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Transferrin-iron and proinflammatory cytokines influence iron status and apical iron transport efficiency of Caco-2 intestinal cell line

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Endogenous factors that regulate the absorption of dietary iron remain unknown. Differentiated cultures of Caco-2 human intestinal cells grown on membrane inserts were used to study the characteristics of transferrin-iron uptake across the basolateral surface, the effects of transferrin-iron uptake on cellular ferritin content, the transport of apical iron across the monolayer, and the influence of proinflammatory cytokines on these processes. Caco-2 cells accumulated transferrin-iron from the basolateral chamber by a temperature-dependent, saturable process that was enhanced in less differentiated cultures and attenuated by pre-exposure to high-iron medium. Exposure of Caco-2 cells to 10 μmol/L diferric transferrin for 36 hr increased cellular ferritin protein 3.4-fold and decreased the transport of apical ^{59}Fe to the basolateral compartment by 45%. Pretreatment of cells with a combination of interleukin-1β, interleukin-6, and tumor necrosis factor-α increased transferrin-iron uptake by 70% and cellular ferritin content by 54%. Also, cytokine treatment decreased apical iron transport across the monolayer by 40% without altering paracellular transport of mannitol. These results suggest that transferrin-iron and proinflammatory cytokines are capable of modulating the iron status and iron transport activity of intestinal epithelial cells. (J. Nutr. Biochem. 8:585–591, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Mammalian iron (Fe) homeostasis is maintained primarily by regulating the absorption of this essential, but potentially toxic, trace metal in the proximal intestine.[1] The efficiency of intestinal absorption of dietary Fe is influenced by both exogenous factors and endogenous conditions. The impact of speciation of Fe in foods and the ability of diverse dietary components to enhance and impede the uptake of the metal from the lumen by enterocytes is well established.[2,3] It is also known that the transfer of Fe from the enterocyte to plasma is stimulated during periods of growth, pregnancy, lactation, hypoxia, and Fe deficiency, whereas high-Fe status and episodes of infection and inflammation are associated with decreased absorption of dietary Fe.[1] Despite extensive efforts, the identification of endogenous factors that modulate the process(es) of Fe absorption remain unknown.

The Caco-2 human cell line represents a useful model for investigating Fe transport and metabolism by intestinal absorptive epithelial cells. After reaching confluency, cultures of Caco-2 spontaneously differentiate into polarized cells that possess many of the ultrastructural and biochemical properties of mature enterocytes.[4] Previous studies with Caco-2 cells have focused primarily on the characteristics of Fe uptake across the apical surface and the expression of specific Fe-binding proteins. Apical uptake of Fe by Caco-2 is enhanced when the metal is reduced to ferrous state either

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by the addition of ascorbic acid[5–10] or by a ferrireductase activity associated with the apical surface.[7,9] In contrast, the addition of inositol phosphates[8,11] and citrate,[6] as well as previous exposure to high-Fe medium,[11] attenuates the cellular acquisition of apical Fe. Several investigators have identified transferrin receptors (TfRs) on the basolateral surface of Caco-2,[12–14] and others have reported that the level of cell ferritin increases in response to incubation of cultures in medium containing high levels of Fe^{3+}-nitrilotriacetic acid (NTA).[5,11] Alvarez-Hernandez et al.[5] reported that maintenance of monolayers of Caco-2 cells in low-Fe medium was associated with an increased transepithelial flux of Fe from the apical to the basolateral compartments. In contrast, Gangloff et al.[11] found that apical uptake, but not short-term transepithelial flux, of ferrous Fe was decreased when Caco-2 cells were maintained chronically in serum-free, high-Fe medium. Although there are some exceptions, the results obtained with Caco-2 cells generally have been similar to the well-described characteristics of Fe uptake and absorption by the intact intestine.

The general goal of our work is to use the Caco-2 cell line as a model to identify endogenous factors that regulate the absorption of dietary Fe. The specific objectives of this study were to define the characteristics of Tf-Fe uptake across the basolateral surface of the cell, to quantify cell ferritin content as the indicator of Tf-Fe accumulation, to examine whether accumulation of Tf-Fe influenced the transfer of apical Fe across the monolayer, and to test the influence of interleukins-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα) on these processes.

Methods and materials

Reagents

Hanks' balanced salt solution (HBSS), tissue culture medium, fetal calf serum (FCS), and glutamine were obtained from Sigma Chemical (St. Louis, MO). Gentamicin and fungizone were purchased from GIBCO (Grand Island, NY). $^{59}FeCl_3$ (specific activity 9.5 GBq/mmol of Fe/mL) and D-[1-^{14}C]mannitol (specific activity 2.04 GBq/mmol) were obtained from Dupont–New England Nuclear (Boston, MA). Human apotransferrin (apo-Tf) and recombinant human IL-6 were purchased from Boehringer Mannheim (Indianapolis, IN). Recombinant human IL-1β and IL-2 were purchased from Genzyme (Cambridge, MA). Recombinant human TNFα was a generous gift from Dr. Phil Pekala, East Carolina School of Medicine. Human liver ferritin, polyclonal rabbit anti-human ferritin, and horseradish peroxidase (HRP)–labeled rabbit anti-human ferritin were purchased from Dako Corporation (Santa Barbara, CA).

Cells

Caco-2 human colonic cells were obtained from American Type Culture Collection (Rockville, MD) and used between passages 25 and 40. Stock cultures were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% FCS, 25 mmol/L glucose, 2 mmol/L glutamine, 50 mg/L gentamicin, and 0.25 mg/L fungizone at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Caco-2 cell monolayers were grown and maintained on Falcon Cyclone membrane inserts (3 μm pores; Becton, Dickinson and Company, Lincoln Park, NJ) in either 6- or 12-well dishes as previously described[10] except that the concentration of FCS in medium was decreased 2 days before use (see below). Cultures were used to study Fe uptake and transport 10–15 days after reaching confluency unless noted otherwise. Cells were fully differentiated, as evidenced by maximal activities of sucrase and alkaline phosphatase[4] and a minimal rate (<0.1%/hr · cm^2) of paracellular flux of ^{14}C-mannitol from the apical to the basolateral chamber.[7]

Two days before initiating experiments, the level of FCS in complete medium was reduced from 10% to 0% and 2% in the medium added to the apical and basolateral compartments, respectively. Medium with the lower level of FCS was added to the basolateral compartment since others have reported that the permeability of the monolayer is increased when cultures are maintained chronically in serum-free medium[11] and medium with high Fe.[5,11] Preliminary studies showed that the rate of transfer of ^{59}Fe (added as 10 μmol/L Fe-NTA) from the apical to the basolateral chamber was inversely related to serum content of the maintenance medium. The rates of transepithelial transport of ^{59}Fe into the basolateral compartment by cultures incubated for 2 days in medium containing 2% and 10% FCS ranged from 4.9 ± 0.5 to 6.5 ± 0.7 (n = 4 experiments) and from 1.4 ± 0.1 to 1.6 ± 0.2 (n = 7 experiments) pmol/(hr · cm^2), respectively. The concentrations of Fe in medium containing 2% and 10% FCS were 1.9 and 5.2 μmol/L, respectively, as determined by flame atomic absorption spectrophotometry. In contrast to the impact of medium FCS on apical to basolateral transport of Fe, paracellular flux of ^{14}C-mannitol from the apical to the basolateral compartment was independent of the amount of FCS in the pretest medium (i.e., 0, 2, or 10% FCS).

For experiments investigating the effects of proinflammatory cytokines on the transport and metabolism of Fe, spent medium in the basolateral compartment of differentiated cultures (10–15 days post-confluency) was replaced with DMEM containing 2% FCS, antibiotics, and indicated quantities of IL-1β, IL-6, and TNFα. Serum-free DMEM without cytokine(s) was added to the apical compartment. After overnight incubation, cultures were washed three times with HBSS before beginning experiments.

Preparation of radiolabelled (^{59}Fe) diferric Tf

Apo-Tf (22.8 mg) was dissolved in 0.84 mL elution buffer (150 mmol/L NaCl, 25 mmol/L HEPES, 5 mmol/L $NaHCO_3$, pH 7.4). ^{59}Fe-NTA complex was prepared by adding 10 μL of 234 mmol/L NTA to an acidified solution (150 μL) containing 0.585 μmol $^{59}FeCl_3$ with 5.6 MBq ^{59}Fe. To prepare $^{59}Fe_2$-Tf, aliquots (30 μL) of ^{59}Fe-NTA solution were added to the apo-Tf solution and mixed well before subsequent additions. The final solution (1 mL) containing 585, 2340, and 292 μmol/L Fe, NTA, and Tf, respectively, was incubated at 37°C for 30 min. To separate ^{59}Fe-Tf from residual ^{59}Fe-NTA complex, we applied the solution to a column (2.5 × 60 cm) containing Sephadex G-25 (Pharmacia, Piscataway, NJ) previously equilibrated with elution buffer. Void volume was collected and analyzed spectrophotometrically. The ratio of A_{465}/A_{280} was 0.046 for these preparations, indicating complete saturation of the two high-affinity Fe-binding sites[15] and is referred to as $^{59}Fe_2$-Tf. Nonradiolabelled Fe_2-Tf was prepared as above, except that ^{59}Fe was not added to the $FeCl_3$ solution.

^{59}Fe uptake from $^{59}Fe_2$-Tf

Spent medium was removed from the apical and basolateral chambers of differentiated cultures, and monolayers were washed three times with DMEM at 37°C. DMEM (1.5 mL) containing 0.2% bovine serum albumin (BSA) instead of FCS and indicated concentrations of $^{59}Fe_2$-Tf (0.2–1.8 μmol/L) was added to basolateral chamber. The apical chamber contained 1.0 mL of DMEM without FCS or BSA. After incubation for 2 hr, medium was removed from both chambers and the cell monolayer was washed

three times with 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L HEPES (pH 7.0) at 0°C to remove ^{59}Fe nonspecifically bound to cell surface. Cells were collected and sonicated as described previously.[10] ^{59}Fe was measured by γ-ray spectrometry.

Cellular uptake and transepithelial transport of apical ^{59}Fe

Methods to assess cellular uptake and transport of apical ^{59}Fe (as 10 μmol/L ^{59}Fe-$(NTA)_2$) to the basolateral chamber have been described.[10]

Cellular ferritin

Ferritin was used as an indicator of cellular Fe status. The amount of ferritin protein was quantified by a "sandwich" enzyme-linked immunoadsorbant assay (ELISA). In brief, wells of 96-well flat-bottomed microtiter plates were coated by addition of 100 μL of polyclonal rabbit anti-human ferritin (IgG fraction, 10 mg/L) in 10 mmol/L phosphate buffer (pH 7.2) and incubated overnight at 4°C. Wells were washed extensively with 10 mmol/L phosphate buffer (pH 7.2) containing 0.1% Tween 20 to remove unbound materials before the addition of diluted test samples and standards (100 μL). After incubation for 2 hr at room temperature, wells were washed five times with 10 mmol/L phosphate buffer with 0.1% Tween 20. Next, 100 μL HRP-labeled rabbit anti-human ferritin (IgG fraction, 65 μg/L) was added to wells. The dish was incubated for 1 hr at room temperature before adding 100 μL of 1,2-phenylenediamine dihydrochloride (4 mmol/L) as substrate. HRP-dependent product formation was quantified by monitoring A_{492} (TECAN Model ATCC 340 plate reader) after quenching the reaction with 150 μL of 1 mmol/L sulfuric acid. Using highly purified human liver ferritin as a standard, the limit of detection was 0.2 ng of human ferritin and absorption was linear between 0.2 and 4 ng.

Analysis of data

Each treatment was assessed in three to four replicate wells for an experiment and each experiment was repeated two to four times. Data (means ± SE) are presented from a representative experiment since absolute values for repeated experiments using cultures at different passages varied by 10–20%. Data from an experiment were analyzed using the general linear model or Student's *t*-test for unpaired samples, as appropriate.[16] Statistically significant differences ($P < 0.05$) shown for the presented experiment were observed for all replicated experiments.

Results

Uptake of Fe from diferric Tf (Fe_2-Tf)

In the initial experiment, DMEM containing 0.2% BSA and 0.45 μmol/L $^{59}Fe_2$-Tf was added to the basolateral chamber of differentiated cultures of Caco-2 and the cellular accumulation of ^{59}Fe was monitored hourly. Cellular acquisition of ^{59}Fe by cultures incubated at 37°C was linear between 0 and 5 hr with a mean rate of 3.4 ± 0.04 pmol (hr · mg protein)$^{-1}$ (*Figure 1A*). This rate was 5.5-fold higher than that observed in cultures maintained at 0°C. Tf-Fe uptake increased linearly ($r = 0.996$) as the concentration of diferric Tf (Fe_2-Tf) in the basolateral chamber was elevated from 0.2 and 1.8 μmol/L (*Figure 1B*). The amount of ^{59}Fe accumulated by Caco-2 cells when $^{59}Fe_2$-Tf was added to the apical compartment was <2% that in cells exposed to similar concentration of $^{59}Fe_2$-Tf in basolateral medium for

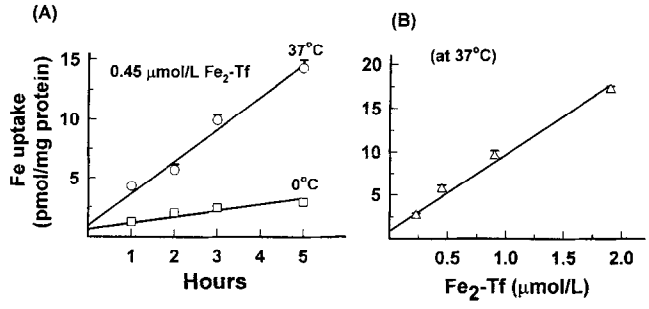

Figure 1 Characteristics of ^{59}Fe uptake from diferric transferrin (Fe_2-Tf) by differentiated cultures of Caco-2 cells. (A) ^{59}Fe uptake from $^{59}Fe_2$-Tf by differentiated cultures of Caco-2 is dependent on temperature and length of incubation. Monolayers of differentiated cultures were washed three times with DMEM at 37°C before addition of DMEM (1.5 mL) containing 0.2% BSA and 0.45 μmol/L of $^{59}Fe_2$-Tf to the basolateral chamber. The apical chamber contained 1.0 mL of DMEM only. Cultures were incubated for 1–5 hr at either 37°C or 0°C and washed three times with 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L HEPES (pH 7.0). Cellular ^{59}Fe content was measured as described in Materials and Methods. The rates of ^{59}Fe uptake at 0°C and 37°C were 0.52 ± 0.04 and 3.4 ± 0.04 pmol (hr · mg protein)$^{-1}$, respectively. (B) ^{59}Fe uptake from $^{59}Fe_2$-Tf is concentration dependent. Conditions were the same as for (A) but cultures were incubated with indicated concentrations of $^{59}Fe_2$-Tf (0.2–1.8 μmol/L) for 2 hr at 37°C. Serum levels of Tf in humans are approximately 30 μmol/L with 30% saturation of Fe. Values are means ± SE from a representative experiment with three replicate wells for each test concentration.

the same period (data not shown). The addition of 100 μmol/L nonradiolabelled Fe_2-Tf to basolateral medium containing 0.45 μmol/L $^{59}Fe_2$-Tf blocked cellular uptake of ^{59}Fe by >99%, indicating that the uptake process was saturable. The degree of cellular differentiation and Fe status affected the ability of cells to acquire ^{59}Fe from $^{59}Fe_2$-Tf. Newly confluent cultures of Caco-2 (3–4 days post-confluency) accumulated 2.5–4 times more ^{59}Fe per hour from $^{59}Fe_2$-Tf than fully differentiated cultures (10–15 days post-confluency). Furthermore, preincubation (20 hr) of fully differentiated cultures in high-Fe medium (20 μmol/L Fe(III)-NTA_2 in apical compartment) decreased Tf-Fe uptake by 30% (13.9 ± 0.3 and 9.5 ± 1.4 pmol/mg protein for control and Fe-NTA-treated cultures, respectively, exposed to 0.75 μmol/L $^{59}Fe_2$-Tf for 2 hr).

Effects of Tf-Fe uptake on cell ferritin content and apical Fe transport

The quantity of ferritin protein in differentiated cultures of Caco-2 maintained in complete DMEM with 10% FCS was 2–3 ng/mg protein. Exposure to 10 μmol/L Fe_2-Tf for 24–36 hr significantly ($P < 0.05$) increased the ferritin content of the cells (*Figure 2A*). Cellular ferritin protein content increased in a curvilinear manner as the concentration of Fe_2-Tf in the basolateral chamber was elevated from 0 to 20 μmol/L (*Figure 2B*). Cellular ferritin content also was significantly increased after overnight incubation of cultures in medium containing 5–25 μmol/L Fe-NTA_2 in the apical chamber (*Figure 2C*). At comparable extracellular concentrations, apical Fe-NTA was more effective than basolateral Fe_2-Tf at increasing the cellular concentration of ferritin.

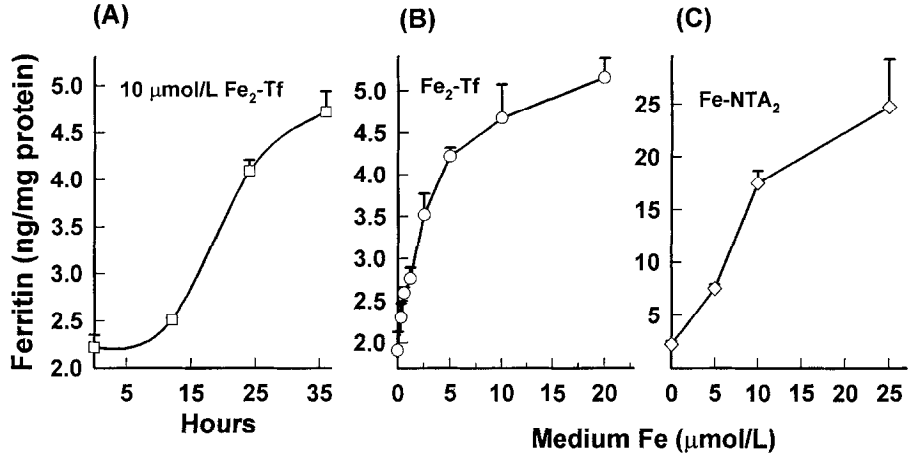

Figure 2 Influence of concentration of diferric transferrin (Fe_2-Tf) in basolateral chamber on ferritin protein levels in Caco-2 cultures. (A) Effect of length of exposure to Fe_2-Tf on the levels of ferritin protein in Caco-2 cells. Differentiated cultures of Caco-2 cells (10–15 days post-confluency) were incubated for 0–36 hr at 37°C with complete DMEM containing 2% FCS plus 10 μmol/L Fe_2-Tf in the basolateral chamber. At designated times, medium was removed and monolayers were washed three times with HBSS at 0°C. Cells were harvested in 10 mmol/L phosphate buffer (pH 7.2) containing 0.1% SDS. Cellular ferritin levels were measured as described in Materials and Methods. The basal levels of ferritin protein were 2–3 ng/mg protein in 10–15 days post-confluent cultures of Caco-2 maintained in complete DMEM with 10% FCS. Concentrations of cell ferritin differ significantly ($P < 0.05$) from control at 24 and 36 hr. (B) Effect of concentration of Fe_2-Tf in the basolateral chamber on ferritin protein in Caco-2 cells. Differentiated cultures of Caco-2 cells were incubated for 20 hr at 37°C with complete DMEM containing 2% FCS and indicated concentrations of Fe_2-Tf in the basolateral chamber. Addition of ≥0.6 μmol/L Fe_2-Tf significantly ($P < 0.05$) increased cellular ferritin content above control level. (C) Influence of apical Fe-$(NTA)_2$ on ferritin protein content in Caco-2 cells. Differentiated cultures of Caco-2 cells were incubated with medium containing DMEM with indicated concentrations of Fe-$(NTA)_2$ in the apical chamber with DMEM plus 2% FCS in basolateral chamber for 20 hr at 37°C. Cellular ferritin protein significantly ($P < 0.05$) increased by addition of ≥5.0 μmol/L Fe-$(NTA)_2$ to apical compartment. All values represent means ± SE from a representative experiment with three wells per treatment.

The impact of exposure of Caco-2 to Fe_2-Tf on the transport of ^{59}Fe from the apical to the basolateral chamber was examined. Preincubation of cultures in medium containing 10 μmol/L Fe_2-Tf in the basolateral chamber for 36 hr decreased the transport of ^{59}Fe from the apical to the basolateral chamber by 45% (*Table 1*). In contrast, paracellular transport of ^{14}C-mannitol from the apical to the basolateral chamber was similar ($P > 0.05$) for cultures that had been preincubated in medium without or with Fe_2-Tf (i.e., 0.064 ± 0.001 and 0.067 ± 0.004% (hr · cm)$^{-1}$, respectively). The decreased transport of ^{59}Fe from the apical to the basolateral chamber in cultures pretreated with Fe_2-Tf was associated with a 3.4-fold elevation in the cellular level of ferritin (*Table 1*).

Table 1 Overnight exposure of Caco-2 cultures to diferric transferrin (Fe_2-Tf) increases cellular ferritin and decreases transport of apical ^{59}Fe to the basolateral chamber

Treatment	^{59}Fe transport (apical to basolateral) (pmol(h · mg protein)$^{-1}$)	Cell ferritin (ng/mg protein)
Control	16.4 ± 1.6	2.9 ± 0.4
With 10 μmol/L Fe_2-Tf	9.1 ± 0.5*	12.7 ± 1.3*

Differentiated cultures of Caco-2 (10–15 days post-confluency) were preincubated in medium containing DMEM with 2% FCS plus either 0 or 10 μmol/L Fe_2-Tf in the basolateral compartment and DMEM only in the apical compartment for 36 hr at 37°C. Monolayers were washed three times with HBSS at 37°C. After the addition of 10 μmol/L ^{59}Fe-$(NTA)_2$ to the apical compartment, the quantity of ^{59}Fe transferred into the basolateral compartment containing DMEM with 2% FCS was monitored hourly for 5 hr. Cellular ferritin was quantified at the end of the transport study. Data are means ± SE for four membrane inserts per treatment from a representative experiment. The asterisk indicates that mean values for the two treatment groups differ significantly ($P < 0.05$) from controls.

Effects of proinflammatory cytokines on Fe transport and cell ferritin

Initially, cultures were incubated for 20 hr in medium containing 2% FCS and various concentrations (0–30 ng/mL) of either IL-1β, IL-6, or TNFα in the basolateral compartment to examine their influence on the cellular uptake of ^{59}Fe from $^{59}Fe_2$-Tf (0.45 μmol/L). Uptake of ^{59}Fe-Tf by cultures pretreated with 10 and 30 ng/mL IL-1β was significantly ($p < 0.05$) greater than that by the control (*Table 2*). Although pretreatment of cultures with 3–30 ng/mL IL-6 increased cellular uptake of Tf-Fe compared to the control, the difference was statistically significant ($P < 0.05$) only at the highest concentration of the cytokine tested (i.e., 30 ng/mL). Addition of either TNFα (*Table 2*) or 30 ng/mL IL-2 (data not shown) to cultures did not affect cellular acquisition of Tf-Fe. Tf-Fe uptake was increased 70% after exposing cultures to medium containing 30 ng/mL of both IL-1β and IL-6 and 500 U/mL TNFα (referred to as a cytokine "cocktail").

Cell ferritin content was 54% higher in cultures treated with the cytokine cocktail than in control cultures (6.0 ± 0.07 vs. 3.9 ± 0.03 ng/mg protein, respectively; $P < 0.05$). Exposure to the cytokine cocktail also decreased the degree to which cell ferritin content declined after Fe-loaded cells were transferred to low-Fe medium. Cultures were loaded with Fe by incubating overnight in medium containing 2%

Table 2 Effect of pretreatment of Caco-2 cells with proinflammatory cytokines on cellular uptake of Tf-Fe

Treatment	Cytokine concentration (quantity/mL)	Fe uptake (pmol/mg protein)
Control	–	5.34 ± 0.26
With IL-1β	3 ng/mL	6.25 ± 0.27
	10 ng/mL	7.42 ± 0.27*
	30 ng/mL	7.69 ± 0.60*
With IL-6	3 ng/mL	6.19 ± 0.21
	10 ng/mL	5.77 ± 0.11
	30 ng/mL	6.78 ± 0.05*
With TNFα	170 U/mL	5.23 ± 0.05
	500 U/mL	5.61 ± 0.11
With IL-1β, IL-6, and TNFα	30 ng/mL each 500 U/mL	9.08 ± 0.48*

Post-confluent (10–15 days) cultures of Caco-2 were incubated in medium containing 2% FCS and indicated quantities of IL-1β, IL-6, and TNFα in the basolateral compartment. DMEM only was added to the apical compartment. After 24 hr, cell monolayers were washed three times with HBSS and the basolateral uptake of Tf-Fe from medium containing 0.45 μM ^{59}Fe-Tf after 2 hr was measured as described in the legend to *Figure 1*. Data are means ± SE from a representative experiment with three wells for each test variable. The asterisk indicates that the mean value differs significantly ($P < 0.05$) from control.

Table 3 Influence of proinflammatory cytokines on Fe status of Caco-2 cells and the efficiency of transport of apical ^{59}Fe across the monolayer

	Control	With proinflammatory cytokines
Apical to basolateral transport of ^{59}Fe (pmol/hr · mg protein)	19.9 ± 0.6	11.9 ± 0.7*
Cellular ^{59}Fe (pmol/mg protein)	272 ± 14	322 ± 15*
Cellular ferritin (ng/mg protein)	10.5 ± 0.2	12.7 ± 0.2*

To examine the impact of exposure to proinflammatory cytokines on the rate of ^{59}Fe transfer from the apical to the basolateral compartment, cultures of Caco-2 cells were incubated initially in medium containing DMEM with 2% FCS alone or plus IL-1β (30 ng/mL), IL-6 (30 ng/mL), and TNFα (500 U/mL) in the basolateral compartment. After overnight incubation (16 hr), 10 μmol/L Fe_2-Tf was added to the basolateral compartment and cultures were incubated for an additional 20 hr. Monolayers were washed and the transport of ^{59}Fe (added as ^{59}Fe-$(NTA)_2$) from the apical to the basolateral compartment was monitored as described in *Table 1*. Cellular content of ^{59}Fe and ferritin content were assessed after 5 hr incubation. Data are means ± SE for a representative experiment using three wells per treatment. The asterisk indicates that values differ significantly ($P < 0.05$) from control.

FCS and 20 μmol/L Fe-$(NTA)_2$ in the apical chamber. This treatment increased the cellular level of ferritin to 33 ± 5 ng/mg protein. After removal of the high-Fe medium, cultures were washed with buffer containing 5 mmol/L bathophenanthroline disulfonic acid and 5 mmol/L sodium dithionite to remove residual Fe from the cell surface.[17] Serum-free DMEM was added to the apical chamber and DMEM containing 2% FCS without or with the cytokine cocktail in the basolateral chamber for 24 hr. The presence of the proinflammatory cytokines attenuated the decline in the level of cellular ferritin in response to incubation in the low-Fe medium (12.4 ± 0.9 vs. 7.0 ± 0.8 ng/mg protein in cytokine-treated and control cultures, respectively; $P < 0.05$).

The influence of the proinflammatory cytokines on the transport of apical Fe was considered. Cultures of Caco-2 were preincubated with or without the cytokine cocktail for 36 hr, and Fe_2-Tf (10 μmol/L) was added to the basolateral chamber for the final 20 hr of this pretreatment period. Exposure of cultures to the proinflammatory cytokines decreased the rate of apical ^{59}Fe transport across the monolayer by 40% (*Table 3*), but did not significantly alter ($P > 0.05$) paracellular transport of ^{14}C-mannitol (data not shown). Decreased transfer of apical ^{59}Fe across the monolayer of cytokine-treated cultures was associated with an 18% increase ($P < 0.05$) in the cellular level of ^{59}Fe and 21% higher quantity of ferritin ($P < 0.05$).

Discussion

While the transfer of newly acquired Fe from the enterocyte to the plasma has been widely investigated, the possibility that enterocytes take up endogenous Fe from plasma has received limited attention. The delivery of plasma Fe to cells is normally mediated by the endocytic Tf receptor (TfR) pathway.[15] TfRs have been identified on the basolateral membrane of epithelial cells lining the intestinal villi of rodents[18,19] and humans.[20] The number of TfRs on the basolateral surface is inversely correlated with the degree of cell maturation, i.e., proliferating cells in the crypt that possess numerous TfRs on the basolateral surface, whereas the numbers of TfRs on columnar epithelial cells lining the villus are low.[18–20] TfR expression by villus epithelial cells also is affected by whole-body Fe status and the concentration of intestinal Fe.[21,22] Some data support the participation of the TfR in the transfer of endogenous Fe to enterocytes. Intact ^{125}I-Tf was detected in intestinal epithelial cells following intravenous injection of the radiolabelled protein; higher levels of ^{125}I-Tf were present in crypt cells compared to absorptive epithelial cells.[21] Moreover, TfR and Tf have been detected in coated pits and endosomes in the basolateral region of mouse enterocytes, suggesting endocytosis of the receptor–ligand complex.[19] Previous reports have indicated that human intestinal Caco-2 cells express TfR on the basolateral surface[13,14] and internalize the Fe transport protein by endocytosis.[12]

Our studies show that differentiated cultures of Caco-2 acquire Tf-Fe from the basolateral compartment. Tf-Fe uptake was temperature dependent, saturable, and inversely related to the degree of differentiation. Tf-Fe uptake was decreased when cells were pretreated in high-Fe medium (20 μmol/L Fe-NTA), suggesting that expression of TfR in Caco-2, as in rat intestinal epithelium, is influenced by cellular Fe status.[23] Moreover, the acquisition of Tf-Fe by Caco-2 cells was associated with a dose-dependent increase in the cellular level of ferritin protein. This finding complements previous reports that the addition of Fe salts to the apical compartment of Caco-2 cultures increased cellular content of ferritin.[2,11,13] We also found that the transport of low-molecular weight non-heme Fe from the apical to the

basolateral compartment was decreased significantly when Caco-2 cells had elevated ferritin after preincubation in medium containing Fe_2-Tf. Although this observation suggests that the enterocyte "senses" whole-body Fe status via the degree of Tf-Fe saturation, we cannot exclude the possibility that apical Fe was diluted by the increased pool of cellular Fe. Recent studies by Tapia et al.[24] with Caco-2 cells have suggested a casual relationship among intracellular Fe content, ferritin, and the regulation of Fe absorption.

Dietary Fe absorption is decreased by infection and inflammation.[25,26] Marked changes in the transport and metabolism of endogenous Fe, such as hypoferremia and elevated levels of ferritin in liver and plasma, are well-recognized responses of the host to acute and chronic episodes of such conditions.[27] *In vitro* studies have demonstrated that proinflammatory cytokines directly modulate the uptake of Tf-Fe and the synthesis of TfR and ferritin in a cell-specific manner. For example, treatment with IL-1β, IL-6, and TNFα increased synthesis of TfR, Tf-Fe uptake, and ferritin synthesis in HepG2.[28] In contrast, treatment with IL-1β and TNFα decreased TfR synthesis and Tf-Fe uptake in the monocytic cell line U937.[29] We are not aware that the potential roles of cytokines as modulators of Fe absorption has been tested previously. Recent studies have demonstrated the presence of cytokine receptors on the basolateral surface of enterocytes[30] and intestinal cell lines.[31,32] In addition, the gut immune system contains up to 40% of total immune cells in the body and the basolateral surface of the enterocyte is exposed to relatively high levels of cytokines in response to localized, as well as systemic, infection and inflammation.[33]

Our data demonstrate that exposure of cells to IL-1β and IL-6, but not TNFα, increased Tf-Fe uptake. Rafferty et al.[34] have suggested that TNFα may require other cytokines to be activated, although Mehran et al.[35] have reported that high concentrations (>100 ng/mL) of TNFα decreased lipid and lipoprotein secretion by Caco-2 cells. Treatment of cultures with all three cytokines decreased the transfer of apical Fe across the monolayer while increasing the cellular content of Fe newly acquired from the apical compartment. These data provide preliminary support that the mucosal block of Fe absorption that occurs during infection and inflammation is mediated by proinflammatory cytokines. More detailed investigations are now required to delineate specific influences of the cytokines on the synthesis of TfR and ferritin and on the apical uptake, intracellular distribution, and utilization of Fe by Caco-2 cells. It is also interesting to speculate that chronically elevated levels of proinflammatory cytokines associated with intestinal parasitic infections and inflammatory bowel disease may contribute to the widespread incidence of Fe deficiency.

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