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Effects of subcytotoxic cadmium on morphology of glial fibrillary acidic protein network in astrocytes derived from murine neural stem/progenitor cells

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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.

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 information on cellular characteristics, which is related to the cell types and states, as well insight into the localization of intracellular and membrane proteins. Recently, automated methods for the analysis of cell morphology based on microscopic images have been developed to evaluate the quality of cultured stem cells (Matsuoka et al., 2013; Mori et al., 2007). The application of image-based analysis on cultured stem cells can provide a rapid and highly sensitive assay system for the assessment of neurodevelopmental toxicity.

Heavy metals enter the body from the atmosphere through respiration, oral ingestion of food and drinks in daily life, and from implanted orthopedic and dental materials (Grandjean and Landrigan, 2006). Among the heavy metals, copper and zinc (Cu^{2+} and Zn^{2+}, respectively) are essential elements for the body. On the other hand, mercury and cadmium (Hg^{2+} and Cd^{2+}) are toxic non-essential elements, which can be deleteriously incorporated in the body. However, the precise mechanisms underlying the toxicity of heavy metals are yet to be completely elucidated.

In this study, we evaluated the developmental neurotoxicity of four heavy metal ions in NSPCs, using cell viability assay and immunocytochemical analysis. Zn^{2+} as an essential element as well

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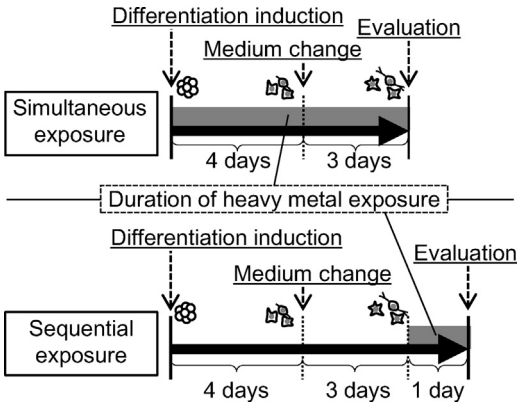

Fig. 1. Schematic illustration of experimental protocols used in exposure of cells to heavy metal ions. Cells were exposed to heavy metal ions either during or after ("simultaneous" or "sequential exposure", respectively) induction of neural differentiation of NSPCs.

as Cu^{2+}, Cd^{2+}, and Hg^{2+}, the neighboring metals in the elemental periodic table were used.

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) CO_2 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 culturing were further cultured on coverslips (Matsunami, Kishiwada, Japan) coated with poly-L-ornithine (Sigma-Aldrich) in the differentiation medium containing DMEM/F12 supplemented with antibiotic-antimycotic mixed solution, 2% (v/v) B27 supplement, and 1% (v/v) fetal bovine serum (FBS, Sigma-Aldrich).

To investigate the effect of heavy metals on neural differentiation, NSPCs were tested using two protocols with different exposure times to heavy metals. One protocol involved simultaneous heavy metal exposure and induction of neural differentiation (Simultaneous exposure, Fig. 1) while the other protocol exposed cells to heavy metals 7 days after induction of neural differentiation (Sequential exposure, Fig. 1).

2.3. Cell viability testing

NSPCs were seeded on 96-well plates at 50,000 cells/mL (100 μL/well), and incubated at 37 °C and 5% (v/v) CO_2 atmosphere for 3 days. PC12 cells were seeded at 200,000 cells/mL. The metal chlorides ($ZnCl_2$, $CdCl_2$, $CuCl_2$, and $HgCl_2$, Nacalai Tesque, Kyoto, Japan) were prepared as stock solution dissolved in distilled water at 25 times the final concentration, and were added to the culture medium after sterilization by filtration. An equal volume of distilled water was given as a control sample. Cells were exposed to culture medium containing $ZnCl_2$, $CdCl_2$, $CuCl_2$, and $HgCl_2$ at concentration ranges of 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 βIII 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 μg/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 (Optiphoto2, 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 ± 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