Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins *in vivo* and *in vitro*

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Maternal iron deficiency during pregnancy induces anaemia in the developing fetus; however, the severity tends to be less than in the mother. The mechanism underlying this resistance has not been determined. We have measured placental expression of proteins involved in iron transfer in pregnant rats given diets with decreasing levels of iron and examined the effect of iron deficiency on iron transfer across BeWo cell layers, a model for placental iron transfer. Transferrin receptor expression was increased at both mRNA and protein levels. Similarly, expression of the iron-responsive element (IRE)-regulated form of the divalent metal transporter 1 (DMT1) was also increased. In contrast, the non-IRE regulated isoform showed no change in mRNA levels. Protein levels of DMT1 increased significantly. Iron efflux is thought to be mediated by the metal transporter protein, IREG1/ferroportin1/MTP1, and oxidation of Fe(II) to Fe(III) prior to incorporation into fetal transferrin is carried out by the placental copper oxidase. Expression of IREG1 was not altered by iron deficiency, whereas copper oxidase activity was increased. In BeWo cells made iron deficient by treatment with desferrioxamine ('deferioxamine'), iron accumulation from iron-transferrin increased, in parallel with increased expression of the transferrin receptor. At the same time, iron efflux also increased, showing a higher flux of iron from the apical to the basolateral side. The data show that expression of placental proteins of iron transport are up-regulated in maternal iron deficiency, resulting in an increased efficiency of iron flux and a consequent minimization of the severity of fetal anaemia.

Key words: caeruloplasmin, divalent metal transporter 1, hephaestin, IREG1, placenta, transferrin receptor.

INTRODUCTION

Anaemia during pregnancy can result in serious problems both for the mother and for her developing fetus. Many studies have shown that it induces fetal growth retardation, and that the effects generated *in utero* and during early development can persist into adulthood (e.g. [1–3]). The degree of deficiency seen in the fetus is not always as severe as that in the mother, but the mechanisms of adaptation which result in the amelioration have not been identified.

During pregnancy, in species with haemochorial placentas, iron is transferred from mother to fetus across the placenta. Some of the stages in the process have been characterized: uptake at the brush border membrane is from iron-transferrin, which binds to the transferrin receptor [4–6]; the complex is internalized into endosomes [7,8]; and iron is released and is transferred to the cytoplasm, presumably through divalent metal transporter 1 (DMT1). Less is known about subsequent processes. The mechanisms of transfer across the syncytiotrophoblast cytoplasm, from apical to basolateral side, have not been characterized. Whether there are chaperone proteins, performing the same function as those identified for copper [9,10], is not known.

We now know a little more about the possible efflux pathway, however. Efflux is probably through the metal transporter protein, IREG1/ferroportin1, as Fe(II) [11–13] (where IRE is iron-responsive element). Investigations on mouse placenta suggest that the protein is located on the basolateral membrane of the syncytiotrophoblast [11]; this has yet to be confirmed in other species. For iron to be incorporated into fetal transferrin, it must be oxidized from Fe(II) to Fe(III). In gut, a protein called hephaestin, a copper oxidase similar to serum caeruloplasmin, probably carries out the oxidase function [14]. We have identified a copper oxidase in placental membranes [15], which we suggest performs the same function, oxidizing Fe(II) to Fe(III), prior to incorporation into fetal transferrin.

In most cells and tissues, iron levels are regulated by iron-regulatory proteins (reviewed in [16]). These bind to IREs on either the 3′-end or the 5′-end of the mRNA. Generally, binding to the 3′-end stabilizes the mRNA and increases translation, whereas binding to the 5′-end inhibits translation (e.g. the transferrin receptor IRE is located at the 3′-end, whereas that of ferritin is at the 5′-end). This is not always the case, however. The IRE for IREG1 is at the 5′-end, and yet translation is increased in iron deficiency in the gut [12].

In order to determine the mechanism(s) of adaptation in the placenta, we have examined the expression of proteins involved in iron transfer in animals given a range of iron-deficient diets. We have collected samples at day 21 of gestation, since this is the period of greatest transfer [4], and hence any changes induced by the diet should be to be maximally expressed.

The effect of altered expression of placental proteins on iron flux across the placenta cannot readily be studied *in vivo*. The number, nature and location of iron pools mean that equilibration with an injected tracer is complex, and the specific activity at different times cannot easily be calculated. Nonetheless, limited data can be obtained using this approach. Several groups, including our own, have examined iron flux in pregnant rats by

Abbreviations used: IRE, iron-responsive element; DMT1, divalent metal transporter 1; BSS, balanced salt solution; DFO, desferrioxamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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injecting ^{59}Fe-labelled transferrin [4]. Iron movement is unidirectional, from mother to fetus, and the absence of a fetus does not alter uptake kinetics into the placenta [4]. Consequently, cell-culture models can be used to study the uptake pathways for iron [4,7]. More recently [17–19], it has been shown that cells in culture can also be used to study efflux pathways. We have, therefore, used BeWo cells in culture to examine the effect of iron status on iron uptake and efflux, as a method for correlating the expression data and the relative concentration data with iron transfer across the placental barrier.

EXPERIMENTAL

Experimental animals and tissue collection

Experiments were performed using weanling female rats of the Rowett hooded lister strain. Female weanling rats ($n = 40$) were fed control diet for 2 weeks, before being randomized into four groups: the first group, 16 animals, remained on control diet (50 mg/kg iron diet), whereas the remaining three groups, 8 animals each, were placed on experimental diets of decreased iron content, 37.5 mg/kg, 12.5 mg/kg and 7.5 mg/kg iron diet. All groups were fed these diets for 4 weeks prior to mating. The rats were mated with males of the same strain. The female rats were maintained on the same diet throughout pregnancy and killed at day 21 of gestation. Dams were killed by stunning and cervical dislocation. Placentas from eight fetuses, chosen from each mother at random, were rapidly dissected, weighed, and frozen in liquid nitrogen, before being stored at -70 °C. All experimental procedures were approved and conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986.

cDNA probes and antibodies

Rat-specific DMT1-IRE, DMT1-non-IRE and transferrin receptor probes, 402 bp, 373 bp and 701 bp respectively, were prepared by reverse transcriptase-PCR from rat placenta RNA using standard protocols and primers designed from rat sequences. The sense and antisense primers, respectively, corresponded to bases 2112–2131 and 2494–5013 of rat natural resistance-associated macrophage protein 2 (DMT1) (GenBank® accession number AF008439), 1644–1663 and 1998–2036 of rat natural resistance-associated macrophage protein 2 (DMT1) alternative splice variant (GenBank® accession number AF029757) and 839–858 and 1520–1539 of rat transferrin receptor (GenBank® accession number M58040). A human transferrin-receptor-specific probe was obtained from the U.K. Human Genome Mapping Project resource centre, I.M.A.G.E. consortium Clone ID 1160912 [20]. Sequence and Northern-blot analysis determined specificity of all probes before being used for slot-blot analysis.

For semi-quantitative PCR, primer sequences were determined from the published cDNA sequences for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank® accession number NM008084) 5′-ATGTCGTGGAGTCTACTGG-3′ and 5′-CTGAGCCGTATTCATTGTG-3′ and mouse IREG1 (GenBank® accession number AF231120) 5′-CATGACTGTATCACTACAGGGTACGC-3′ and 5′-AATCAAAGGACCAAAGACCGATTC-3′.

A commercially available mouse anti-rat CD71 (Serotec, Oxford, U.K.) antibody was used for the detection of transferrin receptor protein. Antibodies to DMT1 were raised in rabbits against the N-terminal of human DMT1, corresponding to amino acids 11–34, DDSVSGDHGESASLGNINPAYSNC.

Northern-blot, slot-blot and semi-quantitative PCR analysis

Frozen tissue samples were transferred directly to TRI reagent (Helena Biosciences, Sunderland, U.K.). RNA was prepared according to the manufacturer's instructions. For the Northern analysis, 20 μg of total RNA was separated on a 1 % agarose gel, transferred to a nylon membrane (Amersham International) by capillary action, and cross-linked with a UV cross-linker (Ultraviolet Products, Upland, CA, U.S.A.). For slot-blot analysis, 5 μg of total RNA was applied to nylon membranes using gentle suction on a slot-blot apparatus (Hoefer Instruments, San Francisco, CA, U.S.A.), then membranes were cross-linked. The cDNA probe was labelled with [α-^{32}P]dCTP with a Megaprime labelling kit (Amersham International). Pre-hybridization was carried out at 42 °C for 30 min in Ultrahyb (Ambion, Abingdon, U.K.). Hybridizations were carried out overnight at 42 °C. The blots were washed to high stringency in 0.05 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) plus 0.1 % SDS at 42 °C, and imaged on a wire proportional counter (Packard Instant Imager; Packard Bioscience Ltd, Pangbourne, Reading, Berks., U.K.). The mRNAs were quantified by measuring the amount of radioactivity hybridizing to the bands on the Northern blot, and corrected for loading by re-probing with a probe for 18 S rRNA.

Reverse transcriptase-PCR was performed using 'rt-to-go' beads (Amersham International) on eight normal control (50 mg/kg Fe) and seven iron-deficient (12.5 mg/kg iron) rat placental RNA samples taken at day 21 of gestation. The cDNA transcript was produced by incubation at 42 °C for 30 min. PCR was performed for 32 cycles (22 cycles for GAPDH) of 94 °C for 30 s, 65 °C (54 °C for GAPDH) for 2 min, 72 °C for 2 min, followed by 72 °C for 10 min in a PTC-100 Thermal cycler (Genetic Research Instrumentation, Braintree, Essex, U.K.). PCR products were stained with ethidium bromide on a 3 % agarose gel and visualized using Fluor-S MultiImager (Bio-Rad Laboratories). Bands were analysed using MultiAnalyst (Bio-Rad Laboratories) image-analysis software.

Measurement of copper oxidase activity

Copper oxidase activity was assayed by measuring the rate of oxidation of *p*-phenylenediamine [15,21]. Briefly, the reaction was initiated by the addition of sample to 200 mM sodium acetate buffer (pH 5.5) containing 1.5 mg/ml *p*-phenylenediamine. The reaction was mixed and incubated at 37 °C. Reactions were halted by the addition of 1.5 M sodium azide (final concentration 225 mM). The colour change was quantified by measurement of attenuance at 530 nm. To account for non-copper oxidase oxidation of *p*-phenylenediamine, parallel reactions were carried with sodium azide present throughout. Activity is expressed as units/mg of protein^{-1}, where 1 unit is 1 absorbance unit change/h.

Western blotting

Frozen placental samples were transferred directly to homogenization buffer (300 mM sucrose and 10 mM Hepes, pH 7.4) and homogenized using an Ultra Turrax homogenizer at low speed for 3 × 30 s periods. Placental homogenate samples (10–50 μg) were solubilized in Laemmli buffer (10 % glycerol, 62.5 mM Tris/HCl, pH 6.8, and 2.3 % SDS) and separated on a 7.5 % resolving SDS gels. The proteins were transferred to nitrocellulose (Amersham International) by semi-dry electrophoresis using a Tris/glycine transfer buffer containing 20 % methanol and 0.2 % SDS. Detection of the specific proteins was performed with specific antipeptide antisera. The blots were incubated with

appropriate secondary antibody (Sigma). Visualization of the immunologically detected proteins was achieved using the Supersignal Western blotting detection system (Pierce, Chester, U.K.). Processed blots were exposed to X-ray film for the optimum exposure time. The relative amounts of proteins were assessed by the intensity of immunoblot staining carried out by densitometry analysis (ImageJ; NIH).

Cell culture

BeWo cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts., U.K.) and routinely cultured in 80 cm^2 flasks (Nunc, Paisley, Renfrewshire, Scotland, U.K.) at 37 °C under air/CO_2 (19:1), 100% humidity, with complete medium (nutrient mixture Ham's F12 Glutamax, containing 10% fetal bovine serum and 2% penicillin/streptomycin; Life Technologies). Cells were sub-cultured every 7 days and the medium changed every 2 days.

Preparation of ^{59}Fe-labelled transferrin

Apo-transferrin (25 nmol; Sigma) was dissolved in 40 μl of balanced salt solution (BSS; 136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 18 mM Hepes, pH 7.4) and 50 μl of $NaHCO_3$ (7.5%). $^{59}FeCl_3$ (50 nmol) in 0.1 M HCl was added dropwise, and the diferric transferrin was stored at 4 °C. Binding to the specific iron-binding sites was confirmed by measurement of the absorption spectrum.

[^{59}Fe]Transferrin uptake in control and iron-deficient BeWo cells

BeWo cells were seeded on to 35 mm diameter culture dishes (Nunc) and grown to 80% confluence. The cells were made iron deficient by incubation with the iron chelator desferrioxamine (DFO) (20 μM), for 40 h, washed three times with ice-cold BSS, and incubated (37 °C) for 1 h with pre-warmed BSS containing 125 pmol $^{59}Fe_2$-transferrin/ml. They were then washed as above and harvested by addition of Pronase (1 mg/ml BSS) [21], incubated on ice for 10 min, aspirated, and centrifuged at 10000 ***g*** at 4 °C for 5 min. The supernatant and cells were counted separately, representing surface-bound and intracellular ^{59}Fe respectively. The cell fraction was resuspended in BSS, homogenized by ultrasonication (Soniprep 150), and an aliquot was kept for DNA analysis. Cell supernatant and cells were counted in a Cobra 5003 Auto-Gamma counter (Packard). The results are expressed as pmol ^{59}Fe/μg of DNA [21].

^{59}Fe efflux from BeWo cells

BeWo cells were grown in 35 mm diameter dishes and made iron deficient by incubation with the DFO (20 μM) for 40 h. The cells were washed three times with ice-cold BSS and incubated with BSS, containing approx. 10 μg of ^{59}Fe-transferrin/ml, for 1 h at 37 °C. The cells were labelled with ^{59}Fe-transferrin for only 1 h in order to avoid iron repletion of the cells. After the labelling, the cells were washed three times with BSS. BSS (1 ml) supplemented with 10 μg of apo-transferrin/ml (Sigma) was added to each plate, and the plates were incubated for increasing times in a water bath at 37 °C. At the end of the incubation period, the medium was aspirated and counted in an Auto-Gamma radiation counter, as described above. The cells were washed three times with ice-cold BSS, harvested with Pronase, and counted as described above. The specific activity of the ^{59}Fe cannot be determined once the ^{59}Fe-transferrin has entered the cell and mixed with the non-labelled intracellular iron. The amount of retained and released ^{59}Fe, therefore, is expressed as c.p.m./μg of DNA. The DNA content of the plates was constant at all time points in all experiments (mean DNA content was 14.9 ± 0.7 μg of DNA/plate).

DNA quantification

The DNA content was quantified using Hoechst dye 33258 (Polysciences Ltd, Warrington, PA, U.S.A.). Calf thymus DNA was used as a standard. Samples, standard and Hoechst Dye were diluted appropriately with TNE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.2 M NaCl). Equal volumes of sample and Hoechst dye (2 μg/ml) were mixed on a 96-well plate (Microfluor black plates; Dynatech Technologies, Ashford, Kent, U.K.) and read at excitation 356 nm and emission 458 nm on a Fluorlite 1000 plate reader (Dynatech Technologies).

Statistical analysis

Data are presented as means $\pm$ S.E.M. Statistical analysis was performed using Excel 6.0 (Microsoft, Seattle, WA). Student's unpaired t test was used to compare two data sets, and one-way ANOVA was used to compare multiple data sets. Significance was assumed at $P \leqslant 0.05$.

RESULTS

The first stage in iron transfer across the placenta is binding to the transferrin receptor. Figure 1 shows the effect of maternal iron deficiency on expression of transferrin receptor mRNA in placentas from animals fed diets with different levels of iron; it is clear there is a dose-dependent increase. The levels of transferrin

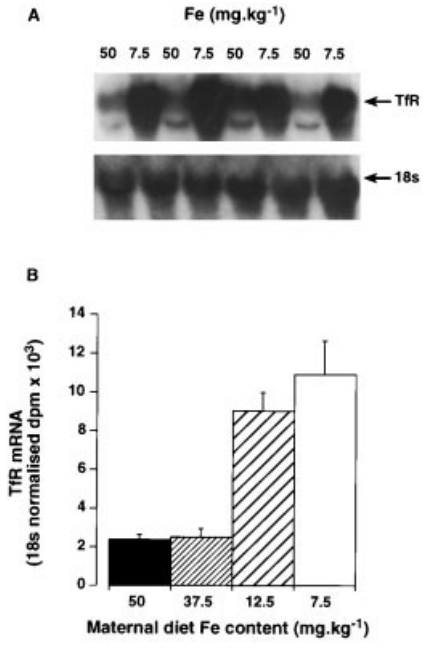

Figure 1 Iron deficiency increases expression of transferrin receptor mRNA in placenta

Placental mRNA was isolated, separated and hybridized as described in the Experimental section. The data show representative Northern blots and corresponding 18 S Northerns (**A**) and data summarized from slot blots (**B**) carried out on a minimum of four and a maximum of fifteen samples ($\pm$ S.E.M.) taken from each dietary condition described in the Experimental section. Statistical analysis was carried out using ANOVA, where $P < 0.0001$. Abbreviation: TfR, transferrin receptor.

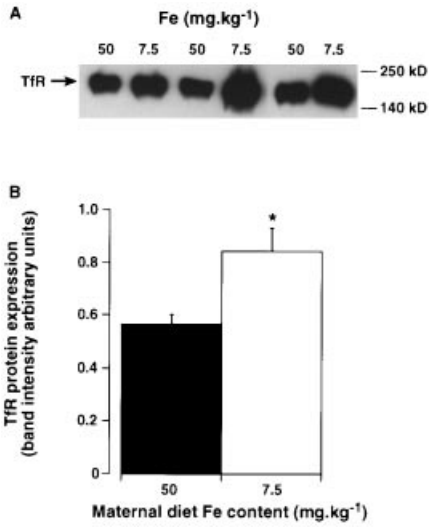

Figure 2 Transferrin receptor (TfR) expression is increased by iron deficiency

The data show Western blots of placental homogenates from animals on control (50 mg/kg) and iron-deficient (7.5 mg/kg) diets treated and separated as described in Experimental section (**A**). The blots were scanned using a densitometer and the summarized data are given in (**B**). The data are the means ± S.E.M. of eight samples in each group. Statistical analysis was carried out using Student's *t* test, $^*P = 0.02$.

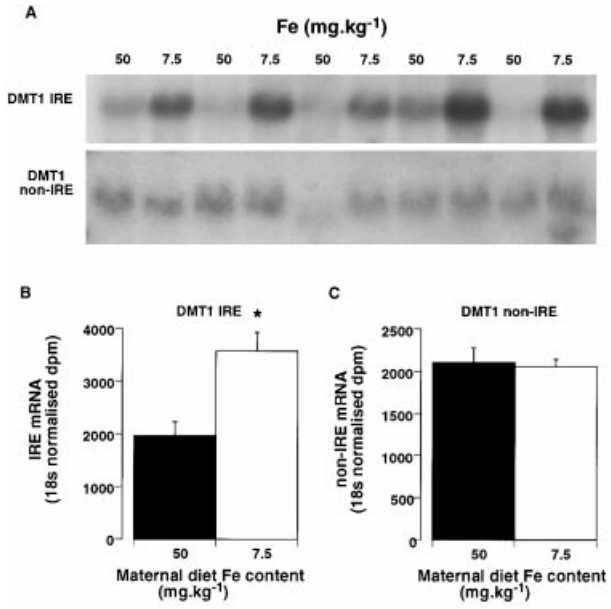

Figure 3 Expression of DMT1 in placentas from animals on different levels of iron

mRNA levels of IRE-containing and non-IRE-containing DMT1 were measured using Northern blotting (**A**). RNA was isolated from placentas and separated as described in the Experimental section. The intensity of the bands was quantified by counting on an Instant Imager and normalized to 18 S counts. The data summarized in (**B**) for IRE-containing DMT1 and in (**C**) for non-IRE-containing are the means ± S.E.M. of five samples from different dams. All statistical analysis was carried out using Student's *t* test, $^*P < 0.02$.

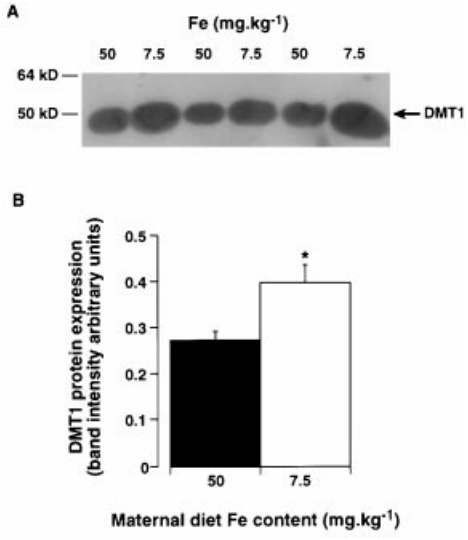

Figure 4 Western blot of placental homogenates taken from control and iron-deficient animals

Samples were treated as described in Experimental section, and incubated with anti-DMT1 antibody. Six samples are shown in (**A**) and the density of the major band was measured in these and eight other samples. The results are summarized in (**B**). Statistical analysis was carried out using Student's *t* test, $^*P = 0.01$.

Table 1 Effect of maternal iron deficiency on placental copper oxidase activity

Copper oxidase activity in the placenta was measured as described in the Experimental section. The results are the means ± S.E.M. of a minimum of four and a maximum of 13 samples taken from each dietary condition. Statistical analysis was carried out using ANOVA, $P < 0.0001$.

Maternal-diet iron level (mg/kg)	Copper oxidase activity (mg/h)
50	1.10 ± 0.24
37.5	1.55 ± 0.36
12.5	1.86 ± 0.09
7.5	1.32 ± 0.13

receptor protein in the placenta also increase with increasing deficiency (Figure 2), although to a lesser proportionate extent than the mRNA.

By analogy with other cell types, we assume that iron is transferred from the endosome into the cytoplasm through DMT1. Levels of DMT1 mRNA increase in iron deficiency. There are two forms of DMT1, only one has an IRE. Figure 3(A) shows a Northern blot of both forms of DMT1 from control and iron-deficient placentas. The data are quantified in Figures 3(B) and 3(C). The increase in DMT1 mRNA levels can completely be accounted for by a rise in the IRE-regulated form (Figure 3B), whereas the non-IRE-regulated isoform does not change (Figure 3C). In parallel with the increase in mRNA levels, there is a rise in protein levels in the placenta (Figure 4). In contrast, IREG1 mRNA levels do not change significantly: IREG1/GAPDH ratio = 0.44 ± 0.09 [means ± S.E.M. for both controls, 50 mg/kg ($n = 8$) and 12.5 mg/kg ($n = 7$) diets]. The activity of copper oxidase increases, however, with decreasing dietary concentrations of iron (Table 1).

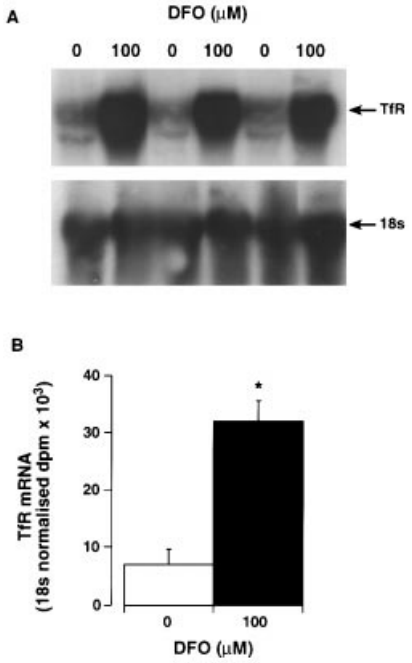

Figure 5 Iron deficiency increases transferrin receptor (TfR) mRNA expression in BeWo cells

BeWo cells were treated and RNA isolated, transferred, and hybridized as described in the Experimental section. Representative Northern blots are shown (**A**) and the normalized densitometric data are represented in (**B**). Results are the means ± S.E.M. of six measurements in each condition. Statistical analysis was carried out using Student's *t* test, $P = 0.004$.

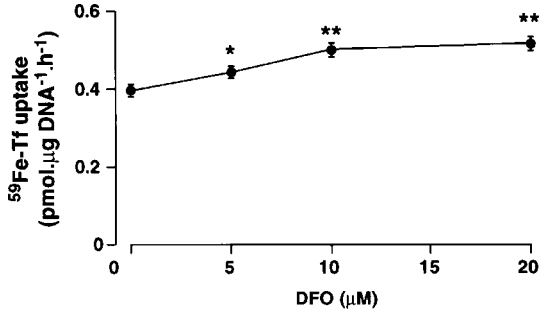

Figure 6 [$^{59}Fe_2$]transferrin uptake increases in iron-deficient cells

BeWo cells were made iron deficient as described in the Experimental section. Cells were incubated with [$^{59}Fe_2$]transferrin (^{59}Fe-Tf) for 1 h, harvested, and DNA content as well as radioactivity were determined for each sample. The increase in uptake is significant for 5 μM DFO ($^*P < 0.05$) and 10 μM and 20 μM DFO ($^{**}P < 0.01$). The results are the means ± S.E.M. of six plates.

These data suggest that the iron-transfer capacity across the placenta would increase in iron deficiency, a view supported by the fact that the degree of deficiency is lower in the fetus than in the mother. It is not possible, however, to test this hypothesis *in vivo*. Consequently, we examined iron uptake and efflux in BeWo cells rendered iron deficient by treatment with DFO.

As in the *in vivo* experiments, iron deficiency in the BeWo cells induced an increase in transferrin receptor mRNA expression (Figure 5). In parallel, there is a significant rise in uptake of ^{59}Fe by the cells (Figure 6). Furthermore, there is an increase in iron efflux from the cells, represented by a greater fall in intracellular

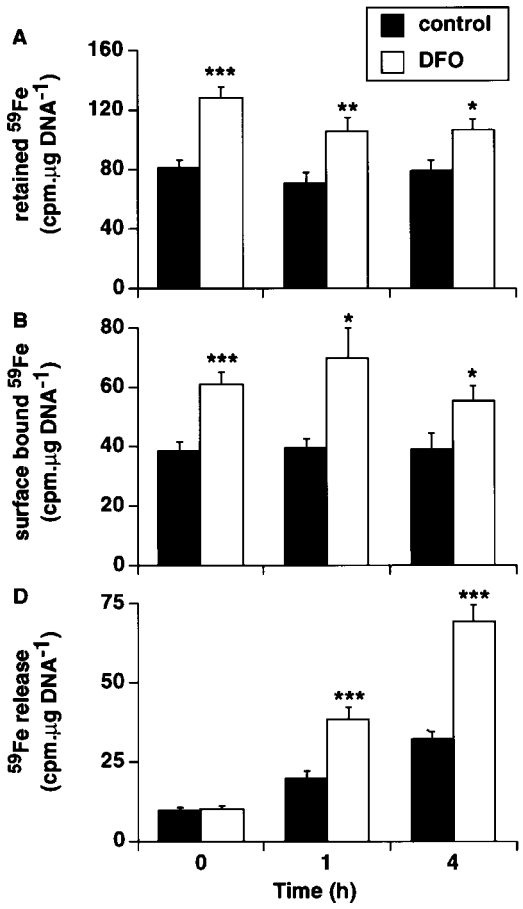

Figure 7 ^{59}Fe efflux is increased in iron-deficient cells

BeWo cells were made iron deficient by incubation with 20 μM DFO as described in the Experimental section. Cells were labelled with [$^{59}Fe_2$]transferrin for 1 h, and ^{59}Fe release was subsequently measured. The data show (**A**) ^{59}Fe retained in the cell, which significantly decreases with time; (**B**) surface-bound ^{59}Fe, which is significantly higher in DFO-treated cells than in control, but which does not change with time; and (**C**) ^{59}Fe released into the medium, which shows increased release from DFO cells when compared with controls. ($^*P < 0.05$, $^{**}P < 0.01$; $P < 0.0001$). The data are the means ± S.E.M., $n = 8$ at each time point.

levels (Figure 7A), no change in membrane-bound ^{59}Fe (Figure 7B), and an increase in the levels of ^{59}Fe in the medium (Figure 7C). These data also correlate with an increase in levels of copper oxidase protein expression in BeWo cells [22].

DISCUSSION

The data presented in the present paper show that iron deficiency in the maternal rat results in compensatory changes in the iron-transport mechanisms of the placenta, which, in turn, minimizes the level of iron deficiency in the fetus. Most, but not all, of the proteins involved in transfer show increases at both mRNA and protein levels, commensurate with the increase in iron flux.

Transferrin receptor mRNA and protein levels both increase in iron-deficient placentas and in the BeWo cells. Interestingly, the extent to which the protein levels increase, in both placenta and BeWo cells, is apparently less than the rise in mRNA. A determination of the relationship between transferrin receptor mRNA and protein levels would itself be a complex study, beyond the scope of the present paper. Furthermore, the balance between protein and mRNA levels is the result of many factors.

For example, both protein and mRNA turnover may be different from each other. Factors, such as translation rate of the mRNA, insertion of the transferrin receptor into the membrane, or availability of amino acids for synthesis, may all change the ratio between the mRNA and protein levels. Consequently, we would be unwise to draw mechanistic conclusions from these data. Increased transferrin receptor levels, concurrent with the rise in DMT1 will, however, mean that the rate of uptake into the syncytiotrophoblast will increase. This would help compensate for the decrease in iron-transferrin concentrations in the maternal serum. The compensation is not perfect, which is not surprising; if it were, the intracellular concentration of iron would return to control levels. This, in turn, would lead to a fall in the transferrin receptor number, DMT1 to fall to control levels, and a lower iron uptake than controls, since maternal transferrin saturation would be significantly lower.

The mechanism of efflux also shows some compensation. At present, the best hypothesis explaining how iron gets into the fetal circulation is that it exits the cell through IREG1/ferroportin1 as the divalent ion [11,12]. It is then oxidized to Fe(III) by the copper oxidase and is incorporated into fetal transferrin. Data from Donovan et al. [11] suggested that the IREG1 is located on the basolateral membrane, work supported by observations in duodenum by McKie et al. [12]. It would be expected that the oxidase would be located close to IREG1 to facilitate oxidation of the Fe(II). However, our data [15] suggest that, at least in cultured placental cells, the copper oxidase is not located on the basolateral membrane. We have localized the protein to an intracellular membrane compartment. Which compartment is not yet clear, but it would seem that the oxidase and the exit channel may not be in the same compartment.

There are three possible explanations for this apparent paradox. Firstly, the location *in vivo* is different from that *in vitro*. Immunolocalization experiments of the copper oxidase in rat placenta have not produced clear-cut results, largely due to problems with cross-reactivity with serum caeruloplasmin (results not shown). So we cannot draw clear conclusions on the localization of the protein in the placenta itself. Secondly, IREG1 may actually be located in a vesicular compartment, and the apparent localization to the basal side in the placenta occurs as a consequence of difficulties in accurate positioning in fixed and stained sections. Thirdly, copper oxidase may not be directly involved in iron release, and that hephaestin actually performs this function on the basolateral membrane; we are currently investigating this possibility. So far, we have been unable to detect hephaestin protein in placental samples, and Northern blots have also been negative. There are very limited data on the localization of hephaestin, but it is interesting to note that Vulpe and co-workers [22] have published one abstract that suggests that, as with the placental copper oxidase [15], the protein is located in the peri-nuclear region, and is not found on the basolateral membrane of gut epithelial cells. Furthermore, although Kingston et al. [23] demonstrated lower haemoglobin levels in sla/Y mice at birth, they showed only a very small decrease in transport of iron across the placenta (approx. 6%). These data suggest that as hephaestin is defective in sla mice [14], the protein can play, at most, a minor role in transplacental iron transfer, although it may be more important in subsequent, unidentified pathways.

IREG1 mRNA levels do not change in the iron-deficient placentas. The regulation of IREG1 is not straightforward: in gut, iron deficiency increases expression of the protein approx. 2.5-fold [12]; however, in contrast with the transferrin receptor and other proteins up-regulated in iron deficiency, its IRE is located on the 5′-end of the mRNA (the same as proteins such as ferritin, which are down-regulated in iron deficiency and up-regulated in iron overload). McKie et al. [12] recognize that these observations are unexpected, but suggest that the explanation may yet be forthcoming. Our data, therefore, are also not necessarily surprising. They suggest that there are other proteins involved in the efflux pathway, probably both in regulation of expression of the actual transport proteins, and also in the relation between the iron-regulatory proteins and the translation apparatus of the syncytiotrophoblast. Importantly, the data also indicate that transfer through IREG1 is not the rate-limiting step for transfer across the placenta.

In summary, our data would appear both to answer questions and to pose additional dilemmas. We correlate increased potential for iron flux with increased iron deficiency. This implies an increased efficiency of transfer, i.e. more of the iron taken up is donated to the fetus rather than the placenta. How this is regulated is still not entirely clear. There are no controversies with uptake; the mechanism is fairly well worked out, but the efflux mechanism has yet to be clarified. Up-regulation of the copper oxidase, rather than IREG1, would imply that this is the rate-limiting reaction, but further investigation is needed. The localization of the two proteins also needs to be studied further. If they are in different membrane compartments, why and how is iron transferred from the IREG1 to the oxidase? Clearly, there are still many questions to be answered before we can properly understand how iron delivery to the developing fetus is regulated.

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