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Pathways for the regulation of body iron homeostasis in response to experimental iron overload

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***Background/Aims*: Secondary iron overload is a frequent clinical condition found in association with multiple blood transfusions.**

***Methods*: To gain insight into adaptive changes in the expression of iron genes in duodenum, liver and spleen upon experimental iron overload we studied C57BL/6 mice receiving repetitive daily injections of iron-dextran for up to 5 days.**

***Results*: Iron initially accumulated in spleen macrophages but with subsequent increase in macrophage ferroportin and ferritin expression its content in the spleen decreased while a progressive storage of iron occurred within hepatocytes which was paralleled by a significant increase in hepcidin and hemojuvelin expression. Under these conditions, iron was still absorbed from the duodenal lumen as divalent metal transporter-1 expressions were high, however, most of the absorbed iron was incorporated into duodenal ferritin, while ferroportin expression drastically decreased and iron transfer to the circulation was reduced.**

***Conclusions*: Experimental iron overload results in iron accumulation in macrophages and later in hepatocytes. In parallel, the transfer of iron from the gut to the circulation is diminished which may be referred to interference of hepcidin with ferroportin mediated iron export, thus preventing body iron accumulation.**

***Keywords*: Iron; Hemochromatosis; Iron absorption; Hepcidin; IRP**

1. Introduction

Secondary iron overload is a frequent clinical condition which can arise from inborn errors of haemoglobin synthesis, myelodysplastic syndromes or chemotherapy induced anaemia, which all require multiple blood transfusions [1,2]. In addition, an inherited form of secondary iron overload is found in Southern Africa which has been linked to a defect in duodenal iron transport together with the consumption of iron rich traditional beer [3].

Secondary iron overload leads to progressive iron accumulation in the reticulo-endothelial system [1] with subsequent negative effects on cell mediated immune function, since iron inhibits interferon-γ inducible pathways in macrophages [4]. Accordingly, subjects with secondary iron overload are at a higher risk for tuberculosis and cancer [3]. In being a catalyst for the formation of highly toxic radicals, iron overload leads to tissue damage and organ failure [5], which is well known in primary iron overload, hereditary hemochromatosis, although with this condition iron is primarily deposited within parenchymal cells [6–8]. However, information of adaptive changes of body iron homeostasis upon secondary or experimental iron overload is rare.

Maintenance of body iron homeostasis is mainly regulated by duodenal iron absorption. Thereby, ferric

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iron is reduced at the luminal site by duodenal cytochrome *b* (Dcytb) [9], and ferrous iron is then transferred into the enterocyte by means of the transmembrane protein divalent metal transporter-1 (DMT-1) [10,11]. At the basolateral site ferrous iron is exported from the enterocyte to the circulation via ferroportin [12–14], and after being oxidised by the membrane bound ferroxidase hephaestin [15] iron is incorporated into transferrin. In addition, up to 50% of absorbed iron may be in the heme form, which is taken up by a yet not characterised duodenal heme receptor [16,17].

The expression of these iron transport genes is strongly regulated by body iron homeostasis, since the duodenal expression of DMT-1, Dcytb, ferroportin and to a lesser extent of hephaestin are increased with iron deficiency anaemia [9,11,15,18–22]. The presence of iron responsive elements (IREs) within the untranslated regions of DMT-1 and ferroportin mRNA suggested that the expression of these proteins may be susceptible to posttranscriptional regulation by iron regulatory proteins (IRP) [23]. However, transcriptional regulation of DMT-1 and ferroportin expression by iron has also been anticipated [24–26].

In terms of regulation of duodenal iron absorption [27] transferrin receptor (TfR1) mediated uptake of circulating iron from the basolateral site of enterocytes and the subsequent modulation of IRP activity was believed to be the pivotal mechanism for sensing the body's needs for iron to the enterocyte [16,17]. The membrane bound protein HFE which is mutated in approximately 80% of subjects with hereditary hemochromatosis [28], modulates the affinity of transferrin for TfR1 but its role in iron absorption remains elusive [29]. Importantly, the recent identification of the liver cationic peptide hepcidin has suggested that this molecule may be the principal regulator of iron absorption. [30–32]. The expression of hepcidin is increased when body iron stores are high [32–34]. Hepcidin over-expression resulted in reduced duodenal iron uptake and the development of anaemia [33,35] while reduced hepcidin expression causes iron overload [36–39]. This, may be traced back to a direct interaction of hepcidin with ferroportin expression, thus affecting iron transfer from the duodenal enterocyte to the circulation [40,41]. The liver derived protein hemojuvelin may have additive effects to those of hepcidin [42].

In the study presented here, we used a mouse model of experimental iron overload to study adaptive changes of iron transport molecules in duodenum, liver and spleen in order to get new insights into regulation of iron homeostasis upon secondary iron overload.

2. Material and methods

2.1. Animal care

Male C57BL/6 mice were kept according to institutional and governmental guidelines on a standard rodent diet in the animal quarter at the University of Innsbruck.

Animals used for experiments were all between 12 and 16 weeks of age, with an average weight of 25 g. For induction of iron overload animals were daily intraperitoneally injected with 1 mg of iron dextran (venofer®) for up to 5 days.

Mice were anaesthetized, blood was collected by orbital puncture, and the animals were then sacrificed. Tissue samples were frozen in liquid nitrogen or fixed in formalin for immunohistochemistry.

2.2. RNA extraction, northern blot analysis and Real Time PCR

Total RNA was extracted from nitrogen frozen tissue samples using a guanidinium–isothiocyanate–phenol–chloroform based procedure as previously described and subjected to northern blotting [43]. For hybridisation we generated radiolabelled cDNA probes applying the oligoprimer procedure using a 2.3 kb EcoRI insert of murine TfR1, a 1.8 kb EcoRI insert of murine DMT-1, a 650 bp EcoRI insert of murine ferroportin, a 950 bp EcoRI insert of murine Dcytb,a 1.5 kb EcoRI insert of murine hephaestin, a 250 bp EcoRI insert of murine Hepcidin, a 900 bp EcoRI insert of murine HFE and a 1.9 kb HindIII insert of chicken beta actin of a PCR2.1 Vector. In parallel, quantitative PCR was carried out exactly as described [43]. The following primers (a, forward primer; b, reverse primer) and TaqMan probes (c, TaqMan probe) were used: **muTfR1**: (a) 5′-CGCTTTGGGTGCTGGTG-3′, (b) 5′-GGGCAAG TTTCAACAGAA GACC-3′, (c) 5′-CCCACACTGGACTTCGCCGCA-3′, **muHFE**: (a) 5′-TCATGAGAGTCGCCGTGCT-3′, (b) 5′-GGCTTGAGGTTTG CTCCA-3′, (c) 5′-AGCCCAGGGCCCCGTGGAT-3′, **muDMT-1(IRE-form)**: (a) 5′-CCAGCCAGTAAGTTCAAGGATCC-3′, (b) 5′-GCGTAGCAGCT GATC TGGG-3′, (c) 5′-TGGCCTCGCGCCCCAACA-3′, **muFerroportin**: (a) 5′-CTACCATTAGAAGGATTGACCAGCT-3′, (b) 5′-CAAATGTCATA ATCTGGCCGA-3′, (c) 5′-CAACATCCTGGCCCC-CATGGC-3′, **muHepcidin**: (a) 5′-TGTCTCCTGCTTCTCCTCCTTG-3′, (b) 5′-AGCTCTGTA GTCTGTCTCATCTGTTGA-3′, (c) 5′-CAGCCT-GAGCAGCACCACCTATCTCC-3′. **muDcytb**: (a) 5′ -TCGCGG-TGACCGGCT-3′, (b) 5′-TTCCAGGTCCATGGCAGTCT-3′, (c) 5′-CGTCTTCATCCAGGGCATCGCC-3′, **muHephaestin**: (a) 5′-TGG-AACT ATGCTCCCAAAGGAA-3′, (b) 5′-CTGTTTTTGCCAGACTT CAGGA-3′, (c) 5′-CAAATCAGACTCTCAACAATGACACAGTGGCT-3′. **muHemojuvelin**: (a) 5′-GGT TTC GTC GGG AGC CA-3′, (b) 5′-TGG TAG ACT TTC TGG TCA ATG CA-3′, (c) 5′-CGC TAC CAC CAT CCG GAA GAT CAC TAT CAT ATT-3′, **muTNFα**: (a) 5′-TTCTATGGCC-CAGACCCTCA-3′, (b) 5′-TTGCTACGACGTGGGCTACA-3′, (c) 5′-CTCAGATCATCTTCTCAAAATTCGA GTGACAAGC-3′, **muIL6**: (a) 5′-GAGGATACCACTCCCAACAGACC-3′, (b) 5′-AAGTGCATCATC GTTGTTCATACA-3′, (c) 5′-CAGAATTGCCATTGCACAA CTCTTT TCTCA-3′.

2.3. Iron regulatory protein activity

Protein-extracts were prepared from nitrogen frozen tissue homogenized in cytoplasmic lysisbuffer (25 mM Tris–HCl pH 7.4, 40 mM KCl, 1% Triton X-100) containing 1 μg/ml of each aprotinin, leupeptin and phenylmethylsulfonyl flouride. For gel retardation assays a ^{32}P-labelled IRE probe was prepared [44], and the analysis of RNA/protein complexes was carried out by non-denaturing gel electrophoresis and subsequent autoradiography as described [45].

2.4. Western blotting

For western blotting 20 μg of protein extracts, prepared as described for the gel retardation assay, were run either on a 10% (for TfR1) or a 15% (for ferritin and β-actin) SDS-polyacrylamide gel. Proteins were transferred onto a nylon membrane (Hybond-P, Amersham-Pharmacia, Vienna, Austria) and blocked in 1×TBS buffer containing 5% dry milk and 0.1% Tween (Merck, Vienna, Austria). The membrane was incubated either with human anti-TfR1-antibody (0.5 μg/ml, Zymed, Vienna, Austria), human anti-ferritin-antibody (2 μg/ml, Dako, Vienna, Austria), murine anti-HFE-antibody (a generous gift by Dr M. Chorney [46]) or human anti-β-actin (2 μg/ml, Sigma, Germany) and further processed as described [43]. β-actin hybridization was used to demonstrate equal protein loading onto gels.

2.5. Immunohistochemistry and Perls' Prussian Blue staining

Formalin fixed, paraffin embedded tissue specimens were used. High temperature antigen unmasking was performed using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). To inactivate endogenous peroxidase, the tissue was incubated with Peroxidase Blocking Reagent (DAKO, USA, Carpinteria, CA) for 30 min followed by a 30 min incubation with diluted goat normal serum using Elite Vectastain ABC Kit (Vector Laboratories) to reduce background staining. Subsequently, tissue sections were incubated at 4 °C over night with polyclonal rabbit anti mouse antibody direct against ferroportin, DMT-1 and Dcytb (Alpha Diagnostic international, San Antonio, USA), respectively. All subsequent steps were performed exactly as suggested by the manufacturer using Peroxidase Substrate Kit AEC (Vector Laboratories, CA). For quality control we performed immunohistochemistry by using only the second antibody, and in addition also by using an isotype-matched control for mouse IgG. In both circumstances, we did not get a specific staining.

Iron in tissue sections was detected by Perls' Prussian Blue staining following a standard protocol [47].

2.6. Quantitative measurement of duodenal iron uptake in vivo

For radioactive iron uptake assays control mice and mice having received five consecutive injections of iron dextran were orally fed with 100 μl of 1.05 mg/ml ^{59}Ferric chloride (specific activity >3 mCi/mg ^{59}Fe) in 0.01 M hydrochloric acid using a gastric tube. All mice were fasted 24 h before initiation of the oral iron uptake assay. Thirty minutes after oral iron administration, mice were anaesthetized, blood was collected by orbital puncture, and the animals were then sacrificed. The proximal 3 cm of the duodenum were cut out and extensively rinsed. Radioactive iron content in serum and duodenum were measured using a gamma-counter. In parallel, the incorporation of ^{59}Fe into duodenal ferritin-core-complexes was investigated as follows: Duodenal slices were subjected to cytosolic protein preparation, as described for gel retardation assay, and separated on a 8% SDS-polyacrylamide gels under non-reducing conditions with subsequent auto-radiography.

2.7. Chemical iron measurements

The concentration of iron in tissues was measured by atomic absorption spectrometry. Liver and spleen iron content was determined after acid digestion of tissue samples followed by iron quantification with atomic absorption spectrometry as described [23]. Values are expressed in microgram per gram wet weight.

2.8. Data analysis

Statistical analysis was carried out using SPSS statistics package and utilized Spearman's rank correlation and one-sided ANOVA.

3. Results

When investigating tissue iron content following iron loading over time we found progressive iron accumulation in liver (Table 1). In the spleen, iron content first increased significantly over 3 days before it descended in spite of continuing iron loading (Table 1).

3.1. Changes in the expression of iron metabolism genes in duodenum, liver and spleen upon experimental iron overload

To get an impression on the dynamic processes occurring after progressive iron loading we performed northern blots for target iron genes. In order to statistically quantify changes in gene expression patterns between baseline and at the end of cumulative iron overload RT-PCR was performed.

TfR1 mRNA levels progressively decreased in the liver as estimated by RT-PCR with iron loading over time, while they significantly increased in the duodenum (Table 2). In the spleen, TfR1 mRNA expression decreased over 3 days of iron administration but increased thereafter (Fig. 1). Thus, TfR1 mRNA levels inversely paralleled the changes in tissue iron content in liver and spleen (Tables 1 and 2).

While no significant change in DMT-1 expression was observed in the liver it decreased with prolonged iron overload in the spleen (Fig. 1, Table 2). In the duodenum, DMT-1 and Dcytb mRNA levels initially decreased upon induction of iron overload but then ascended with prolonged iron substitution (Fig. 1). In contrast, Dcytb mRNA concentration increased with iron loading in the liver and spleen (Table 2).

Ferroportin mRNA levels increased significantly in liver and spleen and decreased in the duodenum upon progressive iron loading (Table 2, Fig. 1), and hephaestin mRNA expression paralleled these changes (Table 2, Fig. 1), although not being significant in the spleen.

In the liver, both hepcidin and hemojuvelin mRNA levels significantly increased with iron loading over time. Hepcidin levels were maximal after 3 days of iron loading and remained high until day 6, thus being significantly higher than at baseline (Table 2). No difference in TfR-2 mRNA expression was observed between control and iron overloaded mice (Table 2).

Table 1
Changes of tissue iron content upon progressive iron loading

	C	*d*1	*d*3	*d*6	*P*
Liver	167 (±39)	636 (±191)	1270 (±693)	2091 (±792)	0.000
Spleen	197 (±48)	357 (±27)	402 (±157)	164 (±5)	0.045

Mice were divided into four different groups ($n=8$ each) receiving daily intraperitoneal injections of iron dextran for 1, 3 and 6 days plus one control group. All mice were sacrificed the same day and total tissue iron content was determined by atomic absorption in liver and spleen tissue slices. Data are expressed as means of microgram iron per gram tissue + SD. *P*-values annotate trends for changes in iron content over time (control to day 6) as estimated by ANOVA analysis.

Table 2
Changes over time in liver, duodenum and spleen mRNA levels of iron metabolism and transport molecules upon experimental iron load in mice

	Duodenum			Liver			Spleen		
	C	6*d*	*P*	*C*	6*d*	*P*	*C*	6*d*	*P*
TfR1	1.14 (±0.14)	2.17 (±0.29)	0.028	2.00 (±0.31)	1.40 (±0.17)	0.036	1.77 (±0.84)	5.51 (±2.07)	0.077
HFE	0.96 (±0.04)	1.60 (±0.18)	0.031	0.97 (±0.05)	1.64 (±0.21)	0.05	1.82 (±0.84)	1.16 (±0.63)	0.118
DMT-1	0.94 (±0.08)	1.15 (±0.16)	0.624	1.03 (±0.16)	1.33 (±0.14)	0.494	1.12 (±0.26)	0.38 (±0.18)	0.056
Ferroportin	1.39 (±0.21)	0.91 (±0.12)	0.033	1.01 (±0.11)	2.75 (±0.24)	0.000	1.03 (±0.06)	3.14 (±1.03)	0.041
Dcytb	2.05 (±0.74)	0.87 (±0.24)	0.041	0.77 (±0.34)	1.58 (±0.68)	0.021	2.72 (±1.5)	4.04 (±1.8)	0.286
Heph	2.84 (±0.37)	1.59 (±0.27)	0.005	0.81 (±0.27)	1.82 (±0.94)	0.02	1.95 (±1.27)	3.29 (±2.84)	0.566
TfR2				1.51 (±0.74)	1.28 (±0.49)	0.337			
Hepcidin				1.03 (±0.22)	4.90 (±0.88)	0.001			
Hemojuvelin				1.07 (±0.71)	1.56 (±0.41)	0.014			

Transferrin receptor (TfR1), HFE, divalent metal transporter-1 (DMT-1,) ferroportin, duodenal cytochrome *b* oxidase (Dcytb), Hephaestin (Heph), Hepcidin and hemojuvelin mRNA levels of eight mice in each group were quantified by RT-PCR analysis. Values for control and 6 days iron loaded mice are expressed as relative abundance normalized to 18S rRNA levels and are presented as mean+SD. *P*-values are calculated by oneway Anova.

Finally we determined IL-6 and TNF-α mRNA levels in spleen and liver samples by means of RT-PCR. We could not found any significant difference in the expression of these cytokines between control and iron loaded animals (data not shown). TfR2 and hemojuvelin were hardly detectable by northern blots technique and their expression was thus only determined by quantitative PCR.

3.2. Determination of intracellular iron availability by bandshift assays

To estimate the effects of secondary iron overload on intracellular iron availability in different tissues, RNA bandshift assays for determination of IRP activity were carried out. In the liver, IRP binding activity decreased over time with iron loading while in the spleen IRP binding affinity initially decreased but then increased after 6 days of iron overload (Fig. 2), thus paralleling the changes in iron content in both tissues. In contrast, IRP binding activity in the duodenum increased with iron administration over time (Fig. 2, Table 1).

3.3. Modulation of organ specific protein expression of critical iron genes by secondary iron overload

In keeping with mRNA data TfR1 protein expression, as determined by western blotting, was inversely regulated in liver and duodenum (Fig. 3). In the spleen, TfR1 protein

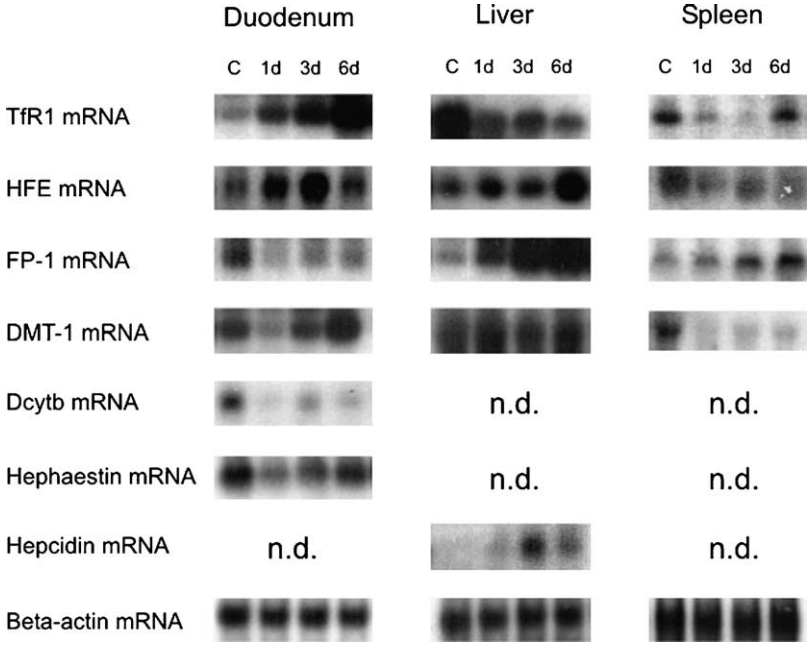

Fig. 1. Duodenal, liver and spleen mRNA levels of iron metabolism and transport molecules in control and secondary iron loaded mice determined by northern blot analysis. Mice divided in four different groups received none (C), one, three or five daily i.p. injections of iron dextran. All mice were sacrificed the same day and duodenal, liver and spleen tissue were subjected to RNA preparation for northern blotting. One of four representative experiments is shown for TfR1, HFE, DMT-1, ferroportin, Dcytb, Hephaestin, Hepcidin and beta-actin, respectively. RNA expression in some tissues was to low for detection by means of northern blot technique. These cases are identified by n.d. for not detected.

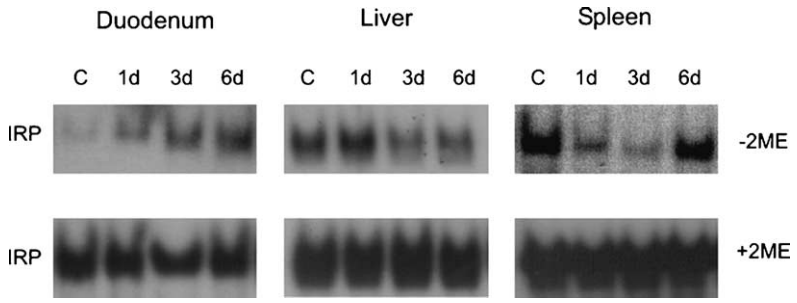

Fig. 2. Effect of secondary iron overload on IRP binding activity in duodenum, liver and spleen. Duodenum, liver and spleen tissue from mice of the four different groups was subjected to cytosolic protein preparation for following bandshift assays. One of four representative experiments is shown. Total IRP amounts were determined by incubation with 2-mercaptoethanol.

expression first decreased and then returned to baseline expression, paralleling changes in IRP binding activity (Fig. 3).

Moreover, HFE protein levels mirrored mRNA levels showing maximum HFE protein expression in liver and duodenum after 3–6 days of iron loading (Fig. 3).

Ferritin protein levels in liver increased with prolonged iron challenge while no significant changes were found in duodenum. Spleen ferritin levels were elevated already after 1 day and remained high thereafter (Fig. 3). In duodenum and spleen, both H-ferritin and L-ferritin, were detectable already in control mice, whereas in liver H-ferritin, reflecting the lower band in the western blot of mice [48], could not be detected until 6 days of iron loading (Fig. 3)

In accordance with mRNA data (Fig. 1) ferroportin protein expression decreased with prolonged iron overload in the duodenum (B1, 5). In the liver of control mice, ferroportin (A1) was detectable in the sinusoidal borders of hepatocytes and Kupffer cells at low levels. During the iron loading phase ferroportin expression strongly increased in Kupffer cells and vanished in hepatocytes (A5). Ferroportin in spleens of iron loaded mice (C5) was restricted to the red pulp, reflecting reticulo-endothelial cells.

DMT-1 tissue expression in liver (A2, A6) and spleen (C2, C6) was not significantly altered by secondary iron overload while the apical DMT-1 staining in the duodenum was higher in iron loaded mice than in controls. In contrast, Dcytb showed no specific staining in the liver (A3, A7) and is therefore not shown in the figure. Perls' Prussian Blue staining showed increased iron accumulation in iron overloaded animals (A8, C8) as compared to controls (A4, C4) in liver and spleen. However, iron accumulation in the spleen was strongest in mice loaded only for 3 days (not shown). The staining could be referred to iron storage within cells of the reticulo-endothelial system. Moreover, an iron deposition in parenchymal liver cells could be found after 6 days of iron loading (Fig. 4).

3.4. Correlations between liver hepcidin mRNA and iron transporter mRNA in different tissues

Spearman rank correlation technique was applied to study the cross-regulatory interactions between hepcidin and iron transporter mRNA expression in the tissues investigated. In control animals, liver hepcidin mRNA levels correlated negatively to duodenal DMT-1 mRNA ($r=-0.673$, $P=0.033$) and positively to hepatic TfR1 mRNA ($r=0.873$, $P=0.000$) levels. Following iron overload we found a positive association between hepcidin and ferroportin levels in liver ($r=0.687$, $P=0.000$) and spleen ($r=0.461$, $P=0.05$) while a negative correlation was evident in duodenum ($r=-0.561$, $P=0.005$).

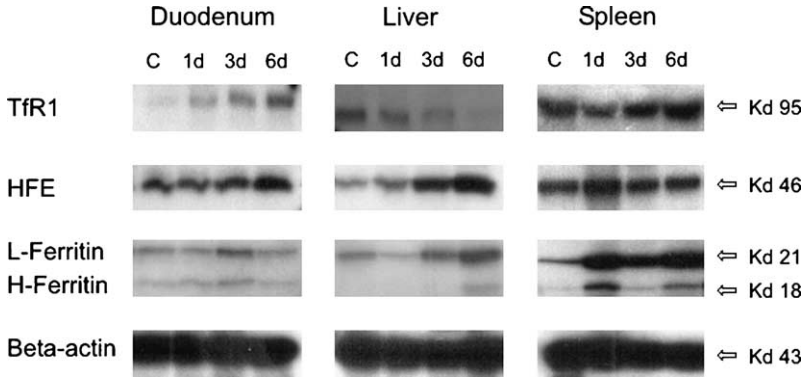

Fig. 3. Effect of secondary iron overload on protein levels of iron metabolism and transport molecules in different tissues. The same tissues as used for RNA preparation were subjected to protein preparation for subsequent western blotting and determination of protein levels of TfR1, HFE, ferritin and beta-actin in duodenum, liver and spleen. One of four representative experiments is shown.

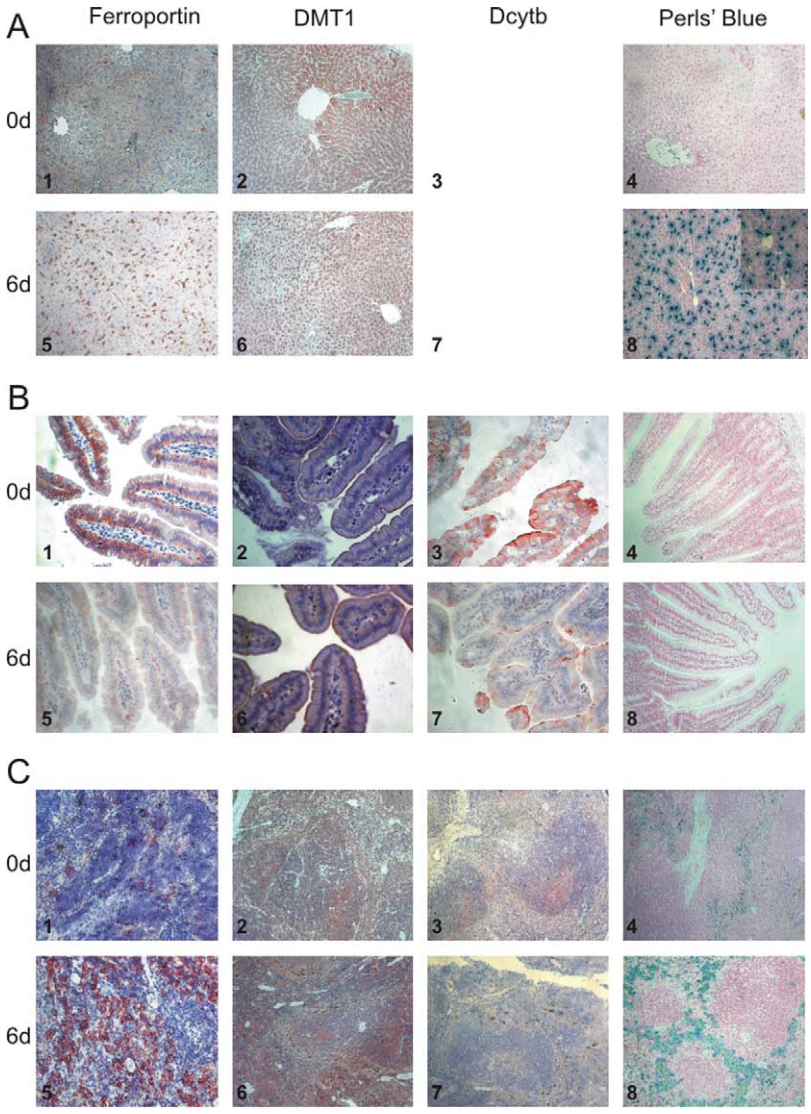

Fig. 4. Tissue distribution of ferroportin, DMT-1, Dcytb and Perls' Prussian Blue staining in liver, duodenum and spleen. Bright field photographs of mouse liver (panel A), duodenum (panel B) and spleen (panel C) immunohistochemistry using affinity purified anti-ferroportin (1, 5), anti-DMT-1 (2, 6), anti-Dcytb antibody (3, 7) and Perls' Prussian Blue staining (4, 8). Control tissue (1–4) and tissue of 6 times injected (cumulative dose 15 mg iron dextran) mice (5–8) correspond to the stated numbers. A3 and A7 are not shown, as there was no specific staining with Dcytb antibody in the liver. All sections were paraffin-embedded. Antibody concentrations were 2.5 μg/ml for anti-ferroportin and anti-Dcytb and 5 μg/ml for anti-DMT-1 antibody. One of eight slides is shown each.

No significant correlations between hepcidin mRNA and HFE, Dcytb or hephaestin expressions, respectively, were observed at any time in the organs investigated (not shown).

3.5. Impact of secondary iron overload on duodenal iron absorption

Control mice and mice after 5 consecutive days of intraperitoneal iron injection were subjected to oral feeding with ^{59}Ferric chloride using a gastric tube. The content of radioactive iron in duodenal slices was significantly higher in iron overloaded than in control mice (Fig. 5a). This was paralleled by an increased incorporation of absorbed radioactive iron within duodenal ferritin in the experimentally iron overloaded group (Fig. 5c). Accordingly, the transfer of iron form the duodenal enterocyte to the circulation was significantly reduced in iron overloaded species as compared to controls (Fig. 5b).

4. Discussion

We herein investigated the dynamic changes in the expression patterns of regulatory iron genes in different organs following experimental iron overload.

As duodenal DMT-1 mRNA and protein levels are surprisingly up regulated with prolonged experimental iron overload, DMT-1 appeared unlikely to be the gate keeper

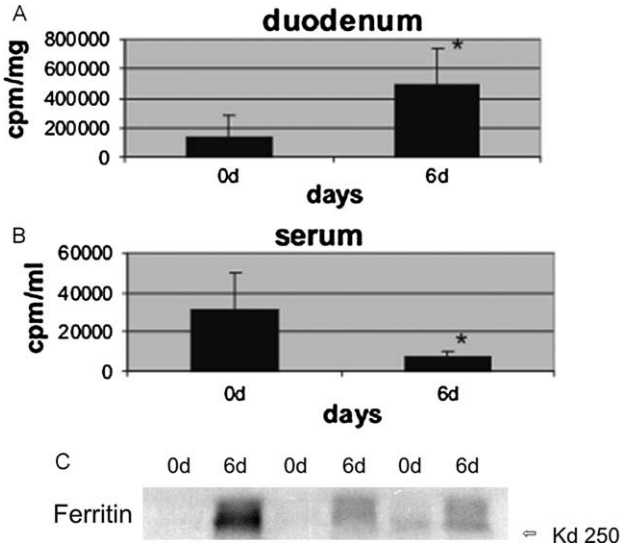

Fig. 5. Effect of secondary iron overload on iron uptake, transfer to the plasma and incorporation of Fe^{59} into ferritin core complex in the enterocyte. Control mice and iron loaded mice (five i.p. injections of dextran) were orally fed 1.05 mg/ml ^{59}Ferric chloride (specific activity >3 mCi/mg Fe) in 0.1 M hydrochloric acid diluted in 100 μl NaCl0.9% using a gastric tube. Mice were sacrificed 30 min later. (A) Duodenal iron uptake was defined as the radioactive iron content measured in extensively rinsed duodenal enterocytes per mg dry weight. (B) Whereas iron transfer was represented by the radioactive serum iron content. Serum samples were taken short before sacrificing the mice. Radioactive content in duodenum and serum were estimated using a gamma counter. Data are presented as mean ± SD of six individuals in each group. *$P < 0.05$ for comparison of iron loaded versus control mice. (C) For the incorporation assay small duodenal of three control and three iron loaded mice were subjected to cytosolic protein preparation. Two hundred micrograms of protein of the cell lysates were loaded on 8% SDS-polyacrylamide gels and electrophoresed under non-reducing conditions using a Tris/Tricine-electrophoresis buffer.

for the control of duodenal iron absorption. Accordingly, an in vivo radioactive iron uptake assay demonstrated that iron uptake into enterocytes is even higher in iron overloaded than in the control animals. Yet, the transfer of the metal from the enterocyte to the plasma was strongly reduced in the iron loaded group, which significantly paralleled the reduced ferroportin expression observed with this condition. Therefore, it is suggestive that iron first accumulates in the enterocyte and promotes ferritin translation with subsequent incorporation of iron into the ferritin core [49] (Fig. 5). This dynamic process will then results in a reduction of metabolically available iron in the cytoplasm with subsequent activation of IRP binding affinity leading to increased expression of DMT-1 and TfR most likely via IRP mediated stabilisation of the respective mRNAs [50]. This is also reflected by our observation of increased IRP activity and high TfR1 and DMT-1 mRNA expression in secondary iron overloaded mice [46,51].

Thus, iron taken up from the duodenal lumen is not transferred to the circulation in our model of experimentally iron overloaded animals which is in accordance with recent data showing that DMT-1 expression is rather affected by luminal iron availability [52], while ferroportin expression responds to systemic iron needs [53] which is also indicative from the negative correlation between duodenal ferroportin mRNA and liver hepcidin mRNA expression levels in iron overloaded mice. Hepcidin interacts with ferroportin mediated iron transfer from the enterocyte as hepcidin induces the internalisation of ferroportin, thereby reducing its iron transport capacity [40]. Recent in vivo data showing that hepcidin injection into rats [53] and mice affect duodenal iron absorption [54] also support this concept.

However, when investigating control animals in our study hepcidin mRNA levels were negatively associated with DMT-1 but not with ferroportin mRNA expression which resembles the observations made by Laftah et al. [54] in normal and iron deficient mice. Thus, hepcidin may exert divergent effects on duodenal iron absorption depending on body iron status [55,56].

This leads to the next question of how excess iron, e.g. acquired by multiple transfusions, is handled within non-hemochromatotic individuals. In this context, macrophages play a decisive role. Following the first i.p. injection the iron content of spleen macrophages and Kupffer cells increased and IRP-activity, as a marker for the intracellular labile iron pool [27] decreased. With progressive iron accumulation we observed a continuous increase in macrophage ferroportin and ferritin expression which may be referred to translational induction of these genes by iron [49]. Thus, after initial accumulation iron was stored within ferritin while the cytoplasmic concentrations of metabolically available iron decreased as indicated by a progressive induction of IRP binding affinity. The latter may also referred to the fact that iron is exported from macrophages/Kupffer cells by ferroportin. Such a strategy would make sense form the immunological point of view since excess iron within cells of the reticulo-endothelial system is associated with an impaired immune response and an increased susceptibility towards infections and cancer [4,57,58].

Hepatocytes respond to excess iron with an increased expression of hepcidin which may then decrease duodenal iron absorption [59–61]. In addition, iron uptake molecules in the liver, such as DMT-1 and TfR1 are down-regulated with experimental iron overload while ferroportin expression is increased [60], however, since whole liver tissue has been investigated we cannot differentiate, at least at mRNA levels, whether these expressional changes are rather related to hepatocytes or Kupffer cells.

In summary, we have demonstrated the dynamic changes occurring in body iron homeostasis with secondary iron overload and enlightened some aspects of the regulatory network which controls iron accumulation in this setting involving metabolic changes in the liver, spleen and the duodenum.

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