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Toxicomics Report

Deubiquitinase USP54 attenuates methylmercury toxicity in human embryonic kidney 293 cells

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ABSTRACT — Deubiquitinases are an important regulator of ubiquitin-mediated signaling pathways because of their ability to cleave the isopeptide bond that connects the ubiquitin chain to the target protein. We previously reported that the expression of many proteins involved in methylmercury toxicity is regulated by the ubiquitin-proteasome system. In this study, double-stranded siRNAs against mRNAs of approximately 60 deubiquitinases were transfected into human embryonic kidney (HEK) 293 cells, and ubiquitin-specific protease 54 (USP54) was identified as a deubiquitinase that increases the sensitivity of HEK293 cells to methylmercury by RNA interference.

Key words: Methylmercury, Deubiquitinase, USP54, RNA interference

INTRODUCTION

Methylmercury is the causative agent of Minamata disease that occurred in the city of Minamata, Kumamoto Prefecture, Japan, in 1956 (Davies, 1991; Harada, 1995). Various neurological disorder symptoms, such as locomotor ataxia, sensory disturbance, visual disturbance, and auditory disturbance, were observed in patients exposed to high concentrations of methylmercury (Hira *et al*., 1982). Methylmercury is one of the most widespread pollutants in the environment. It has become a matter of public health concern because children born to pregnant women who have ingested seafood, in which methylmercury has accumulated through the food chain, show impaired motor and mental development (Al-Ardhi and Al-Ani, 2008; Grandjean *et al*., 1997; Tatsuta *et al*., 2017).

We have previously found numerous proteins involved in methylmercury toxicity in cells, which are regulated by the ubiquitin-proteasome system (Hwang *et al*., 2002; Lee *et al*., 2020; Naganuma *et al*., 2002; Toyama *et al*., 2021b). Ubiquitination of proteins is a major post-translational modification of proteins, which is widely conserved in eukaryotes. It is a reaction in which a small tag protein called ubiquitin is covalently bound to a lysine residue of a target protein through the sequential action of three enzymes: ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, and ubiquitin ligase (Ciechanover, 1994; Hasselgren and Fischer, 1997). Conversely, deubiquitinases are a type of protease that catalyzes degradation of polyubiquitin chains and the removal of ubiquitin mol-

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ecules from ubiquitinated proteins by hydrolyzing and cleaving the peptide or isopeptide bonds at the C-terminus of ubiquitin (D'Andrea and Pellman, 1998; Wilkinson, 2000). Therefore, deubiquitinases have attracted attention as a regulator that acts antagonistically to various cellular responses induced by ubiquitination, such as protein degradation and signal transduction.

In this study, we performed a simple screening by double-stranded siRNA transfection to identify deubiquitinases involved in reducing methylmercury toxicity.

MATERIALS AND METHODS

Cell culture

To search for deubiquitinases involved in reducing methylmercury toxicity, we used human embryonic kidney (HEK) 293 cells that facilitate transfection of double-stranded siRNAs. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Screening of deubiquitinases involved in reducing methylmercury toxicity

Silencer™ Negative Control siRNA and double-stranded siRNAs against approximately 60 deubiquitinases were purchased from Ambion (Austin, TX, USA). Transfection of double-stranded siRNA into HEK293 cells was performed using HiPerFect® transfection reagent (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. First, 2.5 μL of double-stranded siRNA (final concentration: 10 nM) was added to the wells of a 96-well plate. Then, 25 μL of a mixture of HiPerFect® transfection reagent and Opti-MEM (Invitrogen, Carlsbad, CA, USA) was added to the wells, followed by incubation at room temperature for 15 min. Next, 65 μL of cell suspension containing 1×10^4 cells was added to the wells, followed by incubation for 24 hr, addition of 10 μL of methylmercuric chloride (final concentration: 5 μM) at a concentration to inhibit control cell growth by approximately 50%, and then incubation for another 48 hr. Cell viability was examined using alamarBlue® (Invitrogen). Screening was performed using two double-stranded siRNAs with different sequences against one mRNA. The cell survival rate of each siRNA was evaluated by calculating the average of the survival rates.

Confirmation of gene expression

The sequences of double-stranded siRNAs corresponding to USP54 mRNA were as follows: #1, GACTTAGCAGAAGATGTTA; #2, CCAAGTATTGTTAAGCCAA. Cells transfected with double-stranded siRNA (approximately 1×10^6 cells/well in a 6-well plate) were incubated for 24 hr and then washed with PBS. Total RNA was isolated from the resulting cells using Isogen II (Nippon Gene, Toyama, Japan). Single-stranded cDNA was synthesized using a PrimeScrip® RT reagent kit (Takara, Shiga, Japan) in accordance with the manufacturer's protocol. Quantitative real-time PCR was performed with the following primers: sense, 5′-CAGCCAACAAGTGAACAGCC′, antisense, 5′-TGACTCATGGCAGGAAGCAG-3′ for the *USP54* gene, and sense, 5′-GCACCGTCAAGGCTGAGAAC-3′ and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′ for the *GAPDH* gene. The rate of decrease in USP54 mRNA expression was determined from a standard curve after calibration of the assay.

RESULTS AND DISCUSSION

To search for deubiquitinases involved in reducing methylmercury toxicity, double-stranded siRNAs against genes of approximately 60 deubiquitinases commercially available from Ambion were transfected into HEK293 cells and then sensitivity to methylmercury was examined. As a result, cells transfected with double-stranded siRNAs against USP28, USP35, or USP54 mRNAs showed higher sensitivity to methylmercury compared with control cells. However, it was possible that the sensitivity in some of these cells was altered independently of the suppression of gene expression. Therefore, we purchased two double-stranded siRNAs targeting different nucleotide sequences from Sigma-Aldrich (St. Louis, MO, USA) and re-examined the sensitivity to methylmercury. As a result, only cells with suppressed USP54 expression were more sensitive to methylmercury than control cells (Fig. 1A). USP54 mRNA levels were reduced by approximately 80% using double-stranded siRNAs compared with control cells (Fig. 1B). Conversely, cells with suppressed expression of USP28 or USP35 showed no change in sensitivity to methylmercury, which was comparable to control cells (data not shown). These findings suggest that USP54 is a deubiquitinase involved in reducing methylmercury toxicity.

USP54 may be important for overall neuronal development, which has been suggested by deficiency experiments in the fruit fly *Drosophila melanogaster* (Tsou *et al*., 2012). Recently, USP54 was overexpressed in intestinal stem cells and has been shown to promote colon cancer progression, probably by regulating cancer stem cell properties (Fraile *et al*., 2016), its functions remain largely unknown. The Human Protein Atlas database indicates

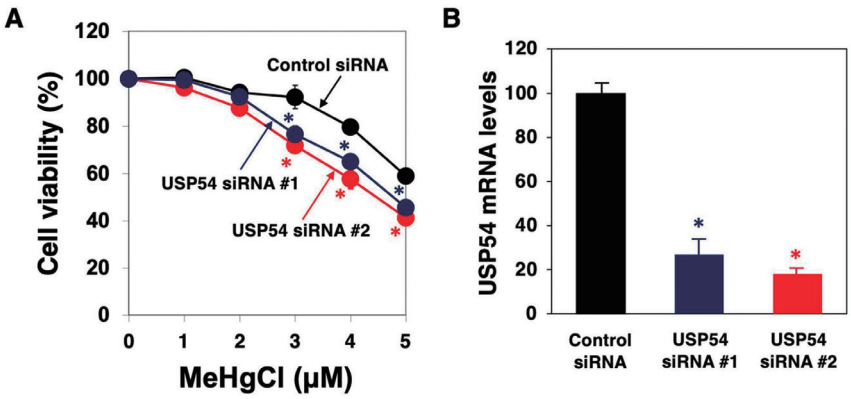

Fig. 1. Effects of suppressing USP54 expression on sensitivity to methylmercury in HEK293 cells. (A) HEK293 cells (1×10^4 cells/well) transfected with two different double-stranded siRNAs against USP54 mRNA were seeded in 96-well plates. Twenty-four hours after transfection, the indicated concentrations of methylmercuric chloride were added and the cells were incubated for an additional 48 hr. Cell viability was measured by an alamarBlue® assay. Each point and bar represents the mean and standard deviation of the results obtained from three cultures. *$p < 0.01$ vs. control siRNA. (B) HEK293 cells transfected with two different double-stranded siRNAs against USP34 mRNA were collected and analyzed for USP54 mRNA expression by quantitative real-time PCR. USP54 mRNA expression was normalized to GAPDH mRNA expression. *$p < 0.01$ vs. control siRNA.

that USP54 is highly expressed in oligodendrocytes and microglia, glial cells of the central nervous system, and is mainly distributed in mitochondria. We have recently found that excessive activation of microglia, immune cells in the brain, is involved in methylmercury-induced central nervous system damage (Hoshi *et al*., 2019; Toyama *et al*., 2021a). Because mitochondria are a main target organelle of methylmercury (Lee *et al*., 2016; Mori *et al*., 2011; Sato *et al*., 2020), USP54 might attenuate its toxicity by suppressing the inflammatory response in the brain induced by methylmercury through an unknown function in mitochondria. In the future, identification of the factors involved in reducing methylmercury toxicity among the substrate proteins recognized by Usp54 is expected to reveal the function of a novel defense mechanism against methylmercury toxicity.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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