

Fig. 5. Effects of Overexpression of Mutant Forms of Rad23 on the Sensitivity of Yeast Cells to Methylmercury

a: Schematic representation of the structural domains of Rad23 and the mutant proteins generated in this study. Rad23 contains a ubiquitin-like (UbL) domain and two ubiquitin-associated (UBA) domains. b: Yeast cells that overexpressed FLAG-Rad23 or mutant derivatives were cultured in SD (-uracil) liquid medium that contained methylmercury at the indicated concentrations.

以上の結果から、Rad23 が有する 2 つの機能のうち、UBA ドメインを介したユビキチン化蛋白質の分解抑制作用はメチル水銀毒性を軽減し、UbL ドメインを介したユビキチン化蛋白質の分解促進作用はメチル水銀毒性を増強するが、Rad23 高発現酵母中では、ユビキチン化蛋白質の分解抑制作用の方が分解促進作用を凌いでいるために、Rad23 高発現酵母はメチル水銀に対して耐性を示すと考えられる。したがって、細胞内にはメチル水銀毒性の軽減に関与し、かつ、UP システムによって分解される蛋白質が存在し、Rad23 はこの蛋白質の分解を抑制することによってメチル水銀毒性に対して防御的に作用していると考えられる。

5. メチル水銀毒性発現における Cdc34 と Rad23 との係わり

Rad23 は、Cdc34 とは逆に、メチル水銀毒性を軽減する蛋白質の分解を抑制することによってメチル水銀毒性を軽減する可能性が示された。しかし、Cdc34 は蛋白質のユビキチン化に関与し、Rad23 はユビキチン化蛋白質のユビキチン鎖と結合することから、Cdc34 と Rad23 がメチル水銀毒性に間接的に係わる同一の蛋白質を基質として認識する可能性も否定できない。そこで、正常酵母と Rad23 欠損

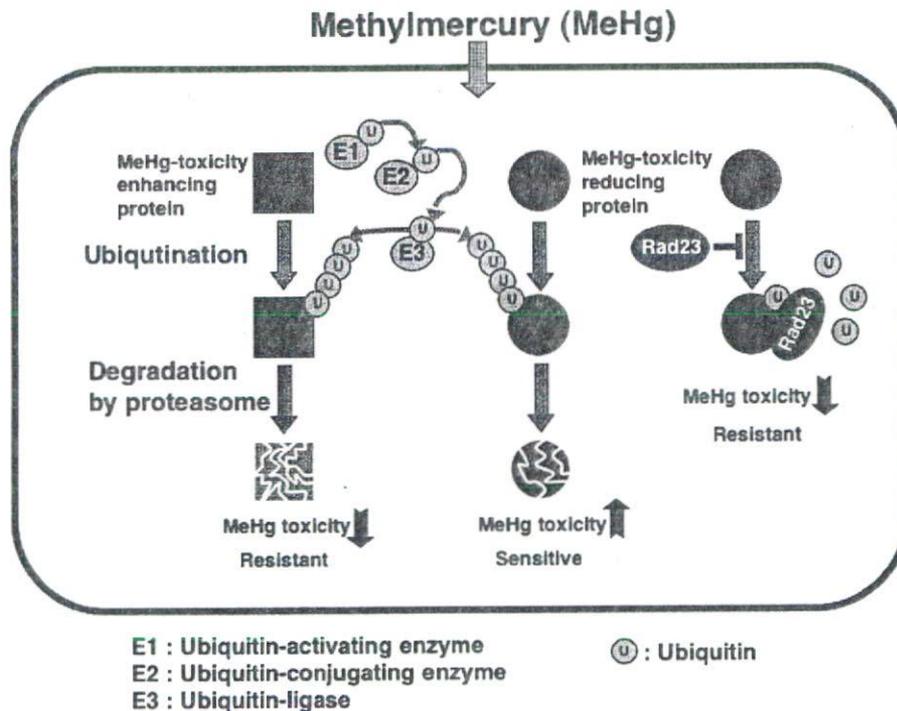


Fig. 6. Regulation of Methylmercury Toxicity by Ubiquitin-proteasome System

酵母にそれぞれ Cdc34 を高発現させたところ、両酵母のメチル水銀に対する耐性度はほぼ同程度に上昇した。このことから Rad23 とユビキチン化蛋白質のユビキチン鎖との結合は何らかの厳密な基質蛋白質認識機構によって調節されており、Cdc34 が E2 として関与する蛋白質分解システムに Rad23 の機能はほとんど影響を与えないと考えられる。細胞内にはメチル水銀毒性を軽減又は増強し、かつ、ユビキチン化を受ける複数の蛋白質が存在し、UP システム及び関連蛋白質はそれら蛋白質の細胞内濃度を複雑に調節することによってメチル水銀毒性の発現程度を規定していると考えられる (Fig. 6)。

#### 6. おわりに

近年、様々な神経変性疾患の発症に UP システムが関わっていることが多く報告されており、UP システムの異常は脳機能に大きな障害をもたらすものと考えられる。一方、メチル水銀も主に中枢神経障害を引き起こすことから、UP システムとメチル水銀毒性との関係も注目すべき事実と考えられる。これまでに筆者らが得た知見は、メチル水銀に対する感受性決定機構として UP システムが重要な役割を果たしている可能性を強く示唆するものである。これらの知見はメチル水銀細胞毒性の発現機構及びそれに対する防御機構の解明に有用な手掛かりを与えただけでなく、UP システムの新しい役割の解明にも大きく貢献し得るものと思われる。今後、Cdc34 及び Rad23 の基質となる蛋白質の中からメチル水銀毒性に係わる蛋白質を同定することによって、UP システムによるメチル水銀毒性調節機構が解明されるものと期待される。

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## Ubiquitin-conjugating enzyme Cdc34 mediates cadmium resistance in budding yeast through ubiquitination of the transcription factor Met4

Gi-Wook Hwang<sup>a</sup>, Takemitsu Furuchi<sup>a,b</sup>, Akira Naganuma<sup>a,c,\*</sup>

<sup>a</sup> Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan

<sup>b</sup> Laboratory of Analytical Chemistry, Graduate School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108-8641, Japan

<sup>c</sup> Tohoku University, 21st Century COE Program "Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation", Sendai 980-8578, Japan

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### Abstract

Overexpression of the ubiquitin-conjugating enzyme Cdc34 conferred strong cadmium resistance on budding yeast. Proteasome activity, which is involved in the degradation of ubiquitinated proteins, was not essential for the acquisition of resistance to cadmium. The overexpression of Cdc34 accelerated the ubiquitination of the transcription factor Met4 and reduced expression of *MET25* gene, which is a target of Met4. A *MET25*-disrupted strain of yeast was more resistant to cadmium than was the wild-type strain, but overexpression of Cdc34 in the *MET25*-disrupted cells did not affect sensitivity to cadmium. Met25 is an enzyme that catalyzes the synthesis of homocysteine from sulfide ( $S^{2-}$ ) and *O*-acetylhomocysteine and we detected the increased production of  $S^{2-}$  upon overexpression of Cdc34. Our results suggest that overexpression of Cdc34 inactivates Met4 and interferes with expression of the *MET25*, with subsequent production of CdS, which has low toxicity, and, thus, a decrease in the cadmium toxicity.

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Cadmium is a major environmental pollutant that is extremely toxic to humans. Overconsumption of cadmium through ingestion of contaminated food damages proximal tubules in the kidney [1]. A high concentration of cadmium interferes with the activities of many enzymes, but the mechanisms that mediate cadmium toxicity remain to be fully characterized.

In mammalian cells, increases in intracellular concentrations of the metal-binding protein metallothionein [2] and of the antioxidant glutathione [3] confer resistance to cadmium. Mechanisms responsible for the resistance of yeast cells to cadmium have been studied extensively, and resis-

tance has been shown to increase upon enhanced excretion of cadmium via the cadmium transporter Cad2 [4]; upon transportation of a Cd–glutathione complex to vacuoles by the S-conjugate pump known as yeast cadmium factor 1 (Ycf1) [5]; and upon the binding of the yeast metallothionein homolog Cup1 to cadmium [6,7].

In yeast cells, cadmium induces the expression of ubiquitin-conjugating enzymes, such as Ubc4, Ubc5, and Ubc7, that operate in the intracellular ubiquitin-proteasome (UP) system for the degradation of proteins [8,9]. Yeast cells with low proteasome activity are very sensitive to cadmium [8], an observation that suggests that the UP system plays a role in cadmium toxicity.

The first step in the UP system is the binding of activated ubiquitin to an ubiquitin-conjugating enzyme. At the same time, the ubiquitin-conjugating enzyme binds to an ubiquitin ligase. Substrate protein is recognized by the

\* Corresponding author. Address: Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan. Fax: +81 22 795 6870.

E-mail address: [naganuma@mail.pharm.tohoku.ac.jp](mailto:naganuma@mail.pharm.tohoku.ac.jp) (A. Naganuma).

ubiquitin ligase, then, many ubiquitin moieties are linked to the substrate protein by the ubiquitin-conjugating enzyme. The ubiquitin chain attached to the ubiquitinated substrate protein acts as a signal for degradation of the substrate by proteasomes [10,11]. Combinations of the many types of ubiquitin-conjugating enzyme and ubiquitin ligase regulate the substrate specificity of the degradative process [12].

It was reported recently that cadmium inhibits the ubiquitin ligase Skp1-Cdc53/cullin-F-box protein (SCF) complex [13,14]. The SCF complex is composed of four subunits, namely Cdc53, Skp1, Hrt1, and F-box protein. There are at least 17 types of F-box protein in budding yeast and they are responsible for the substrate specificity of the SCF complex [15–17]. The F-box protein Met30 inhibits the expression of many genes that are involved in the synthesis of sulfur-containing amino acids [18]. Inhibition is believed to be due to the specific SCF<sup>MET30</sup> complex and the Cdc34-dependent ubiquitination of the transcription factor Met4, which leads to a loss of Met4 function [19,20]. Inhibition of SCF<sup>MET30</sup> by cadmium allows Met4 to remain active and to induce the expression of genes for enzymes involved in the synthesis of glutathione, which protects cells against cadmium toxicity [13,14].

We discovered recently that overexpression of Cdc34, the ubiquitin-conjugating enzyme mentioned above [16], strongly accelerates the ubiquitination of proteins [21]. We postulated that yeast cells that overexpressed Cdc34 would, therefore, be sensitive to cadmium toxicity. Thus, in the present study, we investigated the effects of the overexpression of Cdc34 on cadmium toxicity. Contrary to our expectations, our results showed clearly that Cdc34-overexpressing yeast cells were resistant to cadmium. This observation suggested that the mechanism of cadmium resistance exploits an elevated intracellular concentration of S<sup>2-</sup>, which is triggered by the ubiquitination of Met4 and the resultant reduced expression of MET25.

## Materials and methods

**Yeast strains and media.** *Saccharomyces cerevisiae* strain W303B (*MAT $\alpha$*  his3 can1-100 ade2 leu2 trp1 ura3) was cultured in yeast extract-peptone-dextrose (YPD) medium or in synthetic dextrose (SD) medium at 30 °C. Plasmid DNA was introduced into yeast cells by the lithium acetate procedure [22,23].

**Gene disruption.** Genes were disrupted as described previously [24,25]. The MET16- and the MET25-disrupted strains (*met16 $\Delta$*  or *met25 $\Delta$* ) were generated by one-step PCR-based gene replacement with the HIS3 gene. The *met25::HIS3* vector and the *met16::HIS3* vector were constructed by amplification of the HIS3 gene by PCR using the following primers: MET16-HIS3-F (5'-ATGAAGACCTATCATTGAAATAATGATATA ATTGTCACACAAGAAGAGTTCTCTGGCCTCCTCTAG-3') and MET16-HIS3-R (5'-CTAGGCATCTTGCTTTAAAAATTGCGCGA ATCGGCTGGCTTCATGAATTCTCGTTTCCAGAAATGACACG-3'); and MET25-HIS3-F (5'-ATGCCATCTCATTTCGATACTGTTC AAC TACACGCCGGCCAAGAGAACCCTCTTGGCCTCCTCTAG-3') and MET25-HIS3-R (5'-GTTCTAACATGAACGATGTATGCCAAG TTACCGTAGGCTTCATTGTAGATTCTGTTCCAGAAATGACACG-3'). The products of PCR were introduced into W303B cells.

**Construction of plasmids.** The LEU2-based plasmids pRS425, pRS425-CDC34 (with a wild-type yeast CDC34 gene under the control of the endogenous promoter), and various mutant derivatives of pRS425-CDC34 were constructed as described previously [21]. For construction of the MET25-LACZ fusion gene, a DNA fragment containing a 686-bp fragment of the 5' upstream region of MET25 (from positions -683 to +3) was amplified by PCR with chromosomal DNA from strain W303B as template and the oligonucleotide primers 5'-GGTGAGCATGCCA TTCAATCCTTGGCTTTCAG-3' and 5'-TCCGGATCCTGTATG GATGGGGGTAATAGAATTG-3'. Amplified DNA was introduced into the SphI and BamHI sites of the LEU2-based centromeric plasmid K7. For construction of pKT10-CDC34 and pKT10-MET4-HA, the appropriate genes were cloned by PCR, with chromosomal DNA from strain W303B as template and the following oligonucleotide primers: 5'-A CCAAACAAGGAAAAA-3' and 5'-TTGTCTTCTTTCTTACTGT TC-3' for amplification of CDC34; and 5'-TTCTTTCTTTTCTGC CGT-3' and 5'-TTAAGCGTAATCTGGAACATCGTATGGGTAT TCTTTCTGAACCTTCTCAA-3' for MET4-HA. Amplified DNA was inserted into the blunted PuvII cloning site of the URA3-based plasmid pKT10-GAPDH.

**Northern blotting analysis.** Yeast cells (10<sup>7</sup> cells/ml) were grown in 40 ml of SD liquid medium without leucine (-Leu) for 5 h and harvested. Total RNA was prepared as described previously [19]. The MET16, MET25, and ACT1 genes were cloned by PCR with chromosomal DNA from strain W303B as template and the following oligonucleotide primers: 5'-CAGCAAAGGTATCAACCCAT-3' and 5'-GGTTATATATCGT ACTCTATC-3' for amplification of MET16; 5'-TTCTATTACCCCAT CCATAC-3' and 5'-ACAACCTCATTACGCACAC-3' for MET25; and 5'-TTCTTCCCAAGATCGAAAATT-3' and 5'-AACATACGGCACA AAAGCA-3' for ACT1. The following gel-purified fragments were used as probes: a 563-bp ClaI-EcoRI fragment for MET16; a 271-bp EcoRI-XbaI fragment for MET25; and a 300-bp BglIII-DraI fragment for ACT1. Northern blotting was performed with the DIG system (Roche Diagnostics) according to the manufacturer's instructions. The yeast ACT1 gene was used as an internal control.

**Immunoblotting and immunoprecipitation.** The ubiquitination of cellular proteins was examined by immunoblotting according to the published protocols [21]. Yeast cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and bands of proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and probed with a multiubiquitin-specific antibody (MBL, Nagoya, Japan). The Met4-HA protein was immunoprecipitated from yeast cell lysates with agarose-conjugated monoclonal antibodies against hemagglutinin (clone HA-7; Sigma). After overnight incubation at 4 °C, the beads were washed five times with Tris-buffered saline [20 mM Tris-HCl (pH 7.4), 500 mM NaCl] and proteins were eluted by boiling in sample buffer for SDS-PAGE. The ubiquitination of Met4-HA was visualized with rabbit antibodies against ubiquitin (Sigma) or rat antibodies against HA (Roche, Basel, Switzerland) and peroxidase-conjugated goat antibodies raised against rabbit immunoglobulins (Dako A/S, Glostrup, Denmark) as primary and secondary antibodies, respectively.

**Detection of the production of hydrogen sulfide.** Ten microliters (10<sup>8</sup> cells/ml) of a suspension of yeast cells were spotted on agar-solidified LA medium (0.3% peptone, 0.5% yeast extract, 4% glucose, 0.02% ammonium sulfate, 0.2% lead acetate and 2% agar) in a six-well plate and incubated for 7 days at 30 °C. On such plates, hydrogen sulfide-producing colonies become dark brown in color as a result of the formation of PbS [26].

## Results

### Yeast cells that overexpress Cdc34 are resistant to cadmium

Yeast cells that overexpressed Cdc34 were much more resistant to cadmium than control yeast that harbored an empty vector only (Fig. 1A). We had predicted that

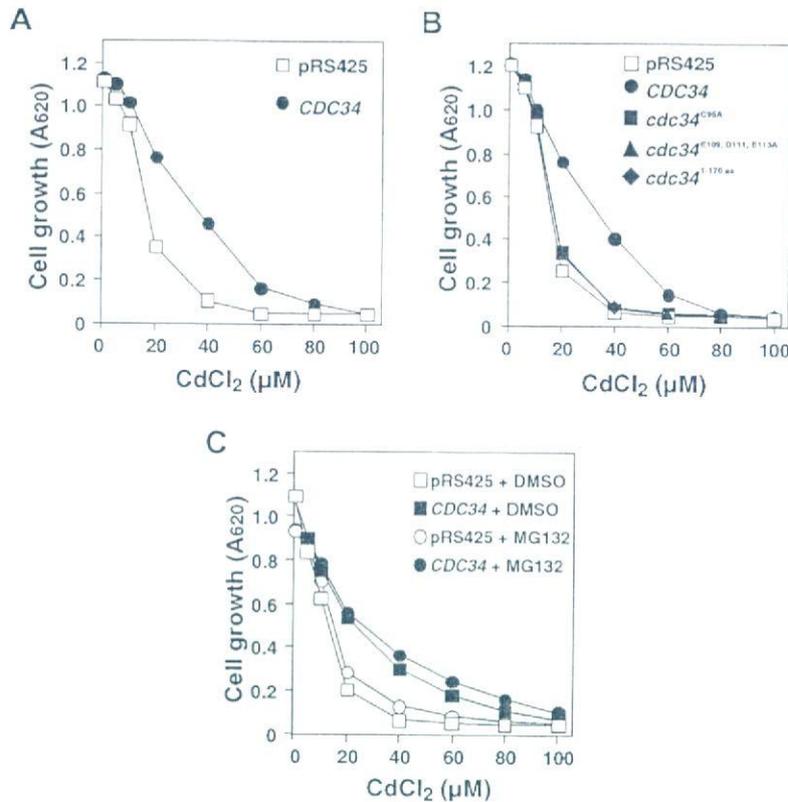


Fig. 1. Cadmium sensitivity of yeast cells that overexpress Cdc34 or mutant forms of Cdc34. (A,B) Yeast cells ( $10^4$  cells/200  $\mu$ l) harboring pRS425 or pRS425-*CDC34* (A); or pRS425, pRS425-*CDC34*, pRS425-*cdc34*<sup>C95A</sup>, pRS425-*cdc34*<sup>E109, D111, E113A</sup> or pRS425-*cdc34*<sup>L170aa</sup> (B) were grown in SD (-Leu) liquid medium that contained cadmium (as CdCl<sub>2</sub>) at the indicated concentrations. After a 48-h incubation, absorbance of cultures at 620 nm (A<sub>620</sub>) was measured spectrophotometrically. Results are shown as means of results of three replicate experiments  $\pm$  SD. The absence of a bar indicates that the SD falls within the symbol. (C) Yeast *erg6 $\Delta$*  cells ( $10^4$  cells/200  $\mu$ l) harboring pRS425 or pRS425-*CDC34* were grown in SD (-Leu) liquid medium, with or without the proteasome inhibitor MG132, in the presence of various concentrations of cadmium. Since MG132 was dissolved in DMSO, controls were treated with appropriate amounts of DMSO, as indicated.

overexpression of Cdc34 would sensitize yeast cells to cadmium by reducing the intracellular concentration of glutathione [13,14]. However, contrary to our expectations, overexpression of Cdc34 was associated with a strong toxicity-reducing mechanism that suppressed any sensitization that might have been caused by lowered levels of glutathione.

#### *The ubiquitin-transfer activity of Cdc34 is involved in the reduction of cadmium toxicity*

To determine whether cadmium resistance conferred by overexpression of Cdc34 was caused by a novel function of Cdc34, we examined the cadmium sensitivity of yeast cells that overexpressed variants of Cdc34 with altered ubiquitin-transfer domains. Replacement of cysteine 95 by alanine [27] at the ubiquitin-binding site resulted in loss of cadmium resistance (Fig. 1B). This observation indicated that a thio-ester binding configuration for the interaction of Cdc34 with ubiquitin is necessary for acquisition of cadmium resistance by Cdc34-overexpressing cells. Cadmium resistance was also lost in yeast cells that overexpressed a mutant Cdc34 in which three amino acids were replaced with alanine (glutamate 109, aspartate 111, and glutamate

113) [28] within a unique insert that is required for the specificity of the activity of Cdc34 and is not found in other ubiquitin-conjugating enzymes. In addition, replacement by alanine of residues required for binding of ubiquitin ligase during substrate recognition in the ubiquitination system [29] also resulted in loss of cadmium resistance (Fig. 1B). These findings suggest that the activity of Cdc34 as a ubiquitin-conjugating enzyme is essential for the acquisition of cadmium resistance by Cdc34-overexpressing yeast cells.

#### *Acquisition of cadmium resistance by Cdc34-overexpressing yeast cells does not require proteasome activity*

Large numbers of ubiquitinated proteins are ultimately degraded by proteasomes. Therefore, we examined the role of proteasomes in the resistance to cadmium of yeast cells that overexpressed Cdc34, using the proteasome inhibitor MG132. Under our experimental conditions, MG132 significantly increased the intracellular concentration of a fusion protein composed of green fluorescent protein (GFP) fused to signal sequences for degradation by ubiquitin-dependent proteolysis [30] (data not shown). However, Cdc34-overexpressing yeast cells were resistant to cadmium

even in the presence of MG132 (Fig. 1C), indicating that protein degradation by proteasomes is not essential for the acquisition of cadmium resistance.

The transcription factor Met4, which is specifically ubiquitinated by Cdc34 and the SCF complex, loses its transcriptional activity by ubiquitination and not as a result of degradation by proteasomes [20,31]. Postulating that Met4 might be involved in the acquisition of cadmium resistance, we examined the effects of overexpression of Cdc34 on the ubiquitination of Met4. Yeast cells that overexpressed Cdc34 and expressed Met4 fused with a hemagglutinin (HA) tag (Met4-HA) had dramatically increased levels of ubiquitinated Met4-HA (Fig. 2).

#### *Cdc34 reduces cadmium toxicity by inhibiting expression of the MET25 gene*

Met4 regulates the expression of a large number of enzymes that are involved in the synthesis of sulfur-containing amino acids [32,33]. One of these enzymes is Met25 (Fig. 3A), which catalyzes synthesis of homocysteine from  $S^{2-}$  and *O*-acetylhomocysteine. The *MET25*-disrupted yeast cells accumulate high intracellular concentrations of  $S^{2-}$  [26]. Cadmium reacts strongly with  $S^{2-}$  to produce the insoluble and minimally toxic CdS and, thus, the accumulation of  $S^{2-}$  is thought to protect cells against cadmium toxicity [34]. We examined the sensitivity to cadmium of yeast cells with disruption of either the *MET25* or the *MET16* gene, which encodes an enzyme involved in the production of  $SO_3^{2-}$  (sulfite; precursor to  $S^{2-}$ ). The *MET16*-disrupted strain was slightly more sensitive to cadmium than the wild-type strain, while the *MET25*-disrupted strain was markedly more resistant to cadmium than the wild-type strain (Fig. 3B). Disruption of *MET16* might lead to a decrease in the intracellular concentration of  $S^{2-}$ , while disruption of *MET25* might

increase the concentration of  $S^{2-}$ , suggesting that intracellularly generated  $S^{2-}$  might be involved in reducing the toxicity of cadmium.

Fig. 3C shows that overexpression of Cdc34 conferred cadmium resistance to wild-type of yeast cells, while the cadmium sensitivity of the *MET16*- and *MET25*-disrupted strains was only minimally affected by overexpression of Cdc34. These observations suggest that Met16 and Met25 are essential for the acquisition of cadmium resistance that is associated with overexpression of Cdc34.

We next examined the effects of the overexpression of Cdc34 on expression of Met16 and Met25. The overexpression of Cdc34 markedly decreased, but did not completely inhibit the expression of the *MET16* gene (Fig. 3D) and the *MET25* genes (Figs. 3D and E). Some  $S^{2-}$  might still accumulate as a result of residual Met16 activity. Indeed, utilization of  $S^{2-}$  was impeded upon inhibition of Met25, such that the concentration of  $S^{2-}$  increased, with resultant cadmium resistance of Cdc34-overexpressing cells. Substantially higher amounts of  $S^{2-}$  were, in fact, detected in yeast cells that overexpressed Cdc34 as compared to amounts in control cells (Fig. 3F).

#### Discussion

The ubiquitin-activating enzyme Uba1 and the ubiquitin ligase SCF complex are important components of the intracellular protein-ubiquitination response that involves Cdc34 [16]. The present study shows, for the first time to our knowledge, that overexpression of Cdc34 accelerates the ubiquitination of Met4 (Fig. 2) and, thus, reduces the transcriptional activity of Met4 (Fig. 3E). Overexpression of the ubiquitin-activating enzyme Uba1 and of Cdc53, Skp1, and Hrt1, constituents of the SCF<sup>MET30</sup> complex, failed to accelerate the protein-ubiquitination response [21]. Thus, it appears that Cdc34 is a rate-determining enzyme in the ubiquitination of Met4.

Cadmium might promote the dissociation of the SCF<sup>MET30</sup> complex, thereby inhibiting the ubiquitination of Met4 and promoting the expression of genes involved in the synthesis of cysteine, which protects cells against cadmium toxicity, and of glutathione, a tripeptide that contains cysteine [13,14]. Therefore, even we though we had expected that overexpression of Cdc34 would increase cadmium sensitivity in yeast, we obtained the opposite result (Fig. 1A). Both Met16 and Met25 were necessary for the acquisition of cadmium resistance upon overexpression of Cdc34 (Fig. 3B), and overexpression of Cdc34 reduced expression of the *MET25* gene through inhibition of the transcriptional activity of Met4 (Figs. 3D and E). These results suggest that cadmium toxicity was reduced as a result of an increase in the intracellular concentration of  $S^{2-}$ , a substrate in the reaction catalyzed by Met25 (Fig. 3F), and formation of the minimally toxic compound CdS.

The reduction in levels of expression of Met25 that was triggered by overexpression of Cdc34 resulted in reduced

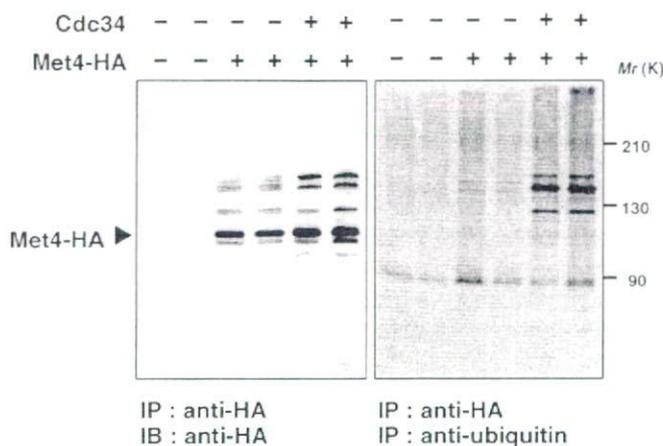


Fig. 2. Effects of overexpression of Cdc34 on ubiquitination of Met4. Yeast cells harboring pKT10-*MET4*-HA and either pRS425 or pRS425-*CDC34* were grown in SD (-Ura, -Leu) liquid medium. Met4-HA was immunoprecipitated (IP) with anti-HA agarose and immunoblotted (IB) with multiubiquitin-specific antibodies (anti-ubiquitin; right panel) or HA-specific antibodies (anti-HA; left panel).

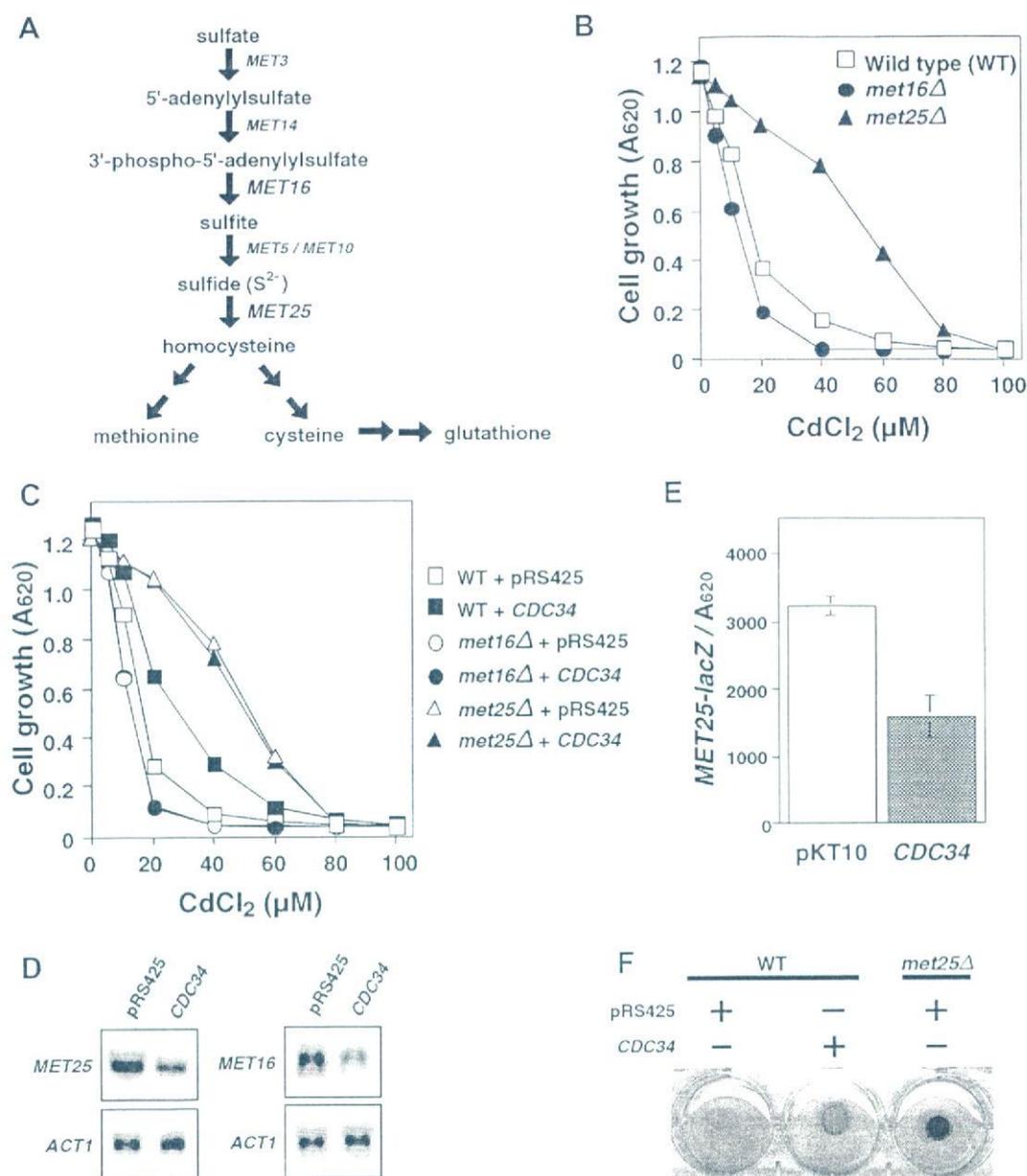


Fig. 3. Roles of the *MET16* and *MET25* genes in Cdc34-mediated resistance to cadmium in yeast cells. (A) Biosynthesis of sulfur-containing amino acids in yeast. (B) Effects of disruption of the *MET16* and *MET25* genes on cadmium sensitivity in yeast. Yeast cells ( $10^4$  cells/200  $\mu$ l), with disrupted a *MET16* (*met16Δ*) or *MET25* (*met25Δ*) genes, were grown in SD (-Leu) liquid medium that contained cadmium at the indicated concentrations. (C) Effects of overexpression of Cdc34 on cadmium sensitivity in yeast cells with a disrupted *MET16* or *MET25* gene. Mutant yeast cells (*met16Δ* or *met25Δ*) harboring pRS425 or pRS425-*CDC34* were grown in SD (-Leu) liquid medium that contained cadmium at the indicated concentrations. For further details, see the legend to Fig. 1. (D) Effects of overexpression of Cdc34 on levels of *Met16* and *Met25* mRNAs. Each lane was loaded with 20  $\mu$ g of total RNA extracted from yeast cells that harbored pRS425 or pRS425-*CDC34*. Act1 mRNA was used as a loading control. (E)  $\beta$ -Galactosidase activity of yeast cells that harbored pKT10 or pKT10-*CDC34* was measured after introduction of the *MET25-LACZ* reporter gene. Results are means  $\pm$  SD. of results from three replicate experiments. (F) Effects of overexpression of Cdc34 on production of  $S^{2-}$ . Yeast cells harboring pKT10-*MET4-HA* and either pRS425 or pRS425-*CDC34* were spotted on agar-solidified medium prepared with lead acetate and incubated for 7 days at 30  $^{\circ}$ C. On this medium, hydrogen sulfide-producing colonies become dark brown in color as a result of the formation of lead sulfide.

synthesis of glutathione and a subsequent increase in the intracellular levels of  $S^{2-}$ . Since  $S^{2-}$  reacts efficiently with cadmium to produce low-toxicity CdS, the effects on the cell of a decrease in the level of glutathione [13,14] are probably unimportant.

The present study showed that overexpression of Cdc34 confers resistance to cadmium in yeast. Cdc34 also affects the toxicity of mercury compounds [19,35] so it is possible

that Cdc34 might play a major role in mitigating or mediating the effects of various metal compounds.

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– Toxicogenomics/proteomics Report –

## siRNA-MEDIATED INHIBITION OF PHOSPHATIDYLINOSITOL GLYCAN CLASS B (PIGB) CONFERS RESISTANCE TO METHYLMERCURY IN HEK293 CELLS

Gi-Wook HWANG<sup>1</sup>, Tatsuya HAYASHI<sup>1</sup>, Kayoko KITA<sup>1</sup>, Tsutomu TAKAHASHI<sup>1</sup>,  
Shusuke KUGE<sup>1</sup> and Akira NAGANUMA<sup>1,2</sup>

<sup>1</sup>Laboratory of Molecular and Biochemical Toxicology,  
Graduate School of Pharmaceutical Sciences, Tohoku University,  
Aoba-ku, Sendai 980-8578, Japan

<sup>2</sup>Tohoku University 21st Century COE Program  
"Comprehensive Research and Education Center  
for Planning of Drug Development and Clinical Evaluation",  
Aoba-ku, Sendai 980-8578, Japan

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**ABSTRACT** — We performed functional gene screening, using a siRNA library targeting 8,500 human genes, to identify proteins that are involved in the susceptibility of cells to methylmercury. Screening revealed that downregulation of the gene for phosphatidylinositol glycan class B (PIGB) by siRNA confers resistance to methylmercury in HEK293 cells.

**KEY WORDS:** Methylmercury, Resistance, siRNA, PIGB, HEK293 cells

### INTRODUCTION

Methylmercury is an important environmental pollutant and causes severe neurological disorders in humans (Clarkson, 2002; Castoldi *et al.*, 2003). However, the molecular mechanism underlying the toxicity of methylmercury remains to be clarified. To identify proteins that are involved in cell susceptibility to methylmercury, we performed functional gene screening using a siRNA library targeting 8,500 human genes (Paddison *et al.*, 2004).

### MATERIALS AND METHODS

#### Transducing siRNA library into HEK293 cells

HEK293 cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified 10% CO<sub>2</sub> atmosphere. A GeneNet™ human 8.5 K siRNA library (System bio-

sciences, Mountain View, CA, USA) that contained 8,500 well-characterized human genes was pre-packaged in pseudoviral particles using the FIV lentiviral expression system. The detailed protocol for the transduction of the siRNA library is available from System Biosciences. Briefly, HEK293 cells (2 × 10<sup>6</sup> cells/plate) were plated on five 10-cm plates and cultured in 10 ml aliquots of medium. After incubation for 24 hr, the culture medium was removed and the cells were transduced with the pseudoviral particles (4 × 10<sup>6</sup> ifu/plate) in plates containing 5 ml of DMEM/3% FBS with polybrene (final 6 µg/ml). After incubation for 12 hr, the culture medium was removed from each plate and replaced by 10 ml of DMEM/10% FBS without polybrene. Cells that had been stably transduced with the siRNA constructs were selected by their resistance to the antibiotic puromycin.

#### Selection of gene-specific siRNAs that confer resistance to methylmercury

The siRNA library-transduced cells (3 × 10<sup>6</sup> cells)

were plated into 6-well plates ( $5 \times 10^5$  cells/well) containing 2 ml medium per well. After incubation for 24 hr, 18  $\mu$ M methylmercury chloride was added to each well and the cells were cultured for additional 48 hr. Then the medium was replaced by fresh medium and after a recovery period, the cells were maintained in DMEM containing the same concentration of methylmercury chloride for a further 48 hr. We obtained one colony that was strongly resistant to methylmercury. Total RNA was isolated from the colony with TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then the purified RNA was reverse transcribed by M-MLV reverse transcriptase (Invitrogen) using a primer (5'-TGCATGTCGCTATGTGTTCTGGGA-3') that was specific to sequence in the pFIV-H1 siRNA expression vector (System bioscience). The siRNA inserts from transduced cells were amplified by PCR by using the primers 5'-AATGTCTTTGGATTGGGAATCTTA-3' and 5'-AAAAGGGTGGACTGGGATGAGTA-3'. The amplified DNA fragments were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). The sequences of the siRNA inserts were verified by sequencing them on an automated sequencer.

#### Construction of an siRNA expression plasmid

For cloning into the pFIV-H1 siRNA expression vector, two DNA oligonucleotides pairs were employed; (PIGB-1: 5'-GATCCGGAATAAGTGCTTTCTTAATTTCAAGCCTTCTGTCAGAGCTTGAATTAGGAAAGCGCTTATTTCTTTTTG-3' and 5'-AATTCAAAAGAAATAAGCGCTTTCCTAATTTCAAGCTCTGACAGGAAGGCTTGAATTAAGAAAGCACTTATTCCG-3'; and PIGB-2: 5'-GATCCCTAGCTCTAGGTTTCTTGTCTTCCTGTCAGAACAGGAACTTAGAGCTGGTTTTTG-3' and 5'-AATTCAAAACCAGCTCTAAGTTTCTGTTCTGACAGGAAGACAAGAACCTAGAGCTAGG-3'), which encoded siRNAs that targeted the 1519-1545 and 1159-1167 regions of the gene PIGB, respectively. The oligonucleotides were annealed, phosphorylated by T4 DNA polynucleotide kinase (Takara, Shiga, Japan), and then ligated into the pFIV-H1 siRNA expression vector. The sequences of oligonucleotide inserts were verified by sequencing them on an automated sequencer. Plasmid DNA was transfected with Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocols.

#### Confirmation of knockdown by real-time PCR

After 48 hr of knockdown with the siRNA expression vectors expressing PIGB-1 and PIGB-2 siRNA, cells were lysed in TRIzol® reagent in order to isolate total RNA. First-strand cDNA synthesis was performed using the PrimeScrip™ RT reagent kit (Takara). We performed real-time PCR reactions (BioRad, iCycler™ thermal cycler) with the following primers: PIGB-F, 5'-TTGACTTGGGAATGGA-CAGA-3' and PIGB-R, 5'-AGACAGAAGTGCT-TGGGCAA-3' for the PIGB gene; and GAPDH-F, 5'-CGGGAAGCTTGTCATCAATGG-3' and GAPDH-R, 5'-GGCAGTGATGGCATGGACTG-3' for the GAPDH gene. The fold decreases in PIGB mRNA levels were determined from standard curves after calibration of the assay.

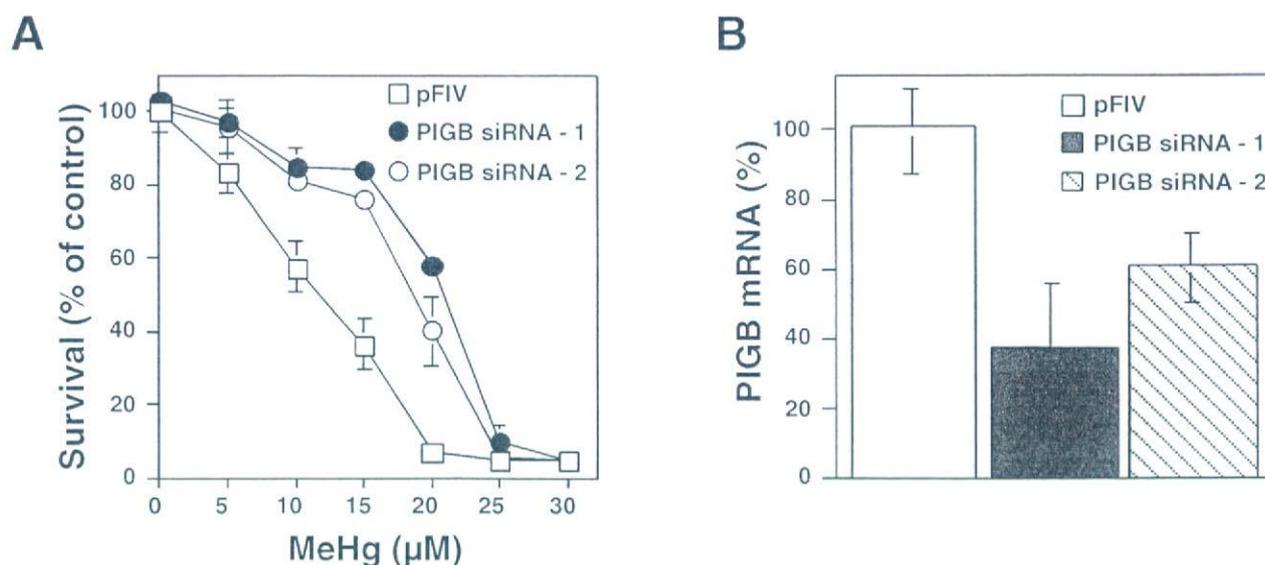
### RESULTS AND DISCUSSION

We introduced an siRNA library (Paddison *et al.*, 2004) that targets 8,500 human genes into HEK293 cells using the FIV lentiviral system. The pooled cells that had been stably transduced with the siRNA library were cultured in medium containing 18  $\mu$ M methylmercury, a concentration that prohibited the proliferation of the parent cells. We obtained one clone that was able to grow in the presence of 18  $\mu$ M methylmercury. An analysis of the nucleotide sequence of the siRNA insert in the resistant clone showed that it corresponded to the region 1519 – 1545 of the cDNA for phosphatidylinositol glycan class B (PIGB). Next, we introduced vectors expressing either the 1519-1555 siRNA (PIGB-siRNA1) or an siRNA (PIGB-siRNA2) against a different region (1159-1167) of PIGB cDNA into HEK293 cells using Lipofectamine™ 2000 reagent. Cells that had been transfected with either PIGB-siRNA1 or PIGB-siRNA2 exhibited significant resistance to methylmercury (Fig. 1a) and decreased levels of PIGB mRNA (Fig. 1b). PIGB is an enzyme involved in the synthesis of the glycosylphosphatidylinositol (GPI) anchor which is a membrane attachment structure for many proteins that occur in a wide variety of eukaryotes from yeasts to mammals (Takahashi *et al.*, 1996). Our finding suggests that the GPI anchor might have an important role in sensitizing human cells to methylmercury.

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Downregulation of PIGB by siRNA confers resistance to methylmercury.



**Fig. 1.** Effects of PIGB knockdown on HEK293 cell sensitivity to methylmercury. (A) HEK293 cells ( $2 \times 10^4$  cells), that had been stably transfected with PIGB siRNA, were plated on 96-well plates and cultured in 100  $\mu$ l aliquots of medium. After incubation for 24 hr, methylmercury chloride (MeHg) was added and cells were cultured for a further 48 hr. Cell viability was determined by treating cells with Alamer Blue (Biosource, Camarillo, CA, USA). Each point and bar represents the mean value and S.D. of the results from three cultures, respectively. (B) Levels of PIGB mRNA in HEK293 cells transfected with PIGB siRNA were analyzed by real-time PCR. The levels of PIGB mRNA were normalized to those of GAPDH mRNA.

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## Downregulation of arginase II and renal apoptosis by inorganic mercury: overexpression of arginase II reduces its apoptosis

Hironori Kanda · Makoto Kikushima · Shino Homma-Takeda · Daigo Sumi · Akiko Endo · Takashi Toyama · Nobuhiko Miura · Akira Naganuma · Yoshito Kumagai

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**Abstract** Inorganic mercury is a toxic metal that accumulates in the proximal tubules of the kidney, causing apoptosis. Arginase II is known to inhibit apoptosis, but its role in the renal apoptosis caused by inorganic mercury is poorly understood. In the present study, we examined the involvement of arginase II in inorganic mercury-dependent apoptosis. A single exposure to mercuric chloride ( $\text{HgCl}_2$ , 1 mg/kg) in rats resulted in a dramatic time-dependent reduction in the activity of arginase II in the kidney; for example, the activity at 48 h after exposure was 31% of the control level. The decrease in arginase II activity was due to a decrease in the protein level, not to a reduction in gene expression or to direct inhibition of the activity itself. More interestingly, diminished arginase II activity was well correlated with the induction of apoptosis as evaluated by renal DNA fragmentation ( $r = 0.99$ ). Overexpression of arginase II in LLC-PK<sub>1</sub>

cells blocked cell death during exposure to inorganic mercury. These results suggest that inorganic mercury causes a reduction in protein levels of arginase II, and that impaired arginase II activity is, at least in part, associated with the apoptotic cell damage caused by this heavy metal.

**Keywords** Inorganic mercury · Arginase II · Apoptosis · Proximal tubule · Accumulation

### Abbreviations

HgCl <sub>2</sub>	Mercuric chloride
NOS	Nitric oxide synthase
NO	Nitric oxide
TE	Tris and EDTA
D-PBS	Dulbecco's PBS
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
ECL	Enhanced chemiluminescent
RT-PCR	Reverse transcription-polymerase chain reaction
DMEM	Dulbecco's modified eagle medium
FCS	Fetal calf serum
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MnCl <sub>2</sub>	Manganese chloride
Mn-SOD	Manganese-superoxide dismutase

H. Kanda · A. Endo · T. Toyama  
Master's Program in Environmental Sciences,  
Graduate School of Comprehensive Human Sciences,  
University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

M. Kikushima · S. Homma-Takeda · D. Sumi · Y. Kumagai (✉)  
Department of Environmental Medicine,  
Doctoral Programs in Medical Sciences,  
Graduate School of Comprehensive Human Sciences,  
University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan  
e-mail: yk-em-tu@md.tsukuba.ac.jp

N. Miura · A. Naganuma  
Laboratory of Molecular and Biochemical Toxicology,  
Graduate School of Pharmaceutical Sciences,  
Tohoku University, Sendai, 980-8578, Japan

*Present Address:*  
S. Homma-Takeda  
Research Center for Radiation Protection,  
National Institute of Radiological Sciences,  
Inage-ku, Chiba, 263-8555, Japan

### Introduction

Inorganic mercury is recognized as a hazardous environmental pollutant. The most serious effect of inorganic mercury is renal toxicity. We have shown that metal predominantly accumulates in the proximal tubules of kidney, thereby causing apoptosis (Homma-Takeda et al. 1999). Apoptosis is a cell death, which ultimately breaks down chromosomes into

fragments containing multiple nucleosomes after caspases activation. Although alterations in apoptotic genes and their transcription factors have been proposed to explain toxic effect of mercury (Buzard and Kasprzak 2000; Shenker et al. 2000), the exact mechanism is unknown.

Arginase, which catalyzes the conversion of arginine to urea and ornithine, exists in two distinct isoforms. Arginase I is expressed almost exclusively in the liver where it serves as an essential enzyme of the urea cycle. In contrast, arginase II is expressed in the kidney and many other extrahepatic tissues (Gotoh et al. 1996; Miyanaka et al. 1998). Although its functions have not been well documented, arginase II has been recognized to participate in the regulation of nitric oxide synthase (NOS) (Jansen et al. 1992). In the kidney, arginase II is distributed in the proximal tubules (Miyanaka et al. 1998), and metabolizes most of the arginine present (Jansen et al. 1992). Interestingly, current consensus is that arginase plays a role in blocking apoptosis as well (Esch et al. 1998; Gotoh and Mori 1999; Estévez et al. 2006). In a preliminary study, we found that exposure to mercuric chloride ( $\text{HgCl}_2$ ) in rats results in a reduction of renal arginase II activity in a dose-dependent manner, suggesting that this enzyme is sensitive to inorganic mercury (Kanda et al. 1998). From these observations, we hypothesized that decreased arginase II levels could be associated with the induction of renal apoptosis *in vivo* following exposure to inorganic mercury. If so, overexpression of arginase II in renal cells could lessen inorganic mercury-dependent apoptosis. In the present study, we addressed these issues using rats and LLC-PK<sub>1</sub> cells stably transfected with this enzyme.

## Materials and methods

### Enzyme preparation

Male Wistar rats (7–8 weeks) were used. The experimental procedures were in accordance with the University of Tsukuba's Regulations on Animal Experiments and Japanese Governmental Law no. 105. The animals were administered  $\text{HgCl}_2$  dissolved in normal saline solution (1 mg/kg, Wako Pure Chemical Industries, Osaka, Japan) once by subcutaneous injection and animals were sacrificed by cardiac puncture under diethylether anesthesia at different times thereafter. Kidney was removed and then stored at  $-70^\circ\text{C}$  prior to use. Control rats were injected with normal saline solution alone. Kidney was homogenized in nine volumes of 10 mM tris-HCl buffer (pH 7.5). Homogenates were centrifuged at 700g for 10 min and supernatant was sonicated using a Tomy UD-200 ultrasonic disruptor at 50 W for 15 s (x6) to rupture the mitochondrial membrane. The resulting mixture was centrifuged at 90,000g for 70 min and each

supernatant was further applied to an Econo-Pac 10DG column to remove low molecular weight components. Thereafter, first eluates were collected to measure arginase II activity *in vivo*. Protein concentrations were measured by the Bradford method (Bradford 1976), with bovine serum albumin as a standard. A partially purified preparation obtained from rat kidney was used in the *in vitro* experiment. The purification included use of heat, CM-Sephadex C-50, ammonium sulfate fractionation (40–60% saturation) and a DE-52 column, which were derived from the methods of Kaysen and Strecker (1973) and Baranczyk-Kuzma et al (1976). The enzyme preparation was incubated with different concentrations of  $\text{HgCl}_2$  (1–1,000  $\mu\text{M}$ ) at  $37^\circ\text{C}$  for 10 min. LLC-PK<sub>1</sub> cells were homogenized in Dulbecco's PBS (D-PBS), followed by the tris-EDTA (TE) buffer. Following centrifugation, the supernatants were homogenized in HEPES-buffered saline with a protease inhibitor and PMSF, and then sonicated. The resulting mixture was centrifuged, and each supernatant was used as cell extracts for the experiments with LLC-PK<sub>1</sub> cells.

### Measurement of mercury level in the kidney

Kidney samples (200 mg) were digested with nitric acid, potassium permanganate, and sulfuric acid at room temperature. Mercury concentrations were then measured as reported previously (Shinyashiki et al. 1998).

### Arginase activity

Arginase activity was measured according to the methods of Slyke and Archibald (1946). The reaction mixture (0.5 ml) consisted of 100 mM arginine, 50 mM glycine-NaOH buffer (pH 9.5), and the enzyme preparation (26–78  $\mu\text{g}$  of protein). Incubation was performed at  $37^\circ\text{C}$  for 10 min. The reaction was terminated by the addition of 0.1 ml of 30% perchloric acid. The reaction mixture was centrifuged at 14,000g for 5 min, and the supernatant (0.4 ml) was mixed with 0.1 ml of alpha-isonitrosopropiophenone and 1 ml of acid mixture ( $\text{H}_2\text{SO}_4:\text{H}_3\text{PO}_4:\text{H}_2\text{O} = 1:3:7$ , by vol.). The mixed sample was heated to  $100^\circ\text{C}$  for 90 min in the dark and then cooled in the dark. The amount of urea formed from arginine was determined as the alpha-isonitrosopropiophenone adduct at 545 nm against the reagent blank set at zero. Given that arginase I is not expressed in the kidney under normal conditions (Ozaki et al. 1999), we considered the arginase activity detected in the present study to represent exclusively arginase II.

### Measurement of nitrite and nitrate

The amount of nitrite and nitrate was determined according to the method of Conrad et al (1993).

### Electrophoresis and immunoblot analysis

For Western blotting, the kidney samples were prepared as described previously (Yu et al. 1995). All operations were done at room temperature. In brief, the supernatant of the rat kidney was subjected to SDS-PAGE (12%). Separated proteins were transferred to a PVDF membrane at 2 mA/cm<sup>2</sup> for 45 min. This was followed by blocking with 3% gelatin in tris-buffered saline 0.1% Tween 20 for 30 min. The membrane was probed with polyclonal antibody against human arginase II for 60 min, and then further incubated with anti-rabbit IgG conjugated to alkaline phosphatase for 30 min. After membrane was washed, rat arginase II was detected with the ECL system and quantitated using an imaging program (Mac BAS, version 2.4), followed by background subtraction under these conditions.

### Isolation of total RNA and reverse transcription-polymerase chain reaction

On the basis of the method described by Chomczynski and Sacchi (1987), total RNA was isolated using an ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). The reverse transcription-polymerase chain reaction (RT-PCR) was performed using an AMV version 2.1 RNA PCR kit (Takara Shuzo, Shiga, Japan) with a standard protocol. The cycling parameters were as follows: 30 cycles, each consisting of 20 s at 94°C, 20 s at 55°C, and 40 s at 72°C. A fragment of the cDNA encoding for arginase II was amplified using as primers, 5'-GGTATCCAGAAGGTC ATG-3' and 5'-TAGGAGTGAAGGTGGT-3', bases 779–796 (sense) and 1107–1124 (antisense), respectively, giving a 345-bp product (Iyer et al. 1998). The samples were run on a 1.2% agarose gel containing ethidium bromide and analyzed by the method described in the immunoblot section.

### DNA fragmentation analysis

Quantitation of DNA fragmentation for the experiment using rat kidney was performed as reported previously (Homma-Takeda et al. 1999). For the experiment using LLC-PK<sub>1</sub> cells, cells were homogenized with ice-cold D-PBS and lysis buffer. After incubation for 20 min at 4°C, the homogenates were centrifuged at 14,000g for 20 min. DNA was extracted with phenol-chloroform, and then treated with RNase (10 mg/ml) for 2 h at 37°C. After the addition of glycogen (5 mg/ml), DNA was purified and dissolved in TE buffer. The resulting specimens were electrophoresed on a 1.5% agarose gel.

### Plasmids

pCAGGS-hAII, a mammalian expression plasmid for human arginase II, was constructed by inserting human *arginase II* cDNA into the *EcoR* I site of the plasmid pCAGGS after linker attachment according to the method of Gotoh and Mori (1999). Additionally, the vector that removed *arginase II* cDNA from the human arginase II-expressing plasmid was used as a control plasmid of highly expressed arginase II. pSVneo possessing a neomycin-resistance gene was also used as a plasmid that has a selective marker.

### Cell culture and DNA transfection

Pig kidney LLC-PK<sub>1</sub> cells (Human Science Research Resources Bank, Tokyo, Japan) were used in the in vitro experiments. Duncan-Achanzar et al. (1996) showed HgCl<sub>2</sub>-induced apoptosis with LLC-PK<sub>1</sub> cells as a model for proximal tubule cells. Cells were cultured at a density of  $2 \times 10^5$  cells/2 ml Dulbecco's-modified Eagle medium (DMEM) containing 60 µg/ml kanamycin and 10% fetal calf serum (FCS)/well in six-well plates at 37°C in 5% CO<sub>2</sub> for 24 h. The transfection of LLC-PK<sub>1</sub> cells with plasmids was carried out using FuGENE6 (Roche, Indianapolis, IN, USA). The arginase II-transfected clones were isolated after selection with 400 µg/ml of geneticin for 2–3 weeks. Cell maintenance was performed in DMEM medium containing 60 µg/ml kanamycin, 400 µg/ml hygromycin B, and 10% FCS (80–90% confluent). The cells were exposed to HgCl<sub>2</sub> (3–75 µM) for 12 h, and then used for observations of morphology, the extraction of DNA fragments and preparation of the enzyme.

### Assessment of cell survival

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used for the determination of cell death. An MTT solution (1/20 volume) was added to the medium, and the mixture was cultured for another 4 h in a CO<sub>2</sub> incubator. The mixture was removed and washed with D-PBS. Dimethyl sulfoxide (1/2 volume of medium) was then added, and MTT formazan was dissolved. The absorbance was measured using a spectral photometer. A microscopic analysis was also conducted.

### Statistics

Data are presented as the means ± standard deviations for each group. A *t* test was carried out; if the two groups showed unequal variance, Welch's test was used.

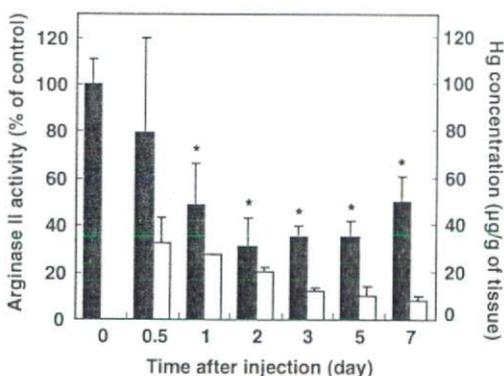
## Results

### Mercury accumulation, arginase activity and nitric oxide in kidney

Figure 1 shows total mercury concentrations and changes in arginase II activity in rat kidney after the subcutaneous injection of  $\text{HgCl}_2$  (1 mg/kg). Once mercury was distributed in the kidney (12 h,  $32.7 \pm 10.6 \mu\text{g/g}$  of tissue), levels of the metal remained at up to  $10.1 \pm 3.99 \mu\text{g/g}$  of tissue even 5 days after exposure. Under these conditions, arginase II activity declined by the injection of  $\text{HgCl}_2$ ; for example, the level of activity at 24 and 48 h was 49 and 31% of the control, respectively (Fig. 1). Subsequently, the activity started on a gradual recovery trend (7 days, 51% of the control). On the other hand, little appreciable change in renal NOS as determined from its enzyme activity (Kanda et al. 1998) and nitric oxide (NO) metabolite levels (data not shown) were seen following exposure to  $\text{HgCl}_2$ .

### Mechanism behind the decrease in arginase II activity

Because inorganic mercury is capable of interacting with thiols, resulting in a loss of enzyme activity (Weinberg et al. 1982), we investigated whether  $\text{HgCl}_2$  suppresses arginase II activity directly. The specific activity of arginase II from rat kidney partially purified by column chromatography and ammonium sulfate fractionation as described in Materials and methods was  $1.82 \mu\text{mol}$  of urea formed/mg/min in the presence of  $\text{MnCl}_2$ . When the arginase II preparation was incubated with  $\text{HgCl}_2$ , the enzyme activity was unaffected (1  $\mu\text{M}$ , 90%; 10  $\mu\text{M}$ , 91%; 100  $\mu\text{M}$ , 87%; 1,000  $\mu\text{M}$ , 84% of control,  $n = 3$ ), while manganese



**Fig. 1** Total mercury concentration and changes in arginase II activity in the kidney. The total concentration of mercury (open square) and arginase II activity (filled square) were measured in rats that received a subcutaneous injection of  $\text{HgCl}_2$  at 1 mg/kg. Zero hour represents the control. Each data represents mean  $\pm$  SD of four animals. The asterisks indicate  $P < 0.05$  compared to the control. Control enzyme activity was  $0.40 \pm 0.06 \mu\text{mol/mg per min}$  ( $n = 4$ )

superoxide dismutase (Mn-SOD) activity was inhibited by approximately half by  $\text{HgCl}_2$  even at 200  $\mu\text{M}$  (Kumagai et al. 1997).

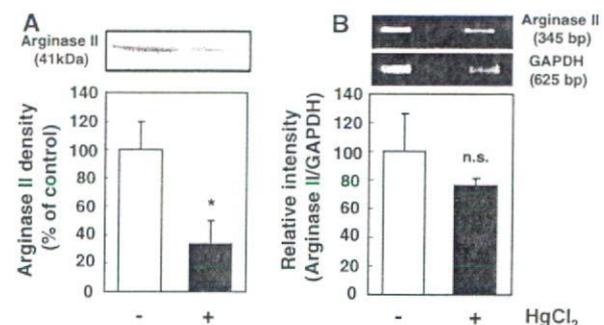
As shown in Fig. 2a, the protein level of arginase II was reduced by 34%, which was comparable with the decrease in enzyme activity at 48 h after exposure to  $\text{HgCl}_2$ . In contrast, renal arginase II gene expression was not significantly altered by  $\text{HgCl}_2$  (Fig. 2b).

### Decreased arginase II activity and apoptosis

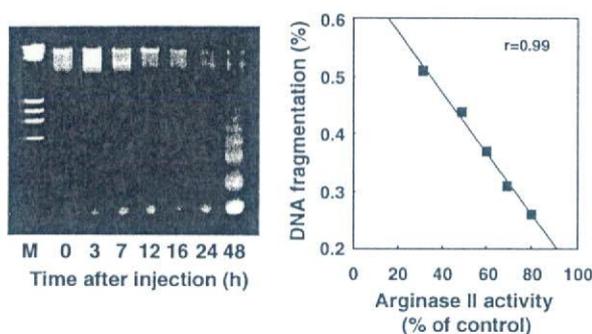
We explored the relationship between the reduction in arginase II activity and induction of apoptosis as measured on the basis of DNA fragmentation in the kidney during exposure to inorganic mercury. Figure 3 illustrates the results of electrophoresis of DNA fragmentation and the correlation with change in arginase II activity caused by  $\text{HgCl}_2$  (1 mg/kg). Interestingly, diminished arginase II activity was well correlated with the induction of apoptosis ( $r = 0.99$ ).

### Effects of overexpression of arginase II in cultured cells on inorganic mercury-mediated cell death

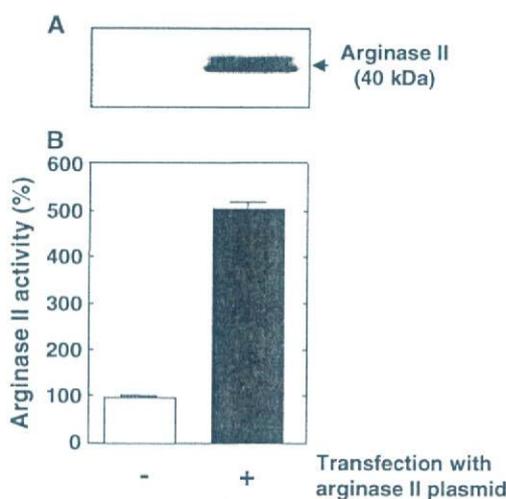
LLC-PK<sub>1</sub> cells were stably transfected with the gene for human arginase II (the pCAGGS-hAII vector). Analysis of the transfected cells by Western blotting indicated that the arginase II protein was overexpressed (Fig. 4). The protein migrated to a point consistent with its expected size, indicating that the human *arginase II* cDNA insert was certainly expressed. Moreover, arginase activity in the transfected LLC-PK<sub>1</sub> cells was fivefold greater than that in the control cells (Fig. 4). Under this condition, these cells were exposed to  $\text{HgCl}_2$  and then cell death was evaluated on the basis of the MTT assay and DNA fragmentation (Figs. 5, 6). Furthermore, we observed the cell morphology



**Fig. 2** Changes in levels of protein content (a) and mRNA (b) of renal arginase II in rats injected with  $\text{HgCl}_2$ . Western blotting and RT-PCR were conducted using renal preparations ( $n = 4$ ) at 48 h after a single subcutaneous administration of  $\text{HgCl}_2$  (1 mg/kg). Arginase II band intensities were normalized to GAPDH band intensities. Each data is mean  $\pm$  SD of four animals. The asterisk and n.s. indicate  $P < 0.05$  and not significant compared to the control, respectively

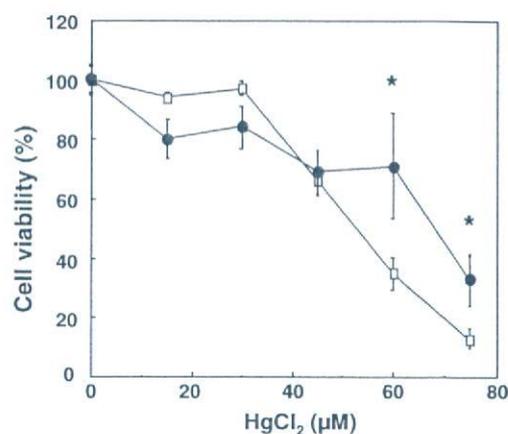


**Fig. 3** Results of electrophoresis of DNA and the correlation with changes in arginase II activity in rat kidney injected with  $\text{HgCl}_2$ . Fragmented DNA was extracted from rat kidney injected with  $\text{HgCl}_2$  (1 mg/kg), and a DNA ladder was detected by electrophoresis using a 1.5% of agarose gel. Zero hour represents the control. *M* shows DNA marker. DNA fragmentation is expressed as a percentage of fragmented DNA versus total DNA (fragmented DNA + intact DNA). Note that the diagram on the right indicates the correlation between arginase II activity and DNA fragmentation illustrated on the left

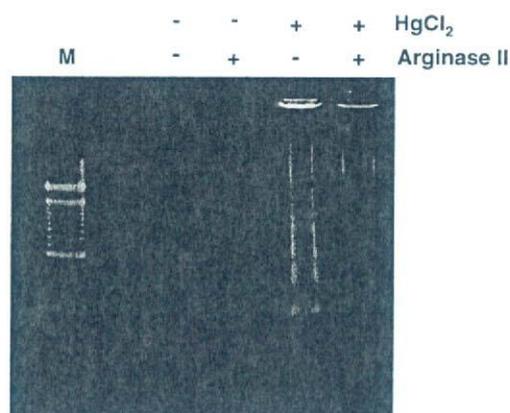


**Fig. 4** Levels of protein (a) and activity (b) of arginase II in LLC-PK<sub>1</sub> cells transfected with the pCAGGS-hAII expression plasmid. Cytosol, as an enzyme preparation, was collected from cells (80–100% confluent;  $n = 3$ ). Arginase II activity in the control group was  $0.16 \pm 0.003$   $\mu\text{mol/mg per min}$

under a microscope at 12 h after exposure to  $\text{HgCl}_2$  (35  $\mu\text{M}$ ). Changes in cell form, which can be described as a collapse of cell after its death, were observed in control cells, but not in cells transfected with *arginase II* cDNA (data not shown). As shown in Fig. 5, concentration-dependent cellular toxicity caused by inorganic mercury was suppressed by overexpression of arginase II. As shown in Fig. 6, apoptosis was not found in the two untreated groups (control cells and arginase II-transfected cells). Although a DNA ladder was found in control at 12 h after exposure to 30  $\mu\text{M}$   $\text{HgCl}_2$ , this was markedly inhibited in cells transfected with *arginase II* cDNA.



**Fig. 5** Effect of overexpression of arginase II on viability in LLC-PK<sub>1</sub> cells exposed to  $\text{HgCl}_2$ . LLC-PK<sub>1</sub> cells (80–100% confluent) were exposed to  $\text{HgCl}_2$  for 12 h. Cell viability was compared by the MTT method ( $n = 8$ ) in control (white circle) and clone transfected arginase cDNA (closed circle). Asterisks indicate  $P < 0.05$  compared to the control



**Fig. 6** Effect of overexpression of arginase II on the DNA ladder in LLC-PK<sub>1</sub> cells exposed to  $\text{HgCl}_2$ . LLC-PK<sub>1</sub> cells (80% confluent) were exposed to  $\text{HgCl}_2$  (30  $\mu\text{M}$ ) for 12 h. DNA was extracted from each cell, and the DNA ladder was detected by electrophoresis using a 1.5% agarose gel. *M* shows DNA marker

## Discussion

The present study indicates that inorganic mercury causes a time-dependent reduction in the activity of arginase II and that the decrease was well correlated with renal apoptosis as determined by measuring the extent of DNA fragmentation in kidney of rats given  $\text{HgCl}_2$ . In contrast, an increased arginase II protein level suppressed cell death during exposure of LLC-PK<sub>1</sub> cells to inorganic mercury.

Exposure of experimental animals to  $\text{HgCl}_2$  causes disturbance of mitochondrial function through thiol modification (Weinberg et al. 1982), because its high affinity for

cysteine thiols (Clarkson 1972). In the present study, incubation of partially purified arginase II with inorganic mercury *in vitro* resulted in little change in the catalytic activity, although there are five cysteine residues in rat arginase II (Gotoh et al. 1996). Thus, it might be difficult for mercuric ions to access the reactive thiol groups of arginase II. Western blotting and RT-PCR indicated that the inorganic mercury-mediated reduction in arginase II activity accompanied a decline in the arginase II protein level rather than mRNA level. These results indicate that the metal-promoted change in arginase II activity *in vivo* is attributable to translational- or post-translational modification. The dosage of HgCl<sub>2</sub> in the present study, which was 1 mg/kg, induced a site-specific apoptosis in the proximal tubules but did not cause tubular injury in rats (Homma-Takeda et al. 1999). Under such conditions, the loss of arginase II activity occurred much earlier than that of the activity of other enzymes as an indicator of mercury-promoted renal damage [e.g., fructose-1, 6-diphosphatase (Phillips et al. 1977; Kroll et al. 1988)].

Inorganic mercury is a heavy metal that accumulates in the proximal tubules of the kidney and causes renal apoptosis (Homma-Takeda et al. 1999; Zalups 2000). One of the hallmarks of apoptosis is the degradation and concomitant compaction of chromatin; however, precise mechanism of the apoptosis induced by inorganic mercury remains to be elucidated. Interestingly, we found that diminished arginase II activity well correlated with renal DNA fragmentation ( $r = 0.99$ ) after the subcutaneous injection of HgCl<sub>2</sub> (1 mg/kg) into rats (Fig. 3). No such inverse correlation (enzyme activity vs. apoptosis) was observed for NOS and SOD isoforms (data not shown), which are known to locate in the proximal tubules of the kidney (Liang and Knox 2000; Gwinner et al. 1998). In addition, protein levels of enzymes responsible for arginine metabolism such as argininosuccinate lyase and argininosuccinate synthetase in the kidney were not affected by the exposure to HgCl<sub>2</sub> (M. Kikushima et al., unpublished observation), although the level of arginase II protein markedly declined. Since these enzymes are also localized in the proximal tubules (Miyataka et al. 1998), our finding about arginase II might result from enzyme- or cell-specific rather than tissue-specific downregulation attributable to the selectively-accumulated inorganic mercury at the site.

Gotoh and Mori (1999) reported that overexpression of arginase II prevented NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. In the case of inorganic mercury (1 mg/kg), however, little enhancement of NOS in terms of its enzyme activity (Kanda et al. 1998) and NO metabolite levels (data not shown) was seen, suggesting that the induction of renal apoptosis *in vivo* caused by inorganic mercury is not directly associated with increased NO production. Rather, decreased activity of arginase II

that negatively regulates apoptosis (Esch et al. 1998; Gotoh and Mori 1999; Estévez et al. 2006) may be responsible for inorganic mercury-dependent apoptosis *in vivo*. To explore this possibility, we constructed a plasmid carrying an arginase II gene and introduced it in LLC-PK<sub>1</sub> cells to investigate the specific effect of arginase II on cell death induced by inorganic mercury. When arginase II activity was five times the control level in cells (Fig. 4), cell death induced by inorganic mercury were blocked (Figs. 5, 6). However, it should be noted that an impressive fivefold upregulation of arginase II activity with transfection is insufficient to completely prevent HgCl<sub>2</sub> toxicity.

Taken together, our observations suggest that the inorganic mercury-mediated induction of apoptosis in the rat kidney is, at least in part, attributable to the downregulation of arginase II activity.

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## Metallothionein proteins expression, copper and zinc concentrations, and lipid peroxidation level in a rodent model for amyotrophic lateral sclerosis

Eiichi Tokuda<sup>a</sup>, Shin-Ichi Ono<sup>a,b,\*</sup>, Kumiko Ishige<sup>c</sup>, Akira Naganuma<sup>d</sup>, Yoshihisa Ito<sup>c</sup>, Takashi Suzuki<sup>a,e</sup>

<sup>a</sup> Laboratory of Clinical Pharmacy, College of Pharmacy, Nihon University, Chiba, Japan

<sup>b</sup> Division of Neurology, Akiru Municipal Medical Center, Tokyo, Japan

<sup>c</sup> Laboratory of Pharmacology, College of Pharmacy, Nihon University, Chiba, Japan

<sup>d</sup> Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan

<sup>e</sup> Department of Pediatrics, Nihon University School of Medicine, Tokyo, Japan

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### Abstract

It has been hypothesized that copper-mediated oxidative stress contributes to the pathogenesis of familial amyotrophic lateral sclerosis (ALS), a fatal motor neuron disease in humans. To verify this hypothesis, we examined the copper and zinc concentrations and the amounts of lipid peroxides, together with that of the expression of metallothionein (MT) isoforms in a mouse model [superoxide dismutase1 transgenic (SOD1 Tg) mouse] of ALS. The expression of MT-I and MT-II (MT-I/II) isoforms were measured together with Western blotting, copper level, and lipid peroxides amounts increased in an age-dependent manner in the spinal cord, the region responsible for motor paralysis. A significant increase was already seen as early as 8-week-old SOD1 Tg mice, at which time the mice had not yet exhibited motor paralysis, and showed a further increase at 16 weeks of age, when paralysis was evident. Inversely, the spinal zinc level had significantly decreased at both 8 and 16 weeks of age. The third isoform, the MT-III level, remained at the same level as an 8-week-old wild-type mouse, finally increasing to a significant level at 16 weeks of age. It has been believed that a mutant SOD1 protein, encoded by a mutant *SOD1*, gains a novel cytotoxic function while maintaining its original enzymatic activity, and causes motor neuron death (gain-of-toxic function). Copper-mediated oxidative stress seems to be a probable underlying pathogenesis of gain-of-toxic function. Taking the above current concepts and the classic functions of MT into account, MTs could have a disease modifying property: the MT-I/II isoform for attenuating the gain-of-toxic function at the early stage of the disease, and the MT-III isoform at an advanced stage.

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**Keywords:** Metallothionein; Copper; Zinc; Oxidative stress; Amyotrophic lateral sclerosis; Superoxide dismutase1

### 1. Introduction

Metallothionein (MT) is a family of low-molecular-weight, cystein-rich, heat-stable and metal binding proteins (Kägi and Schäffer, 1988). MT participates in a broad range of physiological functions such as detoxifi-

\* Corresponding author at: Laboratory of Clinical Pharmacy, College of Pharmacy, Nihon University, 7-7-1 Narashino-dai, Funabashi, Chiba 274-8555, Japan. Tel.: +81 47 465 2397; fax: +81 47 465 2397.

E-mail address: [shin-ono@pha.nihon-u.ac.jp](mailto:shin-ono@pha.nihon-u.ac.jp) (S.-I. Ono).