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Receptor-mediated uptake of ferritin-bound iron by human intestinal Caco-2 cells

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Abstract

Ferritin (Ft) is a large iron-binding protein (~450 kDa) that is found in plant and animal cells and can sequester up to 4,500 iron (Fe) atoms per Ft molecule. Our previous studies on intestinal Caco-2 cells have shown that dietary factors affect the uptake of Fe from ferritin in a manner different from that of Fe from FeSO₄, suggesting a different mechanism for cellular uptake. The objective of this study was to determine the mechanism for Ft-Fe uptake using Caco-2 cells. Binding of ⁵⁹Fe-labeled Ft at 4° C showed saturable kinetics and Scatchard analysis resulted in a K_D of 1.6 μM, strongly indicating a receptor-mediated process. Competitive binding studies with excess unlabelled Ft significantly reduced binding and uptake studies at 37° C showed saturation after 4 h. Enhancing and blocking endocytosis using Mas-7 (a G-protein activator) and hypertonic medium (0.5 M sucrose), respectively, demonstrated that Ft-Fe uptake by Mas-7 treated cells was 140% of control cells, whereas sucrose treatment resulted in a statistically significant reduction in Ft-Fe uptake by 70% as compared to controls. Inhibition of macropinocytosis with 5-(N,N-dimethyl)-amiloride (Na⁺/H⁺ antiport blocker) resulted in a decrease (by ~20%) in Ft-Fe uptake at high concentrations of Ft, suggesting that enterocytes can use more than one Ft-uptake mechanism in a concentration dependent manner. These results suggest that Ft uptake by enterocytes is carried out via endocytosis when Ft levels are within a physiological range, whereas Ft at higher concentrations may be absorbed using the additional mechanism of macropinocytosis.

Keywords

ferritin; ferritin receptor; iron; iron absorption; Caco-2 cells

1. Introduction

Ferritin is an iron-binding protein with very high capacity to bind iron (Fe); up to 4,500 atoms of Fe can be bound to each ferritin molecule [1]. Since several plants used as staple foods express ferritin [2–4], it has been proposed that ferritin could be used for biofortification with Fe [5]. While the natural content of ferritin in staple foods like rice and legumes is relatively low, concentrations can be increased considerably by either conventional plant breeding methods, selecting for high ferritin varieties, or by genetic modification approaches, over-expressing the gene for ferritin [6–10]. If proven successful, this would provide a sustainable

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method for Fe fortification. It is important, however, that the Fe in ferritin is in a bioavailable form so that populations can benefit from this Fe source when being part of their regular diet.

The bioavailability of Fe from ferritin has been assessed in several recent human studies [11–13]. Some early studies showed poor bioavailability of this form of iron, most likely because inappropriate labeling techniques were used [14–16]. In some studies, animal ferritin induced by various methods was used and it is now known that such ferritin is not representative for “normal” ferritin sources. Extrinsic labeling by adding radioiron directly to ferritin was used in some studies, but this added isotope does not equilibrate with the insoluble Fe inside the ferritin core. Using an appropriate and validated extrinsic labeling technique, we have recently shown that human subjects absorb Fe from both animal and plant ferritin well [12,13], and to an extent similar to that from FeSO₄. These results suggest that ferritin may be a useful vehicle for biofortification with Fe.

Iron deficiency and anemia in developing countries are usually due to a combination of low Fe intake and diets containing factors inhibiting Fe absorption. Although Fe intake may be increased by consumption of plant diets with increased ferritin, it is important to assess the effects of dietary factors on Fe absorption from this source. We have recently assessed this *in vitro* [17], using Caco-2 cells, a well established human intestinal cell line that in culture differentiates into enterocyte-like cells. This cell line has been utilized for assessment of Fe bioavailability from various sources [18–25]. Using radiolabeled ferritin and Caco-2 cells in monolayers, we found that phytate, tannic acid and calcium, which are known to inhibit absorption of ferrous Fe [18,21,25–30], and ascorbic acid that enhances Fe absorption [19, 25,31], had significantly less pronounced effect on iron uptake from Ft as compared to that observed for Fe uptake from FeSO₄ [17]. This strongly suggests that Fe is taken up from ferritin via a mechanism different from that for ferrous Fe, which is known to occur via DMT-1 (divalent metal transporter-1) [32,33]. Our *in vitro* digestion experiments suggested that ferritin may survive proteolytic degradation by pepsin and pancreatic enzymes under conditions similar to those of the human gastrointestinal tract.

We have shown in human studies that iron absorption is similar from horse spleen Ft with plant-type mineral and animal-type mineral, and from ferrous sulfate [12]. Further, we have shown in human studies that Fe absorption from soybean Ft was similar to that from FeSO₄ [13]. In this study we explored alternative pathways for Fe uptake using animal Ft and the Caco-2 cell model.

2. Materials and Methods

2.1. Labeling of Ft with ⁵⁹Fe

Animal ferritin (horse spleen) was purchased from Calzyme Laboratories (San Luis Obispo, CA) and then dialyzed to remove the iron core following the protocol previously described [12]. Briefly, iron was removed by thioglycolic acid reduction and dialysis. Iron content of the dialyzed Ft was measured using atomic absorption spectroscopy. The apo-protein shell was then reconstituted using ferrous iron with a radioactive iron tracer. Radioactive iron (⁵⁹Fe as FeSO₄; specific activity 27.7 mCi/mg) was purchased from Perkin Elmer (Boston, MA). Since our primary objective was to study Fe uptake from ferritin, we preferred this method of labeling, particularly as several previous studies have shown validity problems with using ¹²⁵I-labeling of Ft [34,35]. The radiolabel was incorporated to ~90%. Prior to the administration of this radioactive Ft to cells, it was subjected to several buffer exchanges using Centricon filter tubes (30 kD molecular weight cut-off) to ensure removal of loosely bound surface radioactivity. Radioactivity associated with the filtered fractions was measured using a gamma counter (Gamma 8500, Beckman, Irvine, CA) until the non-specific radioactivity was close to background values. Thus, the non-specific radioactivity removal was over 99.9% efficient.

2.2. Cell culture

Caco-2 cells (American Type Tissue Culture Collection, Rockville, MD) were seeded (225,000 cells/cm²) onto cell culture-treated polystyrene plates (used between passages 30–40) and cultured in Minimal Essential Medium (MEM) (Invitrogen Life Sciences, Rockville, MD) containing 10% fetal bovine serum (Sigma), antibiotics (penicillin, 10 units/mL; streptomycin, 1 mg/mL) at 37°C with 5% carbon dioxide. Cell protein was assessed using the Bradford assay [36]. Data are expressed as pmol Fe/μg cell protein.

2.3. Ferritin binding and uptake

Binding studies—To determine the cell stage that allowed for maximal Ft binding, cells on polystyrene plates were treated with ⁵⁹Fe-Ft (1 μM) in serum-free medium for 8 h at 4°C at pre-confluent, confluent, 4 d post-confluent and 7 d post-confluent stages. Results from this pilot study determined the optimum treatment stage for subsequent experiments. In order to demonstrate saturable binding kinetics, cells were treated with ⁵⁹Fe-Ft (0.1–8 μM) in serum-free medium for 8 h at 4°C. In a competitive binding experiment, cells were co-incubated with increasing concentrations of unlabeled Ft (0.5 – 8 μM) to determine binding specificity.

Uptake studies—Cells on polystyrene plates were treated with fixed (1 μM ⁵⁹Fe-Ft) and increasing concentrations (0.1–8 μM ⁵⁹Fe-Ft) in serum-free medium for 16 h and 1 h, respectively, at 37°C. Medium was removed and cells were washed extensively with cold phosphate buffered saline (PBS). Cell-associated radioactivity was quantified in the gamma counter.

2.4. Stimulation of endocytosis

Cells grown on polystyrene plates were treated with 50 μM Mas-7 (Sigma-Aldrich), a G protein activator and a highly potent analog of mastoparan [37,38] for 30 min at 37 °C in PBS containing 1 μM ⁵⁹Fe-Ft and then washed three times with ice-cold PBS to remove any loosely bound radioactivity. Exofacially bound Ft was removed by a brief acid wash (0.15 M NaCl, pH 3.0 for 30 s, on ice) and cellular radioactivity was quantified in the gamma counter. Endosome labeling using Sulfo-Link (sulfo-N-hydroxy-succinimidobiotin) (EZ-Link Sulfo-NHS-Biotin Reagents, Pierce Biotechnology, Rockford, IL) was used as positive control to assess endocytosis via confocal microscopy [39]. This method was used to visually detect an increase in cellular uptake of ⁵⁹Fe-Ft upon incubation with Mas-7, due to lack of a good control for quantitative detection. Cells were biotinylated at the apical membrane by incubation with 0.5 mg/ml Sulfo-Link for 5 min at 4°C. The cells were then washed three times with ice-cold PBS. Biotinylated cells were incubated at 37°C with MEM containing 10% FBS for 30 min (with or without Mas-7). Medium was removed and cells were fixed using 3% paraformaldehyde in PBS for 15 min at room temperature. After a PBS wash, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, followed by three more washes with PBS. Biotin in endocytotic vesicles was detected by incubation with Alexa-488-fluor-streptavidin for 1 h at room temperature. After another PBS wash, cells were exposed to TOPRO for 30 min at room temperature to stain the nuclei and then washed again with PBS. Stained cells sealed under mounted cover slips were visualized by confocal microscopy using an Olympus BX50WI (Olympus America Inc, Melville, NY), with UPlanApo 60X oil lens (NA, 1.35). Digital images were captured using Bio-Rad Radiance 2100 confocal system, LaserSharp 2000 version 4.1.

2.5. Inhibition of endocytosis

Cells grown on polystyrene plates were incubated with 1 μM of radiolabeled Fe as Ft or FeSO₄ (negative control) and co-incubated with or without hypertonic medium containing 0.5

M sucrose for 1 h at 37°C. Medium was removed and cells were washed extensively with cold PBS. Cell-associated radioactivity was quantified in the gamma counter.

2.6. Inhibition of macropinocytosis

Cells on polystyrene plates were treated with 200 μM 5-(N,N-dimethyl)-amiloride for 60 min in PBS containing ^{59}Fe -Ft to block pinocytotic transport. Cells were washed extensively with PBS and solubilized in 1N NaOH. Cell-associated radioactivity was quantified in the gamma counter. Identical experiments were performed using ^{14}C -labeled dextran (0.2 $\mu\text{Ci}/\text{well}$) as positive control. Solubilized cells were diluted into 10 mL EcoLite, shaken vigorously and cell-associated radioactivity was quantified in a β -scintillation counter (Wallac 1410, liquid scintillation counter, PerkinElmer Life and Analytical Sciences, Shelton, CT).

2.7. Effect of Fe internalized from Ft or FeSO_4 on expression of genes involved in cellular Fe metabolism

Confluent Caco-2 cells were treated with 6 μM Fe as mineralized Ft or FeSO_4 (control) or Fe-deficient medium ($<0.1 \mu\text{M}$ Fe) for 24 h at 37°C. Total RNA was extracted and DMT1, FPN, HFE and TfR (transferrin receptor) mRNA levels were determined by real-time RT-PCR as described previously [40]. Data are presented as means $\pm$ SD, $n=3$ wells/treatment; each experiment was done in duplicate.

2.8. Statistical analysis

Results are presented as means $\pm$ SD ($n=3$). Statistical analysis was performed using GraphPad Prism v 4.0 (San Diego, CA). Unpaired Student's *t*-test (GraphPad Prism software) was used to determine whether treatment groups differed significantly from the control groups. Differences were considered significant when $P < 0.05$. Ft/ FeSO_4 uptake and the mRNA expression levels from all groups were subjected to one-way ANOVA and Tukey Test. Means with different letters are significantly different, $p<0.05$.

3. Results

3.1. Ft binding to the apical membrane is saturable and specific

We first determined that Ft binding decreased as cells became differentiated (Figure 1). As cells differentiate, their need for iron uptake decreases, with a corresponding decrease in Ft receptors. We wanted to use a cell stage that presented us with the highest number and density of receptors to ensure optimal binding. As a result, all experiments were conducted at a cell stage when the cells were confluent to ensure formation of tight junctions but had not yet differentiated. Cell growth and differentiation were constantly monitored visually by microscope and by transepithelial electrical resistance (TEER) measurements. Binding studies (Figure 2A) demonstrated saturable binding and Scatchard plot analysis resulted in a binding site density (B_{max}) of 6.28 pmol/ μg protein and a K_D of 1.6 μM (Figure 2B). Binding of radiolabeled Ft was reduced by ~60% when cells were exposed to excess (8-fold) unlabeled Ft (Figure 2A), demonstrating specific binding of Ft to Caco-2 cells.

3.2. Ft uptake into Caco-2 cells increases with time and reaches a maximum by 4 h

Exposing cells to either increasing concentrations (0.1–8.0 μM Ft) for 1 h or to a fixed concentration (1 μM Ft) over a period of 16 h demonstrated uptake being close to saturable at the 8 μM treatment (Figure 2C) and at about 8 h (Figure 2D). These observations lend support to a receptor-mediated uptake process.

3.3. Enhancing endocytosis significantly increases Ft uptake

Effects on Ft uptake were determined in cells treated with Mas-7, a G-protein stimulator. We determined that endocytotic stimulation significantly increased Ft uptake (~140% of control) (Figure 3A). Biotinylated Caco-2 cells were used as positive control and the stimulation of endocytosis with 50 μ M Mas-7 showed diffuse staining at the plasma membrane compared to control cells, confirming enhanced endocytosis (Figure 3B).

3.4. Blocking endocytosis significantly decreased Ft uptake

Treatment of cells with hypertonic medium containing 0.5 M sucrose significantly reduced Ft uptake (by ~70%) (Figure 3C). No effect was observed on FeSO_4 uptake, which is known to enter the enterocyte through a non-endocytotic mechanism via DMT-1.

3.5. Blocking macropinocytosis significantly reduced Ft-Fe uptake at high Ft concentrations

Inhibition of macropinocytosis with 200 μ M 5-(N,N-dimethyl)-amiloride significantly reduced Ft uptake (by ~20%) at Ft concentration of 4 μ M (Figure 4), while no effect was seen on the uptake of 1 μ M Ft. ^{14}C -dextran enters the enterocyte through macropinocytosis on account of its large size and hence was used as positive control for macropinocytosis. It also showed a significant decrease (~40%) when treated with 200 μ M amiloride.

3.6. Uptake of Ft-Fe affects expression of genes involved in cellular Fe metabolism in a manner different from that of FeSO_4

We evaluated the effects of Fe deficient medium and introducing Fe in the form of Ft or FeSO_4 on the expression of genes known to be involved in Fe metabolism in the enterocyte after a 24 h exposure. As previously known, DMT1 increased significantly in Fe deficient cells, however, its expression was unchanged in cells pretreated with Fe either in the form of Ft or FeSO_4 . FPN expression was significantly reduced in Fe-deficient cells and also in cells treated with Ft-Fe but not with FeSO_4 . HFE was dramatically increased in Fe-deficient cells as compared to cells treated with FeSO_4 , but cells treated with Ft-Fe had significantly lower levels of HFE mRNA. TfR expression was similar in cells treated with FeSO_4 , but significantly higher in cells treated with Ft. The underlying mechanisms leading up to the change in expression of these genes are not yet known, but taken together, these results show that Fe taken up by Ft affects the expression of genes involved in cellular Fe metabolism in a manner very different than that of Fe taken up from FeSO_4 .

4. Discussion

In this study, we show saturable binding of Ft and Scatchard plot analysis yielded a dissociation constant (K_D) of 1.6 μ M. While there are no previous data on Ft receptors in enterocytes, binding studies conducted on various other tissues and cell types have shown K_D values ranging from 10^{-7} to 10^{-9} M [41–45], with binding studies on isolated cell membranes showing higher affinity. It is thus possible that the enterocyte Ft receptor has a somewhat lower affinity for Ft than other cell types. On the other hand, the number of receptors per cell may be higher than in other cells as we found a receptor density of 6 pmol/ μ g protein and Hulet et al [44] found a density of only 18 fmol/ μ g protein in brain tissue. Thus, the small intestine may have a relatively large number of Ft receptors, with relatively low affinity. The intestine may therefore have a high capacity for Fe uptake from Ft, although this still needs to be shown in human studies.

We realize that Ft may not pass completely undigested through the gastrointestinal tract. Iron released from the core prior to its intestinal absorption is likely taken up by DMT-1 as discussed in our previous study [17]. The fate of partially digested Ft in the enterocyte is not yet known. We believe that changes in the protein structure may cause a change in receptor-ligand affinity.

How this might affect Ft uptake and the subsequent release of the remaining iron core into the enterocyte is not yet known and needs to be investigated in a different study. However, our previous *in vitro* digestion experiments showed that Ft may survive proteolytic degradation by pepsin and pancreatic enzymes under conditions similar to those in human gastrointestinal tract. Therefore, it is possible for some of the ingested protein to be presented in its intact form to intestinal cells. This study therefore expanded on this possibility and the results demonstrate that this intact Ft binds to the enterocytic surface, followed by its internalization into the enterocyte. Use of excess unlabeled Ft displaced the radiolabeled ligand, thereby indicating specific binding of the ligand. Saturation in the uptake rate of Ft into the cells over time and with increasing Ft concentrations also strongly indicates the presence of a receptor-mediated process. We speculate that given a constant concentration of the Ft ligand, its binding to the receptor might activate internalization of the ligand-receptor complex resulting in a decrease in the number of available cell-surface receptors over time. Similarly, given increasing concentrations of the Ft ligand, a greater number of receptors might complex with the ligand and become internalized, thereby decreasing the number available at the cell-surface. The presence of these mechanisms still remains to be explored.

Blight et al demonstrated receptor-mediated endocytosis of Ft in guinea-pig reticulocytes using electron microscopy [45,46]. Their studies revealed that reticulocytes have specific Ft receptors, causing Ft to bind to the cell surface at 4°C. At 37°C, Ft uptake occurred by endocytosis as Ft accumulated into coated pits which then invaginated to form intracellular vesicles. In a more recent study, Hulet et al identified a saturable and specific binding site in mouse brains [47]. Saturation binding analyses conducted using radiolabeled recombinant human Ft yielded a single class of binding sites found predominantly in the white matter with a K_D of 4.6×10^{-9} M. Although these studies were conducted using non-human tissues, they strongly support the kinetic and mechanistic results obtained in our study. To further verify the Ft uptake mechanism, we conducted experiments where endocytosis was either enhanced or blocked and effects on Ft uptake into cells were observed. Enhancement of endocytosis by Mas-7, a more potent analog of mastoparan (a G-protein activator) caused a significant increase in the uptake of Ft into the cells, providing stronger and more direct evidence in support of endocytosis. Hypertonic medium, on the other hand, has been shown to significantly decrease endocytosis by disrupting clathrin aggregation, thereby preventing internalization of the receptor-ligand complex. When using medium containing 0.5 M sucrose, Ft uptake significantly decreased, strongly suggesting clathrin-mediated endocytosis. This was suggested by the early microscopy analyses [45,46] and also shown in studies on rat oligodendrocyte progenitors using several inhibitors of clathrin-mediated endocytosis [44].

Further investigation into the endocytotic pathways revealed that macropinocytosis also plays a role in the uptake of Ft into enterocytes. However, macropinocytosis seems to be secondary mechanism, triggered only by very high concentrations of Ft in the digestive milieu. It is possible that when Ft is ingested within the context of a mixed plant or meat based diet, Fe may be taken up from Ft by endocytosis. However, in certain meals, such as when consuming foods based on liver, or if the Ft contents of plants can be significantly enhanced by targeted plant breeding or genetic engineering approaches, Fe may also be absorbed by macropinocytosis. Our human studies strongly suggest that Fe is well utilized from diets containing low concentrations of Ft; similar studies are needed for diets with a high content of Ft.

Our results strongly suggest that Ft is taken up by enterocytes via receptor-mediated endocytosis. The fact that expression of genes involved in cellular Fe metabolism is affected differently by Fe from Ft and from FeSO_4 also supports the presence of an uptake process different than that for ferrous Fe. We do not yet know how Ft is processed intracellularly or how Fe is released from Ft. It is possible that Ft is transported to a compartment where the

protein moiety is partially or completely degraded. The iron core of Ft is known to be poorly soluble, but possibly an acidic compartment may facilitate the release of ferrous Fe. It is known that “free” ferrous Fe as that taken up from FeSO₄ will affect expression of genes such as DMT1 by binding to iron-responsive elements (IREs)[33]. The manner by which or to what extent Fe taken up from Ft affects expression of Fe-regulated genes is not yet known. It is possible that Fe is bound to smaller fragments of ferritin protein/peptides, is present as partially soluble Ft iron core, or that the complete release of ferrous Fe from Ft is a slow process and therefore the timing of intracellular processing of this form of Fe is different from that for FeSO₄. These possibilities need to be explored in future studies.

In conclusion, the results from these experiments strongly indicate that enterocytes absorb Ft and that its uptake in these cells occurs via a receptor-mediated mechanism. The results also suggest that macropinocytosis, a secondary mechanism, might be activated at high Ft concentrations. Future research should focus on the regulation of the Ft receptor in the human small intestine to help provide insight into its role in Fe homeostasis.

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Figure 1.

Cells were treated with ^{59}Fe -Ft (1 μM) in serum-free medium for 8 h at 4°C at pre-confluent, confluent, 4 d post-confluent and 7 d post-confluent stages to determine the optimum treatment stage for subsequent experiments. Ft binding decreased as cells became differentiated. Data are presented as pmol bound Ft per μg protein and significant decrease ($p < 0.05$) in Ft binding is indicated with an asterisk.

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 2.

A. Cells treated with ^{59}Fe -Ft ($0.1\text{--}8\text{ }\mu\text{M}$) in serum-free medium for 8 h at 4°C . Data are presented as pmol apical bound Ft per μg protein. Binding specificity, obtained by co-incubation of cells with increasing concentrations of unlabeled Ft ($0.5\text{--}8\text{ }\mu\text{M}$), is also illustrated. B. Scatchard plot analysis of the binding study data. The y-axis represents bound/free ^{59}Fe -Ft and the x-axis represents pmol Ft bound to the apical membrane per μg protein. C. Cells were treated with increasing concentrations ($0.1\text{--}8\text{ }\mu\text{M}$ ^{59}Fe -Ft) in serum-free medium for 1 h at 37°C . Uptake approached saturation at close to $8\text{ }\mu\text{M}$ ^{59}Fe -Ft. Data are presented as pmol ^{59}Fe -Ft per μg protein. D. Cells were treated with $1\text{ }\mu\text{M}$ ^{59}Fe -Ft in serum-free medium

over a period of 16 h at 37°C. Uptake approached saturation by 4 h with the rate leveling off by 8 h. Data are presented as pmol ^{59}Fe -Ft per μg protein.

Figure 3A

Figure 3B

Figure 3C

Figure 3.

A. Cells were treated with 50 μ M Mas-7, a G protein activator and a highly potent analog of mastoparan, for 30 min. at 37° C in PBS containing 1 μ M ⁵⁹Fe-Ft. A significant increase ($p < 0.05$) in Ft uptake (~140% of control) compared to control cells is indicated by an asterisk. B. Endosome labeling using Sulfo-Link was used as positive control to assess endocytosis (uptake of ⁵⁹Fe-Ft upon incubation with Mas-7) by confocal microscopy. Caco-2 cells incubated with 50 μ M Mas-7 for 30 min, demonstrated more diffuse staining at the plasma membrane compared to biotinylated control cells, thereby indicating a significantly higher Ft uptake. C. Cells were incubated with either radiolabeled Ft (1 μ M) or FeSO₄ (1 μ M, negative control) and co-incubated with or without hypertonic media containing 0.5 M sucrose for 1 h

at 37°C. Significant decrease ($p<0.05$) in Fe uptake from Ft as compared to FeSO₄ in sucrose treated cells is indicated with an asterisk.

Figure 4.

Cells were treated with 200 μ M 5-(N,N-dimethyl)-amiloride for 60 min in PBS containing ^{59}Fe -Ft to block pinocytotic transport. Identical experiments were performed using ^{14}C -labeled dextran (0.2 $\mu\text{Ci}/\text{well}$) as positive control. Inhibition of macropinocytosis significantly ($p < 0.05$) reduced Ft uptake at a Ft concentration of 4 μM (indicated with an asterisk), while no effect was seen in the uptake of 1 μM Ft. Amiloride treated ^{14}C -dextran also showed a significant ($p < 0.05$) decrease as indicated with an asterisk.

Figure 5.

Caco-2 cells were treated with 6 μ M Fe as mineralized Ft or FeSO_4 (control) or Fe-deficient medium (<0.1 μ M Fe) for 24 h at 37°C. DMT1, FPN, and TfR (transferrin receptor) mRNA levels were determined by real-time RT-PCR. Data are presented as means $\pm$ SD, n=3 wells/treatment; each experiment was done in duplicate.