Screening for metals exhibiting altered accumulation in Cd-resistant metallothionein null cells using a multitracer technique

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Abstract

Metallothionein (MT) is known to play a predominant role in the protection of cells from cadmium (Cd) toxicity. Recently, we established Cd-resistant cell line (Cd-rB5) from simian virus 40-transformed MT null fibroblasts. These resistant cells exhibited a significant decrease in Cd uptake. To investigate the mechanism of altered Cd accumulation in Cd-rB5 cells, incorporation of various metals was determined simultaneously using a multitracer technique. Cd-rB5 cells exhibited a marked decrease in Mn incorporation as well as that of Cd. The addition of Mn inhibited the uptake of Cd competitively in parental cells but not in Cd-rB5 cells. Similary, Cd effectively inhibited the uptake of Mn of low concentration only in parental cells. These results suggest that Mn and Cd utilize the same transport pathway having a high affinity for both metals, and that the disruption of this pathway in Cd-rB5 cells has caused a marked decrease in Cd accumulation.

1. Introduction

Cadmium (Cd) is an environmental toxicant that causes adverse effects in various organs. Chronic exposure to Cd in animals and humans results in preferential accumulation of Cd in the kidney, thereby leading to nephrotoxicity. Despite detrimental effects of Cd have been well characterized, the mechanism of Cd transport has been poorly understood.

Numbers of animal studies have shown that Cd absorption from the intestine can be affected by metals such as Fe, Zn and Ca [1-4]. In *in vitro* studies, Cd has been extensively used as a potent Ca-channel blocker [5-7] because the ionic radius of Cd is close to that of Ca. On the other hand, cellular uptake of Cd can be inhibited by Ca-channel blockers [8], suggesting that Cd is incorporated into cells at least partly via Ca channels. Other trace elements such as Zn and Cu have also been reported to inhibit Cd uptake in hepatocytes and intestinal cells. These data suggest a possibility that transporters for metals such as Zn, Cu and Fe may also be partly used for Cd incorporation. However, the process of transport of each metal into mammalian cells has not yet been fully elucidated.

One of the most important factor responsible for resistance to Cd in animals is metallothionein (MT). MT is a low-molecular-weight cysteine-rich protein that can be induced by metals including Cd and can attenuate the toxicity of metals by binding them efficiently [9]. Most Cd-resistant cell lines so far established have exhibited high levels of MT protein or mRNA [10-11]. Due to the efficient inducibility of MT, it has been difficult to develop a Cd-resistant cell line that has a different property other than increased levels of MT. Therefore, to explore other factors than MT for Cd resistance, we established Cd-resistant cell lines [12] from SV40-immortalized MT null embryonic fibroblasts [13], which were derived from transgenic mice deficient in MT-I and II, the major isoforms of MT [14]. The Cd-resistant MT null cells we established showed a significant decrease in Cd uptake compared to the parental MT null cells, suggesting that this changes in Cd transport have conferred resistance to Cd [12]. Thus, further characterization of the altered accumulation of Cd in the Cd-resistant MT null cells will promote the elucidation of mechanism of Cd transport in mammalian cells.

In the present study, we investigated the changes in the incorporation of various elements into Cd-resistant cells using a multitracer technique, which enabled us to determine the incorporation of 20 radioactive tracers simultaneously.

2. Experimental procedure

2.1 Measurement of multitracer incorporation into cells

A clone of Cd-resistant MT null cells, Cd-rB5, established from the SV40-transformed embryonic cells of MT null mice were used. Cd-rB5 and their parental cells were preincubated in serum-free media for 30 min, and then were treated with 0.1 ml of multitracer for 120 min. Then, the medium was removed, and cells were washed three times with 4 ml of phosphate buffered saline (PBS) containing 0.05% EDTA. The dish with the washed cells was placed directly on a Ge detector, and the radioactivities of multitracers were determined. The amounts of the element incorporated into cells were expressed as percentages of the total amount added in the medium.

2.2 Measurement of cellular uptake of Mn

Cells were preincubated in serum-free media for 30 min and then exposed to 0.03 μM [^{54}Mn]-$MnCl_2$ for 0, 15, 30, 60 and 120 min. Then, cells were harvested with 1 ml of PBS containing 2% SDS after washing three times with 2 ml of PBS containing 0.05% EDTA, and were transferred to a test tube. The radioactivity of ^{54}Mn was measured by ALOKA auto well γ-counter. Protein concentrations in each sample determined by Lowry's method [17].

2.3 Inhibition of uptake of Cd by Mn and other metals

Cells were preincubated in serum-free media for 30 min, and then treated with 0.03 μM [^{109}Cd]-$CdCl_2$ in the presence of 0, 0.03, 0.06, 0.1 and 0.3 μM $MnCl_2$. After incubation for 15 min, cells were washed three times with 2

ml of PBS containing 0.05% EDTA and harvested with 1 ml of PBS containing 2 % SDS. The radioactivity of ^{109}Cd was measured by ALOKA auto well γ-counter. To examine inhibitory effects of other metals on the uptake rates of Cd, the incorporation of 0.03 μM [^{109}Cd]-$CdCl_2$ during 15 min was determined in the presence of 5-fold excess amount of $MnCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$, $FeSO_4$ or $CuCl_2$. Protein concentrations in each sample were determined by Lowry's method.

2. Results and Discussion

We applied a multitracer technique to identify a metal(s) that exhibits altered accumulation in Cd-resistant MT null cells in which Cd accumulation was significantly suppressed. Figure 1 shows the incorporation of metals in Cd-rB5 and parental cells exposed to multitracer solutions for 120 min. Incorporations of Be, Sc, Cr, Fe, Zn, Se, Rb, Y and Zr were about the same between Cd-rB5 and parental cells. However, the incorporation of Mn in Cd-rB5 cells was as low as approximately 10% of that of parental cells.

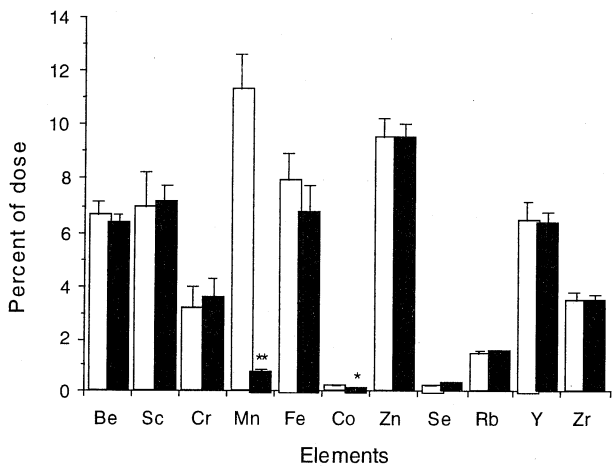

Fig. 1 Incorporation of various elements into Cd-rB5 and parental cells. Cd-rB5 (solid column) and parental cells (open column) cultured in serum-free media were exposed to radioactive multitracers containing 20 elements for 120 min. The incorporation of 13 out of 20 radioactive elements was successfully determined and expressed as a percentage of the amount added in the medium. Values are means ± S.D of three experiments. Asterisks indicate significant differences from parental cells by t-test (*, $p < 0.05$; **, $p < 0.01$).

As shown in Fig. 2, the incorporation of Mn (0.03 μM) into Cd-rB5 cells was markedly suppressed while the parental cells showed a time-dependent accumulation of Mn. At 120 min after the addition of $MnCl_2$ in the medium, approximately 10% of Mn were incorporated into Cd-rB5 cells compared to that in parental cells.

Previously, we established Cd-resistant cell line from MT null mouse fibroblasts that exhibited a marked decrease in the uptake of Cd [12]. Since it is unlikely that there is a specific transporter for uptake of Cd, Cd may transport into cells via pathway for essential elements such as Ca, Zn, Fe or others. The application of multitracer technique in this study revealed that the uptake of Mn in Cd-rB5 cells was reduced to approximately 10% of that of parental cells while no difference in the incorporation of Zn and Fe was observed. As accumulation of Cd in the Cd-rB5 cells was also reduced to 10% of the parental cells, the same mechanism may be responsible for the reduction of the incorporation of both Cd and Mn into Cd-rB5 cells.

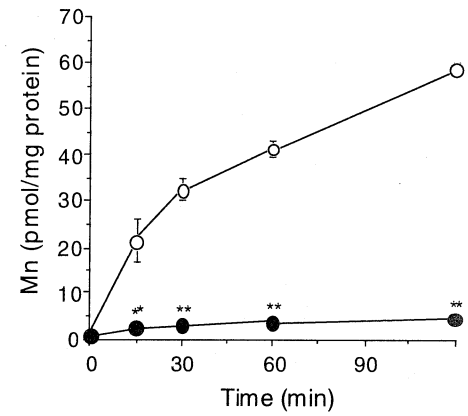

Fig. 2 Time-dependent uptake of Mn in Cd-rB5 and parental cells. Cd-rB5 (closed circles) and parental cells (open circles) were preincubated in serum-free media and exposed to 0.03 μM [^{54}Mn]-$MnCl_2$ for 0, 15, 30, 60 or 120 min.

To test whether the transport of low concentrations of Mn and Cd into cells is mediated through the same pathway, we next examined whether the uptake of Cd and Mn is competitively inhibited by each other. Uptake of Cd by parental cells was inhibited by Mn efficiently in a dose-dependent manner (Fig. 3). However, when Cd-rB5 cells were used, Mn did not inhibit Cd uptake, nor Cd inhibits Mn uptake (data not shown). These results suggest that Mn and Cd share the same process of transport into cells, and that this process is not functioning in Cd-rB5 cells.

To investigate the effects of other metal ions on the uptake of Cd and Mn at low concentrations, the uptake rate of Cd or Mn was determined in the presence of 5-fold excess amount of Zn, Co, Ni, Fe and Cu. As shown in Fig. 4, Cd uptake was inhibited by Zn as well as Mn, but other metals did not exhibit inhibitory effects. Similarly, Mn uptake was also inhibited by both Cd and Zn, but no inhibition by other metals was observed (data not shown). In Cd-rB5 cells, however, Zn did not exert an inhibitory effect on the uptake of Cd or Mn (data not shown). These results suggest that Zn may also have an affinity to the transport system that has a high affinity to Mn and Cd. However, Zn

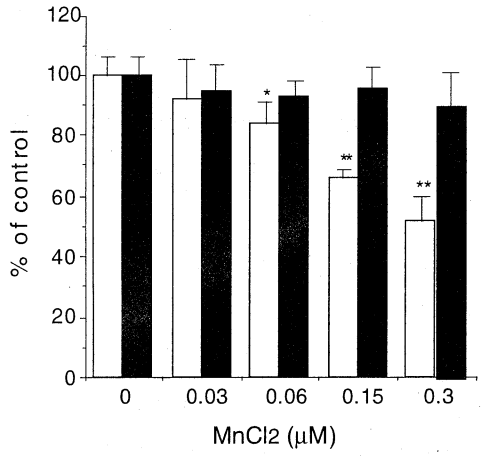

Fig. 3 Dose-dependent inhibition of Cd uptake by Mn in Cd-rB5 (closed column) and parental cells (open column). The rates of ^{109}Cd uptake were expressed as relative percentages to those obtained in the absence of MnCl2.

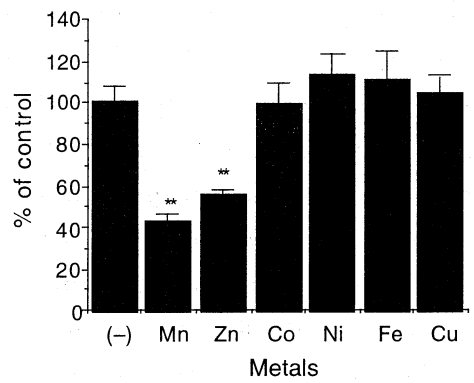

Fig. 4 Effects of various metals on the uptake of Cd in parental cells. The rates of ^{109}Cd uptake were expressed as relative percentages to those obtained in the absence of metal inhibitors.

incorporation was not reduced in Cd-rB5 cells compared to parental cells when Zn was added to the medium as a component of multitracer solution (Fig. 1). Thus, Zn may not be incorporated into cells solely via the high affinity system for Mn-Cd uptake, and may have multiple pathways for its entry into cells.

Conclusion

The application of the multitracer technique and the examination of interaction between Cd and Mn at very low concentrations in the present study have permitted the detection of a novel transport system for both Mn and Cd. To further elucidate the transport system of trace elements in mammaliam cells, these MT-lacking cell line having altered uptake rates of Cd and mn will provide a good tool.

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