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Reduced zinc cytotoxicity following differentiation of neural stem/progenitor cells into neurons and glial cells is associated with upregulation of metallothioneins

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ABSTRACT

We investigated zinc cytotoxicity in mouse neural stem/progenitor cells (NSPCs) and their differentiated progeny (neuronal/glial cells) in correlation with expression of metallothionein (MT) gene. Differentiated cells were less sensitive than NSPCs to $ZnCl_2$ (IC_{50}: 128 μM *vs.* 76 μM). Differentiation of immature NSPCs to the differentiated cells led to an increase in expression of MT family genes (*Mt1*, *Mt2*, *Mt3*, and *Mt4*). Zinc exposure induced a dose-dependent increase in expression level of *Mt1* and that of *Mt2* in both NSPCs and the differentiated cells. Our results showed that the reduced cytotoxicity of zinc associated with differentiation from NSPCs into their progeny was related to the upregulation of MTs.

1. Introduction

Zinc is an essential trace element contained in various tissues such as bone, liver, and brain. Zinc is required for catalytic, structural and regulatory functions of many proteins including transcription factors and enzymes (Szewczyk, 2013). Zinc deficiency produces abnormalities in endocrine, nervous, and immune system function (Bitanihirwe and Cunningham, 2009), so intracellular level of zinc must be tightly regulated by Zn transporters and metal binding proteins such as the metallothioneins (MTs). Zinc in extracellular space acts as a neuromodulator in synaptic transmission. Moreover, zinc exerts neuroprotective effects by acting as a mediator of neuron-glial signaling during brain injury (Chung et al., 2008). Alternatively, excessive levels of zinc can be cytotoxic (Bitanihirwe and Cunningham, 2009; Shuttleworth and Weiss, 2011).

Metallothioneins control the homeostasis of essential trace elements and are ubiquitously expressed across tissues and species (Bitanihirwe and Cunningham, 2009). Four MT isoforms have been identified in mammals: MT1, MT2, MT3 and MT4. In addition to zinc regulation, MTs are involved in heavy metal detoxification and reactive oxygen species (ROS) scavenging (Carpenè et al., 2007; Keen et al., 1997).

Neural stem cells have the capacity for both self-renewal and differentiation into all three major cell types in the central nervous sytem (CNS), neurons, astrocytes, and

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oligodendrocytes (Reynolds et al., 1992). They can be isolated from fetal brain and cultured in suspension as clusters of immature neural cells called neurospheres. They are often called as neural stem/progenitor cells (NSPCs) because it is technically difficult to distinguish neural stem cells, neuronal progenitor cells, and glial progenitor cells each other using specific molecular markers such as nestin and Sox2. They are very useful tools for neurodevelopmental toxicity assays *in vitro* mimicking the assays *in vivo* using brain of the experimental animals in fetal and neonatal stages because the cultured NSPCs retain the original properties of immature neural stem cells *in vivo*.

Little is known about changes in zinc homeostasis and those in cytotoxic sensitivity of zinc during differentiation from NSPCs to their progeny, neurons and glial cells in the CNS. Regulation in metabolism of zinc in a whole body level is important. Regulatory mechanisms including intake of dietary zinc from foods through the digestive tract, blood flow, transport through the blood brain barrier, excretion of zinc out of the body by urine, works cooperatively to keep the adequate level of zinc in the tissue of CNS. In the cellular level of CNS, neuronal and glial cells are influenced by both intracellular and extracellular zinc ion. In this study, we focused on homeostasis and investigated the physiological responses of NSPCs and their differentiated progeny to zinc ion exposure. We provide the first report showing changes in zinc cytotoxicity during differentiation of NSPCs to neurons and astrocytes and an association between these changes and MT expression.

2. Materials and methods

2.1. Materials

Coverslips (φ12 mm) and slide glasses were purchased from Matsunami Glass Ind., Ltd. (Kishiwada, Japan), cell culture plates and 96- and 4-well culture plates from Thermo Fisher Scientific (Yokohama, Japan), and poly-L-ornithine hydrobromide (PLO) and fetal bovine serum (FBS) from Sigma Aldrich (St. Louis, MO, USA). B-27 Supplement, Alexa Fluor 488 anti-mouse IgG, and Alexa Fluor 568 anti-rabbit IgG were purchased from Life Technologies (Carlsbad, CA, USA). Human epidermal growth factor (EGF) and human basic fibroblast growth factor (b-FGF) were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). Normal goat serum was purchased from Vector Laboratories (Burlingame, CA, USA). Anti-tubulin βIII (Tuj1; mouse monoclonal) and anti-glial fibrillary acidic protein (GFAP; rabbit polyclonal) antibodies were from Covance (Princeton, NJ, USA) and Sigma Aldrich, respectively. Solution of 4′,6-diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Mashikimachi, Japan). All other reagents were from Nacalai Tesque (Kyoto, Japan).

2.2. NSPCs culture and differentiation

Mouse NSPCs were isolated from E14 fetal ICR mouse (Japan SLC, Inc., Hamamatsu, Japan) cerebral cortex as previously described (Mori et al., 2011). Experimental procedures for the care and use of animals were approved by Animal Experiment Committee of Osaka Prefecture University and were conducted in accordance with the relevant laws and institutional guidelines for the animal welfare. They were cultured in a growth medium consisting of Dulbecco's modified Eagles medium (DMEM) plus Ham's F-12 supplemented with 1% (v/v) antibiotic-antimycotic mixed stock solution, 2% (v/v) B-27 Supplement, 20 ng/mL EGF, and 20 ng/mL b-FGF at 37 °C in a humidified 5% (v/v) CO_2 atmosphere.

A half volume of the culture medium was changed every 2–3 days. Neurospheres were dissociated once in every 7 days as a passage. Cells were used for experiments after 3–7 passages. To induce differentiation, neurospheres cultured for 3 days were plated either on poly-L-ornithine (PLO)-coated coverslips or PLO-coated culture plates, and then cultured for 7 days in a differentiation medium consisting of DMEM plus Ham's F-12 supplemented with 1% (v/v) antibiotic-antimycotic mixed stock solution, 1% (v/v) FBS, and 2% (v/v) B-27 Supplement. Differentiated cells were cultured for 24 h in the maintenance media described for both zinc cytotoxicity assays and quantitative real-time polymerase chain reaction (qRT-PCR).

2.3. Cytotoxicity tests

Neural stem/progenitor cells were seeded at 5×10^3 cells/well on 96-well culture plates and cultured for 3 days. Differentiated cells were prepared on PLO-coated 96-well plates as previously noted. The same volume of medium was added to the blank wells.

Zinc chloride ($ZnCl_2$) at variable concentrations was added to the medium in each well and an equal volume of distilled deionized water (vehicle) was added to the control wells. After incubation for 24 h, viable cell number was measured by the WST assay kit (Cell Counting Reagent SF, Nacalai Tesque) following the manufacturer's protocol. Absorbance at 450 nm (A_{450}) was measured for each well using a model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). Cell viability was defined as the ratio of viable cell number in the sample well relative to that in the vehicle-treated control well. The zinc IC_{50} (the concentration required to reduce viable cell number to 50% of control) was calculated using Microsoft Excel.

2.4. Immunocytochemistry

Neural stem/progenitor cells were seeded at 0.5×10^5 cells/mL and cultured for 3 days in growth medium. The resultant neurospheres were seeded on PLO-coated coverslips and cultured for 7 days in differentiation medium, and then cultured in medium containing different concentrations of $ZnCl_2$ (20, 50, 100 μM) for 24 h. The cells were fixed with 4% (w/v) paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 20 min at 4 °C.

The fixed samples were washed twice with PBS, permeabilized in PBS containing 0.3% (w/v) Triton X-100 for 5 min, washed twice more with PBS, and blocked with PBS containing 10% (v/v) normal goat serum and 0.01% (w/v) Triton X-100 for 1 h at room temperature. The samples were then incubated for overnight at 4 °C with anti-tubulin βIII (1:500) and anti-GFAP (1:100) antibodies in PBS containing 10% (v/v) normal goat serum and 0.01% (w/v) Triton X-100.