 <http://imagej.nih.gov/ij/>). Exposure to Cd^{2+} for 1 day resulted in a significant reduction
28
29
30 of approximately 30% in the GFAP-positive area of astrocytes.
31
32
33

34
35 The samples prepared by sequential exposure to CdCl_2 and differentiation were stained
36
37
38 with anti-GFAP antibodies and rhodamine-phalloidin to confirm the relationship
39
40
41 between the morphology of actin filaments and the GFAP network. The morphology of
42
43
44 the actin filaments did not show a notable difference despite the shrunken appearance of
45
46
47 the GFAP network in the sample exposed to CdCl_2 (Fig. 4).
48
49
50

51 52 53 54 **4. Discussion** 55 56

57 We used NSPCs derived from fetal mouse cerebrum to evaluated the cytotoxicity of
58
59
60

the essential element, Zn^{2+} as well as its congeners Cd^{2+} , Hg^{2+} , and Cu^{2+} , which is a neighboring element in the periodic table of elements, and estimated the IC_{50} values for cell survival. Previous studies have reported the cytotoxicity of HgCl_2 and CdCl_2 in human NSPCs with IC_{50} values of 10–50 μM (Breier et al., 2008; Moors et al., 2009) while the PC12 neuronal cell line showed IC_{50} values of 10–50 μM (Mundy et al., 2010). We recently reported the cytotoxicity of Zn^{2+} ion against NSPCs and their differentiated cells *in vitro* (Nishikawa et al., 2015). In this study, the IC_{50} values of CdCl_2 , ZnCl_2 , and HgCl_2 in mouse NSPCs were similar to the results of the previous reports described above. The cytotoxicity of ZnCl_2 , CdCl_2 , HgCl_2 , and CuCl_2 in the mouse fibroblast, L929, and mouse osteoblast MC3T3-E1 cell lines was previously evaluated (Yamamoto et al., 1998). In our study, we found that the mouse fetal NSPCs showed lower IC_{50} values following treatment with CdCl_2 and HgCl_2 than they did following incubation with CuCl_2 and ZnCl_2 , which differed from the previous findings with the L929 and MC3T3-E1 cell lines. The order and degree of cytotoxic effects of the different metal ions depended on the cell type.

All minerals enter the body by the oral and respiratory routes, and can be absorbed from the digestive tract. After absorption, minerals are transported by the blood stream to the brain. It was reported that Zn^{2+} ion, an essential metal, is incorporated into cells

1
2
3 by the action of anion transporters on the cell membrane (Gunshin et al., 1997).
4
5
6 Non-essential heavy metals with an ion size similar to that of Zn^{2+} (such as Cd^{2+} and
7
8
9 Hg^{2+}), are commonly incorporated by the same anion transporters (Gunshin et al., 1997;
10
11
12 Nightingale Jr., 1959). These metals were reported to cause cell death by the generation
13
14
15 of reactive oxygen species (ROS) and disruption of mitochondrial function (Belyaeva et
16
17
18 al., 2012). The NSPCs survival curve obtained following incubation with a range of
19
20
21 concentrations of the metals exhibited a moderate decline particularly following
22
23
24 exposure to CuCl_2 and CdCl_2 . However, we observed a steep decrease following
25
26
27 exposure to ZnCl_2 and HgCl_2 in our study. These results suggest the presence of
28
29
30 different metal ion-dependent mechanisms of cytotoxicity.
31
32
33

34
35 In this study, we showed that incubation with HgCl_2 decreased neuronal differentiation
36
37
38 and exposure of NSPCs to CdCl_2 and HgCl_2 after a 1-week neural differentiation
39
40
41 reduced the ratio of neurons generated from the NSPCs (Table 2). In addition, we
42
43
44 showed that exposure to ZnCl_2 and CdCl_2 reduced the GFAP-positive area of astrocytes
45
46
47 (Fig. 3). Gulisano et al. reported that CdCl_2 altered the morphology of glioblastoma
48
49
50 cells and transiently increased their proliferation (Gulisano et al., 2009). Previous
51
52
53 studies by Takiguchi et al. demonstrated that Cd^{2+} is an effective inhibitor of DNA
54
55
56 methyltransferase and induced morphological changes in the TRL 1215 cell line derived
57
58
59
60
61
62
63
64
65

1
2
3 from the rat liver (Takiguchi et al., 2003). Therefore, Cd²⁺ may cause changes in DNA
4
5
6 methylation in astrocytes differentiated from NSPCs. However, simultaneous exposure
7
8
9 of NSPCs to CdCl₂ and ZnCl₂ did not reduce the GFAP-positive area, suggesting that
10
11
12 these compounds do not influence glial differentiation of NSPC but affect astrocytic
13
14
15 formation after cell differentiation. The mechanism underlying the morphological
16
17
18 changes of GFAP in astrocytes remains unknown. Molecular mechanisms involved in
19
20
21 the metal-induced morphological changes of NSPC-derived astrocytes require further
22
23
24 studies in the future. However, the use of changes in morphology of astrocytes that can
25
26
27 be microscopically observed as an index of metal ion toxicity should not be
28
29
30 underestimated as a potentially useful tool in toxicology.
31
32
33

34
35 In the current research environment, there is a strong impetus to devise novel, accurate,
36
37
38 high-throughput *in vitro* methods for performing toxicological evaluations, to satisfy the
39
40
41 increasing demand for a reduction in the number of animal experiments by the
42
43
44 concerned public. Therefore, the morphological analysis of NSPCs and differentiated
45
46
47 cells (neuronal and glial cells) could be a powerful tool for the assessment of
48
49
50 neurodevelopmental toxicity.
51
52
53

54 55 56 57 **5. Conclusions** 58 59 60

1
2
3 We demonstrated the cytotoxicity and developmental toxicity of heavy metal ions,
4
5
6 Zn^{2+} , Cu^{2+} , Cd^{2+} , and Hg^{2+} in cultured mouse NSPCs, and measured the IC_{50} values for
7
8
9 cell viability for each heavy metal ion. By analyzing the cytoskeletal morphology of
10
11
12 neurons and astrocytes differentiated from NSPCs following treatment with heavy metal
13
14
15 ions, we found that CdCl_2 treatment reduced the GFAP-network area of astrocytes.
16
17
18 These results illustrate the importance of image-based morphological analysis in the
19
20
21 assessment of developmental neurotoxicity. This study provides a foundation for a new
22
23
24
25 approach to the evaluation of neurodevelopmental toxicity using stem cells.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

This work was partially funded by the Steel Foundation for Environmental Protection Technology (SEPT) and Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST. We express our gratitude to Ms. Asuka Ohi for technical contributions in the experiments.

References

- Baek, D.H., Kim, T.G., Lim, H.K., Kang, J.W., Seong, S.K., Choi, S.E., Lim, S.Y., Park, S.H., Nam, B.H., Kim, E.H., Kim, M.S., Park, K.L., 2012. Embryotoxicity assessment of developmental neurotoxicants using a neuronal endpoint in the embryonic stem cell test. *J. Appl. Toxicol.* 32, 617-626.
- Bal-Price, A.K., Hogberg, H.T., Buzanska, L., Lenas, P., van Vliet, E., Hartung, T., 2010. In vitro developmental neurotoxicity (DNT) testing: relevant models and endpoints. *Neurotoxicol.* 31, 545-554.
- Belyaeva, E.A., Sokolova, T.V., Emelyanova, L.V., Zakharova, I.O., 2012. Mitochondrial electron transport chain in heavy metal-induced neurotoxicity: effects of cadmium, mercury, and copper. *ScientificWorldJournal.* 2012, 136063.
- Breier, J.M., Gassmann, K., Kayser, R., Stegeman, H., De Groot, D., Fritsche, E., Shafer, T.J., 2010. Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science. *Neurotoxicol. Teratol.* 32, 4-15.
- Breier, J.M., Radio, N.M., Mundy, W.R., Shafer, T.J., 2008. Development of a high-throughput screening assay for chemical effects on proliferation and viability of immortalized human neural progenitor cells. *Toxicol. Sci.* 105, 119-133.
- Gage, F.H., 2000. Mammalian neural stem cells. *Science* 287, 1433-1438.

- Grandjean, P., Landrigan, P.J., 2006. Developmental neurotoxicity of industrial chemicals. *Lancet* 368, 2167-2178.
- Gulisano, M., Pacini, S., Punzi, T., Morucci, G., Quagliata, S., Delfino, G., Sarchielli, E., Marini, M., Vannelli, G.B., 2009. Cadmium modulates proliferation and differentiation of human neuroblasts. *J. Neurosci. Res.* 87, 228-237.
- Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388, 482-488.
- Matsuoka, F., Takeuchi, I., Agata, H., Kagami, H., Shiono, H., Kiyota, Y., Honda, H., Kato, R., 2013. Morphology-based prediction of osteogenic differentiation potential of human mesenchymal stem cells. *Plos One* 8, e55082.
- Moors, M., Rockel, T.D., Abel, J., Cline, J.E., Gassmann, K., Schreiber, T., Schuwald, J., Weinmann, N., Fritsche, E., 2009. Human neurospheres as three-dimensional cellular systems for developmental neurotoxicity testing. *Environ. Health. Persp.* 117, 1131-1138.
- Mori, H., Hara, M., 2013. Cultured stem cells as tools for toxicological assays. *J. Biosci. Bioeng.* 116, 647-652.
- Mori, H., Ninomiya, K., Kanemura, Y., Yamasaki, M., Kino-Oka, M., Taya, M., 2007b.

Image cytometry for analyzing regional distribution of cells inside human neurospheres. J. Biosci. Bioeng. 103, 384-387.

Mori, H., Takahashi, A., Horimoto, A., Hara, M., 2013. Migration of glial cells differentiated from neurosphere-forming neural stem/progenitor cells depends on the stiffness of the chemically cross-linked collagen gel substrate. Neurosci. Lett. 555, 1-6.

Mundy, W.R., Radio, N.M., Freudenrich, T.M., 2010. Neuronal models for evaluation of proliferation in vitro using high content screening. Toxicol. 270, 121-130.

Nightingale Jr., E.R., 1959. Phenomenological theory of ion solvation. Effective radii of hydrated ions. J. Phys. Chem. 63, 1381-1387.

Nishikawa, M., Mori, H., Hara, M., 2015. Reduced zinc cytotoxicity following differentiation of neural stem/progenitor cells into neurons and glial cells is associated with upregulation of metallothioneins. Environ. Toxicol. Pharmacol. 39, 1170-1176.

Reynolds, B.A., Tetzlaff, W., Weiss, S., 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. 12, 4565-4574.

Takiguchi, M., Achanzar, W.E., Qu, W., Li, G.Y., Waalkes, M.P., 2003. Effects of

1
2
3 cadmium on DNA-(Cytosine-5) methyltransferase activity and DNA methylation
4
5
6 status during cadmium-induced cellular transformation. Exp. Cell Res. 286,
7
8
9 355-365.
10

11
12 Theunissen, P.T., Robinson, J.F., Pennings, J.L., van Herwijnen, M.H., Kleinjans, J.C.,
13
14

15
16 Piersma, A.H., 2012. Compound-specific effects of diverse neurodevelopmental
17
18
19 toxicants on global gene expression in the neural embryonic stem cell test (ESTn).
20
21

22 Toxicol. Appl. Pharmacol. 262, 330-340.
23
24

25 Yamamoto, A., Honma, R., Sumita, M., 1998. Cytotoxicity evaluation of 43 metal salts
26
27

28 using murine fibroblasts and osteoblastic cells. J. Biomed. Mater. Res. 39, 331-340.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure legends

Fig. 1 Schematic illustration of experimental protocols used in exposure of cells to heavy metal ions. Cells were exposed to heavy metal ions either during or after (“simultaneous” or “sequential exposure”, respectively) induction of neural differentiation of NSPCs.

Fig. 2 Viability of NSPCs and PC12 cells after exposure to heavy metal chlorides (A)

ZnCl₂, (B) CdCl₂, (C) CuCl₂ and (D) HgCl₂. Open triangles with dotted lines show survival curves of PC12 cells while filled squares with solid lines show data for NSPCs.

Final concentrations of metals were as follows: NSPCs, ZnCl₂ 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 mM; CdCl₂ 0.005, 0.01, 0.0125, 0.02, 0.0275, 0.035, and 0.1 mM; CuCl₂ 0.01, 0.02, 0.05, 0.1, 0.15, and 0.2 mM; and HgCl₂ 0.02, 0.0275, 0.035, 0.05, 0.075, and 0.1 mM; PC12 cell, ZnCl₂ 0.05, 0.1, 0.15, 0.2, 0.35, 0.5, and 1 mM; CdCl₂ 0.0075, 0.01, 0.015, 0.02, 0.035, 0.05, and 0.1 mM; CuCl₂ 0.005, 0.01, 0.05, 0.075, 0.1, 0.2 and 0.5 mM; and HgCl₂ 0.002, 0.005, 0.01, 0.0125, 0.015, 0.02, and 0.05 mM. Incubation in medium with the addition of equal volume of distilled water was used as a control treatment. Results are means $\pm$ SD. (n = 6).

Fig. 3 Image analysis of differentiated NSPCs exposed to heavy metal ions. (A) Fluorescence images of neurons and astrocytes differentiated from NSPCs after exposure to heavy metal chlorides and (B and C) area of astrocytes differentiated from NSPCs, immunostained with GFAP. Cells were exposed to distilled water (control), ZnCl₂, CdCl₂, CuCl₂, and HgCl₂ according to two experimental protocols shown in Fig. 1, involving simultaneous or sequential heavy metal exposure and cell differentiation. Neurons and astrocytes were immunostained with antibodies against tubulin β III (green) and GFAP (red). Cell nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m. Area of cells immunostained with GFAP was measured using an image analysis program. Data are percentage (%) of GFAP-positive area of astrocytes relative to non-treated controls (100%); * $p < 0.05$ compared with control.

Fig. 4 Fluorescence images of GFAP and actin filaments in astrocytes prepared following sequential exposure to CdCl₂. Cells were exposed to distilled water (control) and 2.5 μ M CdCl₂. Astrocytes were stained with antibodies against GFAP (green) and rhodamine-phalloidin (red). Cell nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1

Summary of results (IC₅₀ values) obtained in the cell-viability tests depicted in Fig. 2.

	IC ₅₀ (mM)			
	ZnCl ₂	CdCl ₂	CuCl ₂	HgCl ₂
NSPC	0.082 ± 0.005	0.012 ± 0.006	0.031 ± 0.005	0.048 ± 0.008
PC12	0.196 ± 0.006	0.045 ± 0.018	0.024 ± 0.001	0.020 ± 0.005

IC50 values (mean ± S.D.) were derived from nonlinear regression analysis based on three experimental data.

Table 2

Summary of conditions used in the cell-differentiation test using fluorescent microscopy with immunocytochemistry. Cell types differentiated from NSPCs, concentrations of metal ions, and timing of exposure (simultaneous or sequential exposure, as shown in Fig. 1) are listed. Ratio of tubulin β III-positive cells (neuronal lineage: neuron) to GFAP-positive cells (glial lineage: Astrocyte) was evaluated using fluorescent microscopy.

	Simultaneous exposure		Sequential exposure	
	Neuron (%)	Astrocyte (%)	Neuron (%)	Astrocyte (%)
Control (0 μ M)	3.3 ± 3.0	96.4 ± 3.2	2.7 ± 2.0	96.7 ± 2.5
ZnCl ₂ (75 μ M)	1.8 ± 1.7	97.4 ± 1.9	2.0 ± 1.3	97.8 ± 1.6
CdCl ₂ (2.5 μ M)	3.6 ± 0.3	96.1 ± 0.7	0.9 ± 1.7	94.8 ± 7.8
CuCl ₂ (10 μ M)	7.0 ± 3.1	90.9 ± 3.4	2.2 ± 2.3	97.8 ± 2.3
HgCl ₂ (35 μ M)	0.1 ± 0.4	99.7 ± 0.6	0.06 ± 0.2	99.9 ± 0.2