Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells

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Bannon, Desmond I., Roger Abounader, Peter S. J. Lees, and Joseph P. Bressler. Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells. Am J Physiol Cell Physiol 284: C44–C50, 2003. First published August 22, 2002; 10.1152/ajpcell.00184.2002.—DMT1 (divalent metal transporter 1) is a hydrogen-coupled divalent metal transporter with a substrate preference for iron, although the protein when expressed in frog oocytes transports a broad range of metals, including the toxic metals cadmium and lead. Wild-type Caco-2 cells displayed saturable transport of lead and iron that was stimulated by acid. Cadmium and manganese inhibited transport of iron, but zinc and lead did not. The involvement of DMT1 in the transport of toxic metals was examined by establishing clonal DMT1 knockdown and control Caco-2 cell lines. Knockdown cell lines displayed much lower levels of DMT1 mRNA and a smaller V\textsubscript{max} for iron uptake compared with control cell lines. One clone was further characterized and found to display an ∼50% reduction in uptake of iron across a pH range from 5.5 to 7.4. Uptake for cadmium also decreased 50% across the same pH range, but uptake for lead did not. These results show that DMT1 is important in iron and cadmium transport in Caco-2 cells but that lead enters these cells through an independent hydrogen-driven mechanism.

DMT1 is therefore an interesting candidate for the study of essential and toxic metal absorption from the diet, especially because expression in the proximal duodenum, the site of metal transport, has been shown to be dramatically increased by dietary iron deficiency (8).

The role of iron and iron deficiency has been a consistent theme in the absorption of the toxic metals lead and cadmium. Dietary iron deficiency increases the mucosal uptake of both iron and cadmium in rats, whereas lead transfer, but not uptake, is increased under the same conditions (12). In humans, epidemiological evidence on the effect of iron status on body lead can be contradictory, reporting positive (7) or no association (27), whereas for cadmium there seems to be more uniform animal and human evidence relating iron deficiency and cadmium absorption (12). Furthermore, hereditary hemochromatosis, a disease state in which there is unrestrained uptake of iron, can also be associated with increased cadmium (3) and lead body burdens (6), albeit at low levels. This evident complex nature of lead and cadmium absorption warrants further study, particularly at the molecular level, to clarify both pathways of transport and the role of iron.

The Caco-2 cell line is a human intestinal cell-line that has been used widely as a representative model of mammalian intestinal absorbing cells (21). When confluent, the cells differentiate to form a distinct apical membrane, the outer part of which is enriched in DMT1 (15, 30), along with other markers of differentiation (9, 19). These cells have become useful tools for the study of uptake and transport of nutrients (21, 24), including iron (30) and cadmium (17).
involvement of DMT1 in metal transport has been, until now, based on overexpression of DMT1 by transfection (32) and control of expression using iron treatment (30). Although these approaches represent useful strategies, overexpression can lead to abnormally high expression of the transporter that does not reflect physiological events. Therefore, to study the involvement of endogenous DMT1 in metal transport, we inhibited the expression of DMT1 by means of a U1/ribozyme.

In this study, we demonstrated a saturable, pH-dependent mechanism of iron, lead, and cadmium transport in wild-type Caco-2 cells. We then used a U1/ribozyme to knockdown DMT1 mRNA levels, the functional consequence of which was reduced uptake of iron and cadmium, but not lead, over a range of pH values from 5.5 to 7.4. These result show that lead does not enter Caco-2 cells through DMT1, although the transport pathway seems to be hydrogen coupled.

MATERIALS AND METHODS

Cell culture. Caco-2 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured at 37°C in a humidified atmosphere of 95% air-5% CO2 by using Eagle’s minimum essential medium with Earle’s salts and L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 20% fetal bovine serum, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/l sodium bicarbonate (all from Invitrogen) as recommended by ATCC. Confluent 100-mm plates were split 1:4 by using 0.25% (wt/vol) trypsin-0.03% (wt/vol) EDTA (Invitrogen), and the medium was changed every 3 days. For uptake experiments, the cells were grown for 11–14 days postconfluence on 24-well or 100-mm plates, depending on the sensitivity of the assay.

Construct preparation. The pU1 vector, described elsewhere, was derived from pZEoSV (Invitrogen), which has been modified to contain a U1 snRNA expression cassette and has been used successfully to knockdown the expression of other genes (1, 22). The framework of the construct, U1snRNA (Fig. 1, inset), is an essential component of the nuclear spliceosome complex and is abundant and stable in the mammalian nucleus, and the U1snRNA promoter is potent and constitutively active in mammalian cells. The unusual trimethylguanosine 5’ cap and 5m proteins are thought to signal transport of the U1snRNA back into the nucleus, thus improving the efficiency of the knockdown. The plasmid contains unique EcoR1 and SpeI restriction sites for insertion of a synthesized oligonucleotide/hammerhead ribozyme complex (Fig. 1A) that binds to and cleaves target RNA immediately 3’ of a GUC consensus sequence (16). Specificity is conferred by the two flanking oligonucleotides that bind to their complimentary RNA sequences (Fig. 1).

In practice, the antisense design strategy included systematically finding GUC sequences in DMT1 RNA, extending 20 nucleotides on either side of the C, converting to antiparallel RNA, replacing the G with the ribozyme sequence, and inserting the ribozyme antisense sequence into the U1snRNA. The best candidates for knockdown, based on thermodynamic folding of the RNA structure (33), were those in which there was maximal preservation of the U1snRNA and ribozyme loops as well as maximal accessibility of the antisense sequence to the target RNA (Fig. 1, inset). Antisense/ribozyme sequences were synthesized, annealed, and ligated in the EcoR1 and SpeI sites of the pU1 vector. All ligation junctions were sequenced to verify the orientation of inserts. Two isoforms of DMT1 exits, one containing an iron response element in the 3’ untranslated region of its mRNA (IRE form) and one without (non-IRE form). Although specific antisense oligonucleotides were designed for the IRE and non-IRE forms of human DMT1, only that for the IRE form was used because it is the form found in the intestine that responds to dietary iron (8). Of several candidates, the antisense oligonucleotides to nucleotides 2460–2525 (GenBank accession no. AB004857) was selected and is hereafter referred to as pU1/DMT1.

Transfection and screening. Caco-2 cells were transfected with pU1/DMT1 or pU1 (lacking the ribozyme/antisense) only as control by using Fugene transfection reagent (Roche...

Fig. 1. U1snRNA/ribozyme/antisense construct used to knockdown divalent metal transporter 1 (DMT1) expression. The schematic drawing shows the sequence of oligonucleotides used to inhibit DMT1 in Caco-2 cells, indicating antisense (underlined) and sense (highlighted). The hammerhead ribozyme is shown as the lower loop. The sense sequences are flanked by U1 small nuclear RNA. Arrow indicates ribozyme cleavage sites on the targeted RNA. Inset: representative multifold computer analysis of secondary structure of the entire construct showing the critical preservation of U1snRNA loops and a few antisense hydrogen bonds, ensuring availability of the antisense sequence to bind to the target RNA.
Fig. 2. Uptake of 55Fe and Pb in Caco-2 cells as a function of pH and concentration. Two weeks postconfluent Caco-2 cells were incubated for 10 min at 37°C in Pb or Fe uptake buffer at pH 5.5 or 7.4, followed by washing as described in MATERIALS AND METHODS. A: 55Fe[FeCl3] with 4-fold nitrilotriacetic acid (NTA) and 10-fold ascorbic acid. B: Pb(NO3)2 with 5-fold sodium citrate. Data points, representing means ± SE of 4 replicates, were analyzed using nonlinear regression and a Michaelis-Menten equation.

Molecular Biochemistry) according to the manufacturer’s instructions. Transfected cells were selected in the presence of 100 μg/ml zeocin (Invitrogen), and clonal cell lines were established. Clones were maintained in medium containing zeocin and screened for DMT1 knockdown by Northern analysis.

Northern analysis. Total RNA was isolated using RNeasy according to the manufacturer’s instructions, and Northern analysis was carried out with modifications to a previous procedure (23, 25). Briefly, 10 μg of total RNA were fractionated on a 1% agarose gel by electrophoresis and transferred to Nytran membrane (Schleicher & Schuell, Dassel, Germany). Hybridization, using full-length DMT1 cDNA (from M. Garrick, State University of New York at Buffalo) that was 32P-dCTP-labeled using a random priming kit (Amersham, Amersham, UK), was carried out for 18 h at 42°C. Membranes were washed in 1× SSC/0.1% SDS once for 5 min and then twice for 60 min, followed by exposure to high-performance chemiluminescence film with quantification by densitometry. Blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (1.9 kb) to control for RNA loading, and relative transfer rates and results were normalized to GAPDH mRNA levels. A similar procedure has been used to detect both forms of DMT1 mRNA in Madin-Darby canine kidney cells (23).

Uptake assays. Caco-2 cells were used for assays at 11–14 days post confluence. Uptake buffer consisted of 140 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, and 900 mg (0.09%) of glucose. For pH assays, 10 mM HEPES (pH 7.0 or 7.4), 10 mM PIPES (pH 6.5), or 10 mM MES (pH 5.5) was added to the buffer and the pH was adjusted with HCl or NaOH. Before uptake assays, medium was removed and cells were washed briefly with uptake buffer pH 7.4. To make an 55Fe dosing solution, 10 mM sodium ascorbate was added to a 1:4 Fe-NTA (FeCl3: nitrilotriacetic acid) solution, and 1 ml of this was radiolabeled with 10 μl of a 10 mCi/ml 55FeCl3 (NEN) solution to give a 1 mM 55Fe[Fe-NTA] solution. Fe-NTA solutions maintain iron solubility (31), and ascorbate maintains iron as Fe2+. To initiate dosing, we added aliquots of this 1 mM 55Fe[Fe-NTA] solution to cells bathed in uptake buffer and then incubated cells at 37°C for specific uptake or 4°C for nonspecific uptake. To terminate uptake, we placed cells on ice and replaced the uptake buffer with an ice-cold wash solution consisting of 10 mM HEPES, 1 mM NTA, and 150 mM NaCl to remove nonspecifically bound metal. This was repeated three times for 5 min each. Cells were lysed with 200 mM NaOH for several hours, followed by neutralization with 200 mM HCl and scintillation counting. For 109Cd uptake, aliquots of a 1:15,000 dilution of 109CdCl2 (specific activity 3.893 mCi/ml; NEN) were added to cells in uptake buffer, followed by incubation. With lead transport, aliquots of a 1 mM Pb:citrate [Pb(NO3)2:sodium citrate] solution were added to cells in uptake buffer, followed by incubation. Citrate ions maintain the solubility of lead in solution and have been previously used for lead uptake ex-
UPTAKE OF METALS BY DMT1

Iron and lead uptake by wild-type Caco-2 cells is saturable and pH dependent. We first examined uptake of iron and lead in wild-type cells to establish a benchmark for functional assessment of the knockdown model. In wild-type Caco-2 cells, iron uptake was saturable (Fig. 2A), indicating a limited number of carriers mediating uptake, and at least twofold greater at pH 5.5 than that at pH 7.4, depending on the concentration. For lead, over the same concentration range as iron, uptake was also saturable and increased up to twofold with decreasing pH (Fig. 2B). When the data for both metals were fitted to a Michaelis-Menten equation, the estimated $K_m$ values were 2.9 ± 0.4 μM for iron and 2.5 ± 0.6 μM for lead, whereas the $V_{max}$ was 257 ± 12 fmol·µg protein$^{-1}$·min$^{-1}$ for iron but 80 ± 6 fmol·µg protein$^{-1}$·min$^{-1}$ for lead, which is over threefold less than that for iron.

Iron uptake is affected by changes in divalent iron concentration and the presence of other cations. Acid-stimulated (pH 5.5 vs. 7.4) iron uptake was strongly inhibited by 500 μM iron, manganese, and cadmium (Fig. 3) but not by zinc or lead. This finding demonstrated that acid-stimulated uptake of iron, a property of DMT1, was abrogated by the presence of divalent ions of iron, manganese, and cadmium but was not affected by lead or zinc.

Fig. 4. Fe uptake in knockdown (KD) DMT1 and control Caco-2 cells. KD and control cell lines were developed as described in MATERIALS AND METHODS. Two KD clones (KD1 and KD2) and two control clones (Con1 and Con2) were isolated. A: levels of DMT1 and GAPDH mRNA were examined by Northern analysis. B: concentration-dependent iron uptake of KD1 and Con1. Iron transport was measured in cells that were grown to 13 days postconfluence and then exposed to different concentrations of $^{55}$Fe[FeCl$_3$] with 4-fold NTA and 10-fold EDTA instead of NTA and 10-fold ascorbic acid as described in MATERIALS AND METHODS. Data points represent means ± SE of 3 replicates. Data were analyzed using nonlinear regression. From the fitted model, the $V_{max}$ and $K_m$ values for KD1 are 267 ± 44.7 fmol·µg protein$^{-1}$·min$^{-1}$ and 10.1 ± 3.2 μM, respectively, and those for Con1 are 776 ± 111 fmol·µg protein$^{-1}$·min$^{-1}$ and 13.4 ± 4.4 μM, respectively. C: concentration-dependent iron uptake of KD2 and Con2. Iron transport and data analysis are described in B. The $V_{max}$ and $K_m$ values for KD2 are 319 ± 27.8 fmol·µg protein$^{-1}$·min$^{-1}$ and 9.1 ± 2.2 μM, respectively, and those for Con2 are 558 ± 71.8 fmol·µg protein$^{-1}$·min$^{-1}$ and 15.4 ± 6.4 μM, respectively. D: uptake of iron was examined in the control and KD clones at a substrate concentration of 20 μM iron. The difference between KD and control clonal cell lines was statistically significant (aP < 0.05; ANOVA).
Effect of DMT1 knockdown on uptake of iron. Two clonal knockdown DMT1 cell lines (KD1 and KD2) and two control cell lines (Con1 and Con2) were established from wild-type Caco-2 cells as described in MATERIALS AND METHODS. KD1 and KD2 exhibited much lower levels of DMT1 mRNA than Con1 and Con2 (Fig. 4A). The KD1 and KD2 cell lines also displayed lower levels of iron uptake compared with the Con1 and Con2 cell lines (Fig. 4, B and C). Saturable uptake decreased by 50% in the KD1 cell line compared with the Con1 cell line, with a decrease in $V_{\text{max}}$ from 776.4 to 267.4 fmol·μg protein$^{-1}$·min$^{-1}$. Saturable uptake also declined in the KD2 cell line compared with the Con2 cell line, with a decrease in $V_{\text{max}}$ from 558 to 319 fmol·μg protein$^{-1}$·min$^{-1}$. When the four cell lines were compared at the substrate concentration of 20 μM, the KD cell lines exhibited significantly lower levels of iron uptake than the controls. The control clones were not different from each other, and the knockdown clones were not different from each other (Fig. 4D). Differences in the kinetics of the control clones and the wild-type Caco-2 cells were observed (Fig. 2A) for reasons that are presently unknown.

Effect of pH on the uptake of iron. The pH dependence of iron uptake was compared in the KD1 and Con1 cell lines. The KD1 cell line displayed an ~50% decrease in the uptake of iron that was consistent across a pH range from 5.5 to 7.4 compared with that in the Con2 cell line (Fig. 5). Uptake was inversely proportional to pH, with the highest uptake at pH 5.5.

Effect of DMT1 knockdown on uptake of cadmium and lead. We also examined the effect of diminished expression of DMT1 mRNA on uptake of toxic metals in the KD1 and Con1 cell lines. An ~50% decrease in the uptake of 1 μM cadmium across a pH range was observed (Fig. 6A). When cells were exposed to 10 μM lead, uptake increased with decreasing pH up to that at pH 5.5, as would be expected in cells expressing DMT1, but there was no consistent difference between the knockdown and control cell lines (Fig. 6B), suggesting that DMT1 is not a major pathway by which lead enters these cells. This is consistent with the data in Fig. 3, showing that lead does not inhibit the uptake of iron in wild-type Caco-2 cells whereas cadmium strongly inhibits iron uptake.

DISCUSSION

Previous studies have shown that DMT1 mediates the transport of iron in Caco-2 cells (30), making it a suitable model for the study of essential and toxic metal acquisition in humans. In our experiments, uptake of iron depends on hydrogen ion concentration and was saturable, properties associated with DMT1. Knockdown clonal DMT1 Caco-2 cell lines were established displaying lower levels of DMT1 mRNA compared with control cell lines. The functional conse-
quence of the knockdown was a reduction in the $V_{\text{max}}$ for iron, indicating a decrease in the number of transporters, as well as reduced uptake of iron across a pH range from 5.5 to 7.4. We also found a decrease in the transport of cadmium across a pH range from 5.5 to 7.4 and competition between cadmium and iron in uptake. Our studies corroborate previous studies suggesting that DMT1 mediates the transport of Cd (11, 29). The competition by cadmium during iron transport would decrease the amount of iron absorbed during digestion. Lower levels of body iron would likely result in increase in DMT1 expression in the intestine (2) because of the IRE located at the 3'-end of DMT1 mRNA. Hence, the competition between cadmium and iron provides a molecular mechanism to explain both the process of iron deficiency induced by chronic exposure to cadmium (12) and the increased absorption of dietary cadmium shown in humans who are iron deficient (26).

In wild-type cells, the effect of pH and concentration was similar for both lead and iron, providing evidence that lead could be transported by DMT1. However, lead did not inhibit iron uptake or vice versa (data not shown). Furthermore, the knockdown DMT1 Caco-2 cell line displayed levels of lead uptake similar to those of the control cell line. Collectively, our evidence demonstrates that DMT1 is not the major pathway by which lead enters Caco-2 cells, even though the properties are consistent with hydrogen-coupled transport. Similar to lead, zinc does not inhibit transport of iron and does not appear to be transported by DMT1 in Caco-2 cells (30). In contrast, transport of zinc in intestinal brush-border membrane vesicles from rats exhibited properties similar to a DMT1-mediated mechanism (18). Furthermore, in one of the original studies describing DMT1, a broad range of metals, including zinc and lead, were transported in frog oocytes expressing DMT1 (14). Finally, increased transport of lead was observed in yeast and human fibroblasts overexpressing DMT1 (5). To reconcile these disparate observations, we suggest that high levels of DMT1 are needed for the DMT1-mediated transport of lead and zinc. Caco-2 cells, rather, express low levels of DMT1 but express other transporters in sufficient amounts to transport lead and zinc. Unfortunately, the identity of the transporters mediating the uptake of lead in Caco-2 cells is unknown.

Previous studies had shown that iron uptake in DMT1-transfected COS cells reaches a pH optimum at 6.75 (32), but our work consistently demonstrated that metal uptake increases with increasing hydrogen ions up to pH 5.5, in agreement with findings of other workers (13, 30). These differences might be explained by the different cell lines expressing DMT1. Interestingly, the KD DMT1 cell line displayed a diminished capacity to transport iron and cadmium across the pH range, rather than only at an acidic pH. Thus DMT1 appears to transport iron at physiological pH, albeit at lower amounts, suggesting the possible involvement of DMT1 in the transport of iron in cell types other than intestinal cells, where the extracellular fluid is at a physiological pH.

In summary, our results provide evidence for the direct involvement of DMT1 in Caco-2 cells for the uptake of iron and cadmium. Our results explain why iron deficiency is a risk factor for cadmium poisoning. Interestingly, the transport of lead was not mediated by DMT1 in Caco-2 cells, suggesting the presence of a different transporter.

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