Iron regulatory proteins 1 and 2 in human monocytes, macrophages and duodenum: expression and regulation in hereditary hemochromatosis and iron deficiency

Stefania Recalcati
Alessandra Alberghini
Alessandro Campanella
Umberto Gianelli
Elisa De Camilli
Dario Conte
Gaetano Cairo

Background and Objectives. The functions of the iron regulatory proteins (IRP1 and IRP2), which control cellular iron homeostasis are similar but not identical. As an inappropriate up-regulation of total IRP activity has been found in the duodenum and monocytes of patients with hereditary hemochromatosis (HH), we investigated the respective roles of IRP1 and IRP2 in these settings.

Design and Methods. Specific antibodies were used in RNA-supershift, immunoblotting and immunohistochemistry assays to evaluate IRP1 and IRP2 separately in monocytes, macrophages and duodenum of control subjects, and patients with HH or iron-deficiency anemia.

Results. The activity of both IRP1 and IRP2 and the levels of IRP2 were: (i) higher in monocytes and macrophages of HH patients than in those of control subjects; (ii) increased in the duodenal samples of the patients with HH and iron-deficiency anemia. IRP2 levels increased when monocytes differentiated to macrophages. Under all of the examined conditions, IRP2 was induced to a greater extent. In the duodenum of HH and anemic patients, IRP1 was shifted from the aconitase form (present in controls) to the apoform, whereas the IRP1 in monocytes/macrophages was always in the apoform, in both the patients and controls. The RNA-bound fraction of IRP1 was small in all of the samples. Both IRP were expressed more in the villi than in the crypts of the duodenum, with no differences in localization or expression between the patients and controls.

Interpretation and conclusions. These findings of the first extensive investigation of the comparative expression of the two IRP in human tissues and blood cells indicate that IRP2 is the major regulator of intracellular iron homeostasis in humans.

Key words: iron, hemochromatosis, reticuloendothelial cells, duodenum.

Haematologica 2006; 91:303-310

From the Institute of General Pathology (SR, AA, GC), Department of Medical Sciences (AA, DC) and Department of Medicine, Surgery and Odontoiatry, University of Milan (UG, EDC), Dibit, IRCCS H. San Raffaele, Milan, Italy (AC).

Correspondence:
Gaetano Cairo,
Institute of General Pathology
University of Milan,
via Mangiagalli 31,
20133 Milan, Italy.
E-mail: gaetano.cairo@unimi.it

Iron is necessary for a number of essential cell functions but, as excessive amounts can be toxic, iron metabolism is carefully controlled at both cellular and systemic levels. The key proteins of iron homeostasis are the cytoplasmic iron regulatory proteins (IRP1 and IRP2) which, in response to fluctuations in the cell iron pool, post-transcriptionally control the expression of iron uptake and storage proteins such as the transferrin receptor (TfR) and ferritin.[1,2] When cell iron levels are low, IRP1 and IRP2 bind to iron responsive elements (IRE) in untranslated regions of transcripts, stabilizing TfR mRNA while also decreasing the translation of ferritin mRNA and thus increasing cell iron availability. The opposite occurs when cell iron levels are high: as the affinity of IRP1 and IRP2 for IRE decreases, the translation of ferritin mRNA is enhanced and the stability of TfR mRNA is reduced, thus preventing the intracellular formation of potentially toxic excess iron.

IRP1 is the cytosolic counterpart of mitochondrial aconitase and is regulated by a post-translational switch between an apoprotein form capable of binding mRNA and a form endowed with a 4Fe-4S cluster that possesses aconitase activity; although highly homologous to IRP1, IRP2 is unable to assemble an Fe-S cluster and is regulated by means of proteasome-mediated protein degradation.[1,2] IRP1 and IRP2 have similar but not identical functions. Both bind to consensus IRE sequences with similar affinity and specificity, but IRP2 can preferentially recognize an exclusive IRE subset such as that present in ferritin mRNA,[3] and is specifically regulated under pathophysiologic conditions such as cell proliferation, oxidative stress and inflammation.[1,4] Furthermore, gene targeting studies have shown that IRP2 may play a predominant role *in vivo*, as IRP1$^{-/-}$ mice have a mild phenotype, whereas IRP2$^{-/-}$ animals misregulate iron metabolism in the liver, brain and duodenum.[5-8] Given their pivotal role in controlling iron metabolism, IRP also regulate iron transport and systemic iron balance,

and hence play a role in iron metabolism disorders. Abnormalities in IRP activity have been found in the duodenum and monocytes/macrophages of patients with hereditary hemochromatosis (HH). In particular, we have previously shown that total IRP1 plus IRP2 activity is inappropriately up-regulated in these two compartments,[9-11] which suggests that these cells are paradoxically iron-deficient in iron overloaded subjects. As the two IRP cannot be distinguished in human cells by means of the common bandshift assays,[12] these previous studies[9-11] were unable to determine whether there was a particular modulation of IRP1 vs IRP2 in the duodenum or monocytes/macrophages of HH patients. However, the demonstration that inflammatory agents simultaneously activate IRP1 and repress IRP2 in the J774 mouse macrophage cell line suggests that these proteins may be differentially regulated in HH,[13] and this may be highly relevant for the expression of IRP-controlled mRNA because IRP1 and IRP2 may preferentially bind different IRE-containing mRNA.[3] In fact, iron homeostasis in J774 cells exposed to inflammatory agents is regulated by nitric oxide-mediated IRP2 down-regulation rather than IRP1 activation.[13] The above considerations indicate that it may be important to analyze the activity patterns of the individual IRP in order to unravel the abnormalities of iron metabolism in HH.

In the present study, we used antibodies specific for IRP1 and IRP2 in supershift and immunoblotting assays to evaluate the level and the activity of IRP1 and IRP2 separately in monocytes, macrophages and duodenum, which are crucial for iron recycling and absorption, taken from control subjects, and HH and anemic patients. We also performed immunohistochemistry experiments to analyze the pattern of expression of these proteins in the duodenum, and made use of a novel assay to assess the distribution of IRP1 between its two functional forms.

Design and Methods

Subjects

The 51 study subjects gave their informed consent, and the study protocol was approved by the Ethics Committee of the University of Milan. The subjects were unrelated.

Control group. Monocytes were purified from 16 healthy blood donors (10 men and 6 women, aged 31-65 years) with no clinical history of iron metabolism disorders and normal serum iron indices. Duodenal biopsy samples were obtained from 8 patients (5 men and 3 women, aged 34-61 years) undergoing upper gastrointestinal tract endoscopy for dyspepsia, with no evidence of abnormal findings at histology.

*Hereditary hemochromatosis group. M*onocytes were purified from 12 patients (9 men and 3 women, aged 26-59 years; 5 untreated and 7 on a phlebotomy program) diagnosed as having HH on the basis of previously reported standard criteria;[9] all were homozygous for the major C282Y mutation in the *HFE* gene. Duodenal biopsy samples were obtained from 9 patients (6 men and 3 women, aged 28-62 years) undergoing endoscopy for dyspepsia.

Iron deficiency anemia group. Duodenal biopsy samples were obtained from 6 patients (4 men and 2 women, aged 29-70 years) with anemia secondary to gastric or duodenal ulcers.

Biochemical evaluations

Hemoglobin and the serum iron and transferrin saturation indices were determined using standard, previously reported techniques.[9] Serum ferritin was measured by means of an enzyme immunoassay (Enzymuntest, Boehringer Mannheim, Milan, Italy).

Monocyte isolation and culture

The monocytes were purified as previously described.[10] Buffy coats were prepared from venous heparinized blood, and mononuclear cells were separated on Ficoll-Paque solution (Amersham Co. Milan, Italy). The monocytes were then separated from lymphocytes by density gradient centrifugation on a solution consisting of RPMI 1640 medium (54%) and 285 mOsm Percoll (46%) (Amersham Co. Milan, Italy). The monocyte yield, purity, viability and recovery were as previously reported.[10] The cells were either pelletted and stored in aliquots at -80 °C or cultured. To induce their differentiation to macrophages, the monocytes were resuspended in RPMI 1640 medium containing 2 mM glutamine, antibiotics and 10% serum and kept in 5% CO_2 at 37 °C for 6 days in the presence of 100 μg/mL granulocyte-macrophage colony-stimulating factor (PeproTech EC, London, UK). The medium and all of the reagents were free of endotoxins.

Immunoblot analysis

The monocytes or macrophages and the biopsy samples were lysed in the buffer described by Leibold and Munro.[14] The lysate was centrifuged at 800×g for 5 min and 70 μg protein aliquots of the supernatant were electrophoresed in 10% acrylamide-SDS gels, electroblotted to Hybond membranes (Amersham Co. Milan, Italy) and incubated with a rabbit polyclonal anti IRP2 antibody (raised against a conserved sequence in the IRP2 degradation domain, 1:100 dilution)[15] and a monoclonal antibody against β-actin (Sigma Chemical Co., Milan, Italy) to check equal protein loading. After incubation with the appropriate secondary antibodies, proteins were detected by means of chemiluminescence using an immunodetection kit (ECL Plus, Amersham Co. Milan, Italy) according to the manufacturer's instructions, and quantified by laser densitometry.

RNA-protein bandshift and supershift assays

The cells were lysed in the buffer described by Leibold and Munro,[14] the lysate was centrifuged at 16,000×g for 5 min at 4°C, and the supernatant was used for RNA-protein bandshift assays. Equal amounts of protein were incubated with a molar excess of IRE probe transcribed *in vitro* with T7 RNA polymerase from the pSPT-fer plasmid containing the IRE of the human ferritin H chain[16] in the presence of 100 μCi of (α-^{32}P) UTP (800 Ci/mmol) (Amersham Co. Milan, Italy) and sequentially treated with RNase T1 and heparin as pre-

viously described.[17] For the supershift experiments, the lysates were incubated for 10 minutes at room temperature with saturating amounts of mouse antibody raised against recombinant human IRP1 (rIRP1)[18] before being incubated with the IRE probe. After separation on 6% non-denaturing polyacrylamide gels, the IRP1 and IRP2 RNA-protein complexes were visualized autoradiographically and separately quantified by means of direct nuclear counting using an InstantImager (Packard Instruments Co. Milan, Italy).

Determination of aconitase activity

Aconitase activity was determined spectrophotometrically at 240 nm by monitoring the disappearance of *cis*-aconitate, as previously described.[19] The incubation (1 mL of final volume) contained lysates in Leibold and Munro buffer (100 μg protein) and 0.1 mM *cis*-aconitate in 0.3 M NaCl (pH 7.0), at 37°C; one mU was defined as the amount of enzyme that consumed 1 nmol of *cis*-aconitate/min.

Immunoblotting analysis of IRP1 conformations

The extracts were prepared and analyzed as previously described 18. Briefly: the cells and biopsy samples were homogenized in 20 mM Tris-HCl pH 7.4 and 250 mM sucrose in the presence of 0.007% digitonin. The extracts were first centrifuged at low speed (1500×g for 10 min) to remove nuclei and cell debris, and then the supernatants were centrifuged at 10,000×g for 10 min to remove membranes and obtain cytosolic preparations. This fraction was further centrifuged at 100,000×g for 60 min at which point the supernatant was referred to as a soluble cytosolic preparation. The pellets from the 10,000×g and 100,000×g centrifugations were mixed and resuspended in 20 mM Tris-HCl pH 7.4, 250 mM sucrose, 1% Triton X-100; these samples are referred to as cytosolic precipitates. The samples containing the protein extracts from the various subcellular fractions or rIRP1 were separated on non-denaturing 7.5% polyacrylamide gels in Tris-glycine buffer or in non-denaturing 6% polyacrylamide gel in TBE buffer. Using a semi-dry blotting apparatus the proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Co. Milan, Italy), which were incubated with mouse anti-IRP1 antibody (diluted 1:750) and secondary, peroxidase-labeled anti mouse Ig diluted 1:4000 (Sigma Milan, Italy). Bound activity was revealed using a Super Signal West Pico system (Pierce Biotechnology Inc., Rockford IL, USA).

Immunohistochemistry

Immunohistochemistry assays were performed on formalin-fixed, paraffin-embedded specimens of normal appearing mucosa of distal duodenum. The deparaffined slides were stained using the automated staining system Genomix i-6000 (BioGenex, San Ramon CA, USA) with anti-IRP1[18] and IRP2[15] antibodies at a dilution of 1: 400 in 0.5% bovine serum albumin and sodium azide. Heat-induced antigen retrieval was obtained using a 0.01 M citrate solution at pH 6.0 in a microwave oven at 750 W (2 cycles×5 minutes). The reaction was revealed with the Dako ChemMate EnVision Detection Kit (Dako A/S, Glostrup, Denmark) according to the manufacturer's instructions. Slides incubated with normal goat serum instead of the primary antibody were used as negative controls.

Table 1. Serum iron indices in the study groups (monocyte isolation and duodenal biopsies).

	n°	Hb (g/dL)	Serum iron (μg/dL)	Transferrin saturation (%)	Serum ferritin (μg/L)
Monocyte isolation					
Control	16	13±2	77±35	19±7	33±21
HH	12	14±1	134±50*	61±23*	1132±641*
Duodenal biopsies					
Control	8	13±1	90±26	22±9	38±18
HH	9	14±2	166±47*	67±25*	1478±972*
Iron deficiency anemia	6	9±2*	27±7*	8±3°	12±3°

*Mean values ± SD; *p≤0.001 vs controls; °p< 0.05 vs controls*

Statistical analysis

The data are expressed as mean values ± SD. Inter-individual variability in the control groups was below 10%. The significance of the differences was evaluated by means of the t test using the Stat View 4.0 program (Abacus Concept Inc. Berkeley, CA, USA).

Results

IRP activity and levels in monocytes and macrophages

Monocytes were purified from 16 control and 12 HH subjects whose hematologic and serum iron parameters are shown in Table 1, and the RNA binding activity of IRP1 and IRP2 evaluated by means of bandshift and supershift assays (Figure 1A). As human IRP1 and IRP2 co-migrate, RNA bandshift assays showed that their combined activity was greater in the monocytes of the HH patients than in those of the controls (Figure 1B), in agreement with previous findings.[10,11] To determine the contributions of the two IRP to the total binding activity we incubated cytosolic extracts with a saturating amount of the anti-IRP1 antibody and evaluated the intensity of the supershifted IRP1 band; the amount of RNA binding activity that was not supershifted was taken as an indication of IRP2 activity (Figure 1A).[15] Both IRP were more active in the monocytes of HH patients than in those of the controls (Figure 1A and B). The binding activity of IRP2 was less than that of IRP1 (Figure 1A) but was induced to a greater extent (Figure 1B), thus suggesting that IRP2 is more sensitive to alterations in iron availability. As IRP2 is mainly controlled at the level of protein stability,[1,2] we also evaluated its content in monocytes by means of immunoblotting (Figure 1C), which allows more precise quantification than supershift assays. In agreement with the results obtained by evaluation of the binding activity, analysis of the monocyte lysates, normalized for actin content, showed that IRP2 protein levels were about two times

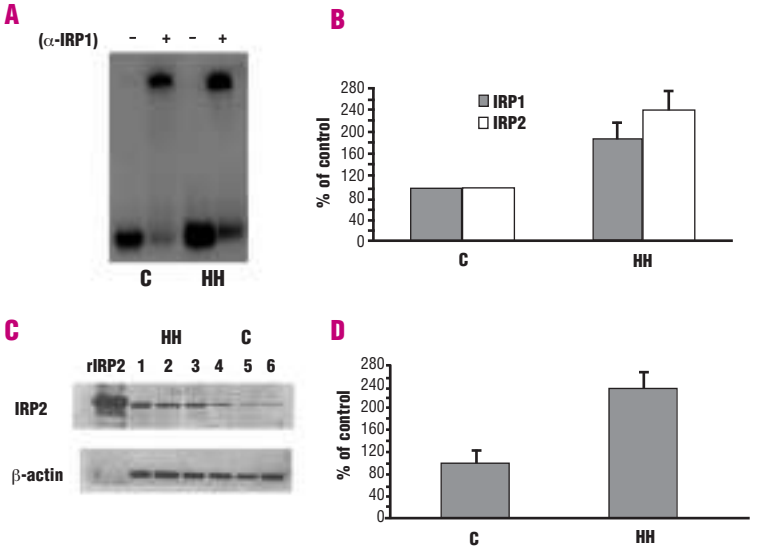

Figure 1. Analysis of IRP1 and IRP2 in monocytes. Panel A. RNA bandshift analysis of IRP activity. Cytoplasmic extracts of monocytes from one control subject and one HH patient were incubated with an excess of a ^{32}P-labeled iron-responsive element probe and RNA-protein complexes separated on non-denaturing polyacrylamide gels. For supershift analysis, the extracts were pre-incubated with an antibody against IRP1 (α-IRP1) before binding. A representative autoradiogram is shown. Panel B. Quantitative analysis of IRP binding activity in all study subjects indicated in Table 1: mean percentages ± SD of control values. The IRP1 and IRP2 bands were quantified by direct nuclear counting as described in the *Design and Methods*. Panel C. Immunoblot analysis of IRP2 content in monocytes. Equal amounts of proteins from the monocyte lysates were loaded onto SDS polyacrylamide gels and immunoblotted with an antibody against IRP2. This latter antibody recognized histidine-tagged rat recombinant IRP2 (rIRP2). The blots were reprobed with an antibody against β-actin as a loading control. A representative result with monocytes taken from three control subjects and three HH patients is shown. Panel D. Quantitative analysis of IRP2 levels in all study subjects indicated in Table I: mean percentages ± SD of control values.

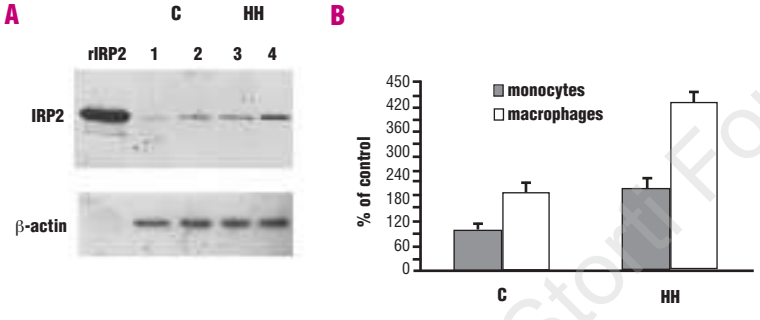

Figure 2. Immunoblot analysis of IRP2 content during monocyte-macrophage differentiation. Panel A. Monocytes isolated from one control subject and one HH patient were lysed immediately after purification (lanes 1 and 3) or allowed to differentiate to macrophages *in vitro* (lanes 2 and 4). Equal amounts of proteins from the lysates were loaded onto SDS polyacrylamide gels and immunoblotted with antibodies against IRP2. The antibody recognized histidine-tagged rat recombinant IRP2 (rIRP2). The blots were reprobed with an antibody against β-actin as a loading control. The result is representative of independent experiments with monocytes purified from four controls and four HH patients. Panel B. Quantitative analysis of IRP2 levels: mean percentages±SD of control values.

higher in the HH patients than in controls (Figure 1D). We also investigated the behavior of IRP2 during cell differentiation. Figure 2A illustrates that IRP2 content increased to the same extent when both the control and HH monocytes were allowed to differentiate to macrophages, thus showing that the quantitative differences found in monocytes were maintained in macrophages (Figure 2B).

IRP activity and levels in duodenum

We also investigated both IRP in the duodenum which, as the site of iron absorption, plays a key role in iron homeostasis. Duodenal cytoplasmic extracts from 8 controls, 9 patients with HH and 6 patients with iron deficiency anemia (Table 1) were processed for bandshift and supershift assays as described above (Figure 3A). The activity of both IRP1 and IRP2 was greater in the biopsy samples taken from the HH and anemic patients than in those from control subjects. As in the case of monocytes, the binding activity of IRP2 in the duodenum was induced to a greater extent than that of IRP1 (Figure 3B). Moreover, immunoblotting analysis of the cell lysates (Figure 3C) showed that IRP2 protein levels were higher in the HH patients than in the controls, and were similar to those found in anemic patients (Figure 3D).

Immunohistochemical evaluation of IRP in the duodenum

The availability of specific antibodies prompted us to analyze the expression and distribution of the two IRP in the duodenal specimens of control subjects, HH and anemic patients by means of immunohistochemistry. As both antibodies expressed the same pattern of immunoreactivity, and there were no significant differences in localization or expression between the control subjects and the patients, only the results obtained by incubating duodenal samples of the control subjects with the anti-IRP1 antibody are shown (Figure 4). The intensity of the immunoreaction staining in the epithelium always decreased from the apical portion of the villus to the basal cripts (Figure 4A). In particular, there was intense granular positivity in the cytoplasms of the enterocytes, whereas, despite a mild degree of aspecific background, the goblet cells were always negative (Figure 4B), and there was no immunoreactivity in the stromal cells of the lamina propria or the epithelial cells of Brunner's glands. No nuclear immunoreactivity was observed. The preferential expression of both IRP in epithelial cells is in agreement with the different IRP levels described in various tissues[20,21] and between cell types within specific tissues,[6] and is possibly related to the different roles of the various intestinal cell types in iron metabolism.

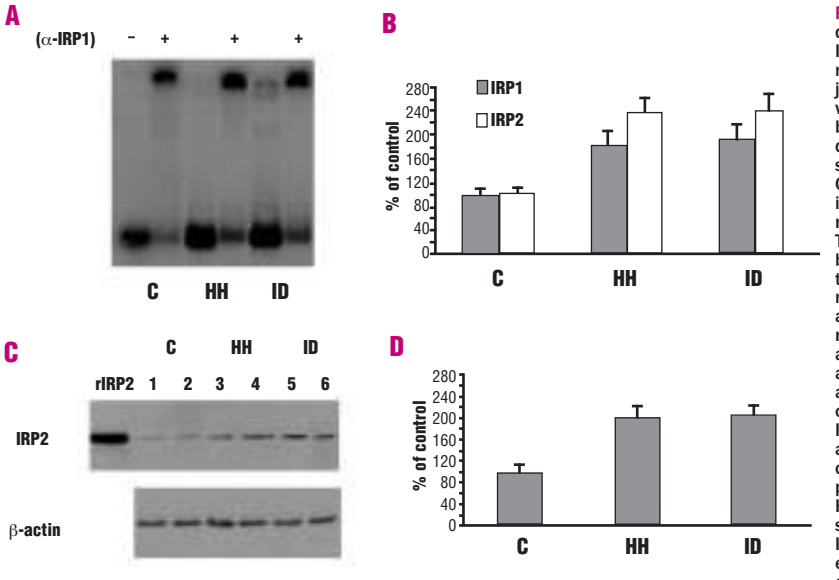

Figure 3. Analysis of IRP1 and IRP2 in duodenum. Panel A. RNA bandshift analysis of IRP activity. Cytoplasmic extracts of duodenal biopsy samples from one control subject, one HH patient and one anemic patient were assayed for IRP activity by means of bandshift and supershift assays as described in the legend to Figure 1. A representative autoradiogram is shown. Panel B. Quantitative analysis of IRP binding activity in all study subjects indicated in Table I: mean percentages ± SD of control values. The IRP1 and IRP2 bands were quantified by direct nuclear counting as described in the *Design and Methods*. Panel C. Immunoblot analysis of IRP2 content. Equal amounts of proteins from the lysates of monocytes taken from controls, and anemic and HH patients were loaded onto SDS polyacrylamide gels and immunoblotted with antibodies against IRP2. The antibody recognized histidine-tagged rat recombinant IRP2 (rIRP2). The blots were reprobed with an antibody against β-actin as a loading control. A representative result with samples taken from two control subjects, two HH patients and two anemic patients is shown. Panel D. Quantitative analysis of IRP2 levels in all the study subjects indicated in Table 1: mean percentages ± SD of control values.

Analysis of IRP1 conformations

We have recently developed an immunoblotting-based method that allows simultaneous detection of the different IRP1 conformations present in a cell.[18] As this novel assay distinguishes the iron-containing and iron-free forms in the soluble fraction, as well as the RNA-bound form in the precipitate fraction, we used it to obtain further information about the status of IRP1 in the monocytes, macrophages and duodenum of the study subjects. The blots in Figure 5 show that the faster moving band, corresponding to the 4Fe-4S cluster-containing aconitase/IRP1,[18] was prominent in the control duodenal samples (Figure 5A, left), but undetectable in the control monocytes and macrophages (Figure 5B and C). Conversely, the slowly migrating band corresponding to the iron-free apoform was more evident in the duodenal extracts of the HH and anemic patients than in those of the controls (Figure 5A left), and was the only detectable form in the monocyte/macrophage extracts (Figure 5B and C). In line with these findings, aconitase enzymatic activity was 80% less in the duodenal samples of the HH and anemic patients than in those of the controls (Figure 5A), and was unmeasurable in the monocytes and macrophages of all of the subjects. These results indicate that duodenal IRP1 shifts from the aconitase form in normal subjects to the apoform in HH and anemic patients (Figure 5A), whereas practically all the IRP1 in monocytes/macrophages is always in the apoform, without any significant differences between patients and controls (Figure 5B and C). In line with these observations, treatment of the soluble cytosolic extracts with a reductant (2-mercaptoethanol), which enables reliable detection of all of the soluble iron-free and iron-bound forms of IRP1,[18] showed that the amount of IRP1 was not significantly different in the various duodenal (Figure 5A right) and monocyte/macrophage samples (Figure 5B and C). The bands of IRP1 detectable after RNase-A treatment of the cytosolic precipitates were very faint and similarly intense in all of the samples, thus indicating that the RNA-bound fraction of IRP1 is small and not significantly different in the different subjects, even in the duodenal biopsies of HH and anemic patients in whom IRP1 is switched to the cluster-less apoform.

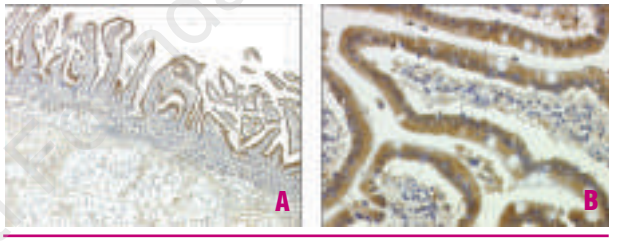

Figure 4. Immunohistochemical analysis of IRP in duodenum. The tissue sections of the duodenal biopsies of control subjects were prepared as described in the *Design and Methods*, and immunostained with the antibody against IRP1. The results shown are representative of all studied subjects indicated in Table 1. Panel A. Intense immunoreactivity in the epithelial cells of the duodenal mucosa; the stromal cells of the lamina propria and the epithelial cells of Brunner's glands are negative (DAB, 5×). Panel B. Intense granular positivity in the cytoplasms of enterocytes; the goblet cells were always negative. No nuclear immunoreactivity was observed (DAB, 40×).

Discussion

Iron regulatory proteins are simultaneously key regulators of iron homeostasis and sensors of iron levels, and hence provide information about iron status. However, the mechanisms underlying the IRE/IRP-mediated control of iron homeostasis were mainly discovered by means of cell culture studies and, because *in vivo* tissue oxygen concentrations are in the 3-5% range and the sensitivity of the two IRP to oxygen tension is very different,[22] the results obtained in cultured cells grown in 20% O_2 may have little relevance to normal (patho)physiology. Indeed, only recently has the targeted disruption of IRP1 and IRP2 in mice revealed that the latter may play a more impor-

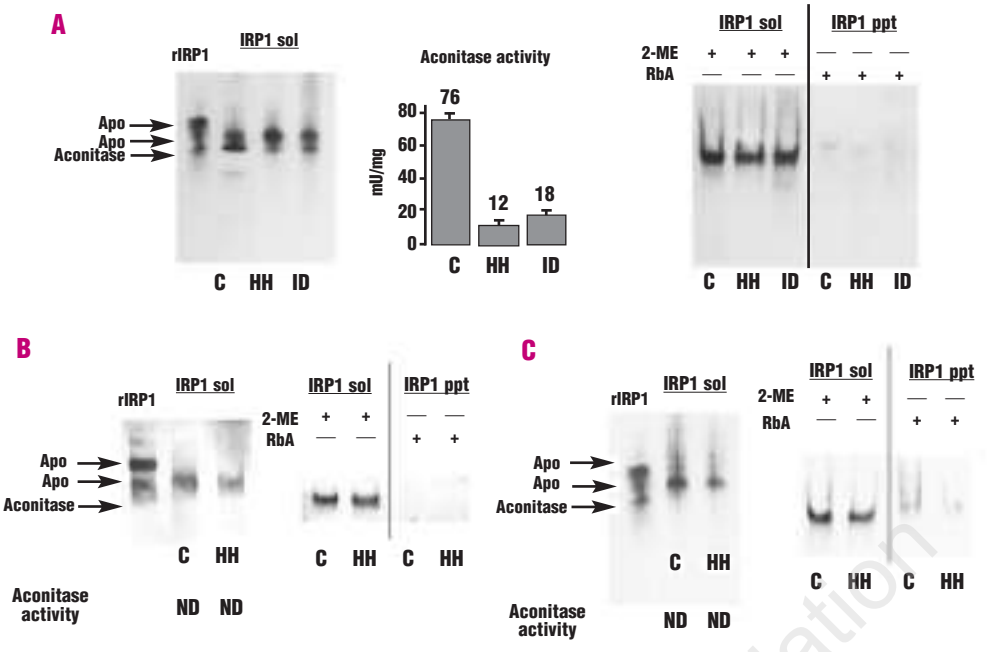

Figure 5. Immunoblot analysis of IRP1 on native gels, and aconitase activity. Panel A. Analysis of IRP1 in the duodenal biopsies of controls (C), HH patients (HH) and iron deficiency anemia patients (ID). Left: the soluble cytosolic fractions (IRP1sol) were analyzed by means of non-denaturing polyacrylamide gel electrophoresis and blotted with the antibody against IRP1. Recombinant IRP1 (rIRP1) was used as a control for the localization of the different bands. rIRP1 separates into three different bands corresponding to a partially oxidized apoform (upper band), the major form of apo-IRP1 present also in cells (middle band) and the Fe-S cluster form endowed with aconitase activity (lower band). Middle: aconitase enzymatic activity was determined as described in Design and Methods in all study subjects indicated in Table 1. Mean values ± SD. Right: Samples of soluble cytosolic (IRP1sol) and precipitate fractions (IRP1ppt) were respectively treated with 2-mercaptoethanol (2-ME) or ribonuclease A (RbA) before being separated on non-denaturing polyacrylamide gels, blotted and incubated with anti-IRP1 antibody. The results shown are representative of all study subjects indicated in Table 1. Panel B. Analysis of IRP1 in monocytes of controls (C) and HH patients (HH). The soluble cytosolic and precipitate fractions were analyzed for aconitase activity and IRP1 as described above. ND: not detectable. The results shown are representative of all study subjects indicated in Table 1. Panel C. Analysis of IRP1 in macrophages of controls (C) and HH patients (HH). The soluble cytosolic and precipitate fractions were analyzed for aconitase activity and IRP1 as described above. ND: not detectable. The results shown are representative of independent experiments with monocytes purified from four controls and four HH patients.

tant role in iron homeostasis.[5-8] Moreover, given the importance of IRP in iron metabolism, surprisingly little is known about their *in vivo* roles in human clinical settings. The analysis of *ex vivo* material, such as biopsy samples or freshly isolated monocytes may therefore reveal more about the role of IRP in iron metabolism, and this latter's implications in diseases such as HH. In the present study, we investigated duodenal cells and monocytes/macrophages because they are responsible for dietary absorption and recycling of iron, and hence play central roles in iron homeostasis. The use of specific antibodies against the individual IRP in a variety of assays allowed us to demonstrate that the activity of both IRP is significantly higher in the monocytes of HH patients than in those of controls, and that duodenal IRP1 and IRP2 activity and content are both increased in patients with HH or iron deficiency anemia. These results confirm and extend our previous findings showing inappropriate up-regulation of total IRP activity in monocytes/macrophages and duodenal cells of HH patients,[9-11] which indicated that key cells of iron transport are paradoxically iron-deficient in iron overloaded subjects. In HH patients the impairment of hepcidin-mediated ferroportin degradation may increase iron export from intestinal absorptive cells and from macrophages,[23] thus leading to iron deficiency in these cells. The reduction in the iron pool, which may also be caused by a disruption in the normal function of the TfR-HFE complex as demonstrated by the normalization of the iron-deficient phenotype in the monocytes of HH patients transfected with the wild type *HFE* gene,[24] seems to affect the two IRP differently, with preferential IRP2 activation indicating that IRP2 is more sensitive to changes in iron levels. The *ex vivo* conditions analyzed here may represent the reduced oxygen levels present *in vivo*, which have been shown to favor IRP2 over IRP1.[22] In turn, the higher IRP activity may represent the molecular basis underlying the altered expression of IRP-controlled mRNA in the duodenum of HH patients, such as reduced ferritin content[25-27] and upregulation of the proteins involved in dietary iron transport.[28-30]

Analysis of the conformations of IRP1 in the duodenum of control subjects showed that this protein is mainly in the aconitase form, as previously reported.[18] On the other hand, the Fe-S containing form of IRP1, which was found to be a minor but still detectable component in our previous analysis of a smaller number of samples,[18] was practically undetectable in both monocytes and macrophages. The difference in cytoplasmic aconitase levels between duodenal and monocytes/macrophages may be related to this protein's role as a substrate supplier of cytoplasmic

isocitrate dehydrogenase, whose activity affects the formation of NADPH and contributes to the redox and/or metabolic balance of the cell.[31] In fact, the aconitase-dependent increase in NADPH would provide reducing equivalents and also favor the Fe(II) state, thus promoting safe iron storage in ferritin and/or heme. Moreover, our present findings indicate that IRP1 is poorly bound to target mRNA (as revealed by the analysis of RNase-treated extracts) in both the duodenum of HH and anemic patients (in whom it is shifted to the apoform) as well as in the monocytes/macrophages of all subjects, in which it is constitutively present as apoprotein. This situation in pathophysiologic conditions of relative iron deficiency differs from that observed in cells exposed to an iron chelator, in which the switch to the iron-free apoform corresponds to increased RNA binding.[18] Altogether, our analysis of IRP1 conformations seems to indicate that IRP1 can sense iron deficiency but is marginally involved in regulating iron metabolism, at least in the presence of physiological levels of IRP2.

The immunohistochemical analysis of the duodenal biopsy samples showed that both IRP have a gradient of expression, and that there are no differences in their content or distribution between normal subjects and HH or anemic patients. With regard to the first point, the increasing expression along the cript-villus axis, and the finding that IRP2 is more expressed when monocytes are allowed to become macrophages (Figure 2), suggest that IRP expression is controlled and upregulated during differentiation, which is in line with the fact that increased RNA binding activity of both IRP1 and IRP2, accompanied by higher expression of the *IRP1* gene, has been observed during *in vitro* differentiation of adipocytes.[32] Our findings are also in agreement with other studies of the duodenal expression of IRP-controlled mRNA showing the crypt to tip gradient of TfR expression.[29,33] The recent demonstration that IRP2 inactivation increases ferritin expression more in the villi than in the crypts[8] also suggests that the role of IRP2 (and hence its content) is higher in the villi, in accordance with our findings. More generally, a number of other iron-related proteins have been shown to be regulated during differentiation of a variety of cells. In fact, ferritin and TfR have also been shown to be up-regulated during differentiation of monocytes[34,35] and other cell types.[36,37] In addition, increased ferritin H expression has been demonstrated in a number of model systems of hematopoietic differentiation.[38] With regard to the comparison between control subjects and HH and anemic patients, immunohistochemistry showed not only a similar IRP localization but also no differences in IRP expression. The discrepancy between the unchanged IRP2 levels revealed by immunohistochemistry, and the increased levels shown by immunoblotting (Figure 3) can be tentatively explained by the limitation of immunohistochemistry as a quantitative assay. The findings discussed above also indicate that iron and differentiation control IRP1 in different ways: the similar IRP1 levels in normal subjects and HH patients (also demonstrated by the findings shown in Figure 5) are in agreement with the accepted model of post-translational IRP1 regulation that implies a switch between its aconitase and RNA-binding forms without any changes in protein levels, whereas differentiation increases IRP1 protein expression, at least in the duodenum, which is in line with the results obtained in the adipocyte differentiation model.[32]

In conclusion, the results of this first study of the comparative expression of the two IRP in human tissues and blood cells indicate that IRP2 is the master regulator of iron metabolism, as its binding activity was induced to a greater extent than that of IRP1 under all conditions. Moreover, IRP1 is often in the aconitase form and, even when present as an apoprotein that should be endowed with IRE-binding activity, binds poorly to target mRNA. Our findings in human subjects in pathophysiological settings therefore confirm those previously obtained in cell cultures[22,39] and animal models of IRP deficiency[5-8] indicating that IRP2 is the major sensor and modulator of iron homeostasis.

GC was the principal investigator and takes primary responsibility for the paper. SR and GC conceived and designed the study. SR and AA isolated monocytes/macrophages and performed the bandshift analysis of IRP binding activity and immunoblotting assays of IRP2 levels. AC analyzed IRP1 conformations. UG and EDC performed the immunohistochemistry experiments. DC was involved in design of the study, provision of study patients and critical manuscript review. SR and GC analyzed the data and wrote the manuscript.

Figures 1, 2, and 3 were prepared by SR and AA, Figure 4 was prepared by UG and EDC, Figure 5 was prepared by AC. Table 1 was prepared by SR and DC. All the authors gave final approval for submission of the paper and jointly decided the order of authorship. The authors declare that they have no potential conflicts of interest.

We wish to thank Dr. L. Kuhn for the plasmids encoding the IRE probe and Dr. E. Leibold for the plasmid coding for IRP2. This study was supported by grants from MIUR (FIRB and COFIN) to G.C. and IRCCS Ospedale Maggiore Milano to D.C.

Manuscript received October 18, 2005. Accepted December 23, 2005. Prepublished online on February 17, 2006. PII: 03906078_9334.

References

1. Cairo G, Pietrangelo A. Iron-regulatory proteins in pathobiology. Biochem J 2000;352:241-50.
2. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell 2004;117:285-97.
3. Ke Y, Wu J, Leibold EA, Walden WE, Theil EC. Loops and bulge/loops in iron-responsive element isoforms influence iron regulatory protein binding. Fine-tuning of mRNA regulation? J Biol Chem 1998;273:23637-40.
4. Cairo G, Recalcati S, Pietrangelo A, Minotti G. The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage. Free Radic Biol Med 2002; 32: 1237-43.
5. LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, et al. Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nat Genet 2001;27:209-14.
6. Meyron-Holtz EG, Ghosh MC, Iwai K, LaVaute T, Brazzolotto X, Berger UV, et al. Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. Embo J 2004;23:386-95.
7. Cooperman SS, Meyron-Holtz EG, Olivierre-Wilson H, Ghosh MC, McConnell JP, Rouault TA. Microcytic anemia, erythropoietic protoporphyria, and neurodegeneration in mice with targeted deletion of iron-regulatory protein 2. Blood 2005;106:1084-91.
8. Galy B, Ferring D, Minana B, Bell O, Janser HG, Muckenthaler M, Schumann K, Hentze MW. Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). Blood 2005;106:2580-9.
9. Pietrangelo A, Casalgrandi G, Quaglino D, Gualdi R, Conte D, Milani S, et al. Duodenal ferritin synthesis in genetic hemochromatosis. Gastroenterology 1995;108:208-17.
10. Cairo G, Recalcati S, Montosi G, Castrusini E, Conte D, Pietrangelo A. Inappropriately high iron regulatory protein activity in monocytes of patients with genetic hemochromatosis. Blood 1997;89:2546-53.
11. Recalcati S, Pometta R, Levi S, Conte D, Cairo G. Response of monocyte iron regulatory protein activity to inflammation: abnormal behavior in genetic hemochromatosis. Blood 1998; 91:2565-72.
12. Hentze MW, Kuhn LC. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci USA 1996;93:8175-82.
13. Recalcati S, Taramelli D, Conte D, Cairo G. Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. Blood 1998;91: 1059-66.
14. Leibold EA, Munro HN. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. Proc Natl Acad Sci USA 1988;85:2171-5.
15. Alberghini A, Recalcati S, Tacchini L, Santambrogio P, Campanella A, Cairo G. Loss of the von Hippel Lindau tumor suppressor disrupts iron homeostasis in renal carcinoma cells. J Biol Chem 2005;280:30120-8.
16. Mullner EW, Neupert B, Kuhn LC. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell 1989;58:373-82.
17. Cairo G, Pietrangelo A. Transferrin receptor gene expression during rat liver regeneration. Evidence for post-transcriptional regulation by iron regulatory factor B, a second iron-responsive element-binding protein. J Biol Chem 1994;269:6405-9.
18. Campanella A, Levi S, Cairo G, Biasiotto G, Arosio P. Blotting analysis of native IRP1: a novel approach to distinguish the different forms of IRP1 in cells and tissues. Biochemistry 2004; 43:195-204.
19. Minotti G, Ronchi R, Salvatorelli E, Menna P, Cairo G. Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. Cancer Res 2001;61:8422-8.
20. Mullner EW, Rothenberger S, Muller AM, Kuhn LC. In vivo and in vitro modulation of the mRNA-binding activity of iron-regulatory factor. Tissue distribution and effects of cell proliferation, iron levels and redox state. Eur J Biochem 1992;208:597-605.
21. Guo B, Brown FM, Phillips JD, Yu Y, Leibold EA. Characterization and expression of iron regulatory protein 2 (IRP2). Presence of multiple IRP2 transcripts regulated by intracellular iron levels. J Biol Chem 1995;270:16529-35.
22. Meyron-Holtz EG, Ghosh MC, Rouault TA. Mammalian tissue oxygen levels modulate iron-regulatory protein activities in vivo. Science 2004; 306:2087-90.
23. Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, et al. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metab 2005;1:191-200.
24. Montosi G, Paglia P, Garuti C, Guzman CA, Bastin JM, Colombo MP, et al. Wild-type HFE protein normalizes transferrin iron accumulation in macrophages from subjects with hereditary hemochromatosis. Blood 2000;96:1125-9.
25. Whittaker P, Skikne BS, Covell AM, Flowers C, Cooke A, Lynch SR, et al. Duodenal iron proteins in idiopathic hemochromatosis. J Clin Invest 1989; 83:261-7.
26. Fracanzani AL, Fargion S, Romano R, Piperno A, Arosio P, Ruggeri G, et al. Immunohistochemical evidence for a lack of ferritin in duodenal absorptive epithelial cells in idiopathic hemochromatosis. Gastroenterology 1989; 96: 1071-8.
27. Pietrangelo A, Rocchi E, Casalgrandi G, Rigo G, Ferrari A, Perini M, et al. Regulation of transferrin, transferrin receptor, and ferritin genes in human duodenum. Gastroenterology 1992; 102:802-9.
28. Zoller H, Pietrangelo A, Vogel W, Weiss G. Duodenal metal-transporter (DMT-1, NRAMP-2) expression in patients with hereditary haemochromatosis. Lancet 1999;353:2120-3.
29. Barisani D, Parafioriti A, Armiraglio E, Meneveri R, Conte D. Duodenal expression of a putative stimulator of Fe transport and transferrin receptor in anemia and hemochromatosis. Gastroenterology 2001;120:1404-11.
30. Stuart KA, Anderson GJ, Frazer DM, Powell LW, McCullen M, Fletcher LM, et al. Duodenal expression of iron transport molecules in untreated haemochromatosis subjects. Gut 2003; 52:953-9.
31. Narahari J, Ma R, Wang M, Walden WE. The aconitase function of iron regulatory protein 1. Genetic studies in yeast implicate its role in iron-mediated redox regulation. J Biol Chem 2000; 275:16227-34.
32. Festa M, Ricciardelli G, Mele G, Pietropaolo C, Ruffo A, Colonna A. Overexpression of H ferritin and up-regulation of iron regulatory protein genes during differentiation of 3T3-L1 pre-adipocytes. J Biol Chem 2000; 275: 36708-12.
33. Banerjee D, Flanagan PR, Cluett J, Valberg LS. Transferrin receptors in the human gastrointestinal tract. Relationship to body iron stores. Gastroenterology 1986;91:861-9.
34. Testa U, Petrini M, Quaranta MT, Pelosi-Testa E, Mastroberardino G, Camagna A, et al. Iron up-modulates the expression of transferrin receptors during monocyte-macrophage maturation. J Biol Chem 1989;264:13181-7.
35. Testa U, Kuhn L, Petrini M, Quaranta MT, Pelosi E, Peschle C. Differential regulation of iron regulatory element-binding protein(s) in cell extracts of activated lymphocytes versus monocytes-macrophages. J Biol Chem 1991; 266:13925-30.
36. VanLandingham JW, Levenson CW. Effect of retinoic acid on ferritin H expression during brain development and neuronal differentiation. Nutr Neurosci 2003;6:39-45.
37. Bevilacqua MA, Faniello MC, Iovine B, Russo T, Cimino F, Costanzo F. Transcription factor NF-Y regulates differentiation of CaCo-2 cells. Arch Biochem Biophys 2002;407:39-44.
38. Torti FM, Torti SV. Regulation of ferritin genes and protein. Blood 2002; 99:3505-16.
39. Recalcati S, Conte D, Cairo G. Preferential activation of iron regulatory protein-2 in cell lines as a result of higher sensitivity to iron. Eur J Biochem 1999;259:304-9.