IRON HOMEOSTASIS DURING TRANSFUSIONAL IRON OVERLOAD IN β-THALASSEMIA AND SICKLE CELL DISEASE: Changes in Iron Regulatory Protein, Hepcidin, and Ferritin Expression

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Hypertransfusional (≥8 transfusions/year) iron in liver biopsies collected immediately after transfusions in β-thalassemia and sickle cell disease correlated with increased expression (RNA) for iron regulatory proteins 1 and 2 (3- to 11-fold) and hepcidin RNA: (5- to 8-fold) (each \( p < .01 \)), while ferritin H and L RNA remained constant. A different H:L ferritin ratio in RNA (0.03) and protein (0.2–0.6) indicated disease-specific trends and suggests novel post-transcriptional effects. Increased iron regulatory proteins could stabilize the transferrin receptor mRNA and, thereby, iron uptake. Increased hepcidin, after correction of anemia by transfusion, likely reflects excess liver iron. Finally, the absence of a detectable change in ferritin mRNA indicates insufficient oxidative stress to significantly activate MARE/ARE promoters.

**Keywords** ferritin, hepcidin, hypertransfusion iron overload, iron regulatory proteins, sickle cell disease, β-thalassemia

The prevalence and importance of iron overload in many clinical conditions is increasingly recognized. Many of the earliest studies focused on hereditary hemochromatosis [1]. However, the iron burden of hypertransfusions in both β-thalassemia major (THL) or sickle cell disease (SCD) is much less studied [2]. Both DNA and mRNA are targets for regulatory changes related to iron overload. For example, ferritin is part of the phase II responses that are regulated by antioxidant inducers via DNA MARE/ARE promoter sequences also found in thioredoxin reductase, NADPH-quinone reductase, heme oxygenase, and β-globin [3]. In addition, iron uptake genes, such as transferrin receptor1, ferritin, and ferroportin, are regulated by iron via the mRNA-IRE (iron response element) family and IRP (iron regulatory protein) repressors; IRPs regulate IRE-RNA translation initiation or degradation, depending on the IRE-mRNA [4, 5]. For example, IRP/IRE interactions can increase iron uptake by stabilizing the transferrin receptor1 mRNA. At the same time, ferritin synthesis will decrease since the translation of ferritin mRNA will be blocked by IRPs. Since ferritin protein is not full of iron under most conditions, the iron entering via the transferrin receptor can still be accommodated inside ferritin. In contrast, in hypertransfusion iron overload, both increased iron uptake and iron storage continue to occur in the liver. To investigate this observation, we compared 5 iron homeostatic genes in both THL and SCD liver tissue: iron regulatory proteins (IRP 1 and 2), ferritin (FTH and FTL), and hepcidin (HAMP). Our results show no changes in ferritin RNA, increases in hepcidin RNA, and a novel increase in IRP1 and IRP2 RNA.

**PATIENTS AND METHODS**

Clinically indicated liver biopsies (\( n = 6 \) SCD, \( n = 6 \) THL), obtained 1–3 days after red cell transfusion, were obtained at Children’s Hospital & Research Center Oakland, with IRB approval and informed consent, and were the source of the tissue analyzed in this study. The tissue was flash frozen in the operating suite. The diagnosis of thalassemia or sickle cell
disease was based on globin isoelectric focusing and family studies. Chart review confirmed that there were not any statistically significant differences for SCD vs. THL between number of transfusions and/or amount of deferioxamine therapy during the preceding 5 years, or between mean hemoglobin levels (10.7 ± 0.35 vs. 11.2 ± 0.60, \( p = .47 \)), white cell counts (10.2 ± 0.29 vs. 12.5 ± 1.8, \( p = .24 \)), or C-reactive protein (0.35 ± 0.33 vs. 0.06 ± 0.05, \( p = .13 \)).

RNA for QC-PCR (quantitative competitive PCR) was isolated from liver tissue (0.5–2.9 mg) using RNaqueous 4-PCR kit (Ambion, Austin, TX). Primers were designed to allow amplification of both native and competitor RNA molecules [6], distinguishable by a 20- to 36-bp size differential, and to allow detection of all splice variants where multiple splice forms were known. Primers used were glyceraldehyde-3 phosphate dehydrogenase (GAP): 5′-GTCATCCATGACAACKTTTGGTGATC-3’, 5′-AGTACAGGCGGAGGATGTGTTCT-3’; IRP1: 5′-AGAAGGGCCAGACACTTTAGAAG-3’, 5′-CATCCTGATCAAATACCTCCTG-3’; IRP2: 5′-CGCCTTTAGTACCATTGAAAC-3’, 5′-GCTTTGTTTGGTTTCCAGTCT-3’; FTL: 5′-TTCTGGAGACTCACTTAGAT-3’, 5′-TTAGGCAGAAGCCCTATTAC-3’; FTH1: 5′-CTCTACGCCTCCCTACGTTAAGCCTATATCCTGGAAG-3’. All samples were normalized to GAPDH expression. Hepcidin mRNA analyses used semiquantitative RT-PCR in an ABI 7700 Sequence Detector with SYBR Green (PE Biosystems, Foster City, CA), described previously [7], normalized to GAPDH. The liver RNA control was a mixed sample from 25 Caucasian tissue specimens (Clontech, Mountain View, CA).

Liver tissue ferritins were quantitated in liver extracts from flash-frozen SCD or THL biopsy or control autopsy tissue that was free of historic or histological disease (Liver Tissue Procurement and Distribution System, Minneapolis, MN), by Western blotting. Detection of peroxidase-coupled sheep antibodies to human ferritin (Binding Site, San Diego, CA) used swine anti-goat, chemiluminescence-labeled IgG (Roche–Basel, Switzerland), and Amersham-ECL+ (Chalfont St. Giles, UK). Quantitation employed phosphoimagery with ImageQuant (V5.1, Molecular Dynamics, Sunnyvale, CA, USA). Human liver ferritin (Calzyme, San Luis Obispo, CA) was included in each analysis to allow interblot normalization. Liver iron was analyzed by inductively coupled plasma mass spectroscopy (Mayo Clinic, Rochester, MN).

Comparisons were analyzed by a two-sided \( t \) test or rank sum testing, as appropriate; statistical significance of difference values were a minimum of \( p < .05 \). Statistical analyses used STATA 9.2 SE (StataCorp, College Station, Texas).

RESULTS AND DISCUSSION

Published data on the effects of hypertransfusional iron overload in THL and SCD have described the effects on liver iron, urinary hepcidin, and
FIGURE 1 Hypertransfusion changes liver expression of iron homeostasis genes in β-thalassemia and sickle cell disease. The RNA concentrations are the means of 6 β-thalassemia (THL) and 7 sickle cell disease (SCD) liver samples and the error is the standard deviation. Lines are the normalization value (1.0) for control (see Patients and Methods). Note that hepcidin values are significantly greater in SCD and THL than in hepatitis C [7]; (p < .007). *Significantly different from control (normal) values, p < .01.

serum ferritin, or, for THL, liver hepcidin RNA (reviewed in De Domenico [8]), but not liver IRPs. In our samples of heavily transfused patients, liver iron values were 8.2 ± 6.6 (THL), 12.9 ± 6.9 (SCD), and 1.0 ± 0.47 (normal autopsy samples) mg/g dry weight; liver histology (Perl’s stain): hepatocytes and Kupffer cells had similar iron scores (data not shown.) Serum ferritin values were 1923 ±1045 (THL) and 2651 ±1149 (SCD) ng/mL.

Despite the iron overload seen on histology, and increased measures of total iron content, ferritin H and L RNA in liver was unchanged by hypertransfusion (Figure 1). These findings contrast with administration of chronic, elemental iron in animal models (reviewed in references [4, 5]), where chronic exposure to high concentrations of inorganic iron salts increased ferritin L mRNA. Such differences mean that either the animal models differ from humans, or chronic red cell transfusions to near normal hematocrits change iron homeostasis differently than inorganic iron salts, or both.

Our observations of a 5- to 8-fold increase in hepcidin (Figure 1) in both SCD and THL confirms a previous THL study [8]. However, in another study in THL mice, liver hepcidin was decreased by excess iron [9]; in that study, the source of iron was elemental and administered orally, rather than from red cells administered intravenously during hypertransfusion. Possibly the different iron forms and routes of administration induce different effects. In still another recent report, liver hepcidin levels correlated with hemoglobin levels rather than with liver iron [10]. Our data would support such a correlation, as chronically transfused patients in our program have relatively high hemoglobin values at the time of biopsy. By contrast, in normocytic women, there was no relationship detected between serum or urinary prohepcidin and iron loading [11]. It may be that the hemoglobin levels at
the time of biopsy are a significant variable in determining liver hepcidin RNA values. In our study, transfusions preceded biopsy, thus increasing oxygenation at the time of biopsy and possibly causing a rapid change in signals that regulate hepcidin expression. The relationship of hepcidin expression to liver iron, hemoglobin, and possibly disease-specific features is clearly complex and understanding is still emerging.

The increases in liver hepcidin mRNA likely reflect the dominance of signals from increased iron, over anemia signals, since the anemias had been corrected by transfusion 3 days before the biopsy. The constancy of liver ferritin H and L mRNA in liver with excess iron from transfusions fits well with the greater sensitivity of human ferritin genes to oxidant stress than to iron, and the presence of MARE/ARE (antioxidant response element) promoters in ferritin genes: antioxidant inducers such as tert-butylhydroquinone and heme were 5× as effective as inorganic iron in changing ferritin gene transcription [3].

The IRP1 and IRP2 mRNAs were significantly elevated by hypertransfusion, \( p < .01 \) (3-fold for IRP1 and 9- to 11-fold for IRP2; Figure 1). Excess elemental iron is known to decrease IRP1 and IRP2 activity (RNA binding) and protein concentrations (immunoanalysis), and changes mRNA splicing in cultured cells [12–14]; however, that most studies of IRP expression do not analyze amounts of IRP RNA. In the case of hypertransfusion overload, there have been no studies of IRP RNA expression to our knowledge. Our observation of increased IRP1 and IRP2 RNA is unpredicted based on models for typical cells where excess iron decreases IRP protein [4, 5]. However, in transfusional iron overload, hepatocytes are not typical cells, since they need to maintain or even increase iron uptake released by senescent, transfused red cells even though the iron will increase IRP protein turnover [4, 5]; in such cells, increased IRP transcription and translation is needed to produce the IRP needed to stabilize the transferrin receptor1 mRNA. The small increase in ferritin protein observed (2-fold) is likely a consequence of the increased expression of IRP. Fortunately, ferritin protein is usually only partly filled with mineral, and space for increased iron remains available.

Comparison of the H:L subunit ratio in ferritin mRNA and protein (Figure 2), showed an unpredicted effect. The H:L subunit ratio of mRNA was 8- to 20-fold lower than the H:L subunit ratio of protein in SCD, THL and normal tissue. Such data indicate either more efficient translation of H ferritin mRNA, or more breakdown of L ferritin protein, or both. In addition, the H:L subunit ratio of ferritin protein decreased (Figure 2) in transfusional iron overload, which has also been observed in hereditary hemochromatosis [15]; the trend was larger in SCD than THL.

Taken together, the increased liver expression of IRP1, IRP2, and HAMP and the unchanged expression of FTH and FTL RNA with changes in
FIGURE 2 Hypertransfusion decreased the H:L ratio of liver ferritin mRNA and protein. Average ferritin H:L subunit ratios in RNA and standard deviations used the RNA data in Figure 1, and for protein, used data from Western blots (see inset). The liver ferritin content of the samples studied (average and standard deviation) was 4.7 ± 1.6 (THL), 3.1 ± 1.8 (SCD), and 2.2 ± 1.4 (normal, adult autopsy samples) mg/100 mg protein. *Significantly different from control (normal) values, $p < .01$; **$p < .05$.

the ratio of H:L protein subunits, observed here, show the importance of both transcriptional and posttranscriptional regulation of iron homeostasis during hypertransfusion iron overload of SCD and THL. The constant level of liver ferritin H and L RNA observed in hypertransfusion iron overload, for both SCD and THL, may indicate insufficient oxidative stress in the liver from excess iron to induce the phase II antioxidant responses of ferritin genes [3]. Differences in damage to other organs observed between thalassemia and sickle cell disease patients [16, 17] may reflect different responses to increased iron, such as less IRP expression, that differ from liver and point to the need for further studies.

REFERENCES


