Brief report

Hepcidin in iron overload disorders

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Hepcidin is the principal regulator of iron absorption in humans. The peptide inhibits cellular iron efflux by binding to the iron export channel ferroportin and inducing its internalization and degradation. Either hepcidin deficiency or alterations in its target, ferroportin, would be expected to result in dysregulated iron absorption, tissue maldistribution of iron, and iron overload. Indeed, hepcidin deficiency has been reported in hereditary hemochromatosis and attributed to mutations in *HFE*, transferrin receptor 2, hemojuvelin, and the hepcidin gene itself. We measured urinary hepcidin in patients with other genetic causes of iron overload. Hepcidin was found to be suppressed in patients with thalassemia syndromes and congenital dyserythropoietic anemia type 1 and was undetectable in patients with juvenile hemochromatosis with *HAMP* mutations. Of interest, urine hepcidin levels were significantly elevated in 2 patients with hemochromatosis type 4. These findings extend the spectrum of iron disorders with hepcidin deficiency and underscore the critical importance of the hepcidin–ferroportin interaction in iron homeostasis. (Blood. 2005; 105:4103-4105)

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Introduction

Hepcidin, a 25-amino acid peptide produced by hepatocytes,[1] is a key iron-regulatory hormone.[2] Hepcidin acts by inhibiting intestinal iron absorption and iron release from hepatic stores and from macrophages recycling senescent erythrocytes.[2-4] Increased hepcidin levels have been associated with anemia of inflammation,[5,6] and hepcidin deficiency may be the common pathogenic feature of hereditary hemochromatosis.[2] Inflammation and iron loading induce hepcidin synthesis, whereas anemia/hypoxia suppresses it.[5,7]

Iron absorption in humans is regulated by the combined influences of the erythropoietic demand for iron, tissue oxygenation, and the body's iron stores. Hepcidin, regulated by hypoxia and iron, is at the point of convergence of the "erythroid" and "stores" regulation. In a number of clinical syndromes, including atransferrinemia, thalassemia syndromes, congenital dyserythropoietic anemias, and sideroblastic anemias, erythroid iron needs appear dominant over the inhibitory signals from iron stores,[8,9] and iron absorption is inappropriately stimulated despite massive iron overload. To assess the opposing effects of anemia and iron loading on hepcidin as their common target, we analyzed hepcidin levels in thalassemia syndromes and congenital dyserythropoietic anemias, diseases that often lead to iron overload.

Hepcidin levels were also analyzed for the first time in patients with hemochromatosis type 4 (ferroportin disease), a disease caused by mutations in iron-exporter ferroportin.[10] Ferroportin is directly regulated by hepcidin: hepcidin binds to ferroportin and causes its internalization.[11] However, in contrast to all other types of hemochromatosis (caused by mutations in *HFE, TFR2, HAMP,* and *HJV*), which are characterized by hepcidin deficiency,[12-15] we found increased hepcidin levels in the 2 patients with ferroportin disease.

Study design

Patients

Approval for these studies was obtained from the institutional review boards of the affiliated institutions. Informed consent was provided according to the Declaration of Helsinki. Twenty-one patients with iron overload attributed to various abnormalities (7 with β-thalassemia intermedia, 8 with β-thalassemia major, 2 with congenital dyserythropoietic anemia type 1 [CDAI], 2 with *HAMP*-associated juvenile hemochromatosis with the 93delG mutation, and 2 with hemochromatosis type 4 caused by the Val162del mutation of the *SLC40A1* gene) and 1 patient with iron-deficiency anemia participated in the study. Table 1 displays the patients' relevant demographic and laboratory data.

Urinary hepcidin assay

Urinary creatinine concentrations were measured at the University of California at Los Angeles Clinical Laboratories. Cationic peptides were extracted from patients' urine by CM Macro-prep (Bio-Rad Laboratories, Hercules, CA), eluted from the matrix with 5% acetic acid, lyophilized, and resuspended in 0.01% acetic acid. Immunodot assay was used to quantitate hepcidin in the urine extracts.[16] Extracts equivalent to 0.1 to 0.5 mg creatinine were dotted on Immobilon-P membrane (Millipore, Bedford, MA), along with a range of synthetic hepcidin standards (0-40 ng).

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Table 1. Clinical data and urinary hepcidin levels in patients with iron overload disorders

Patient	Disease	Sex	Age, y	HCT, %	Hb, g/dL	MCV, fL	Fe, μg/dL	Ferritin, ng/mL	Hepcidin, ng/mg creatinine	Transfusions	Chelation
1	β-TI	F	24	24	7	67	120	170	4	N	N
2	β-TI	M	32	23	6	63	110	850	3	Occasional	DFO
3	β-TI	F	45	28	7.4	59	126	720	< 2	N	N
4	β-TI	M	45	29	7.5	63	110	680	9	N	N
5	β-TI	M	52	27	8	68	145	650	7	Occasional	DFO
6	β-TI	M	37	29	8.2	62	156	260	69	N	N
7	β-TI	F	44	32	9.1	65	120	180	< 2	N	N
8	β-TM	M	28	33	9.2	68	110	2600	6	Y	DFO
9	β-TM	F	23	33	9.2	65	125	4200	< 2	Y	DFO
10	β-TM	F	28	29	7.9	93	87	3200	10	Y	DFO
11	β-TM	M	29	33	9.1	78	110	1300	< 2	Y	DFO
12	β-TM	M	30	33	9.5	78	68	1250	35	Y	DFO
13	β-TM	M	36	32	9	85	120	9200	124	Y	DFO/L1
14	β-TM	M	35	30	9.5	75	-	6500	5	Y	DFO/L1
15	β-TM	F	26	32	9	82	110	3250	231	Y	DFO/L1
16	CDAI	M	42	23	6.3	115	80	1300	< 2	N	DFO
17	CDAI	F	47	26	7.2	115	145	1030	< 2	Occasional	DFO
18	ID	M	67	32	9	61.2	18	30	< 2	N	N
19	JH (HAMP)	F	32	45	15.4	88	269	64	< 2	N	N
20	JH (HAMP)	F	35	42	14.5	91	229	56	< 2	N	N
21	HH type 4	M	70	43	14	92	80.9	2352	673	N	N
22	HH type 4	M	31	36.5	12	94	49.6	1396	552	N	N

β-TI indicates β-thalassemia intermedia; β-TM, β-thalassemia major; CDAI, congenital dyserythropoetic anemia type 1; ID, iron deficiency; JH (HAMP), juvenile hemochromatosis (HAMP 93delG mutations); HH type 4, hemochromatosis type 4 (Val162del); DFO, desferioxamine; and L1, deferiprone.

Hepcidin was detected on the blots using rabbit anti–human hepcidin antibody,[5] with goat anti–rabbit horseradish peroxidase (HRP) as a secondary antibody. Dot blots were developed by the chemiluminescence detection method (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL) and were quantified with the Chemidoc cooled camera running Quantity One software (Bio-Rad Laboratories). Hepcidin quantity in each sample was then normalized using urinary creatinine concentrations and was expressed as nanogram hepcidin per milligram creatinine.

Within-assay precision was determined by performing 8 separate carboxymethyl ion exchange matrix (CM) extractions of the identical urine aliquots and analyzing them by immunodot assay in duplicate; the resultant intra-assay coefficient of variation [(mean of the standard deviations/grand mean) × 100] was 6%.

Accuracy of the assay was determined by spiking urine with synthetic hepcidin 0 ng/mL to 1600 ng/mL, followed by CM extraction and immunodot assay. The recovery of synthetic hepcidin was more than 90%.

Day-to-day variations of hepcidin levels in urine were assessed by measuring hepcidin levels in 3 healthy volunteers who collected first morning urine for 8, 15, or 21 days. Hepcidin levels were 50 ± 26, 18 ± 8, and 55 ± 22 ng/mg creatinine.

Results and discussion

Hepcidin is a key regulator of iron homeostasis and a mediator of anemia of inflammation. Its deficiency is the likely cause of most types of hereditary hemochromatosis. Hepcidin is, therefore, emerging as a fundamental diagnostic parameter; however, hepcidin measurements in a variety of human disease states are still lacking. In this study, we analyzed hepcidin levels in a spectrum of iron overload disorders.

Thalassemia syndromes represent the major cause of iron overload in Mediterranean countries. In thalassemia major, iron overload is attributed mainly to blood transfusions required for the treatment of the disease, but it is partly also caused by increased iron absorption. In contrast, patients with thalassemia intermedia, who have a milder form of anemia and remain for the most part transfusion independent, nevertheless also experience iron overload because of increased iron absorption. In the murine model of human thalassemia, the $Hbb^{th3/+}$ mice, hepcidin mRNA expression was found to be decreased.[17] In this study, hepcidin was measured in 8 patients with thalassemia major and 7 with thalassemia intermedia (Table 1). In healthy subjects, hepcidin levels ranged between 10 and 200 ng/mg creatinine and the ratio of urinary hepcidin (adjusted for creatinine) to serum ferritin was close to 1.[5,15] In contrast, patients with thalassemia had very low urinary hepcidin levels, despite high serum ferritin levels that reflected systemic iron overload. Several patients with thalassemia had no detectable hepcidin. In 4 patients (patients 6, 12, 13, and 15), urinary hepcidin concentrations were in the normal range, but even this likely represented an inadequate response to iron loading, considering the patients' ferritin levels. The variations in hepcidin concentrations among the patients with thalassemia may reflect the effects of other genetic or environmental variables, including possibly the effect of concomitant minor infections or other inflammatory stimuli. Hepcidin was also undetectable in 2 patients with congenital dyserythropoietic anemia type 1 (CDAI), another disease associated with ineffective erythropoiesis and concomitant iron overload (Table 1, patients 16 and 17). Decreased or absent urinary hepcidin is the expected response in patients with iron-deficiency anemia, such as patient 18 with anemia caused by gastrointestinal blood loss.

Findings in patients with thalassemia and CDAI indicate that even though hepcidin functions as a mediator of "erythroid" and "stores" signals, erythroid drive exerts the dominant influence in these conditions. Because hepcidin normally acts to retain iron in the liver[18] and the spleen,[3] the lack of hepcidin in the high-iron milieu could expose other organs to iron loading. Patients with thalassemia major indeed frequently experience iron-induced heart disease and endocrinopathies.[19]

We also analyzed hepcidin levels in 2 patients with type 4 hemochromatosis (ferroportin disease) who carry the Val162del mutation[20] and were untreated. Ferroportin disease is caused by the

autosomal dominant mutations in the *SLC40A1* gene encoding the iron export protein ferroportin/IREG1/MTP1.[10] Distinct from what occurs in other forms of hemochromatosis, in ferroportin disease iron accumulates predominantly in reticuloendothelial macrophages. Clinical features include early increases in serum ferritin levels (in spite of low-normal transferrin saturation) and marginal anemia, sometimes with poor tolerance of phlebotomy.[10]

How the heterozygous ferroportin mutations contribute to the development of the disease has been unclear. A loss-of-function mechanism has been proposed by which the mutations impair iron release from macrophages, resulting in inadequate iron supply to the bone marrow, which would in turn increase iron absorption.[21] If this were the sole effect of the ferroportin mutation, hepcidin should be suppressed. However, measurements of urinary hepcidin in our 2 patients showed hepcidin was dramatically increased (Table 1, patients 21 and 22). The increased hepcidin levels in ferroportin disease are of particular interest given that hepcidin binds to ferroportin and causes its internalization.[11] It remains to be determined whether disease-inducing, dominantly acting ferroportin mutations interfere with hepcidin binding or internalization.

Hepcidin increases in patients with ferroportin mutations are in contrast to all other types of hemochromatosis (caused by mutations in *HFE*, *TfR2, HAMP,* and *HJV*) in which hepcidin deficiency was observed.[12-15] In the current study, we confirmed by direct measurement of urinary hepcidin that the 93delG mutation in the hepcidin gene[14] results in hepcidin deficiency (Table 1, patients 19 and 20). The 93delG mutation generates a frameshift in prohepcidin peptide after residue 31.

Our findings highlight the potential usefulness of hepcidin measurement as a diagnostic tool and the need for further analysis of hepcidin expression in a wider spectrum of iron disorders.

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