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Review

Cellular Cadmium Uptake Mediated by the Transport System for Manganese

Seiichiro Himeno, Takahiro Yanagiya,[1] Shuichi Enomoto,[1] Yukihiro Kondo[2] and Nobumasa Imura

Kitasato University, School of Pharmaceutical Sciences, Department of Public Health and Molecular Toxicology, Tokyo 108–8641,
[1]*The Institute of Physical and Chemical Research (RIKEN), Wako 351–0198, and*
[2]*Nippon Medical School, Department of Urology, Tokyo 113–8602*

Himeno, S., Yanagiya, T., Enomoto, S., Kondo, Y. and Imura, N. *Cellular Cadmium Uptake Mediated by the Transport System for Manganese.* Tohoku J. Exp. Med., 2002, **196**(1), 43–50 —— The mechanism of cellular cadmium (Cd) uptake has been poorly understood. Recently, we developed Cd-resistant cell lines from metallothionein null mouse cells and showed that the Cd resistance of these cells was conferred primarily by a reduced Cd accumulation. Surprisingly, the uptake rate of manganese (Mn) was also markedly reduced in Cd-resistant cells. Subsequent studies on the kinetics of Cd and Mn uptake by Cd-resistant and parental cells revealed that the Mn transport system with high affinity for Mn is used for cellular Cd uptake, and that this pathway is suppressed in Cd-resistant metallothionein null cells. This is the first indication that the transport system for Mn is used for Cd uptake in mammalian cells. Divalent metal transporter 1 (DMT1) is the only known mammalian transporter involved in the uptake of both Cd and Mn. However, the high-affinity Mn/Cd transport system we found seems to be distinct from DMT1 because of the difference in optimal pH and substrate specificity. On the other hand, various types of Mn transporters have been shown to play an important role in cellular Cd uptake in non-mammalian species such as yeast, plants and bacteria, suggesting the existence of Mn transporters other than DMT1 in mammals. ———— cadmium; resistance; metallothionein; manganese; transport

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Address for reprints: Seiichiro Himeno, Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences, Kitasato University, 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan.
e-mail: himenos@pharm.kitasato-u.ac.jp

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Cadmium (Cd) is an environmental contaminant that has adverse effects on health. The major routes of Cd intake among humans are diet ingestion and tobacco smoking (WHO 1992). Although the intestinal Cd uptake rate is less than 5%, a substantial amount of Cd is accumulated chronically by human tissues, particularly by the kidneys. The half-life of Cd in the human body is estimated to be many years owing to its low excretion rate from the body (WHO 1992).

Although the precise mechanism of Cd uptake by and excretion from cells has not been fully elucidated, it has been assumed that the cellular Cd uptake is mediated by pathways for other essential elements such as calcium (Ca) or iron (Fe). Since the ionic radius of Cd is similar to that of Ca, Cd has been extensively utilized as an antagonist for Ca transport via Ca channels (Tsien et al. 1987; Lopez et al. 1989). Feeding animals a Ca-deficient diet is known to cause increased accumulation of Cd via the intestinal tract (Omori and Muto 1977; Washko and Cousins 1977). Also, it has been shown that dietary Fe deficiency promotes the intestinal Cd uptake (Valberg et al. 1976). However, the total amount of Cd taken up by cells could not be fully explained by the pathways for Ca and Fe. Furthermore, the existence of an intracellular metal-binding protein, metallothionein (MT), which efficiently binds to and can be readily induced by Cd (Suzuki et al. 1993), makes the dynamics of cellular Cd transport greatly complicated. The existence of MT has also hindered the precise determination of the rates of influx and efflux of Cd.

In a recent study, we demonstrated that the transport system for divalent manganese (Mn) is a new candidate for the pathway of Cd uptake by cells (Yanagiya et al. 2000). To explore non-MT factors for Cd resistance, we established Cd-resistant MT null cells, and found that these cells showed a decreased accumulation of Cd, which was ascribed primarily to a markedly decreased rate of Cd uptake by cells (Yanagiya et al. 1999). The application of a new technology called "multitracer technique" has revealed that the uptake of Mn by Cd-resistant MT null cells was also markedly suppressed. Kinetic and competition studies revealed that Cd and Mn share a common pathway for entering cells and that the suppression of this pathway causes the decrease in Cd accumulation in Cd-resistant MT null cells (Himeno 2002; Yanagiya et al. 2000). This is the first indication that the transport system for Mn is utilized for cellular Cd uptake in mammals. In this review, we explored the possible interactions of Cd and Mn in both mammalian and non-mammalian species. Compared with scant knowledge on the interactions of Mn and Cd in mammals, there is much evidence indicatng that the Mn transport system is an important pathway for cellular Cd uptake by several types of bacteria, yeast and plants.

Establishment of Cd-resistant metallothionein null cells

A drug-resistant cell line is a useful tool for studying the cellular strategies for protection against drug toxicity. In general, the acquisition of drug resistance can be achieved by the following: 1) lowering cellular drug concentration by preventing the entry of drugs or by enhancing the excretion of drugs, 2) sequestering intracellular drugs and thereby inactivating them, and 3) enhancing repair systems for the damage caused by drugs. Most Cd-resistant cell lines thus far established have shown enhanced gene expression of MT, leading to an efficient sequestration of intracellular Cd ions (Grady et al. 1987). Even if the transport of Cd could be altered in Cd-resistant cells in addition to the increase in MT level, it is difficult to examine the accurate rates of Cd influx and efflux in the presence of large amounts of MT that trap Cd efficiently. To overcome this problem, we established Cd-resistant cell lines from MT null mouse cells.

Kondo et al. (1999) introduced cDNA of simian virus 40 into primary cultured embryonic

cells obtained from MT null and control mice. The established MT null cells, which showed characteristics of fibroblast cells, were apparently immortalized and displayed higher sensitivity to Cd than MT-containing control cells. By continuously exposing of these MT null cells to Cd in the medium, we were able to develop two subclones of Cd-resistant MT null cells, Cd-rA7 and Cd-rB5 (Yanagiya et al. 1999). These cells showed about five times higher resistance to Cd than the parental MT null cells. Cd accumulation in Cd-rA7 and Cd-rB5 cells was markedly lower than that in parental cells, while other factors such as glutathione content or antioxidant enzyme activity did not differ between Cd-resistant and parental cells. Thus, the utilization of MT null cells led to the establishment of a Cd-resistant cell line that acquired Cd resistance due primarily to a decreased Cd accumulation for the first time.

The reduced accumulation of Cd may be caused by either reduced uptake or enhanced excretion of Cd, or both. The time-course experiment on Cd uptake by Cd-rA7 and Cd-rB5 cells revealed that the uptake rate of Cd by these cells was about 10% of that by parental cells at all dose levels examined (Yanagiya et al. 1999). Also, the release rate of Cd from Cd-rA7 and Cd-rB5 cells was higher than that from parental cells. Thus, both the reduced uptake and enhanced excretion of Cd might have contributed to the decrease in Cd accumulation in Cd-rA7 and Cd-rB5 cells. The enhanced excretion of Cd from these cells might be ascribed to the activation of an efflux pump for Cd, but the transporter for Cd efflux from cells has not yet been identified. In contrast, the mechanism of the reduced uptake of Cd by Cd-resistant MT null cells has been investigated in more detail.

Involvement of Mn in Cd uptake by mammalian cells

Since cellular Cd uptake might be mediated by the transport systems for other essential elements, we hypothesized that the uptake of other elements by Cd-rA7 and Cd-rB5 cells may also be suppressed. To test this hypothesis, we utilized the "multitracer technique" developed by the Institute of Physical and Chemical Research (RIKEN) in Japan. The multitracer solution contains more than 20 radioactive elements, and the uptake of these elements by cells can be analyzed simultaneously by a germanium detector and a subsequent computer analysis (Ambe et al. 1995; Enomoto et al. 1996). The cellular uptake of Zn, Cu or Fe during the 2 hours after the addition of multitracer solution did not show any difference between Cd-resistant MT null cells and parental cells. Unexpectedly, however, the uptake of Mn by Cd-resistant MT null cells was as low as 10% of that by parental cells (Yanagiya et al. 2000). Therefore, more detailed analyses on dose-dependent and time-dependent uptake of Mn by Cd-resistant MT null cells and parental cells were performed by using a single tracer of ^{54}Mn. The uptake rate of Mn by Cd-resistant MT null cells, as determined by the cellular uptake of ^{54}Mn during the initial 15 minutes after the addition of $^{54}MnCl_2$ into the medium, was markedly suppressed compared with that by parental cells at low concentrations of Mn (less than 3 μM), but not at concentrations higher than 10 μM, suggesting the existence of at least two components in the Mn transport system (Yanagiya et al. 2000). In accordance with our observations, other group have also reported the existence of both high- and low-affinity pathways for Mn uptake by mammalian cells (Galeotti et al. 1995; Chua et al. 1996). The suppression of the high-affinity transport system for Mn uptake in Cd-resistant MT null cells might have caused the reduced uptake of Mn as well as Cd.

Kinetic and competition analyses were performed to test whether Cd and Mn share the same pathway for entering cells. The addition of Mn into the medium inhibited Cd uptake by parental cells dose-dependently, and Cd inhib-

ited Mn uptake in the same manner. However, no mutual inhibition of Cd and Mn uptake by Cd-resistant MT null cells was observed. These data suggest that Cd and Mn share the same transport system for entering cells, and that this transport system is suppressed in Cd-resistant MT null cells. The Ki value of Mn for the inhibition of Cd uptake was 0.14 μM, indicating that the Mn/Cd transport system has a high affinity for both Mn and Cd (Yanagiya et al. 2000). However, a specific transporter for cellular Mn uptake has not yet been isolated in mammals.

Discovery of divalent metal transporter 1

In general, cellular Fe uptake is mediated by the endocytosis of transferrin, which binds trivalent Fe (Fe[III]), via a transferrin receptor. However, it has been assumed that the transferrin-independent divalent Fe (Fe[II]) transporter plays an important role in the uptake of Fe from the intestinal lumen where transferrin concentration is very low. In 1997, Gunshin et al. (1997) isolated a transferrin-independent Fe(II) transporter from rat intestine, and named it divalent cation transporter 1, (later re-named divalent metal transporter1, DMT1). The predicted amino acid sequence of DMT1 showed a significant homology to that of natural resistance-associated macrophage protein 1 (Nramp1), which was isolated as a mutated gene responsible for the sensitivity of a host animal to intracellular parasites such as mycobacteria (Vidal et al. 1993). Nramp1 was expressed exclusively in macrophages, whereas DMT1 is expressed in large amounts in the intestine. DMT1 was found to be identical to rat Nramp2, which was isolated as the gene responsible for the genetic anemia in Belgrade rats that had a defect in intestinal Fe absorption (Fleming et al. 1998). Gunshin et al. (1997) demonstrated that DMT1 is a proton symporter that has an optimal pH of 5.5, and that DMT1 has broad substrate specificity covering Fe(II), Co, Cu(II), Ni, Mn, Pb, Zn and Cd. Thus, DMT1 is the first mammalian transporter to be involved in the cellular uptake of both Mn and Cd, and therefore, it was the most probable candidate transporter that could be responsible for the high-affinity Mn/Cd transport in mouse cells.

A novel transport system for Mn and Cd distinct from DMT1

To test the above hypothesis, we examined the inhibition of Cd and Mn uptake by other divalent metals using parental cells. The addition of Co, Ni, Fe(II), or Cu(II) did not show any inhibitory effects on the uptake of Cd or Mn by parental cells, although the addition of Zn partly inhibited the uptake of both Mn and Cd (Yanagiya et al. 2000). Furthermore, DMT1 has an optimal pH of 5.5, while the highest uptake of Cd and Mn was achieved at neutral pH in parental cells in our experiment. These data suggest the existence of a high-affinity Mn/Cd transport system that is distinct from DMT1 in mouse fibroblast cells.

The high-affinity Mn/Cd transport system may exist ubiquitously among mammalian cells since the mutual inhibition of Cd and Mn uptake was also observed in HeLa, PC12, and Caco-2 cells (Yanagiya et al. 2000). However, although more than half of the Cd uptake by these cells was inhibited by the addition of five times excess amount of Mn in the medium, the total amount of Cd uptake could not be explained solely by the Mn uptake system. Since the expression of intestinal DMT1 is enhanced by dietary Fe deficiency (Gunshin et al. 1997), the known enhancement of intestinal Cd uptake due to Fe deficiency may be explained by the up-regulation of DMT1. It seems likely that the acidic environment of the intestinal lumen serves as a driving force for Cd uptake by the proton symporter, DMT1, whereas the high-affinity Mn/Cd transporter that has an optimal pH of 7.4 may not be so efficient in the intestine. In excitable cells, some kinds of Ca channels may also be used for Cd uptake. Further

studies are required to clarify tissue- or cell-type-specific roles of each transporter involved in Cd uptake.

Interactions of Mn and Cd in non-mammalian species

In bacteria, yeast and plants, several types of transporters for Mn uptake have already been isolated. Interestingly, most of the Mn transporters in these species are also capable of incorporating Cd into cells.

Mn and Cd uptake by yeast

A kinetic study on Mn uptake by *Saccharomyses cerevisiae* demonstrated that there are two Mn uptake pathways having high affinity (Km=0.3 μM) and low affinity (Km=62 μM) for Mn (Gadd and Laurence 1996). Supek et al. (1996) found that the *SMF1* gene that can complement the mutation of mitochondrial-processing peptidase, which requires Mn for its activity, is involved in the high-affinity Mn uptake by *S. cerevisiae*. The disruption of the *SMF1* gene caused a decrease in Mn uptake, whereas the overexpression of it enhanced Mn uptake. The predicted amino acid sequence of the *SMF1* gene showed a homology to those of mammalian Nramp family proteins. The phenotypes of the *smf1* null mutant strain were complemented by the expression of mammalian Nramp2 (DMT1), and not Nramp1 (Pinner et al. 1997).

The expression of *SMF1* was negatively regulated by the *BSD2* gene (Liu et al. 1997). The *bsd2* null mutant strain, in which *SMF1*-dependent Mn uptake is up-regulated, exhibited an elevated uptake of Cd, and consequently, a hypersensitivity to Cd. The hypersensitivity to Cd as well as the increased uptake of Cd in the *bsd2* null mutant strain was completely abolished by the deletion of *SMF1*. Thus, the yeast Mn transporter, *SMF1*, is also involved in the uptake of and sensitivity to Cd. Interestingly, the *bsd2* null mutant yeast also showed elevated uptake of Cu and Co, suggesting the role of the *Smf1* protein as a transporter of multiple divalent metals in yeast (Liu et al. 1997).

Mn and Cd uptake by Arabidopsis thaliana

The *smf1* null mutant yeast was utilized for the identification of the plant Mn transporter. Since Mn plays a crucial role in the photosynthetic reaction, this element is essential for the survival of plants and photosynthetic organisms (Westphal et al. 2000). However, the mechanism of Mn uptake by plant cells has not been fully elucidated until recently. Korshunova et al. (1999) found that the defect in Mn transport in the *smf1* null strain of *S. cerevisiae* was restored by IRT1, the Fe(II)-transporter gene derived from *Arabidopsis thaliana*. The enhanced uptake of Mn by IRT1 in *smf1* null mutant yeast was inhibited by the addition of Cu, Fe(II), Zn or Cd, suggesting the role of IRT1 in the transport of these metals. When *A. thaliana* plants were grown in an Fe(II)-deficient medium, the expression of IRT1 in the root tissue was enhanced, and the uptake of Co, Mn and Zn by root tissues was elevated. Earlier studies have also shown that the accumulation of Cd and Mn in root tissues of pea and *A. thaliana* plants was enhanced by Fe deficiency in the growth media (Rodecap et al. 1994; Cohen et al. 1998).

Mn and Cd uptake by Lactobacillus plantarum

In bacteria such as *Bacillus subtilis* (Que and Helmann 2000), *Staphylococcus aureus* (Perry and Silver 1982) and *Lactobacillus plantarum* (Archibald and Duong 1984), Cd competes with Mn for cellular uptake, while Cd competes with Zn for cellular uptake in *E. coli* (Laddaga and Silver 1985a). Since the intracellular concentration of Mn in *L. plantarum* is particularly high (30 mM), *L. plantarum* was used for studying the mechanism of cellular Mn uptake. An early study showed that when *L. plantarum* was grown in an Mn-depleted medium, the uptake rate of radiolabeled Mn was inhibited by Cd, but not by Fe(II), Cu(II), Co, Mg, Zn or Ca (Archibald and Duong 1984). The depletion of

Mn from the growth medium induced the rapid uptake of both Mn and Cd by *L. plantarum*. Kinetic analyses of the inhibition of Mn and Cd uptake revealed that the two elements are incorporated into the cells by a common pathway.

Recently, the transporter gene for uptake of Mn and Cd was isolated from *L. plantarum* (Hao et al. 1999a). The nucleotide sequence of the isolated gene for Mn transport (mntA) showed that this gene belongs to a family of P-type cation-translocating ATPases. The expression of MntA in *E. coli* conferred enhanced uptake of Cd, which was inhibited by the addition of Mn to the medium. When *L. plantarum* was grown in the Mn-depleted medium, the mRNA level of mntA as well as the uptake rate of Mn was increased. A mutant strain of *L. plantarum* that had a defect in Mn uptake (Hao et al. 1999b) did not cross-react with the antibody for MntA protein (Hao et al. 1999a). This mutant strain also exhibited a reduced uptake rate of Cd. Thus, MntA appears to be the transporter responsible for the uptake of Mn and Cd in *L. plantarum*.

Mn and Cd uptake by Bacillus subtilis

A common system for Cd and Mn uptake was also recognized in *Bacillus subtilis*. An early study established a Cd-resistant strain of *B. subtilis* from a plasmidless parental strain (Laddaga et al. 1985b). The resistant strain showed a reduced uptake rate of Cd compared with the parental strain. The addition of Cd and Mn into the medium inhibited the uptake rates of Mn and Cd, respectively, by the parental strain, but not by the Cd-resistant strain. Kinetic analyses showed that the Cd uptake by parental strain is mediated by the same system as that for Mn uptake, and this system seems to be disrupted in the Cd-resistant strain probably by a chromosomal mutation. It is noteworthy that these features of the Cd-resistant strain are similar to those found in Cd-resistant MT null mouse cells (Yanagiya et al. 2000).

Recently, it was demonstrated that the mutation of *B. subtilis* mntR, which is a homologue of DtxR, a divalent metal ion-dependent repressor that controls Fe transport in *Corynebacterium diphtheriae*, conferred hypersensitivity to both Mn and Cd (Que and Helman 2000). The suppressor gene that abolishes the hypersensitivity to Mn and Cd of the mntR null mutant strain was screened and isolated. The predicted amino acid sequence of the suppressor gene (mntH) was homologous to those of the Nramp family proteins. The mutation of mntH resulted in a decrease in the survival rate of *B. subtilis* at low Mn concentration. Thus MntH, the bacterial homologue of Nramp, plays an important role in Mn and Cd uptake by *B. subtilis*.

The above-mentioned findings suggest that various types of Mn uptake systems play an important role in cellular Cd uptake in non-mammalian species. However, the structure of the transporter varies from species to species. *SMF1* of *S. cerevisiae* and MntH of *B. subtilis* belong to the Nramp family, wheres MntA of *L. plantarum* belongs to a P-type cation-translocating ATPase. IRT1 of *A. thaliana* has no homology to either Nramp or P-type ATPase. In *B. subtilis*, a Cd-resistant strain exhibited a decrease in the uptake rate of Mn and Cd due to the depression of a common pathway for Mn and Cd uptake, similar to that observed in Cd-resistant MT null mouse cells. It seems likely that some kinds of Mn transporters similar to those found in non-mammalian species are evolutionarily preserved in mammalian cells. Isolation and characterization of the high-affinity Mn/Cd transporter distinct from DMT1 in mammalian cells are required to further elucidate the mechanism of cellular Cd uptake.

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