Adaptive changes of duodenal iron transport proteins in celiac disease

Donatella Barisani, Antonina Parafioriti, Maria Teresa Bardella, Heinz Zoller, Dario Conte, Elisabetta Armiraglio, Cristina Trovato, Robert O. Koch, and Günter Weiss

1Department of Experimental and Environmental Medicine and Medical Biotechnology, University of Milano-Bicocca, 20052 Monza; 2Department of Pathology, Istituto Ortopedico Gaetano Pini, 20122 Milan; 3Department of Medical Sciences, IRCCS Ospedale Maggiore, 20122 Milan, Italy; and 4Department of Internal Medicine, University of Innsbruck, A6020 Innbruck, Austria

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Barisani, Donatella, Antonina Parafioriti, Maria Teresa Bardella, Heinz Zoller, Dario Conte, Elisabetta Armiraglio, Cristina Trovato, Robert O. Koch, and Günter Weiss. Adaptive changes of duodenal iron transport proteins in celiac disease. Physiol Genomics 17: 316–325, 2004. First published March 30, 2004; 10.1152/physiolgenomics.00211.2003.—Iron deficiency is a manifestation of celiac disease (CD) usually attributed to a decreased absorptive surface, although no data on the regulation of iron transport under these conditions are currently available. Our aim was to evaluate divalent metal transporter 1 (DMT1), duodenal cytochrome b (Dcytb), ferroportin 1 (FP1), hephaestin, and transferrin receptor 1 (TIR1) expression, as well as iron regulatory protein (IRP) activity in duodenal biopsies from control, anemic, and CD patients. We studied 10 subjects with dyspepsia, 6 with iron-deficiency anemia, and 25 with CD. mRNA levels were determined by real-time PCR, protein expression by Western blotting or immunohistochemistry, and IRP activity by gel shift assay. Our results showed that DMT1, FP1, hephaestin, and TIR1 mRNA levels were significantly increased in CD patients with reduced body iron stores compared with controls, similar to what was observed in anemic patients. Protein expression paralleled the mRNAs changes. DMT1 protein expression was localized in differentiated enterocytes at the villi tips in controls, whereas with iron deficiency it was observed throughout the villi. FP1 expression was localized on the basolateral membrane of enterocytes and increased with low iron stores. TIR1 was localized in the crypts in controls but also in the villi with iron deficiency. These changes were paralleled by IRP activity, which increased in all iron-deficient subjects. We conclude that duodenal DMT1, FP1, hephaestin, and TIR1 expression and IRP activity, thus the iron absorption capacity, are upregulated in CD patients as a consequence of iron deficiency, whereas the increased enterocyte proliferation observed in CD has no effect on iron uptake regulation.

divalent metal transporter 1; transferrin receptor 1; ferroportin 1; iron absorption

Iron absorption occurs mainly in the duodenum through the differentiated enterocytes present at the tip of the villi, as demonstrated in animal models (29) and human samples (44). Recently, several proteins involved in intestinal iron absorption have been cloned, allowing identification of the steps involved in this process (1, 13, 18, 27, 28, 46). Intestinal iron absorption includes uptake across the apical membrane via an energy-dependent process through the divalent metal transporter 1 (DMT1) a highly hydrophobic integral membrane glycoprotein able to transport Fe2+ and other divalent cations by a proton-coupled process (18, 39). DMT1 duodenal expression is regulated by the body’s needs for iron, as supported by the observation that its mRNA and protein expression in the duodenum are increased with iron-deficiency anemia (9, 18, 48–50). The mechanism underlying this regulation may be linked to the presence of a specific RNA hairpin structure called an iron-responsive element (IRE) within the 3′-untranslated region (UTR) of DMT1 (14). IREs are targeted by iron-regulatory proteins (IRP 1 and 2); IRP binding affinity to IREs is increased by iron deficiency, and this may result in mRNA stabilization and increased protein expression (22).

The iron present in the diet is mainly in the ferric form, and thus needs to be reduced before its uptake via DMT1. This process may occur through the duodenal cytochrome b (Dcytb), a reductase which is expressed at the apical membrane of villous enterocytes (28, 52), although its role in iron absorption has not been confirmed yet. Other proteins are involved in iron efflux from intestinal cells; in particular ferroportin 1 (FP1), a transmembrane protein situated on the basolateral surface of the enterocytes (1, 13, 27) that is essential for iron transfer across the epithelial barrier. FP1 bears an IRE in its 5′-UTR that has been demonstrated to be able to bind IRPs (1). Finally, hephaestin, a membrane-bound ceruloplasmin homolog, has been implicated in iron export from enterocytes (46). The role of this protein in iron efflux has also been shown in the mouse model of sex-linked anemia, characterized by a defect in iron efflux from intestinal cells caused by a mutation in the hephaestin gene (46).

The expression of the proteins involved in iron uptake responds to the body’s needs for iron, and it has been suggested that iron requirements are sensed by the undifferentiated enterocytes of the crypts, which then predetermine the level of expression of these proteins in the differentiated cells present at the top of the villi. However, it is still unclear how the need for iron is sensed by the duodenal enterocytes. It has been suggested that TIR1-mediated iron uptake from the basolateral side of the intestinal crypt cells determines the level of the expression of iron transport proteins (4). Conversely, TIR1 could also be regarded as an index of cell proliferation, since it
is necessary to supply iron needed to enter the cell cycle (24). The expression of TIR1 has been reported to be increased in untreated CD patients, although no information was available as to whether this is related to body iron stores (26).

A protein that may shed light on the regulation of iron absorption has recently been discovered and named hepcidin (30, 34, 35). Hepcidin is a peptide hormone synthesized in the liver and secreted as 20–25-amino acid peptides. Liver hepcidin expression has been detected increased in iron overload in rats (35), whereas, in the liver of hemochromatotic subjects, its mRNA has either been reported decreased (8) or unchanged (16) compared with controls. The role of this molecule as an iron absorption regulator is also supported by data obtained in genetically modified mice, since hepcidin knockout animals develop severe iron overload (30), whereas transgenic ones show iron deficiency (31). Moreover, in rats, an inverse relationship has been demonstrated between hepatic hepcidin mRNA levels and the duodenal expression of DMT1, FP1, and hephaestin (15). Thus hepcidin could represent the signal peptide secreted by the liver that intervenes in the regulation of the expression of iron transporters in the duodenum, although the mechanisms of this regulations remains elusive.

In the present study we thus evaluated the expression of DMT1, Dcytb, FP1, hephaestin, and TIR1, as well as IRPs activity in duodenal biopsies of CD patients, and their relationship to body iron stores, and we compared the results with those obtained in normal subjects and patients with iron-deficiency anemia.

PATIENTS AND METHODS

Duodenal biopsies from a total of 41 subjects who underwent upper gastrointestinal endoscopy with duodenal biopsy for diagnostic purposes were investigated after having obtained their informed consent. The study was approved by the pertinent ethics committee (IRCCS Ospedale Maggiore, Milan, Italy).

As a control we used 10 patients (8 women and 2 men) with dyspepsia, with normal endoscopic and histological findings. We also studied six patients (4 women and 2 men) with iron-deficiency anemia due to menorrhagia (n = 3), polyposis coli (n = 2), and gastric ulcer (n = 1). Patients were considered iron deficient when ferritin levels were <15 ng/ml for men and <10 ng/ml for women.

The CD patient group consisted of 20 women and 5 men. Diagnosis of CD was based on positive anti-endomysium antibody testing, duodenal histology (Marsh’s classification grade 3) (20) and response to a GFD. Twelve patients were enrolled at diagnosis (10 with iron deficiency and 2 with normal iron stores), while 13 (2 with iron deficiency and 11 with normal iron stores) had been on a GFD for a mean period of 6.9 yr (range 2–14 yr). In this latter subgroup patients were reevaluated by endoscopy, at the time of this study, to assess treatment effectiveness, according to our own current policy. In all cases the response to GFD was assessed by histology, which showed a Marsh’s classification grade 0 or 1. For the purpose of the study, CD patients were divided according to their serum ferritin levels as described in the normal and iron deficiency groups. Age and iron-related indexes of the above groups are given in Table 1.

All subjects’ biopsies were analyzed by immunohistochemistry, whereas 30 underwent mRNA assessment by quantitative PCR, 13 underwent protein expression evaluation by Western blotting, and 15 underwent IRPs activity analysis by gel retardation assay. All subjects underwent two or more different analyses (immunohistochemistry and molecular assays), according to the number of frozen biopsies available.

Biopsy processing. Three biopsies were taken from the distal duodenum, fixed in a 10% solution of paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) and embedded in paraffin. Sections were mounted on polylysine- or silane-treated slides (Sigma, Milan, Italy) for hematoxylin and eosin staining and immunohistochemistry. Histological findings in CD patients were classified using Marsh’s criteria (25). Duodenal biopsies were also frozen in liquid nitrogen for RNA isolation or protein extraction and gel retardation assay.

Antibody purification. Anti-DMT1 antiserum, which recognizes both the IRE and non-IRE form of the protein, and anti-hephaestin antiserum were generated as previously described (21, 50, 52). Anti-ferroportin antiserum was generated by immunizing rabbits with the synthetic peptide FACGPDAKEVRKENANTSV. Antibodies were purified by affinity chromatography using a Sepharose-4B column linked to the specific peptides used for the immunization.

Western blotting of duodenal extracts and immunohistochemistry of duodenal biopsy slides performed in presence of the blocking peptide did not detect any signal (50, 52). Anti-Dcytb antiserum was generated as previously described (28) and kindly provided by Dr. A. McKie.

Immunohistochemistry. Immunohistochemistry was performed as previously described (7) on all the subjects enrolled in the study. Briefly, slides were pretreated with 3% H2O2 for 15 min to inhibit endogenous peroxidase and with blocking agent (Normal Reagent; Vector, Burlingame, CA) for 30 min to prevent background staining. Incubation with primary antibodies was performed for 1 h for anti-DMT1 and anti-FP1 (10 μg/ml), anti-Ki67/Mib1 (10 μg/ml; Dako, Glostrup, Denmark), and anti-TIR1 Ab (7 μg/ml; Zymed, San Francisco, CA), respectively. Detection of the hybridization signal was performed with biotinylated secondary antibodies (anti-rabbit IgG and anti-mouse IgG for DMT1, FP1, and TIR1 or Ki67/Mib1, respectively; Vector Elite Kit, Vector), followed by peroxidase-conjugated streptavidin and diaminobenzidine solution (ABC detection system, Vector). Counterstaining was performed with Harris hematoxylin. Sections were examined independently by a gastroenterologist (D. Barisani) and pathologists (A. Paraforitii and E. Armiraglio) unaware of patients’ body iron stores. In particular, proliferation rate was assessed on serial slides by measuring the number of positive nuclei for the proliferative index Ki67/Mib1 using an image analysis computer system (Image-Pro Plus; Media Cybernetics, Silver Spring, MA). In each subject, the entire slide was evaluated at ×400 magnification; the number of positive epithelial cells was assessed by two independent pathologists (A. Paraforitii and E. Armiraglio) in five different fields.

Total RNA isolation and cDNA synthesis. Total RNA was prepared from the duodenal biopsies as previously described (50). One microgram of total RNA was reverse transcribed using random hexamers (Roche, Mannheim, Germany) and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers’ instructions.

Quantitative PCR. Six controls, 18 CD patients (9 with low and 9 with normal ferritin values), and 6 patients with iron-deficiency anemia were analyzed by TaqMan quantitative PCR. Primers and probes were designed using the Primer Express Software (Perkin-Elmer, Monza, Italy) to span introns to avoid coamplification of

Table 1. Age, hemoglobin, and serum ferritin in controls, patients with iron-deficiency anemia, and those with celiac disease

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 10)</th>
<th>Iron-Deficiency Anemia (n = 6)</th>
<th>Low body iron stores (n = 12)</th>
<th>Normal body iron stores (n = 13)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>37.3 ± 10.3</td>
<td>39.6 ± 14.6</td>
<td>37.1 ± 18.6</td>
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<tr>
<td>Hb, g/dl</td>
<td>14.5 ± 1.5</td>
<td>10.5 ± 1.0</td>
<td>11.0 ± 0.8</td>
<td>13.8 ± 1.2</td>
</tr>
<tr>
<td>SF, ng/ml</td>
<td>80.1 ± 18</td>
<td>105.2 ± 2.2</td>
<td>7.4 ± 2.1</td>
<td>48.3 ± 38.2</td>
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Values are means ± SD. Hb, hemoglobin; SF, serum ferritin.
genomic DNA. All primers and probes were purchased from Microsynth (Baglang, Switzerland). Quantification of DMT1, Dcytb, hephaestin, ferroportin 1, and TfR1 cDNA was performed as previously described (50, 52); to minimize intra-assay and interassay variability due to differences in RT efficiency, their cDNA levels were normalized to the amount of GAPDH cDNA in duodenal biopsies.

Western blotting. Duodenal expression of proteins involved in iron transport was analyzed by Western blotting in four controls and three subjects for each group. Briefly, proteins were extracted from duodenal biopsy specimens using RIPA buffer (150 mmol/l NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/l Tris-HCl, pH 8.0, 0.2 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin), and 10 μg of total protein was used for immunoblotting. Blots were incubated with 500 ng/ml affinity-purified anti-Dcytb, anti-DMT1, anti-FP1, and anti-hephaestin antibody and with 200 ng/ml anti-TfR1 for 2 h at room temperature and, after washing, with secondary horseradish peroxidase-conjugated antibodies (anti-rabbit IgG for Dcytb, DMT1, FP1, and hephaestin, and anti-mouse IgG for TfR1; Pierce, Rockford, IL) at 1:10,000 dilution for 1 h. Signal detection was performed using the WestPico detection reagent (Pierce). Equal loading of the lanes was assessed by hybridization with anti-actin antibodies (Sigma, Milan, Italy). Quantification of Western blots was performed with a FluorS Multilager using the Quantity One 4.1.1 Software Package, both from Bio-Rad (Milan, Italy).

Generation of a 32P-labeled IRE probe and gel retardation assay. Detergent extracts were obtained from frozen biopsies (2 controls, 2 iron-deficiency anemia, 5 CD patients with reduced body iron stores, and 6 with normal body iron stores) and, after an initial homogenization, prepared as described previously for cultured cells (20). A 32P-labeled ferritin IRE probe was generated using T7 RNA polymerase, purified by gel electrophoresis (15% of 20:1 acrylamide/bisacrylamide; 6 mol/l urea), followed by probe elution and phenol/chloroform extraction. The DNA template had the following sequence: 5'-GGGATCCGTCCAAGCACTGTTGAAGCAGGATCC-CTATAGTGAATCGTAATTA-3'. Approximately 15,000 cpm of this transcript was incubated with 10 μg of cytoplasmic protein extracts at room temperature. After 20 min, heparin (final concentration, 3 mg/ml) was added for 10 min, and analysis of RNA/protein complexes was performed by nondenaturing gel electrophoresis and autoradiography. Autoradiographs were analyzed using the OptiQuant system. Relative IRP activity was calculated as the ratio between the IRP activity in the analyzed samples and their corresponding total IRP activity obtained by preincubation of the samples with 2% 2-mercaptoethanol.

Other methods. Hemoglobin was measured by standard methods, and serum ferritin was measured by RIA (liso-Phase; Lepllet, Milan, Italy). Anti-endomysial antibodies were measured by immunofluorescence using a commercially available kit (Eurospital, Trieste, Italy). Statistical analysis. Statistical evaluation was performed with the SYSTAT software package (SPSS, Chicago, IL). Due to the nongaussian distribution of the values for ferritin and DMT1, TfR1, Dcytb, hephaestin, FP1, and GAPDH cDNAs, values were converted into logarithms for statistical evaluation. Presence of significant differences between the various groups was assessed by Student’s t-test with Bonferroni correction for multiple comparison. Correlation between cDNAs ratios with serum ferritin was assessed by Spearman with Bonferroni correction for multiple comparison. Correlation between cDNAs ratios with serum ferritin was assessed by Spearman rank test.

RESULTS

Duodenal mRNA expression of proteins involved in iron transport. Total RNA extracted from duodenal biopsies obtained from subjects with dyspepsia, as well as from patients with CD (subdivided according to their body iron stores) or iron-deficiency anemia, was reverse transcribed into cDNA and used as template for TaqMan quantitative PCR.

An increased level of DMT1 mRNA was detected in subjects with low body iron stores. As depicted in Fig. IA, DMT1/GAPDH cDNAs ratio was significantly higher in patients with CD and low body iron stores and in subjects with iron-deficiency anemia compared with controls or CD patients with normal iron status (P < 0.05 for all comparisons). The expression of Dcytb mRNA was also increased in iron-deficient subjects compared with controls, but the difference did not reach statistical significance (Fig. 1B). Dcytb cDNA expression was also similar in CD patients with or without low body iron stores.

With regard to the proteins involved in iron transfer across the basolateral membrane, an increase in the mRNA levels of FP1 and hephaestin was observed in subjects with low body iron stores, independently from the presence of CD (Fig. 1, C and D). In particular, a pronounced increase in FP1 mRNA expression was present both in patients with CD and low body iron stores and iron-deficiency anemia subjects compared with controls (P = 0.019 and 0.037, respectively). These differences were also detected for hephaestin mRNA, which still reached statistical significance (P = 0.014 and P = 0.016, respectively). Similarly, an increased TfR1 mRNA expression was detected, as depicted in Fig. 1E, with significantly higher levels of the TfR1/GAPDH ratio in iron-deficiency anemia compared with controls or CD patients with normal iron stores (P = 0.04 and P = 0.034, respectively). TfR1 mRNA levels were also significantly increased in CD patients with low body iron stores compared with control subjects (P = 0.035).

To understand the coordinate expression of iron transporters and oxidoreductases, statistical correlation coefficients between mRNA levels of DMT1, FP1, Dcytb, TfR1, and hephaestin were calculated. A significant positive correlation was observed between the various cDNAs ratios, as assessed by the Spearman rank test (DMT1 and FP1, r = 0.814, P < 0.001; DMT1 and TfR1, r = 0.56, P = 0.002; DMT1 and hephaestin, r = 0.713, P < 0.001; DMT1 and Dcytb, r = 0.726, P < 0.001; FP1 and hephaestin, r = 0.388, P = 0.04; FP1 and Dcytb, r = 0.852, P < 0.001; TfR1 and hephaestin, r = 0.708, P < 0.001). In contrast, the expression of Dcytb and hephaestin or TfR1 (r = 0.379, P = 0.051; and r = 0.380, P = 0.0505, respectively) and FP1 and TfR1 (r = 0.379, P = 0.051) were not correlated, although the calculated correlation coefficients showed a borderline significance.

We further evaluated the possible correlation between the level of expression of the various mRNAs described above and the level of body iron stores, as assessed by serum ferritin levels. A significant negative correlation with the serum ferritin levels was detected for all cDNAs ratios but Dcytb (Spearman rank test, r = −0.568, P = 0.004 for DMT1; r = −0.384, P = 0.047 for FP1; r = −0.652, P < 0.001 for hephaestin; r = −0.463, P = 0.015 for TfR1; r = −0.162, P = 0.412 for Dcytb).

Iron transport protein expression. The increased DMT1 mRNA level detected with quantitative PCR was paralleled by an increased protein expression, as shown by Western blotting (data not shown) and immunohistochemistry (Fig. 2). In controls the DMT1 antisense showed staining of the apical part of the villi (Fig. 2A), with a specific pattern of distribution on the microvillus membrane (Fig. 2A, inset). DMT1 expression was increased in patients with iron-deficiency anemia, in whom also the middle and lower third of the villi showed staining (Fig. 2B). In presence
of low body iron stores, DMT1 staining was not limited to the microvillous membrane but was also present in the cytoplasm, although a stronger staining was observed on the microvilli and in microvillous membrane but was also present in the cytoplasm, of low body iron stores, DMT1 staining was not limited to the receptor 1 (TfR1) cDNA levels in duodenal biopsies from controls (C), patients with iron-deficiency anemia (IDA), and celiac disease patients with low (CD low Fe) or normal (CD) body iron stores. DMT1/GAPDH cDNA ratio (A), Dcytb/GAPDH cDNA ratio (B), FP1/GAPDH cDNA ratio (C), hephaestin/GAPDH cDNA ratio (D), and TfR1/GAPDH cDNA ratio (E) are shown on a logarithmic scale; median (horizontal line), interquartile range (boxes), and minimal and maximal values (vertical lines) are shown for each patient group. *P < 0.05 compared with controls. #P < 0.05 compared with nonanemic CD patients.

![Graphs showing DMT1, Dcytb, FP1, hephaestin, and TfR1 expression levels.](image)

**Fig. 1.** Divalent metal transporter 1 (DMT1), duodenal cytochrome b (Dcytb), ferroportin 1 (FP1), hephaestin, and transferrin receptor 1 (TfR1) cDNA levels in duodenal biopsies from controls (C), patients with iron-deficiency anemia (IDA), and celiac disease patients with low (CD low Fe) or normal (CD) body iron stores. DMT1/GAPDH cDNA ratio (A), Dcytb/GAPDH cDNA ratio (B), FP1/GAPDH cDNA ratio (C), hephaestin/GAPDH cDNA ratio (D), and TfR1/GAPDH cDNA ratio (E) are shown on a logarithmic scale; median (horizontal line), interquartile range (boxes), and minimal and maximal values (vertical lines) are shown for each patient group. *P < 0.05 compared with controls. #P < 0.05 compared with nonanemic CD patients.

The apical part of the cytoplasm, as depicted in Fig. 2 inset, in CD patients with reduced body iron stores, DMT1 protein was hyperexpressed in the epithelium covering the villi remnants in those with atrophic mucosa (Fig. 2C), whereas in those with normal appearing mucosa the protein was present throughout the entire villus (Fig. 2D). No staining was ever detected in the crypts. In CD patients with normal body iron stores, the intensity of the staining obtained with anti-DMT1 antibody was comparable to that observed in control subjects, being localized on the surface epithelium or at the top of the villi (Fig. 2, E and F, respectively).

Dcytb protein expression, assessed by Western blotting, was slightly increased in subjects with decreased body iron stores (about 50%, as assessed by densitometric analysis), either with or without CD, as shown in Fig. 3A; similar findings were also detected when hephaestin expression was considered (Fig. 3A). Ferroportin 1 expression was about two times higher in CD patients with low body iron stores and in iron-deficiency anemia patients (Fig. 3A), as assessed by densitometric analysis and normalization of the data obtained by actin hybridization (Fig. 3B). The increased expression was detected by immunohistochemistry only in patients with low levels of hemoglobin, showing the localization of FP1 on the basolateral membrane of the villi enterocytes (data not shown).

TfR1 expression was also regulated by body iron stores. In controls, most prominent TfR1 expression was observed in the crypts (along the basal and lateral membranes and in the basal cytoplasm), with some staining detected throughout the entire villus and no staining at the apical membrane (Fig. 4A). In patients with iron-deficiency anemia, the above crypt-to-villus gradient was lacking, and TfR1 was overexpressed throughout the entire villus (Fig. 4B). In CD patients, TfR1 expression was increased in crypt and villous enterocytes in both untreated (Fig. 4C) and treated (Fig. 4D) patients with low body iron stores. On the other hand, TfR1 protein expression in untreated and treated CD patients with replenished iron stores was similar to that observed in controls (Fig. 4, E and F, respectively).

**IRP binding activity.** The analysis of 15 patients (2 controls, 2 iron-deficiency anemia, 5 CD patients with reduced body iron stores, and 6 with normal body iron stores) revealed an increased IRP activity in non-CD subjects with iron-deficiency anemia (Fig. 5A, lanes 3 and 4) compared with controls (Fig. 5A, lanes 1 and 2). In CD patients with low body iron stores, an increased IRP activity comparable to that of anemic patients was observed (Fig. 5A, lanes 5 and 6), whereas in those with normal body iron stores IRP activity was reduced and thus similar to that observed in controls (Fig. 5A, lanes 7 and 8). Upon addition of 2-mercaptoethanol to the samples, total IRP activity was similar in all patients (Fig. 5B), indicating that differences in IRP activity reflect post-translational modifications.

The notion that IRP activity in enterocytes reflects body iron stores is supported by the finding of a highly significant inverse correlation (r = −0.731, P = 0.0014) between serum ferritin levels (expressed as logarithmic values) and relative IRP activity...
in duodenal biopsies, the latter being quantified by densitometric analysis of gel shift autoradiographies, as shown in Fig. 5C.

**Ki67/Mib1 expression.** To analyze whether the increased TfR1 expression could be attributed to different proliferation rates rather than to normal or decreased body iron stores, cell proliferation was assessed by determination of Ki67/Mib1 expression (data not shown). Cell proliferation rate, assessed by counting the number of positive nuclei of epithelial cells, was increased in CD patients with atrophic mucosa compared with both controls and CD patients with normal appearing mucosa. Moreover, Ki67/Mib1 protein expression was similar in untreated CD patients independently of the amount of total body iron stores, as also observed in treated CD patients with normal appearing mucosa. Overall these findings rule out the possibility that in our patients the observed increase in TfR1 expression reflects differences in cell proliferation rates.

**DISCUSSION**

The present study investigated DMT1, Dcytb, FP1, hephaestin, and TfR1 expression, as well as IRP activity in controls, in patients with iron-deficiency anemia and with CD, demonstrating for the first time that in the last group the expression of...
these proteins depends primarily on body iron stores rather than being affected by CD per se.

Iron-deficiency anemia represents one of the most frequent manifestations of CD at diagnosis, affecting about 65% of patients (2). Various underlying causes may take part in its development, including reduced iron absorption due to villous atrophy or chronic gastrointestinal bleeding (14). Although iron supplementation could be necessary, GFD alone was demonstrated able to correct the anemia and to normalize body iron stores in about 95 and 55% of patients, respectively (5).

The data presented here indicate that DMT1, FP1, hephaestin, and TIR1 expression (and to a lesser extent Dcytb) are similarly regulated in CD and non-CD patients and that these parameters are mainly affected by body iron stores. In controls, DMT1 was localized to the apical microvillous membrane of the enterocytes at the tip of the villi, similarly to what was previously observed in humans (50), rats (45), and mice (9, 10). DMT1 localization is consistent with the role of this transporter in the absorption of Fe$^{2+}$ from the gut, as supported by transport studies (41, 47). In the present study DMT1 expression (both at the mRNA and protein level) was increased in anemic CD patients and returned to normal after GFD and normalization of body iron stores. Thus DMT1 expression in CD is likely influenced by the amount of iron in the body and not by the changes in duodenal mucosa occurring in CD. It must be noted, however, that in iron-deficient subjects the staining was present also in the cytoplasm of enterocytes, a finding different from what previously reported by Zoller et al. (50) in humans and Trinder et al. (45) in rats. However, similar findings were observed by Canonne-Hergaux et al. (9, 10) in mice, where a cytoplasmic staining was observed in iron-deficient animals. The concordance between DMT1 mRNA and protein expression agrees with data obtained in rats (33) and humans (50), thus suggesting that the increased protein production depends on an increase in steady-state mRNA. Dcytb expression was not significantly increased either at the mRNA or protein level in celiac subjects with decreased body iron stores. Although this may suggest an effect of the mucosal damage on the reductase expression, this hypothesis is counteracted by the observation that Dcytb mRNA and protein

Fig. 3. Western blot analysis of Dcytb, hephaestin, and FP1 expression. A: representative results obtained in controls, iron-deficient CD patients, CD patients with normal body iron stores, and patients with iron-deficiency anemia (IDA). B: quantitative analysis of Dcytb, hephaestin, and FP1 expression. Densitometric values obtained for these protein were divided by densitometric values for actin measured in the corresponding sample. Median (horizontal line), interquartile range (boxes), and minimal and maximal values (vertical lines) are shown for each patient group. *$P < 0.05$ compared with controls. **$P < 0.05$ compared with nonanemic CD patients.
levels were comparable to controls also in subjects with iron-deficiency anemia (who did not show any alteration in duodenal histology), a finding similar to that observed in another cohort of anemic patients (41).

With regard to proteins involved in iron efflux or uptake across the basolateral membrane of the enterocytes, FP1 and hephaestin or TIR1, respectively, their mRNA levels were significantly increased both in celiac subjects with decreased body iron stores as well as in subjects with iron-deficiency anemia. This finding was also supported by the detection of increased protein expression for these molecules, data which agree with those previously observed by Rolfs et al. (38) and Zoller et al. (50). The increased expression of FP1 could be detected by immunohistochemistry, confirming the expression of this protein on the basolateral surface of the differentiated enterocytes, as described in mouse (13) and humans (50). On the contrary, TIR1 was present in the basolateral portion of the cytoplasm of the crypt enterocytes, which is in accordance with observations made in rats (23) and humans (6). However, the data presented here have been obtained on paraffin-embedded tissue, which may cause an altered distribution of the staining obtained by immunohistochemistry. TIR1 distribution is more reliably assessed on frozen sections, and a different distribution of staining using anti-TIR1 antibodies on either frozen or paraffin-embedded sections has been reported in rats (32), although the authors employed an antibody different from that used in the present study. TIR1 expression was low in controls and CD patients with normal body iron stores, data which are

Fig. 4. Duodenal expression of TIR1 in controls, patients with iron-deficiency anemia, and CD patients. A: for controls, staining was mainly localized in the crypts. B: for patients with iron-deficiency anemia, staining was strongly increased throughout the entire villus. C: for patients with untreated CD and low body iron stores, TIR1 staining was present both in the crypts and on the surface epithelium. D: for patients with treated CD and low body iron stores, TIR1 staining was present throughout the entire villus. E: for patients with untreated CD and normal body iron stores, TIR1 staining was present in the crypts. F: for patients with treated CD and normal body iron stores, TIR1 was expressed mainly in the crypts. Original magnification was ×200 for A–F.
in accordance with a study by Pietrangelo et al. (36), which detected increased TR1 mRNA levels only in patients with severe anemia and suggested a causal relationship between TR1 expression and entocyte iron content. The localization of TR1 on the basolateral portion of the crypt enterocytes could also have a pivotal role in the sensing of body iron by these cells and in determining the level of expression of the other proteins involved in iron transport (4). However, the data presented here do not allow us to exclude the presence of other regulation mechanisms, such as that involving hepcidin, which may act directly on differentiated enterocytes regulating the expression of proteins involved in iron uptake (13, 30).

We also checked the possibility that TR1 expression may rather reflect a stimulated proliferation by the evaluation of cell proliferation rates using Ki67/Mib1 expression. As previously reported (26, 37) untreated CD patients showed a higher cell proliferation rate, but proliferation rates per se cannot explain the differences observed in the expression of the analyzed iron transport proteins, in particular TR1, since Ki67/Mib1 protein levels were similar in patients with or without iron deficiency.

A significant positive correlation was observed between the mRNA levels of all the molecules analyzed, excluding Dcytb, whereas an inverse correlation was observed between mRNA levels and ferritin levels. These findings further confirm the regulation of the expression of these proteins by body iron stores, a fact that represents the molecular basis of the increased iron absorption observed in iron deficiency in human duodenal biopsies (12, 17). Furthermore, they demonstrate the presence of similar regulatory mechanisms both in celiac subjects with reduced body iron stores and subjects with iron-deficiency anemia.

Both DMT1 and TR1 mRNA have at least one IRE element in their 3′-UTR that may regulate their expression at the posttranscriptional level through IRP/IRE interaction. Although a previous study (11) demonstrated the need for more than one IRE element to obtain an IRP/IRE-dependent stabilization of the respective mRNA in vitro, there is increasing evidence in favor of the ability of the DMT1 IRE to bind IRPs (19). In our series, the increased DMT1 and TR1 expression was also associated with an increased IRP activity. Thus reduced body iron stores will increase IRP activity, as supported by the finding of a significant inverse relationship between serum ferritin levels and IRP activity in duodenal cells. Since the human biopsy samples employed in the present study for IRP activity measurement included cells derived from both crypts and villi, it was not possible to assess the relative contributions of these two cell populations to the total IRP activity. This possible bias, however, seems to be excluded from data obtained in iron-deficient rats (40), which demonstrated that IRP activity was equally increased both in crypt and villi duodenal enterocytes. Interestingly, in the same rat iron-deficient model (40), infusion of parenteral iron normalized IRP activity first in crypt enterocytes and then in villous cells, supporting the idea of the “iron sensor” being located in the crypts. IRPs may then target the 3′-UTR of TFRC and DMT1 mRNAs and stabilize these mRNAs, subsequently leading to an increased expression of these proteins in the duodenum. In addition, DMT1 expression appears to be regulated by cellular iron availability by an as yet not elucidated transcriptional mechanisms (43, 51).

Fig. 5. Iron regulatory protein (IRP) activity in duodenal biopsies from controls, patients with iron-deficiency anemia, and CD patients. A: IRP activity was assessed by gel retardation assay in duodenal samples from controls (C, lanes 1 and 2), patients with iron-deficiency anemia (IDA, lanes 3 and 4), celiac patients (CD) with reduced body iron stores (CD low Fe, lanes 5 and 6), or CD patients with normal body iron stores (CD, lanes 7 and 8). B: total IRP activity was assessed by gel retardation assay after preincubation of the corresponding samples shown in A with 2% 2-mercaptoethanol. C: activated IRP/total IRP ratio evaluated by densitometric analysis in 2 controls, 2 iron-deficiency anemia patients, 5 CD patients with reduced body iron stores, and 6 CD patients with normal body iron stores. Median (horizontal line), interquartile range (boxes), and minimal and maximal values (vertical lines) are shown for each patient group.
In summary, the present findings indicate that, in CD patients, there is a positive correlation among DMT1,FP1, hephaestin, and TfR1 expression, as well as IRP activity. Moreover, the expression of these proteins involved in iron transport is inversely correlated to body iron stores, thus demonstrating the presence of normal iron absorption regulatory mechanisms even in patients affected by CD.

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