

Functional and molecular responses of human intestinal Caco-2 cells to iron treatment¹⁻⁴

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ABSTRACT

Background: Divalent metal transporter 1 (DMT1), HFE, and stimulator of iron transport (SFT) are transmembrane proteins that have been implicated in the regulation of iron homeostasis.

Objective: The objective of this study was to investigate whether absorption and transepithelial movement of iron correlated with gene expression of DMT1, HFE, and SFT in an experimental model of human absorptive enterocytes.

Design: Caco-2 cells were exposed to iron-supplemented media in either the presence or the absence of serum for 24, 72, and 168 h. At each time point, the uptake and transepithelial movement of iron were examined and gene expression of DMT1, HFE, and SFT was measured. Manganese and zinc absorption was also examined at 168 h.

Results: Iron treatment in the presence or absence of serum reduced the uptake and transepithelial movement of iron by $\approx 50\%$ after 72 and 168 h. No effect was observed at 24 h. The uptake and transepithelial movement of manganese were similar to those of iron at 168 h, whereas the effects on zinc were less pronounced. In the absence of serum, iron treatment was associated with a reduction of DMT1 expression by 50% at 72 and 168 h. HFE expression was dependent on serum, but iron treatment did not alter HFE expression. SFT expression was not affected by iron.

Conclusions: Iron treatment decreased cellular uptake of iron, manganese, and zinc, suggesting that these metals may utilize the same apical transporter. The transepithelial movement of iron and manganese, but not of zinc, was reduced across iron-treated Caco-2 cells, suggesting that iron and manganese are regulated by the same mechanism at the basolateral membrane. The gene expression of DMT1, HFE, and SFT did not fully correlate with the functional responses of Caco-2 cells. This may have been a result of posttranscriptional regulation of these genes or regulation of other genes involved in the uptake and transepithelial movement of iron in Caco-2 cells. *Am J Clin Nutr* 2000;72:770-5.

KEY WORDS Iron, manganese, zinc, intestinal absorption, Caco-2 cells, divalent metal transporter 1, DMT-1, HFE, stimulator of iron transport, SFT

INTRODUCTION

Although it is well established that mammalian cells have an absolute requirement for iron, disturbances in the cellular handling

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of iron contribute to the pathogenesis of diseases such as hemochromatosis, Parkinson disease, diabetes mellitus, and cancer (1-4). Despite the prevalence of these disorders, the mechanism by which dietary iron is absorbed into the body is poorly understood. Recently, the identification of several novel genes that are involved in genetic disorders of iron metabolism has provided new insights into the regulation of iron movement across the intestinal epithelium.

Divalent metal transporter 1 (DMT1; also known as Nramp-2 and DCT1) is a transmembrane, proton-coupled transporter that is believed to be responsible for the transport of nonheme iron across the apical membrane of enterocytes. Duodenal expression of DMT1 is up-regulated by iron deficiency and hemochromatosis (5-8). Functional studies in *Xenopus* oocytes showed that DMT1 can mediate the uptake of various divalent metal ions, including iron, manganese, and zinc (5). It has also been suggested that DMT1 is responsible for iron transport across endosomal membranes (9).

Feder et al (1) reported a candidate gene for hereditary hemochromatosis that was identified by positional cloning and that encodes a novel major histocompatibility complex class I-like protein called HFE. In parenchymal cells, HFE interacts with the transferrin receptor (TfR) to inhibit the uptake of transferrin-bound iron (10, 11). The transport of iron across the basolateral membrane of enterocytes may also be regulated by HFE (1). Although the mechanism by which this occurs has not been determined, Waheed et al (12) proposed that HFE allows intestinal crypt cells to sense body iron stores and to program migrating enterocytes to absorb more or less dietary iron via DMT1.

Stimulator of iron transport (SFT) encodes a transmembrane protein that facilitates uptake of divalent and trivalent iron into RNA-microinjected *Xenopus* oocytes (13, 14). The nature of the SFT-mediated transport is similar to the uptake of non-transferrin

¹From the Departments of Nutrition and Internal Medicine, University of California, Davis.

²Presented in part at Experimental Biology '99, Washington, DC, 17-21 April 1999 (abstract LB205).

³Supported in part by the Foundation of Henning and Johan Throne-Holst (JT).

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Received July 16, 1999.

Accepted for publication March 28, 2000.

bound iron (15), but the localization of SFT to endosomes suggests that SFT is also involved in transport of iron across intracellular membranes (13, 14). Experiments with human HeLa cells *in vitro* showed that SFT mRNA is up-regulated by iron depletion and down-regulated by iron supplementation (16). It was also shown that SFT is up-regulated in the liver of persons with hemochromatosis (16). The role of SFT in intestinal iron absorption has not been explored.

Although some insights have been made into the functions of these recently discovered genes, regulation of the expression of the genes in an experimental model of human absorptive enterocytes has not been well studied. Furthermore, how this regulation affects the absorptive process of iron and other essential minerals is unknown. In the present study, Caco-2 cells were treated with iron, and kinetic experiments were performed with iron, manganese, and zinc. DMT1, HFE, and SFT concentrations were measured to determine whether the expression of these genes correlated with changes in the kinetics of the metals. Experiments were performed in the presence and the absence of serum to determine whether serum factors influence gene expression and alter the cellular uptake and transepithelial movement of iron, manganese, and zinc. Intracellular iron concentration and TfR mRNA concentrations were measured to determine the effects of various treatments on cellular iron status.

MATERIALS AND METHODS

Chemicals

$^{59}\text{FeCl}_3$ (specific activity: 0.14 GBq/mg Fe^{2+}), $^{54}\text{MnCl}_2$ (specific activity: 0.94 GBq/mg Mn^{2+}), and $^{65}\text{ZnCl}_2$ (specific activity: 0.04 GBq/mg Zn^{2+}) were obtained from DuPont NEN (Boston). Nitrilotriacetic acid (NTA), fetal bovine serum (FBS), FeSO_4 ($7\text{H}_2\text{O}$), and *N*-2-hydroxyethyl-piperazine-*N'*-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Company (St Louis). Cell culture media, antibiotics (penicillin and streptomycin), and Hank's Balanced Salt Solution (HBSS) were obtained from Gibco BRL through Life Technologies (Grand Island, NY).

Cells and media

The Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown in minimum essential medium (MEM; Gibco, Rockville, MD) with Earle's salts (Gibco), L-glutamine, 10% FBS, and 1% penicillin and streptomycin at 37°C with 5% carbon dioxide. Cells between passages 45 and 50 were harvested at 80% confluence and split either into new tissue culture flasks (for gene expression studies) or onto permeable polycarbonate filters (for uptake and transepithelial movement studies) at a density of 225 000/cm². After 3 wk, the cells were treated for 24, 72, or 168 h with one of the following culture media: serum-free medium (MEM; 1 $\mu\text{mol/L Fe}^{2+}$), iron-supplemented serum-free medium (MEM + Fe; 6 $\mu\text{mol/L Fe}^{2+}$), serum-containing medium (MEM + FBS; 6 $\mu\text{mol/L Fe}^{2+}$), or iron-supplemented serum-containing medium (MEM + FBS + Fe; 200 $\mu\text{mol/L Fe}^{2+}$). Iron was supplemented as FeNTA_4 . The concentrations of iron in the media were determined by using inductively coupled axial plasma spectrometry (ICAP-AES) as described by Golub et al (17).

Intracellular iron

Caco-2 cells were seeded onto permeable polycarbonate filter supports and treated for 168 h with the various media as

described above. The cells were rinsed 3 times with HBSS and the filters were gently cut from the supports. The cells were digested for 3 d in 3 mL ultrapure nitric acid at room temperature and then analyzed by using ICAP-AES. Filters without cells were treated as described above and used as background ($n = 6/\text{group}$).

Transport experiments

Experiments were carried out at 37°C in HBSS (pH 7.4) containing 25 mmol HEPES/L as described previously (18). Briefly, the Caco-2 cells were rinsed and incubated for 45 min with prewarmed HBSS. After preincubation, the HBSS was discarded and the filter supports with the monolayers were transferred to new wells containing 1.5 mL of 20 $\mu\text{mol/L}$ human apotransferrin in HBSS; 0.5 mL HBSS containing 20 $\mu\text{mol } ^{59}\text{Fe}^{2+}$, $^{54}\text{Mn}^{2+}$, or $^{65}\text{Zn}^{2+}/\text{L}$ and 2 mmol L-ascorbate/L was added to the apical chamber and the monolayers were incubated at 37°C for 3 h. At the end of the experiment, radioactivity in the basolateral chamber was measured by using β - or γ -spectrometry. The cumulative fraction (Fr_{cum}) of metal movement from the apical to the basolateral side of the monolayers versus time was determined according to the following equation:

$$\text{Fr}_{\text{cum}} = \text{Cbl}_i / \text{Cap}_0 \quad (1)$$

where Cbl_i is the concentration of metal on the basolateral side at the end of the time interval i and Cap_0 is the concentration of metal on the apical side at the beginning of the time interval.

To determine the amount of metal taken up into the cells during the 3 h of incubation, the monolayers were carefully rinsed with 1 mL ice-cold HBSS. The filters were removed and the Caco-2 cells were dissolved in 5 mL of 0.5 mol NaOH/L overnight; this was followed by β - or γ -spectrometry.

Assessment of monolayer integrity

The integrity of monolayers was confirmed by measuring the transepithelial electrical resistance with a Millicell Electrical Resistance System device (Millipore, Bedford, MA). Measurements were made before the transport experiments in HBSS, according to the method of Artursson et al (19). Monolayers with resistances < 180 Ω/cm^2 were not used in the experiments. Background resistance was determined by measuring the transepithelial electrical resistance across a filter without cells in HBSS.

Gene expression studies

Total RNA was isolated from Caco-2 cells by using the method of Chomczynski and Sacchi (20); 12 μg of total RNA per lane was loaded on a formaldehyde agarose gel. Hybridizations were performed at 68°C in Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's specifications and followed by washes at high stringency. Signal intensity was quantified by using phosphor imaging and IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA). Gene expression values were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and expressed in units relative to the control.

Probes

Human cDNA probes for DMT1, SFT, and GAPDH were generated by using reverse transcriptase-polymerase chain reaction with Caco-2 cell RNA as a template. Poly(A)⁺ RNA was isolated and reverse transcribed by using the Micro Fast Track kit (Invitrogen, Carlsbad, CA) and Superscript II reverse transcriptase (Life

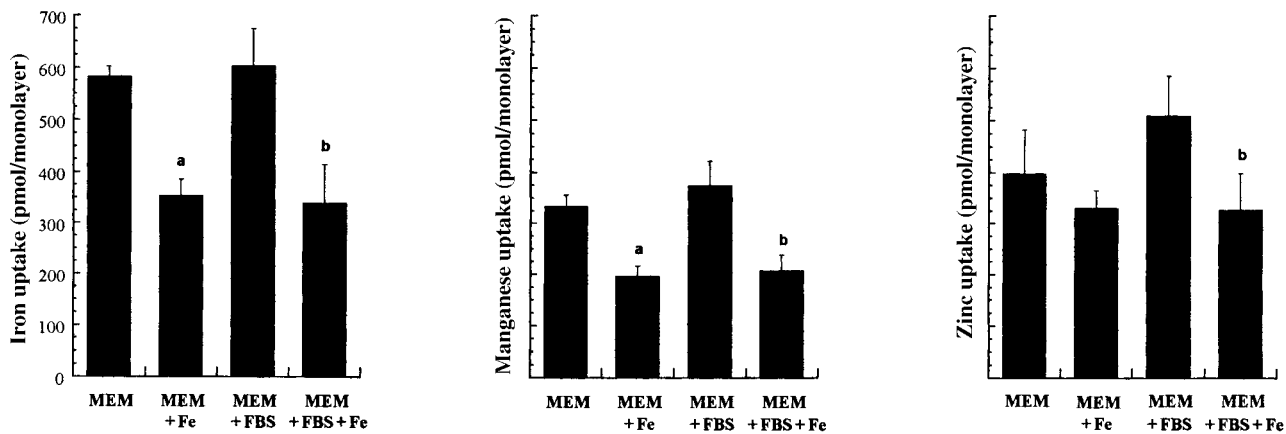


FIGURE 1. Uptake of iron, manganese, and zinc into Caco-2 cells after 3 h of incubation at 37°C in the presence of 20 $\mu\text{mol } ^{59}\text{Fe}^{2+}$, $^{54}\text{Mn}^{2+}$, or $^{65}\text{Zn}^{2+}/\text{L}$ and 2 mmol L-ascorbate/L in the apical chamber and 20 μmol human apotransferrin/L in the basolateral chamber. Before the transport experiments, the monolayers were allowed to differentiate for 21 d and then treated for 72 h in serum-free minimum essential medium (MEM), iron-supplemented MEM (MEM + Fe), serum-containing medium (MEM + FBS), or iron-supplemented, serum-containing medium (MEM + FBS + Fe) as described in Materials and Methods (24- and 168-h data not shown). The data are means \pm SDs of 5–6 monolayers obtained from 2 separate experiments. ^aSignificantly different from MEM, $P < 0.05$. ^bSignificantly different from MEM + FBS, $P < 0.05$.

Technologies, Grand Island, NY). DMT1 primer sequences were 5'-TGTCACCGTCAGTATCCCAA-3' (forward) and 5'-GTGCAATGCAGATTCAATG-3' (reverse). This set of primers recognizes both iron response element (IRE) and non-IRE containing DMT1 transcripts. SFT primer sequences were 5'-TGGACATCTGCA TCTTCAGC-3' (forward) and 5'-TTAATTATCAGCCGGTTGG-3' (reverse). GAPDH primer sequences were 5'-GACCACAGTC CATGACATCACT-3' (forward) and 5'-TCC ACCACCT GTTGCTGTAG-3' (reverse). The cDNA-probe for HFE was obtained by using polymerase chain reaction for human genomic DNA with primer sequences 5'-TGGCAAGGGTAAACAGATCC-3' (forward) and 5'-CTCAGGCACTCCTCTCAACC-3' (reverse). A 2-kb probe to human TfR was generated by using polymerase chain reaction from pCDTR1 (ATCC, Manassas, VA) with primers 5'-CGAGCAGCTTTTAATACAGGG-3' (forward) and 5'-GACACTAAGAACCTGAAGAGACCC-3' (reverse). The probes were labeled with $\alpha^{32}\text{P}$ -dCTP by using the Rediprime II random prime or Megaprime DNA labeling systems (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

The significance of the differences between the 2 iron-treated groups and their respective controls was analyzed by two-factor analysis of variance with interaction. Significance was set at $P < 0.05$.

RESULTS

Transport experiments

Before all the experiments, Caco-2 cell monolayers were treated for 24, 72, or 168 h with media containing various amounts of iron in the presence or the absence of serum, as described above. The resistance across the monolayers was not significantly different among groups. Intracellular iron concentrations were significantly greater in cells treated with MEM + Fe than in those treated with MEM (0.133 ± 0.003 and

0.038 ± 0.001 $\mu\text{g Fe/L}$, respectively) and significantly greater in cells treated with MEM + FBS + Fe than in those treated with MEM + FBS (0.266 ± 0.009 and 0.044 ± 0.001 $\mu\text{g Fe/L}$, respectively) at 168 h.

Both the uptake and the transepithelial movement of iron by cells treated with the iron-supplemented media (MEM + Fe and MEM + FBS + Fe) were $\approx 50\%$ of those of their respective controls (MEM and MEM + FBS) at 72 and 168 h (Figures 1 and 2). However, neither the uptake nor the transepithelial movement of iron was affected 24 h after iron supplementation. The response to iron supplementation was not dependent on the presence of serum. The effects of iron supplementation on the uptake and the transepithelial movement of manganese were similar to those on the uptake and transepithelial movement of iron. The uptake of manganese was less than that of iron, but transepithelial movement was greater (Figure 1). Transepithelial movement of zinc across the Caco-2 cell monolayers was lower than that of iron and manganese. Zinc uptake was slightly lower with supplementation in the absence of serum but the difference was not significant. In the presence of serum, iron supplementation was associated with significantly lower zinc uptake. The absence of serum had no significant effect on zinc movement across iron-supplemented Caco-2 cells. However, in the presence of serum, iron supplementation was associated with increased zinc movement from the apical to basolateral compartment. This occurred despite reduced cellular uptake of zinc under these conditions.

Gene expression studies

The relative gene expression of DMT1, HFE, and SFT was measured by using northern hybridization to RNA extracted from Caco-2 cells treated similarly to those in the transport experiments. TfR expression was generally low and was detected only in cells grown in the presence of serum. Under these conditions, iron treatment decreased TfR expression to 58%, 36%, and 35% of that of the control after 24, 72, and 168 h, respectively (Figure 3). Iron supplementation decreased DMT1 expression by 50% under

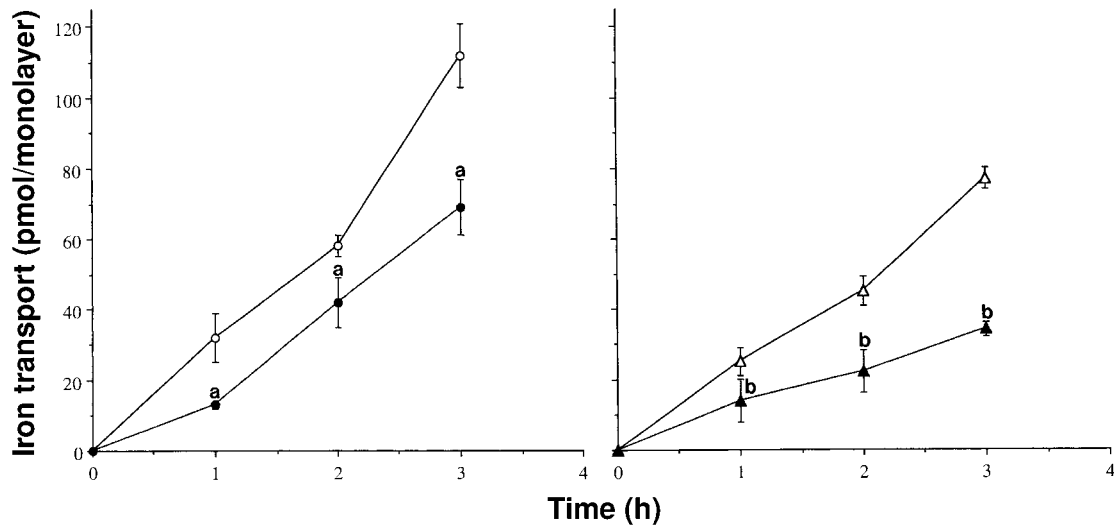


FIGURE 2. Transepithelial movement of iron across monolayers of Caco-2 cells incubated for 3 h at 37°C in the presence of 20 $\mu\text{mol } ^{59}\text{Fe}^{2+}/\text{L}$ and 2 mmol L-ascorbate/L in the apical chamber and 20 μmol human apotransferrin/L in the basolateral chamber. Before the transport experiments the monolayers were allowed to differentiate for 21 d and then treated for 168 h in serum-free minimum essential medium (MEM; \circ), iron-supplemented MEM (MEM + Fe; \bullet), serum-containing medium (MEM + FBS; \triangle), or iron-supplemented, serum-containing medium (MEM + FBS + Fe; \blacktriangle) as described in Materials and Methods (24- and 72-h data not shown). The data are means \pm SDs of 5–6 monolayers obtained from 2 separate experiments. ^aSignificantly different from MEM, $P < 0.05$. ^bSignificantly different from MEM + FBS, $P < 0.05$.

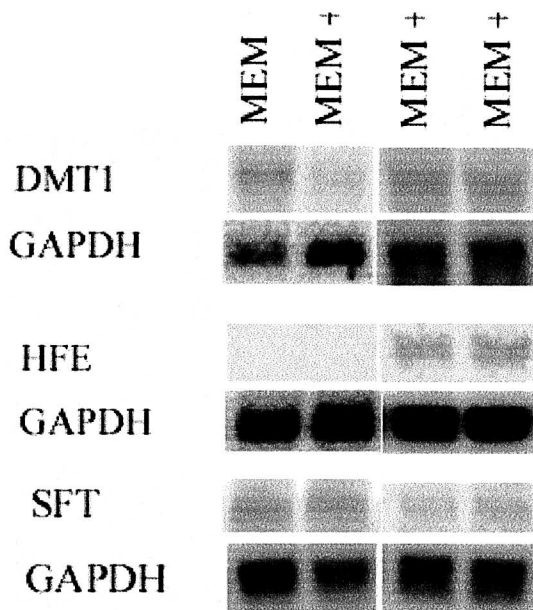


FIGURE 3. Gene expression of divalent metal transporter 1 (DMT1), HFE, stimulator of iron transport (SFT), and transferrin receptor (TfR) in Caco-2 cells treated for 72 h in serum-free minimum essential medium (MEM), iron-supplemented MEM (MEM + Fe), serum-containing medium (MEM + FBS), or iron-supplemented, serum-containing medium (MEM + FBS + Fe) as described in Materials and Methods. Total RNA was isolated from the Caco-2 cells and analyzed for DMT1, HFE, SFT, and TfR mRNA by using northern hybridizations and phosphor-imaging. Gene expression values were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and expressed in units relative to controls.

serum-free conditions. Similar to the transport studies, this effect was not present until after 24 h of treatment. In the presence of serum, DMT1 expression was unchanged by iron supplementation at all time points. HFE expression was detectable only in Caco-2 cells treated with serum-containing media. Supplementation of the cell culture medium with iron did not alter the expression of HFE at any of the time points. SFT was expressed in both MEM and MEM + FBS. Iron supplementation under either of these conditions did not affect SFT gene expression.

DISCUSSION

In the present study, the iron status of Caco-2 cells was modulated and correlations were sought between metal transport and expression of several genes implicated in iron metabolism. Intracellular iron status was determined by direct measurement and by assessing the regulation of TfR gene expression. Similar to the findings of others, we found that iron supplementation reduced iron uptake (21, 22). Our results also showed that uptakes of manganese and zinc were reduced by iron supplementation of Caco-2 cells. However, the reduction with zinc was only moderate.

DMT1 expressed in *Xenopus* oocytes was shown to transport divalent metal ions, including iron, manganese, and zinc (5). DMT1 was implicated in intestinal iron absorption when it was identified as the gene mutated in the microcytic anemia (*mk*) mouse and the phenotypically similar Belgrade (*b*) rat (6, 9, 23). In these 2 animal strains, orthologous mutations (Gly185Arg) in the DMT1 gene results in severely reduced absorption of dietary iron.

Our results showed that the expression of DMT1 is down-regulated by iron in the absence of serum. The down-regulation


of DMT1 correlated with a decreased uptake of iron into Caco-2 cells. The changes in DMT1 expression we observed were small compared with those observed *in vivo* under iron-deficient conditions but were similar in magnitude to the changes in TfR. Alternative splice forms of DMT1 that differ in the carboxy terminal amino acids and the 3'-untranslated region have been identified. One of these transcripts contains a sequence consistent with an IRE similar to those found in the 3'-untranslated region of TfR (5, 24). *In vivo* studies showed that the putative IRE-containing transcript predominates in intestine and that this form is up-regulated in iron deficiency. In addition, the 5' flanking region of the DMT1 gene contains several sequences consistent with metal regulatory elements. The posttranscriptional regulation of TfR mediated by the binding of iron regulatory proteins to multiple IREs in the TfR transcript has been well established. Whether the regulation of DMT1 expression *in vivo* or *in vitro* is mediated through promoter elements, IRE binding, or both has yet to be determined. In fact, *in vitro* data show that DMT1 expression does not necessarily correlate with iron regulatory protein binding activity (25).

Our study also showed that iron treatment in the presence of serum decreased cellular iron uptake, which was not accompanied by a decrease in DMT1 expression. This suggests either that DMT1 is posttranscriptionally modified at high intracellular iron concentrations or that other transporters are involved.

In addition to uptake, the transepithelial movement of iron across the Caco-2 cells was significantly reduced by iron treatment. The same effect was observed for manganese, suggesting that the mechanism by which intestinal iron is absorbed into the circulation is operative for manganese as well. However, the transepithelial movement of zinc was not affected by iron supplementation under serum-free conditions, suggesting that this step is mediated by a separate mechanism. This is consistent with the recent finding that absorption of zinc requires specific zinc transporters to facilitate efflux from enterocytes (26). In contrast, we found that transepithelial movement of zinc was significantly increased by iron supplementation in the presence of serum. One possibility is that high intracellular iron induces these zinc transporters.

The results of ferokinetic studies suggest that HFE is involved in the transport of iron across the basolateral membrane of the enterocyte. The identification of HFE as the mutated gene responsible for hereditary hemochromatosis constitutes an important piece of the iron regulatory puzzle (1). Interestingly, it has been shown that the expression of DMT1 is up-regulated in the intestines of persons with hereditary hemochromatosis and in HFE-knockout mice (7, 8). In the present study, we found that serum is required for HFE expression in Caco-2 cells. Han et al (27) reported previously that HFE is up-regulated by iron loading and down-regulated by iron depletion in Caco-2 cells, suggesting reciprocal regulation with DMT1. In contrast, our experiments did not show any effects of iron supplementation on HFE expression. One potential explanation for these different results is the stage of differentiation. Whereas Han et al used cells that were 13 d postconfluent, we used fully differentiated cells that were 21 d postconfluent.

Although it remains unclear whether the novel iron transport gene SFT plays a role in the regulation of dietary iron absorption, the results of the present study show that the SFT gene is present in Caco-2 cells. However, no differences were observed in gene expression as a result of iron supplementation to the media.

In conclusion, iron treatment in both the presence and the absence of serum resulted in a decreased uptake of iron and manganese and, in the presence of serum, of zinc into Caco-2 cells, but only in the absence of serum did this effect correlate with DMT1 expression. Otherwise, correlations between the uptake and the transepithelial movement of iron and the gene expression of DMT1, HFE, or SFT in Caco-2 cells were not observed, indicating that these genes are regulated posttranscriptionally or that other genes are involved. Genes not investigated in this study, including heme oxygenase 1 and hephaestin, may be involved in the regulation of uptake and transepithelial movement of iron (28, 29). Further investigations are needed to determine the mechanism by which DMT1 expression is regulated by intracellular iron and to identify other genes that may be involved in the regulation of iron absorption. 

We acknowledge the technical assistance of Melissa Crawford, Department of Internal Medicine, University of California Davis.

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