A role for tumour necrosis factor α in human small bowel iron transport

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Cytokines are integral to the development of anaemia of chronic inflammation. Cytokines modulate hepcidin expression and iron sequestration by the reticuloendothelial system but their direct effects on small bowel iron transport are not well characterized. The aim of the present study was to examine the local effects of TNF α (tumour necrosis factor α) on small bowel iron transport and on iron transporter expression in the absence of hepcidin. The effects of TNF α on iron transport were determined using radiolabelled iron in an established Caco-2 cell model. The effect of TNF α on the expression and localization of the enterocyte iron transporters DMT-1 (divalent metal transporter 1), IREG-1 (iron-regulated transporter 1) and ferritin was determined utilizing Caco-2 cells and in a human *ex vivo* small bowel culture system. TNF α mediated an early induction in both iron import and iron

export, which were associated with increased DMT-1 and IREG-1 mRNA and protein expression ($P < 0.05$). However, by 24 h, both iron import and iron export were significantly inhibited, coinciding with an induction of ferritin heavy chain ($P < 0.05$) and a decrease in DMT-1 and IREG-1 to baseline levels. In addition, there was a relocalization of IREG-1 away from the basolateral cell border and increased iron deposition in villous enterocytes. In conclusion, TNF α has a direct effect on small bowel iron transporter expression and function, leading to an inhibition of iron transport.

Key words: anaemia, Caco-2 cell, cytokine, iron transport, small bowel, tumour necrosis factor α .

INTRODUCTION

Iron is an essential dietary element and its dysregulation is common to a number of pathological conditions [1]. As there is no physiological excretion of iron, its absorption from the small bowel must be tightly regulated, and the small bowel is therefore a vital site in the regulation of iron metabolism.

Ferrous iron is taken up into the enterocyte by the multi-ion DMT-1 (divalent metal transporter 1; also known as Nramp2 (natural-resistance-associated macrophage protein 2) [2] and DCT-1 (divalent cation transporter 1) [3]), which is located on the apical villous membrane of the duodenum and proximal jejunum [3]. Once in the enterocyte, iron has two possible fates: it may be stored bound to ferritin, or is exported out of the enterocyte by the basal IREG-1 (iron-regulated transporter) [4], also known as ferroportin [5] and MTP-1 [6].

Anaemia of chronic inflammation represents a reorganization of the internal iron turnover, probably as an adaptive response to chronic inflammation or infection, iron being essential for the metabolism of pathogens [7]. This condition is characterized by the sequestration of iron in reticuloendothelial cells with resultant hypoferraemia and reduced erythropoiesis in the face of adequate body iron stores [8].

Although the pathway of iron absorption has been described, its regulation is not well understood. Recently, the hepatic antimicrobial peptide hepcidin has been identified as the putative central regulator of iron absorption [9,10]. The regulation of hepcidin expression in response to the body's iron requirements is becoming well established [11]. An intriguing feature of hepcidin expression is its modulation by cytokines, especially IL-6 (interleukin-6) [12]. In a recent elegant study, Nemeth et al. [13] demon-

strated that, in humans, IL-6 is able to induce hepcidin expression and significantly lower serum iron levels. This occurs probably by a combination of decreased small bowel iron absorption and increased sequestration of iron into the reticuloendothelial system. In addition, the proinflammatory cytokine interferon γ has been shown to increase the uptake and decrease the export of iron in a monocytic cell line [14]. This establishes two likely roles for cytokines in the anaemia of chronic disease: up-regulation of hepcidin and direct effects of cytokines on the reticuloendothelial system.

Although there is evidence suggesting a role for cytokines in modulating hepatic hepcidin expression and iron transporter expression in monocytes, little is known regarding possible local effects of cytokines on small bowel iron transport.

In the present study, we have used two small bowel cell models, namely the cell line Caco-2 and a human small bowel *ex vivo* culture system, to investigate the direct effects of the proinflammatory cytokine TNF α (tumour necrosis factor α) on the expression of the main proteins involved in enterocyte iron transport: DMT-1, IREG-1 and ferritin. The Caco-2 cell line was selected since the cells have an enterocyte-like phenotype, expressing a number of small intestinal brush border enzymes and nutrient transporters [15,16]. In addition, this cell line expresses the key iron transport proteins, including DMT-1 [17], IREG-1 [18] and ferritin [19], as well as being the most commonly used model to study iron transport.

We have clearly shown that TNF α can dynamically regulate the expression and localization of iron transporters, ultimately having an impact on iron transport, and have provided evidence for a novel mechanism whereby cytokines can contribute to the anaemia of chronic inflammation.

Abbreviations used: DMT-1, divalent metal transporter 1; ferritin H, ferritin heavy chain; IEL, intraepithelial lymphocyte; IRE, iron-responsive element; IREG-1, iron-regulated transporter 1; TAMRA, tetramethylrhodamine; TNF α , tumour necrosis factor α .

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Table 1 Real-time PCR probe and primers

Abbreviation: FAM, fluorescein.

	Probe (5'-FAM 3'-TAMRA)	Forward primer (5'-3')	Reverse primer (5'-3')
DMT-1 IRE +ve	CTCTATCAGGCTTAGGA TTCTTTGAACCTATTCC ACTTT	CCATATGAAATATAAA ATGAAGAGACACCTA	CCCCTCTTAACCTCCAC TGAGAAA
DMT-1 IRE - ve	CCCACCCATAACAGTCA TACACTCCCAGAGT	TGGGAAGGGTGTTCAC AACTG	CCATCAGAGGCCAATC GTTTA
DMT-1 exon 1A	AGGCAGCTCCACACTGT GAACAAAATCATA	CATGAGGAAGAAGCAG CTGGAGA	TGTTCAGGACCCAGCAC CAT
DMT-1 exon 1B	TTCTAAGAACTCAGCCA CTCAGGTATCCACC	TGCGGAGCTGGTAAGA ATCA	TGTTCAGGACCCAGCAC CAT
IREG-1	AGGATTGACCAGTTAACC AACATCTTAGCCCC	AGCAAATATGAATGCC ACAATACG-	CAAATGTCATAATCTGG CCAACAG
Ferritin H	CCAACGAGGTGGCCGA ATCTTCCTT	GGAACATGCTGAGAAA CTGATGAA	CATCAGAGCTGGTTTC TTGATATCC
Hepcidin	AGCTGCAACCCAGGA	CCCACAACAGACGGGA CAA	TCTGGAACATGGGCATC C

MATERIALS AND METHODS

Cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Paisley, U.K.) with 10% (v/v) foetal calf serum supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin, and grown to 14 days post-confluency before experimentation. A dose-response curve for the effect of TNF α on DMT-1 mRNA expression in Caco-2 cells was generated to identify the optimal dose for stimulation experiments (5 ng/ml; results not shown). Cells were challenged with or without TNF α for 1–24 h and then processed for the determination of iron transporter expression levels or iron uptake studies.

Ex vivo small bowel cultures

Endoscopic duodenal biopsies were collected from adult patients undergoing upper gastrointestinal endoscopy for dyspeptic symptoms. All biopsies were confirmed to be histologically normal and, in addition, all patients had normal serum iron levels (14.3–35.8 μ M). Endoscopic duodenal biopsies ($n = 60$) were cultured *ex vivo* for up to 6 h as described previously [20]. Each biopsy was divided into two and cultured in Medium 199 (Gibco), supplemented with 10% foetal calf serum, 1 μ g/ml insulin, 500 units/ml streptomycin and 250 units/ml penicillin in a 95% O₂/5% CO₂ atmosphere at 37 °C for up to 6 h in the presence or absence of TNF α (0.5 ng/ml, optimum dose; results not shown). At two time points (1 and 6 h), biopsies were processed to determine iron transporter expression levels and localization by real-time PCR, Western blotting and immunohistochemistry respectively. Viability of biopsies was assessed by the lactate dehydrogenase assay before its inclusion in the present study as described previously [21].

Real-time PCR

Real-time PCR was performed as described previously [22]. Briefly, all reactions were performed using 18 S rRNA as an internal standard (PE Biosystems, Foster City, CA, U.S.A.; Roche, Indianapolis, IN, U.S.A.), and contained one of the sets of probes and primers listed in Table 1, in the presence of 1 $\times$ Mastermix (PE Biosystems), 50 nM 18 S 5'- and 3'-primers and 200 nM 18 S probe [5'-VIC[®], 3'-TAMRA (tetramethylrhodamine) labelled]

and 0.25 μ l of cDNA (equivalent to 12.5 ng of reverse transcribed RNA) in a 25 μ l reaction mixture. Reactions without cDNA were included as negative controls. All reactions were performed in triplicate. Gene expression was normalized to the 18 S and represented as Δ Ct values. For each sample, the mean for three Δ Ct values was calculated. Comparison of gene expression between control and treated samples was derived by subtracting control Δ Ct values from treatment Δ Ct values to give a $\Delta\Delta$ Ct value, and relative gene expression was calculated as $2^{-\Delta\Delta Ct}$. Relative gene expression is normalized to 1.0 (100%) of controls. Each experiment was performed in triplicate.

Western blotting

Caco-2 cells after subjecting to stimulation were processed for Western blotting as described previously [22] with monoclonal antibodies raised against either DMT-1 [5 μ g/ml NRAMP24-A (recognizing IRE +ve/-ve isoforms); Alpha Diagnostic International (ADI), San Antonio, TX, U.S.A.; IRE stands for iron-responsive element], IREG-1 (5 μ g/ml MTP11-A; ADI), ferritin H (ferritin heavy chain; 1:500; Abcam, Cambridge, U.K.) or Cytokeratin 19 (1:2,000; Oncogene Research Products, Cambridge, MA, U.S.A.), the latter being employed for normalization of epithelial protein loading. Immunoreactive bands were then subjected to densitometry using the software NIH Image 1.62. Before reprobing with the different antibodies, the membranes were incubated in stripping buffer containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol in 62.5 mM Tris/HCl (pH 6.8) for 30 min at 70 °C.

Immunocytochemistry

To ensure that there was no inter-patient variability between treated and non-treated samples, each duodenal endoscopic biopsy was initially divided into two and cultured for 6 h in the presence or absence of TNF α . Biopsies were then fixed in formalin, embedded into paraffin and 7 μ m sections were processed for immunohistochemistry. Sections were dewaxed and then incubated in 0.1% Tween 20 containing 1 mM EDTA (pH 8.0) for 16 h at 65 °C. Sections were then incubated for 1 h with either DMT-1 [10 μ g/ml NRAMP24-A (recognizing IRE +ve/-ve isoforms); ADI], IREG-1 (10 μ g/ml MTP11-A; ADI) or ferritin H (1:500; Abcam). Immunoreactivity was detected by the

avidin-biotinylated secondary antibody method (Dako ABC kit; Dako, Carpinteria, CA, U.S.A.) and visualized with diaminobenzidine reagent. Sections were counterstained with haematoxylin. Omission of primary antibody was employed as a negative control. Stained sections were scored independently by three observers (N.S., C.T. and T.I.) with regard to cellular localization. Images were visualized from paraffin sections using a Nikon Eclipse E600 microscope and digital image was taken using a Nikon DXM1200F camera (Nikon, Surrey, U.K.). The Nikon ACT-1 version 2.62 software was used for image acquisition.

Immunofluorescence

Cells were fixed in methanol/acetone, blocked [20 % (v/v) goat serum in 1 % BSA/PBS] and incubated with either monoclonal DMT-1 [100 μ g/ml NRAMP24-A (recognizing IRE +ve/-ve isoforms); ADI], IREG-1 (100 μ g/ml MTP11-A; ADI) or ferritin H (1:50; Abcam) antibodies for 1 h before labelling with FITC goat anti-mouse (1:500; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.). Cells were washed and incubated in DAPI (4,6-diamidino-2-phenylindole; 1:10 000) for 1 min before visualization. Omission of primary antibody was employed as a negative control. Images were visualized using an Olympus BX40 microscope and digital image was taken using a Sensys Photometrics camera (Middlesex, U.K.). Desksoft Smart-Capture 2 software was used for image acquisition (Desksoft, www.desksoft.com).

Prussian Blue staining of *ex vivo* small bowel biopsies

Paraffin sections were dewaxed and incubated in 1 % HCl containing 1 % ferrous cyanate for 20 min. Sections were washed and counterstained with neutral Red before visualization. Images were visualized from paraffin sections using a Nikon Eclipse E600 microscope and digital image was taken using a Nikon DXM1200F camera. Nikon ACT-1 version 2.62 software was used for image acquisition.

Iron uptake studies

Caco-2 cells (1×10^4) were seeded into the apical chamber of a transwell plate (Costar, High Wycombe, Bucks., U.K.) and cultured in 1.0 ml of growth media. Upon reaching 14 days post-confluency, the medium from the apical chamber was removed and cells were briefly washed in PBS several times. Cells were then cultured in 1.0 ml of growth media with or without TNF α (5 ng/ml) for up to 24 h. In all experiments, the basal chamber contained 1.5 ml of growth medium alone. The medium was then removed and the cells were washed with PBS followed by culture in 1 ml of growth medium containing 1 μ M iron as $^{55}\text{FeCl}_3$ (37 kBq/ml; Polatom Radioisotope Centre, Otwock-Swierk, Poland) and $^{56}\text{FeSO}_4$ in a 1:10 molar ratio, with a 100-fold molar excess of sodium ascorbate, and 20 mM 2-(*N*-morpholino) ethanesulphonic acid-buffered salt solution (pH 5.5). After 1 h culture, aliquots were removed from the basal and apical chambers. In addition, the cell monolayer was lysed in 1 % Triton X-100/0.1 M NaOH. All aliquots were then subject to scintillation counting. Iron import was calculated as the total counts in both the cell layer and basal chamber. Iron export was calculated as the counts within the basal chamber alone. Each experiment was performed in triplicate at each time point.

Caco-2 monolayer permeability studies

Caco-2 cells (1×10^4) were cultured in the apical chamber of a transwell plate as described for the iron uptake studies above. Upon reaching 14 days post-confluency, the medium from the

apical chamber was removed and cells were briefly washed in PBS several times. Cells were then cultured in 1.0 ml of growth media containing FITC-labelled dextran (200 μ g/ml; Sigma-Aldrich) in the presence or absence of TNF α (5 ng/ml) for 24 h. The media from the apical and basal chambers were then assessed for fluorescence intensity (excitation wavelength = 495 nm and emission wavelength = 525 nm), using a fluorescence spectrophotometer F-4500. Control experiments included (i) a standard curve for fluorescence detection and (ii) studies to determine whether FITC-labelled dextran was able to cross the membrane of the transwell plate in the absence of cells. Each experiment was performed in triplicate.

Statistical analysis

All experimental errors are shown as 1 S.E.M. Statistical significance was calculated by unpaired Student's *t* test using SPSS version 10.0 (SPSS, San Rafael, CA, U.S.A.). Significance was accepted at $P < 0.05$.

Ethics

The present study has been carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was obtained from the Sandwell and West Birmingham LREC (Local Research Ethics Committee; no. 03/05/633). All patients have provided informed written consent.

RESULTS

Effects of TNF α on iron uptake in Caco-2 cells

To investigate the effect of TNF α on iron uptake, Caco-2 cells were challenged with TNF α for up to 24 h (Figure 1). Treatment with TNF α resulted in a significant early induction in both iron import (Figure 1A) and iron export (Figure 1B) at 6 h ($P < 0.05$). However, by 24 h of culture with TNF α , a significant repression in both iron import and export was observed ($P < 0.05$). At 24 h, the ratio of iron export to import is also significantly reduced in TNF α -treated cells, indicating that more iron is being incorporated within the cell, probably bound to ferritin (Figure 1C). To demonstrate that these changes in iron import/export were not a TNF α -mediated effect on monolayer integrity, a non-transported marker, FITC-labelled dextran, was utilized. In these experiments, it was shown that regardless of the presence of TNF α , less than 0.1 % of the starting FITC-dextran was detected in the basal chamber and the remaining 99.9 % remained in the apical chambers (control, apical chamber, 99.9 ± 0.01 %; basal chamber, 0.06 ± 0.02 %; TNF α -treated, apical chamber, 99.9 ± 0.01 % and basal chamber, 0.05 ± 0.006 %). There was no statistical difference in the level of fluorescence in the basal chambers of TNF α -treated or control ($P > 0.12$). In addition, when FITC-labelled dextran was placed into the apical chamber of a transwell plate (in the absence of cells), it was demonstrated that the FITC-dextran was able to cross the membrane and, by 24 h, there was equilibration between the apical and basal chambers.

The TNF α -mediated effects on iron transport in Caco-2 cells is hepcidin-independent

To determine if the TNF α -mediated effects on iron transport were a consequence of hepcidin, hepcidin mRNA expression was determined in both TNF α -treated and control Caco-2 cells ($n = 6$) (Figure 2). Figure 2 demonstrates that, whereas human liver ($n = 6$) has an abundance of hepcidin mRNA expression, Caco-2 cells, both control and TNF α -treated, failed to demonstrate any

Figure 1 Iron import and export in Caco-2 cells in response to TNF α

Caco-2 cells grown to 14 days post-confluency were cultured with 5 ng/ml TNF α (---) or culture media alone (—). Cells were then incubated in the presence of ^{55}Fe . Iron import (A) and export (B) were determined by scintillation counting and expressed as d.p.m. (DPM). (C) The ratio of iron export to iron import in control (—) and TNF α treated (---) samples. * $P < 0.05$, Student's t test. Error bars represent 1 S.E.M.

significant mRNA expression even after 40 cycles of amplification.

The effect of TNF α on DMT-1, IREG-1 and ferritin H expression in Caco-2 cells

To investigate whether the TNF α -induced changes in iron import/export were mediated by changes in the expression of iron transporter proteins, we determined the mRNA and protein levels of DMT-1, IREG-1 and ferritin H in Caco-2 cells.

Stimulating Caco-2 cells with TNF α resulted in changes in DMT-1, IREG-1 and ferritin mRNA expression (Figure 3). Since four DMT-1 splice variants (exon 1A, exon 1B, IRE-containing and non-IRE-containing) have been described, we examined the effects of TNF α on these spliced variants initially (Figures 3A–3D). Our results demonstrate that there was a significant elevation at 1 h ($P < 0.05$) in only the DMT-1 IRE-containing and DMT-1 exon 1A variants (Figures 3A and 3C) with no changes observed for either the exon 1B-containing or the non-IRE-containing

Figure 2 Hepcidin mRNA expression in human liver and Caco-2 cells

Caco-2 cells grown to 14 days post-confluency were cultured with 5 ng/ml TNF α or culture media alone and then subject to real-time PCR for hepcidin (A) and 18S rRNA (B) expression, the latter being utilized as an internal control. Human liver was utilized as a positive control and demonstrated an abundance of hepcidin expression, whereas Caco-2 cells, both treated and untreated, failed to show any mRNA expression. ○, human liver samples ($n=3$); ▲, control Caco-2 samples ($n=3$); and □, TNF α -treated Caco-2 samples ($n=3$). Results are representative of three independent experiments.

variants (Figures 3B and 3D). However, these elevations were only short-lived and by 8 and 24 h the message levels had returned to baseline. Similarly, IREG-1 and ferritin mRNA were also elevated following TNF α stimulation with significant elevations at 1 and 8 h respectively, with both the transcripts returning to baseline by 24 h (Figures 3E and 3F).

To ensure specificity of TNF α treatment, we examined E-cadherin expression as previously reported [23] and observed a dramatic repression in E-cadherin mRNA over a 24 h time period (results not shown).

To confirm these observations, Western blotting was performed to examine protein levels (Figure 4). Exposure of Caco-2 cells to TNF α resulted in a significant increase in DMT-1 by 8 h ($P < 0.05$), with protein levels returning to baseline by 24 h (Figure 4A). IREG-1 protein was significantly induced at 3 h ($P < 0.05$), with levels returning to background at 8 and 24 h (Figure 4B). Additionally, ferritin H protein expression was significantly induced at 24 h only ($P < 0.05$), supporting our mRNA observations (Figure 4C).

The effect of TNF α on DMT-1, IREG-1 and ferritin H expression in *ex vivo* small bowel cultures

We sought to verify our results obtained in Caco-2 cells by using an *ex vivo* small bowel culture model system (Figure 5). Consistent with our Caco-2 cell findings, challenging small bowel cultures ($n=40$) with TNF α resulted in a significant elevation in the DMT-1 IRE-containing and the DMT-1 exon 1A variants

Figure 3 Transcriptional modulation of DMT-1, IREG-1 and ferritin H by TNF α

Caco-2 cells were challenged with 5 ng/ml TNF α and DMT-1 IRE-containing (A), DMT-1 non-IRE (B), DMT-1 exon 1A (C), DMT-1 exon 1B (D), IREG-1 (E) and ferritin H (F) mRNA levels determined at 1, 8 and 24 h time intervals. Error bars represent S.E.M. * $P < 0.05$, Student's t test. Results shown are the means for three independent experiments, each set performed in triplicate. ■, TNF α -treated cells; □, control unstimulated cells.

($P < 0.05$), with no significant change in either DMT-1 IRE -ve or the DMT-1 exon 1B variants (Figures 5A–5D). Similarly, IREG-1 and ferritin H mRNA were also both elevated following 1 h culture with TNF α compared with untreated small bowel cultures (Figures 5E and 5F).

Western blotting demonstrated that, by 6 h of culture in the presence of TNF α , there was no change in either DMT-1 or IREG-1 protein expression (Figures 6A and 6B) but there was a significant induction in ferritin H expression (Figure 6C).

The effect of TNF α on DMT-1, IREG-1 and ferritin H localization in Caco-2 cells

Results from the iron uptake assay (Figures 1A and 1B) clearly demonstrate that at 24 h there was a marked repression in iron

import and export. This is associated with a decrease in DMT-1 and IREG-1 expression to baseline levels (Figures 3 and 4). However, the repression of iron import and export below baseline levels suggests an additional aberration of function of these proteins. One possible mechanism for this aberration of function might be explained by a possible redistribution of these proteins. To investigate whether this was a possible explanation, immunolocalization for DMT-1, IREG-1 and ferritin H was performed on Caco-2 cells and *ex vivo* small bowel cell cultures. Challenging Caco-2 cells for 24 h with TNF α failed to cause any relocalization of DMT-1 (Figures 7A–7C). However, IREG-1 was lost from the cell borders with immunoreactivity being predominantly weak and cytoplasmic (Figures 7D–7F). Ferritin H was also strongly relocalized to the perinuclear compartment (Figures 7G–7I).

Figure 4 Modulation of DMT-1, IREG-1 and ferritin H protein expression by TNF α

Caco-2 cells were challenged with 5 ng/ml TNF α and DMT-1 (A), IREG-1 (B) and ferritin H (C) and protein expression levels were determined relative to the internal epithelial marker cytokeratin 19 (CK-19) at 3, 8 and 24 h. A representative Western blot for each protein is shown (D). Error bars represent S.E.M. * $P < 0.05$, Student's t test. Results shown are the means for three independent experiments, each set performed in triplicate. ■, TNF α -treated; □, control unstimulated cells.

The effect of TNF α on DMT-1, IREG-1 and ferritin H localization in *ex vivo* small bowel cultures

Immunolocalization studies were performed in *ex vivo* small bowel cultures ($n = 10$) treated for 6 h with or without TNF α . *Ex vivo* small bowel cultures challenged with TNF α did not cause a relocalization in DMT-1 expression. DMT-1 remained localized in the apical surface of villous enterocytes (Figures 8A–8C). However, culturing in the presence of TNF α did result in a

dramatic relocalization of IREG-1 (Figures 8D–8F). In untreated cultures, IREG-1 was localized predominantly on the basolateral border of crypt enterocytes. However, exposure to TNF α caused a loss of this discrete basolateral border immunoreactivity with only diffuse cytoplasmic immunoreactivity remaining. In untreated cultures, ferritin H was weakly localized within the cytoplasm, whereas culture with TNF α resulted in strong immunoreactivity in the apical cytoplasmic pole of the villous enterocytes (Figures 8G–8I). To examine whether the increase in ferritin H expression observed with TNF α treatment leads to an increase in enterocyte iron loading, we stained *ex vivo* small bowel sections with Prussian Blue (Figures 8J and 8K). In the untreated cultures, no Prussian Blue staining was discernible; however, in treated cultures, Prussian Blue staining was clearly observed in the apical poles of the villous enterocytes in agreement with the pattern of immunoreactivity observed for ferritin H.

DISCUSSION

Previous studies have suggested a role for TNF α in the modulation of iron transport proteins. Torti et al. [24] and Ludwiczek et al. [14] have demonstrated that ferritin and DMT-1 respectively can be induced by TNF α . Furthermore, murine studies have suggested a physiological role for TNF α in regulating small bowel iron absorption by the effect of dietary iron on small bowel IELs (intraepithelial lymphocytes) [25,26]. In the present study, we have demonstrated that, in a hepcidin-free environment, exposure to TNF α is associated with a rapid and dynamic modulation of iron transporter expression in both an established cell line model and an *ex vivo* small bowel culture system. Importantly, this modulation was associated with a coincident effect on iron transport.

Culturing Caco-2 cells in the presence of TNF α led to an initial induction in iron import and export, whereas prolonged exposure (24 h) led to a dramatic repression in both. The net effect of prolonged exposure was cellular iron accumulation. To explain these observations, we examined mRNA and protein levels of DMT-1, IREG-1 and ferritin H in both Caco-2 cells and in *ex vivo* small bowel cultures after TNF α treatment.

Since, in total, four DMT1 splice variants have been described so far [27], we initially examined what effect TNF α had on these DMT-1 splice variants in the Caco-2 cell line. Utilizing probes to the 5' variants (exon 1A or 1B) and 3'-untranslated region variants (IRE +ve or IRE -ve), we have been able to demonstrate that responsiveness to TNF α was exhibited by the exon 1A (IRE +ve) splice variant of DMT-1. This variant is also believed to be the main iron-responsive form of DMT-1 in the small bowel [27]. Little or no response to TNF α was observed with the exon 1B and IRE -ve splice variants.

In Caco-2 cells, the elevation in DMT-1 and IREG-1 mRNA was maximal at 3 h, whereas maximal ferritin H expression occurred at 8 h. Interestingly, TNF α -mediated increase in DMT-1 and IREG-1 mRNA in Caco-2 cells was short-lived, transcripts being restored to baseline after 8 h culture. In contrast, expression of ferritin H mRNA decreased to baseline at 24 h. These changes in mRNA level were accompanied by similar changes in protein expression for all three proteins.

In the *ex vivo* small bowel culture system, exposure to TNF α resulted in a similar pattern of mRNA expression. However, the time course was significantly different compared with the Caco-2 cell model with all the three transcripts [DMT-1 exon 1A (IRE +ve), IREG-1 and ferritin] significantly increased at 1 h. In addition, by 6 h, there was no change in DMT-1 or IREG-1 protein expression but there was a significant induction in ferritin H. This suggests that, at this later time point, the induction in DMT-1 and

Figure 5 Modulation of DMT-1, IREG-1 and ferritin H mRNA by TNF α in *ex vivo* cultures

Ex vivo small bowel cultures were cultured in the presence ($n = 40$) or absence of TNF α ($n = 40$) for 1 h. DMT-1 IRE-containing (A), DMT-1 non-IRE (B), DMT-1 exon 1A (C), DMT-1 exon 1B (D), IREG-1 (E) and ferritin (F) mRNA levels were compared with control unchallenged biopsies. Error bars represent S.E.M. * $P < 0.05$, Student's t test.

IREG-1 had occurred and returned to baseline, whereas ferritin H induction, which in the cell model was a later event, was up-regulated as anticipated. Alternatively, it could be that, in these *ex vivo* cultures, 6 h was too short a time period to observe changes in DMT-1 and IREG-1 protein expression. The variation in the time course of these events probably reflects inherent differences between the two model systems utilized in the present study. It appears likely that the *ex vivo* system is a more representative model of the *in vivo* scenario due to conservation of cell function and a villous architecture. Indeed, we believe we have uniquely demonstrated that this is a valid model for the future study of iron transporter regulation.

The biological relevance of the early induction in DMT-1 and IREG-1 is unclear. Both promoter sequences contain putative

TNF α response elements and the rapidity of their induction suggests a direct effect on the promoter of these transporters. Alternatively, a TNF α -mediated inhibition of mRNA degradation might also be a possible cause of this early increase in mRNA expression. The later repression of both proteins may reflect a second competing pathway, which becomes dominant at later time points. This hypothesis is supported by results from a recent study that demonstrated a repression of DMT-1 in Caco-2 cells after 72 h TNF α exposure, an effect that appeared to be promoter-independent [19]. However, in this study, the DMT-1 exon 1B variant was utilized, which we have demonstrated is not TNF α -responsive for shorter time periods.

Although, in our study, it appears that the IRE-containing form of DMT-1 is up-regulated in response to TNF α , the changes

Figure 6 Modulation of DMT-1, IREG-1 and ferritin H protein by $\text{TNF}\alpha$ in *ex vivo* cultures

Ex vivo small bowel cultures were cultured in the presence ($n=4$) or absence of $\text{TNF}\alpha$ ($n=4$) for 6 h, and DMT-1 (A), IREG-1 (B) and ferritin H (C) protein expression levels were determined relative to CK-19. Error bars represent S.E.M. * $P < 0.05$, Student's *t* test.

in expression are unlikely to be modulated by changes in IRP (iron-responsive protein) function. The IRE for DMT-1 is found on the 3'-end of its transcript, whereas the transcript of IREG-1 contains a 5'-IRE. As such, $\text{TNF}\alpha$ -modulated DMT-1 and IREG-1 expression would have been reciprocal if it were IRP/IRE-dependent. Clearly, further studies are required to delineate the mechanisms underlying the early mRNA increases and the opposing effects on the temporal expression of both DMT-1 and IREG-1.

The dynamic changes in protein expression correlate well with the effects seen on iron transport in Caco-2 cells. The early *de novo* induction in DMT-1 and IREG-1 resulted in early induction in iron import and export. However, the repression in iron import and export observed at 24 h was over and above that which could be explained by the decrease in DMT-1 and IREG-1 protein expression. It is likely that the relocalization of IREG-1 away from the basolateral membrane combined with increased ferritin H expression allows for decreased iron export and increased accumulation within enterocytes. Indeed Prussian Blue staining of *ex vivo* cultures after exposure to $\text{TNF}\alpha$ demonstrated iron deposition within the apical portion of the villous enterocytes in a similar pattern as that observed for ferritin H staining.

Thus we believe that, in our hepcidin-free system, enterocyte iron trapping and ultimate repression of iron transport is $\text{TNF}\alpha$ -driven and that $\text{TNF}\alpha$ is potentially mediating its effect partially through IREG-1 relocalization (rather than changes in IREG-1 protein expression). In this regard, it is with great interest that a recent elegant study by Nemeth et al. [28] has demonstrated using tissue culture models that hepcidin can bind IREG-1 and this causes internalization and subsequent degradation of IREG-1, leading to decreased export of cellular iron. This raises the possibility that cytokines and hepcidin can regulate enterocyte iron transport through a common protein, IREG-1.

Figure 7 Localization of DMT-1, IREG-1 and ferritin H in Caco-2 cells stimulated with $\text{TNF}\alpha$

(A–C) Exposure of Caco-2 cells to $\text{TNF}\alpha$ for 24 h resulted in *de novo* localization of DMT-1 to the cell borders. (D–F) IREG-1 was predominantly localized at cell borders in control cells (white arrows); however, $\text{TNF}\alpha$ exposure resulted in a loss of cell-border immunoreactivity with only weak diffuse cytoplasmic immunoreactivity remaining. (G–I) Weak nuclear immunoreactivity was observed for ferritin H; however, $\text{TNF}\alpha$ exposure resulted in strong perinuclear cytoplasmic immunoreactivity. Magnification, $\times 100$.

Figure 8 Localization of DMT-1, IREG-1 and ferritin H in TNF α -challenged *ex vivo* cultures

Ex vivo small bowel cultures ($n = 10$) were cultured in the presence or absence of TNF α for 6 h. DMT-1 was predominantly localized in the apical surface of villous enterocytes in both control and treated cultures (A–C; magnification, $\times 60$). IREG-1 was localized on the basolateral border of crypt enterocytes in control biopsies (black arrows). Only a weak diffuse cytoplasmic immunoreactivity was evident after exposure to TNF α (D–F; magnification, $\times 60$). Ferritin H was weakly localized within the cytoplasm in control culture, whereas culture in the presence of TNF α resulted in strong immunoreactivity in the apical cytoplasmic pole of the villous enterocytes (G–I; magnification, $\times 60$). This latter observation is supported by Prussian Blue staining. Blue deposits were observed in the apical poles of the villous enterocytes (black arrows) in only treated small bowel cultures (J, K; magnification, $\times 100$). To ensure no inter-patient variability between treated and non-treated samples, each duodenal endoscopic biopsy was initially divided into two and cultured for 6 h in the presence or absence of TNF α ; this Figure represents a single patient.

Recent murine studies have suggested a possible physiological role for TNF α in regulating small bowel iron absorption. Small bowel IELs release TNF α in response to dietary iron [25]. Blocking the effects of TNF α by utilizing a TNF receptor-2 knockout mouse led to splenic iron overload [26]. In the same study, utilizing an HFE (mutated in hereditary haemochromatosis) knockout mouse led to hepatic iron overload and also removed the ability of IELs to produce TNF α on dietary iron challenge.

Moreover, there is a growing body of evidence suggesting a role for TNF α in the anaemia of chronic inflammation. In a murine study, recombinant TNF α induced serum iron deficiency by iron

sequestration within the reticuloendothelial system [29]. In addition, TNF α has been shown to directly inhibit erythropoiesis in patients with active rheumatoid arthritis, an effect that was reversed by anti-TNF α therapy [30,31]. Neither study characterized changes in small bowel iron transporter expression as a consequence of systemic inflammation.

We believe that, in the present study, we have been able to delineate the local effects of TNF α from the systemic effects of hepcidin. Our hypothesis is that, in normal physiology, local TNF α release, probably by IELs, is able to modulate a rapid induction in iron transport, which is then repressed with chronic

exposure to TNF α , causing a block on iron absorption. However, why there should be this early induction still remains unexplained. In the anaemia of chronic inflammation, it is likely that there is a local and systemic TNF α effect in addition to hepcidin, both culminating in a block on iron absorption.

The effects of the pleiotropic agent TNF α on iron transport *in vivo* is likely to be complex with the interplay of multiple signal-transduction pathways and other effectors of iron metabolism. Clearly, it would be enlightening to extend this work to a whole organism to characterize the interplay between the small bowel, TNF α and these other known regulators of iron metabolism, namely hepcidin.

In summary, utilizing two model systems, we demonstrate that TNF α has a dynamic effect on iron transporter expression in enterocytes, resulting ultimately in a block in iron transport and increased iron storage. It is likely that, in chronic inflammation, an increase in systemic TNF α would cause a more sustained block on iron absorption, leading ultimately to anaemia. Thus it is interesting to speculate on the future of anti-TNF α therapy in the treatment of the anaemia of chronic inflammation.

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