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Band 3/anion exchanger 1/solute carrier family 4 member 1 expression as determinant of cellular sensitivity to selenite exposure

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ABSTRACT

Selenium is a chalcogen element that is essential in animals, but is highly toxic when ingested above the nutritional requirement. Selenite is used as a supplement in patients receiving total parenteral nutrition. However, the therapeutic and toxic doses of selenite are separated by a narrow range. This ambivalent character of selenite implies the presence of cellular mechanisms that precisely control selenite homeostasis. Here, we investigated mechanisms that determine cellular susceptibility to selenite exposure. The resistance to selenite exposure was significantly different among cell lines. We determined the expression levels of *TPMT* (thiopurine *S*-methyltransferase) and *SLC4A1* (solute carrier family 4 member 1), which encode selenium methyltransferase and selenite transporter, respectively. We also examined the effect of inhibition of Band 3 protein activity, which is encoded by *SLC4A1*, on the cellular sensitivity to selenite. The data suggest that the expression level of *SLC4A1* is the determinant of cellular sensitivity to selenite.

1. Introduction

Selenium (Se) is a chalcogen element that is essential in animals, but is highly toxic when ingested above the nutritional requirement [1,2]. Se naturally occurs in the form of two inorganic salts, selenite (+IV, SeO_3^{2-}) and selenate (+VI, SeO_4^{2-}), as well as in various organic forms in a manner that is in part similar to those of sulfur. Selenite undergoes oxidation and reduction to result in the generation of oxidative and reductive stresses. Se has a lower redox potential than sulfur, and is driven through redox cycles. Selenite is a strong oxidant and induces oxidative stress. In addition, selenite is easily reduced with intracellular glutathione to generate a reduced form of Se, hydrogen selenide (H_2Se). The hydrogen selenide is highly reactive and causes reductive stress. These characteristics of selenite, but not selenate, are expected to be useful in anticancer therapy [3–5]. As an essential trace element, selenite is used for Se supplementation in patients receiving total parenteral nutrition [6]. However, the therapeutic and toxic doses of selenite have a narrow range, i.e., 10 times the average plasma concentration of Se leads to chronic toxicity [2,4]. This ambivalent character of selenite implicitly suggests that animal cells possess mechanisms for selenite homeostasis.

The toxicity of Se is largely dependent on its chemical form, and methylation is the major process for the detoxification and excretion of Se. We have recently identified thiopurine *S*-methyltransferase (TPMT) as a Se methyltransferase [7]. TPMT catalyzes both mono- and di-methylation to produce methaneselenol and dimethylselenide from non- and mono-methylated Se compounds, respectively. Because dimethylselenide is less toxic than selenite, the transient transfection of *TPMT* promotes cellular survival after selenite exposure. TPMT was also shown to catalyze the methylation of selenocysteine [8].

The incorporation of selenite in erythrocytes has been well studied; there are reports that the incorporation is mediated by Band 3 protein [9–11]. Band 3 protein, also called anion exchanger 1 or solute carrier family 4 member 1 (SLC4A1), is encoded by *SLC4A1* and is an anion transporter that mediates the electroneutral exchange of bicarbonate for chloride on the plasma membrane of erythrocytes [10]. Selenite, whose pKa_2 ranges from 7.3 to 8.5 [12,13], is transported as a monovalent hydrogen selenite ion ($HSeO_3^-$) that has structural similarity to bicarbonate. Although Band 3 protein can transport selenate at pH 5.0 as a monovalent anion, the Band 3-mediated selenate transport is inhibited at physiological pH [10]. Selenate, whose pKa_2 is 1.8 [13], is present as SeO_4^{2-} at physiological pH, and is taken up via sulfate transporters [14].

Previous work has shown that cellular susceptibility to selenite differs among cell lines [15]. However, no reasonable explanations for the

Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; SLC4A1, solute carrier family 4 member 1; TPMT, thiopurine *S*-methyltransferase.

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specific susceptibility of the cell lines have been presented. As mentioned above, two mechanisms, i.e., the methylation and the transporter of selenite, may contribute to the susceptibility. Here, we investigated a mechanism that determines the cellular susceptibility.

2. Materials and methods

2.1. Cell culture

HepG2, A549, and HEK293 cells were purchased from RIKEN Bio-Resource Research Center. The cells were maintained in DMEM, high glucose (Sigma-Aldrich, D5796) supplemented with 10% fetal bovine serum (FBS) except specifically noted.

2.2. MTS cell viability assay

Cells were seeded in 96-well cell culture plates that are coated with poly D-lysine hydrobromide or collagen type I (Iwaki, 4020-010) containing 100 μL of DMEM/5% FBS. After 24 h, the medium was exchanged with serum-free DMEM, and the cells were exposed to sodium selenite (Nacalai Tesque, 31824-02) or sodium selenate (FUJIFILM Wako Pure Chemical Corporation, 191–09242) for 24 h. To each well, 20 μL of CellTiter 96 AQueous One Solution (Promega, G3582) was added, and the cells were incubated at 37 °C in 5% CO_2 atmosphere for 1 h. The production of colored formazan from MTS tetrazolium was measured at 490 nm with SpectraMax ABS (Molecular Devices), and cell viability was calculated. Samples were prepared in triplicate or more in each experiment. For the inhibition of Band 3 protein, the selenite exposure was performed in the presence or absence of 25 μM DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, Sigma-Aldrich, D3514). Samples were prepared in quadruplicate in each experiment. Statistical values were calculated from more than eight samples. The Tukey-Kramer test was performed with statsmodels v0.12.1 in Python 3.8.5.

2.3. Quantitative real-time PCR

Cells were cultured until reaching semiconfluence in the absence of selenite. Cells were collected and lysed in Isogen II (Nippon Gene, 311–07361), and total RNA was prepared according to the manufacturer's instructions. The total RNA was diluted to 90 ng/μL, and cDNA was synthesized with Bio-Rad T100 Thermal Cycler and ReverTra Ace qPCR RT Master Mix (TOYOBO, FSQ-201) according to the manufacturer's instructions. The PCR product was diluted by 50-fold, and 2 μL of the dilution was mixed with 2 pmol each of forward and reverse primers and made up to 5 μL. One volume of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent, 600892) was added, and gene expression was quantitated with CFX Connect Real-Time PCR System (BioRad). The reaction was preheated at 94 °C for 5 min, and the PCR cycle of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min was repeated for 50 cycles, followed by incubation at 72 °C for 7 min. *GAPDH* was used as internal control. The PCR primers were as follows: *SLC4A1* forward, 5'-GGT GAT GGA CGA AAA GAA CCA-3'; *SLC4A1* reverse, 5'-AAG ACT CTA CGC AGC TCT AGG-3'; *GAPDH* forward, 5'-GTC TCC TCT GAC TTC AAC AGC G-3'; and *GAPDH* reverse, 5'-ACC ACC CTG TTG CTG TAG CCA A-3'. The PCR products were electrophoresed, and accurate amplification was confirmed (data not shown). The expression level of *SLC4A1* was calculated by CFX Maestro 1.1 (Bio-Rad) and normalized to that of *GAPDH*. The *SLC4A1/GAPDH* ratio for HEK293 cells was used as 100% control. The results were expressed as means ± standard deviation in the graph. n indicates the number of samples.

2.4. Antibodies and western blots

HEK293, HepG2, and A549 cells were cultured until reaching semiconfluence in the absence of selenite, lysed in Laemmli's SDS-sample buffer, and probed with the following antibodies: TPMT (Abcam, ab155284, EPR10820) and GAPDH (Santa Cruz Biotechnology, sc-47724). The band intensities of western blots were quantitated with ChemiDoc XRS+, and the signal intensity was normalized to that of GAPDH. The lysates from HEK293 were prepared in duplicate in each experiment, and the TPMT/GAPDH ratio for HEK293 was used as 100% control. The results were expressed as means ± standard deviation in the graph. n indicates the number of samples.

3. Results and discussion

3.1. Differences in resistance to selenite exposure among three cell lines

We compared the resistance to selenite exposure among three cell lines: A549, HepG2, and HEK293. A549 and HepG2 are lung and liver carcinoma cell lines, respectively. HEK293 is a cell line that was derived from human embryonic kidney cells. A549 was the most tolerant, and HEK293 was the most susceptible (Fig. 1A). We also examined the cellular resistance to selenate exposure. Selenate was less toxic than selenite to the cell lines, and the difference in susceptibility to selenate among the cell lines was much smaller than the case of selenite (Fig. 1B). Although there were some significant differences in cell viability at higher concentrations of selenate, apparent differences in the susceptibility to selenate among the cell lines were not observed. Our findings are consistent with the previous result showing that the IC_{50} values of selenite and selenate are 61 and 880 μM, respectively, in colorectal carcinoma cell line [16]. These data clearly indicated that selenite had cell-specific toxicity.

3.2. Methylation capacity of Se does not correlate with selenite resistance

Recently, we showed that TPMT catalyzed the mono- and di-methylation of Se, and that the exogenous expression of *TPMT* promoted cellular survival after selenite exposure [7]. These results indicated that differences in susceptibility to selenite would depend on the expression of *TPMT*. Then, we examined the expression level of *TPMT* in each cell line. HepG2 cells showed the highest expression, and A549 cells, the lowest expression (Fig. 2). These data indicated that the expression level of *TPMT* did not correlate with the cellular resistance to selenite exposure, and suggested that factors other than Se methylation contributed to the susceptibility to selenite.

In our previous work, we showed that the overexpression of *TPMT* in HEK293 cells promoted cellular survival after selenite exposure. However, the expression level of *TPMT* in each cell line did not correlate with the resistance to selenite (Figs. 1A and 2). These results suggested that TPMT actually contributed to the detoxification of selenite in cells, but was not the primary factor to explain the difference in susceptibility to selenite among the cell lines.

3.3. Band 3 protein is involved in differences in cellular resistance to selenite exposure

The toxicity of selenite, but not selenate, varied among the cell lines (Fig. 1), and selenite and selenate are incorporated into the cells through specific transporters [10,14]. We hypothesized that the transporters would affect cellular resistance to selenite exposure. We examined the expression of *SLC4A1*, which encodes Band 3 protein. HEK293 cells showed the highest expression, and A549 cells, the lowest expression (Fig. 3). This finding raised the possibility that the expression level of *SLC4A1* was the determinant of cellular sensitivity to selenite.

To confirm the possibility mentioned above, we examined the effect of inhibition of Band 3 protein activity on the sensitivity of HEK293 cells to selenite. DIDS, an inhibitor of Band 3 protein, has been shown to inhibit Band 3-mediated incorporation of selenite into red blood cells [9,17]. The treatment with DIDS ameliorated cellular survival after exposure to selenite (Fig. 4), demonstrating that Band 3 protein actually contributed to the influx of selenite, and that the incorporation of

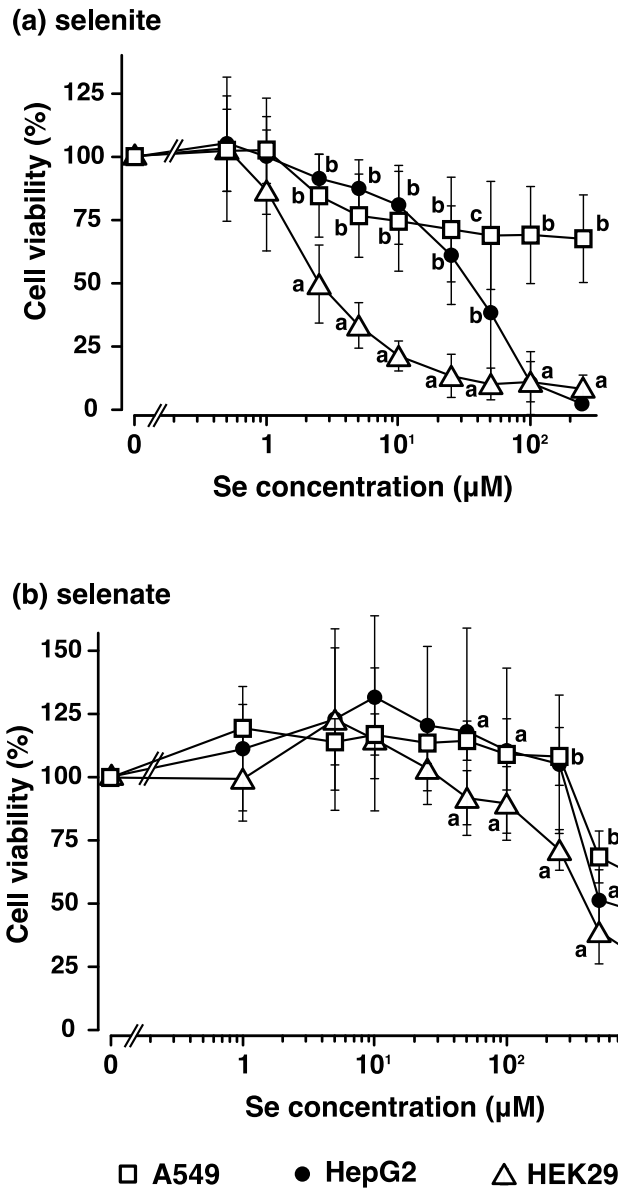

Fig. 1. Cellular resistance to selenite exposure.
A549, HepG2, and HEK293 cells were exposed to the indicated concentration of selenite (a) or selenate (b) for 24 h, and cell viability was examined by the MTS assay. The graph represents results of more than three independent experiments. Adjusted *p* values were calculated by the Tukey or Tukey-Kramer test. Different letters indicate significant differences among cell lines ($p < 0.01$). Bars indicate standard deviation.

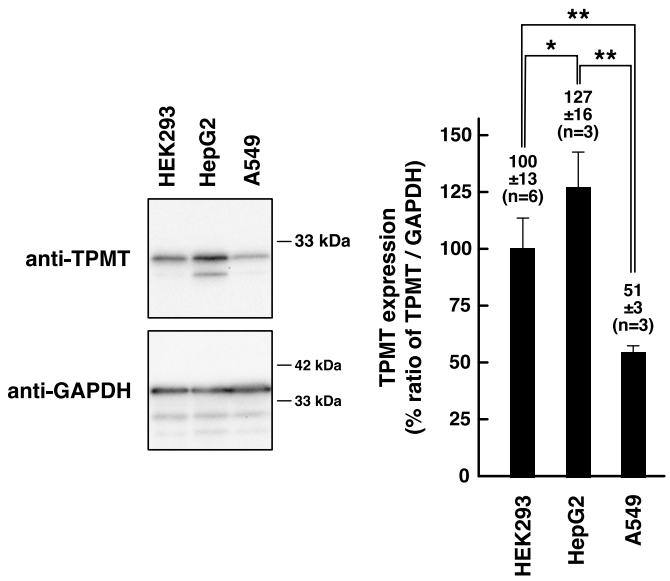

Fig. 2. Expression level of TPMT protein.
The expression level of TPMT was examined in A549, HepG2, and HEK293 cells. Cells were lysed, and the cell lysate was blotted with antibodies against TPMT and GAPDH. The graph represents results of three independent experiments. Adjusted *p* values were calculated by the Tukey–Kramer test. Bars indicate standard deviation. *, $p < 0.05$. **, $p < 0.01$.

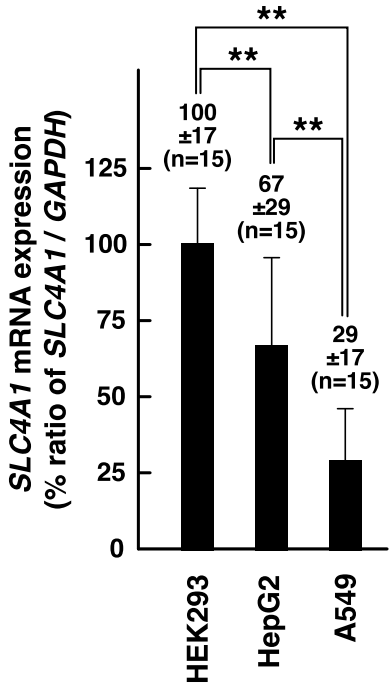

Fig. 3. Expression level of SLC4A1.
The expression level of *SLC4A1* was examined in A549, HepG2, and HEK293 cells. Total RNA was extracted, and mRNA of *SLC4A1* was quantitated by qPCR. The expression levels of *SLC4A1* were normalized to those of *GAPDH*. The graph represents results of three independent experiments. Adjusted *p* values were calculated by the Tukey test. Bars indicate standard deviation. **, $p < 0.01$.

selenite played a key role in the selenite toxicity. These data led us to conclude that the incorporation of selenite, rather than methylation, plays a critical role in the different cellular sensitivities among the cell lines.

SLC4A1 is involved in the exchange of chloride and bicarbonate in kidney. Mammalian plasma contains two major Se-containing proteins, selenoprotein P (SELENOP) and glutathione peroxidase 3 (GPX3). SELENOP and GPX3 are biosynthesized and are secreted from a liver and a kidney, respectively [18,19]. A liver incorporates dietary selenium and synthesizes SELENOP, and SELENOP is delivered to other organs as a selenium source. A kidney incorporates SELENOP and synthesizes GPX3 under physiological conditions. Almost all of Se in plasma is in the form of SELENOP or GPX3 [1]. However, in SELENOP knockout mice, a selenite administration can rescue the selenium concentration in the kidney and glutathione peroxidase activity in plasma to the same extent as those of wild-type mice [20]. This suggests that selenite is incorporated by SLC4A1 into a kidney to be used as another selenium source in addition to SELENOP.

Mutations and polymorphisms of *SLC4A1* are often found in patients with distal renal tubular acidosis [21], hereditary spherocytosis [22],

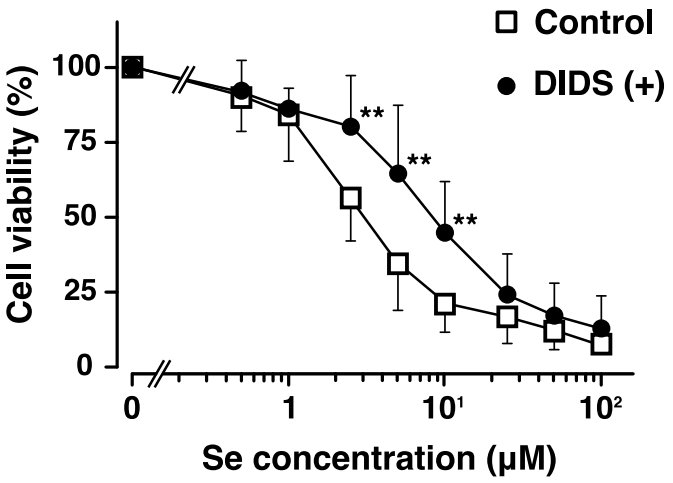

Fig. 4. Inhibition of Band 3/SLC4A1 protein improved cell viability after selenite exposure.
HEK293 cells were exposed to the indicated concentration of selenite for 24 h in the presence or absence of 25 μM SLC4A1 inhibitor, DIDS. Cell viability was examined by the MTS assay. The graph represents results calculated from eight samples in two independent experiments. *p* values were calculated by the Student's *t*-test. Bars indicate standard deviation. **, $p < 0.01$.

and other disorders in red cell membrane [23,24]. Selenite is used as supplementation to total parenteral nutrition [6], and as a nutritional aid and a food additive for patients who have lost the ability to ingest food, patients with short bowel syndrome [25], and infants receiving specially formulated milk [26]. However, as the nutritional and toxic doses of selenite are quite similar [2,4], clinical selenite supplementation has to be conducted carefully. Our data suggest that clinical selenite supplementation should be carried out with careful attention given to the activity of Band 3 protein and the polymorphisms of *SLC4A1*. On the other hand, some Se compounds including selenite are expected to have anticancer and anticarcinogenic activities, although the clinical use of Se compounds for these purposes has not been achieved [4]. Our observations suggest that the evaluation of *SLC4A1* mutations and polymorphisms would be useful to predict the effect of selenite on chemotherapy. Exploration of genotype-phenotype correlation of *SLC4A1* is expected to prevent selenite toxicity in nutritional supplementation and to promote the application of selenite as an anticancer agent.

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Declaration of interests

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Data availability

No data was used for the research described in the article.

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