

Hepcidin Is Produced by Lymphoplasmacytic Cells and Is Associated With Anemia in Waldenström's Macroglobulinemia

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Abstract

Waldenström's macroglobulinemia (WM) patients often present with anemia as their primary disease manifestation that may be related to hepcidin, an important regulator of iron homeostasis. We therefore determined hepcidin levels in 53 WM patients, and 20 age-matched healthy patient donors by hepcidin-25 ELISA. Serum hepcidin levels were elevated in WM patients versus healthy patients ($P = .04$), and correlated with BM disease involvement ($P = .004$), beta-2-microglobulin levels ($P = .029$), and inversely with hemoglobin ($P = .05$). No correlation with serum iron indices was observed, though in patients with high hepcidin levels, increased iron deposition in bone marrow macrophages was observed. Importantly, hepcidin transcripts and protein were produced by primary WM cells. Hepcidin levels correlated with serum IL-6 ($P < .001$) and C-Reactive Protein ($P = .033$) levels. The results of this study implicate hepcidin as a contributor to anemia in WM, and suggest that an iron re-utilization defect accompanies hepcidin overproduction leading to its sequestration in WM patients.

Introduction

Anemia is a common problem in patients with Waldenström's macroglobulinemia (WM), and is considered among the foremost of

reasons for initiation of therapy.¹ While bone marrow replacement by disease is an important component for the production of anemia in WM patients, it alone as an etiology is unlikely to explain anemia in many patients.¹ Other etiologies which could be contributory include dilutional effect resulting from the intravascular osmotic draw imposed by high IgM levels; hemolysis due to cold as well as warm antibodies; and bone marrow injury inflicted by prior chemotherapy exposure and/or treatment related myelodysplasia. Despite thorough evaluation for these etiologies, anemia in WM patients is often out of proportion to the level of disease involvement.¹

Hepcidin is a peptide hormone that regulates iron metabolism.^{2,3} Though primarily produced by hepatocytes, monocytes and lymphocytes have recently been shown to produce hepcidin.³⁻⁵ Hepcidin exerts its regulatory function by binding to and mediating the internalization and subsequent degradation of the iron export protein ferroportin, which is found on enterocytes, monocytes, and macrophages.^{6,7} Upon ligation by hepcidin, ferroportin is internalized, ubiquitinated, and the degraded.^{7,8} By inhibiting ferroportin, hepcidin prevents gut enterocytes from secreting absorbed iron into the hepatic portal system, thereby limiting iron absorption. Iron release from monocytes and macrophages is also prevented by hepcidin which then build up iron stores. Several cytokines have been implicated in hepcidin induction, including interleukin-6 [IL-6], interleukin-1 α [IL-1 α], interleukin-1 β [IL-1 β], and bone morphogenetic proteins (BMPs), particularly BMP6.⁹⁻¹¹ We therefore investigated hepcidin as a potential factor for anemia in patients with WM.

Patients and Methods

Study Design

We randomly selected patients presenting with and without anemia with the clinicopathologic diagnosis of WM who were not on therapy, and for whom stored serum was available for these studies. Normal institutional range for hematocrit for males is 38.4%-

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Table 1 Clinical Characteristics for 53 WM Patients in This Study

| Characteristic | Median | Range |
|--------------------------------------|----------|-----------|
| Age, Years | 63 | 45-81 |
| Untreated (%) | 43 (81%) | NA |
| Male:Female | 1.4 | NA |
| Beta-2 Microglobulin, mg/dL | 2.5 | 1.3-9.9 |
| Bone Marrow Involvement, % | 40 | 5-90 |
| Serum IgM, mg/dL | 1930 | 671-7220 |
| Median Hematocrit, % | 34.2 | 25.6-47.5 |
| Anemic, % | 44 (83%) | NA |
| Serum Iron | 67 | 14-170 |
| Total Iron Binding Capacity (TIBC) | 321 | 193-435 |
| Iron Saturation (Serum Iron/TIBC), % | 20.7 | 5.5-48.9 |
| Ferritin, µg/L | 55.5 | 18-843 |
| C-Reactive Protein, mg/L | 6.7 | 0-101.6 |
| Median Platelets, 1000/µL | 270 | 42-567 |

48.2% and for females is 34.8%-43.6%. The study was approved by the Dana Farber Cancer Institute/Harvard Cancer Center Institutional Review Board. Fifty-three WM patients were identified, whose baseline characteristics are depicted in Table 1. Twenty age- and sex-matched healthy donors were used as controls in these studies. Their baseline characteristics were: median age, 63.5 years (range, 42-88 years); and male/female ratio of 1.5.

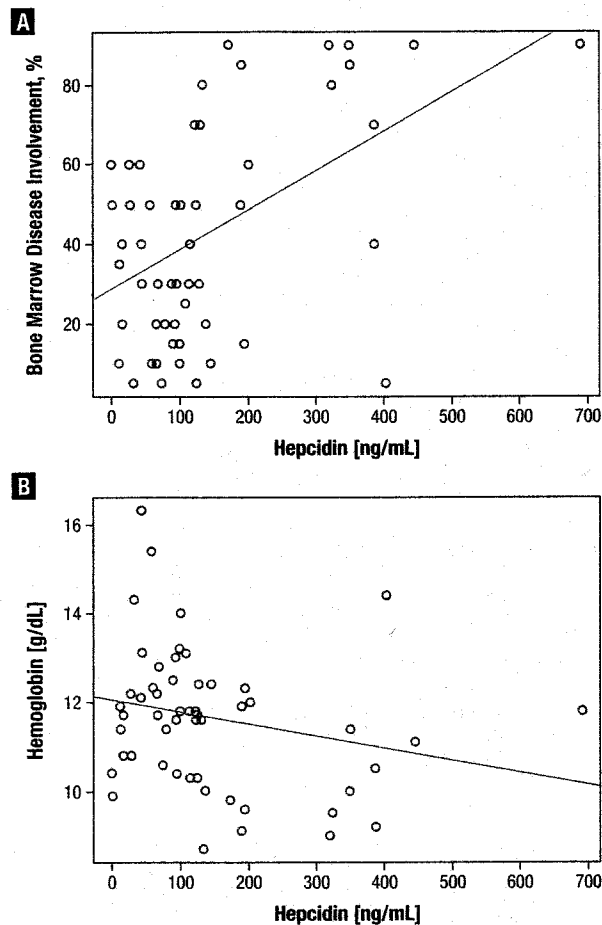
Assays

Plasma levels of hepcidin were determined in duplicate using the Hepcidin-P competitive ELISA for hepcidin-25, the biologically-active form of hepcidin (Intrinsic Lifesciences, La Jolla, CA, USA). Serum iron, total iron binding capacity (TIBC), ferritin, beta 2 microglobulin (sB2M) and C-reactive protein (CRP) testing were performed by the Brigham and Women's Hospital. Plasma levels of IL-6 and soluble CD27 (Bender Medsystems, La Jolla, CA), and BMP6 (R&D Systems, Minneapolis, MN) were performed in duplicate by commercial ELISA kit per the manufacturer's instructions.

Bone Marrow Evaluations

Bone marrow aspirates (BMA) were obtained from WM patients undergoing diagnostic testing, and normal, age-matched healthy individuals undergoing primary, elective total joint arthroplasty after written consent. BMA clots were obtained by allowing aspirate to coagulate at room temperature for approximately 3 hours. BMA clots were then fixed in ice-cold, 10% neutral-buffered formaldehyde for 1 hour. Fixed clots were washed 3 times for 10 minutes in phosphate-buffered saline followed by centrifugation to remove excess fixative, after which clots were bathed in 70% ethanol and stored in the dark at 4°C until use. Fixed clots were processed into paraffin wax-embedded blocks for histology by typical methods within 1 month of initial processing. Sections were cut at 4 µm using a microtome and floated onto Colorfrost-Plus microscope slides (Thermo Fisher Scientific, Wilmington, DE). Morphological assessment was performed after standard hematoxylin and eosin

Figure 1 Relationship of Plasma Hepcidin Levels in 53 WM Patients to (A) Bone Marrow Disease Involvement [$P = .004$, Spearman's $Rho = 0.385$], and to (B) Hemoglobin Levels [$P = .05$, Spearman's $Rho = -0.269$] for 53 WM Patients

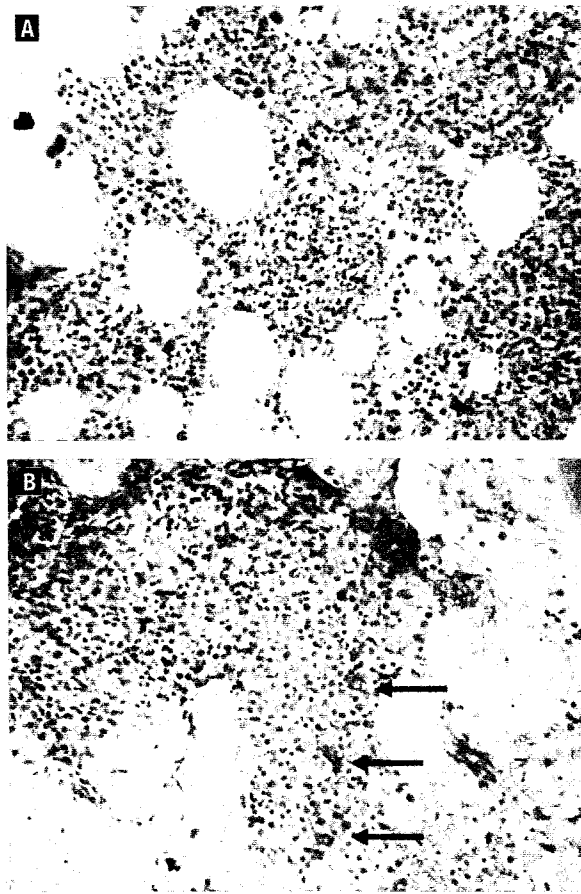


staining, and hemosiderin-containing cells were identified following Perls Prussian blue iron stain.

Molecular Studies

CD19+ B-lymphocytes were immunomagnetically isolated from purified WM patient and healthy donor bone marrow mononuclear cells (Miltenyi Biotech, Auburn, CA). Total RNA was extracted from the enriched, CD19+ bone marrow mononuclear cells of WM patients and healthy donor controls using the RNeasy Mini Kit (Qiagen, Valencia, CA). Gene expression profiling was performed using the Affymetrix Human Genome HG-U133a Plus 2.0 microarray platform in accordance with the manufacturer's instructions (Affymetrix, Santa Clara, CA). Raw microarray chip data was analyzed using the dChip software program. Hepcidin expression was confirmed by qRT-PCR in accordance with the manufacturer's instructions. Taqman probes for Hepcidin Antimicrobial Peptide [probe # hs01037160_g1] and the TATA Box Protein [probe # hs99999910_m1], a housekeeping gene, were purchased from Applied Biosystems, and semi-quantitative RT-PCR analysis was performed in triplicate using the manufacturer's reagents and the

Figure 2 Bone Marrow Aspirate Clot Section Stained With Perls Prussian Blue Iron Stain From (A) Healthy Patient and (B) Representative WM Patient With Elevated Hepcidin Levels



Arrows show iron deposition in bone marrow macrophages in the patient. 400x magnification.

7300 real-time PCR System in accordance with the published instructions (Applied Biosystems, Carlsbad, CA). Western blot analysis was performed from cell lysates obtained from CD19+ sorted bone marrow lymphoplasmacytic cells from 3 WM patients, BCWM.1 cells, and 3 healthy donors. Anti-hepcidin-25 antibody (Abcam, Cambridge, MA) was used to probe western blots.

Statistical Analysis

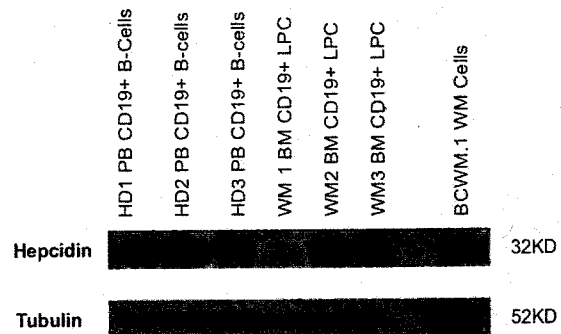
Unless otherwise stated, the comparison of all parameters was performed using a 2-tailed Student's t test on Microsoft Excel software; a P value of $\leq .05$ was deemed to be significant for the above studies. A nonparametric Spearman's rank correlation coefficient [rho] was also calculated for linear correlation studies between plasma hepcidin levels and various serologic factors.

Results

Hepcidin Levels Are Elevated in WM Patients Presenting With Anemia

Clinical characteristics for the 53 WM patients are shown in Table 1. As shown in Figure 1, hepcidin levels positively correlated

Figure 3 Western Blot Analysis of Lysates Demonstrating Hepcidin Protein Production in Peripheral Blood B-Cells in 3 of 3 Healthy Donors (HD 1-3); Bone Marrow Lymphoplasmacytic Cells in 2 of 3 WM Patients (WM 1-3); and in the BCWM.1 WM Cell Line



with bone marrow disease involvement [$P = .004$, Spearman's rho = 0.385], and negatively correlated with hemoglobin [$P = .05$, Spearman's rho = -0.269] among all WM patients. Hepcidin levels were also positively correlated with beta-2-microglobulin [$P = .029$, Spearman's rho = 0.300]. In contrast, no correlation between hepcidin levels and age, gender, familial disease status, previous treatment status, serum IgM or the surrogate disease marker sCD27 were observed (data not shown).

In comparison to healthy donors [median, 91.8; range, 12.2-211.6 ng/mL], the median plasma levels for all WM patients was elevated [median, 107.5 ng/mL; range, 0-689 ng/mL; $P = .04$]. Among the 44 WM patients who presented with anemia, the median hepcidin levels were higher at 118.5 [range, 0-689 ng/mL] versus the 9 non-anemic WM patients in whom the median hepcidin levels were 66.5 [range, 31.7-401.8 ng/mL; $P < .001$]. No correlation between hepcidin levels serum iron levels, total iron binding capacity (TIBC), iron saturation (serum iron/TIBC), or ferritin levels was observed (data not shown). Morphological examination following Perls Prussian blue iron stain in 8 patients displaying elevated hepcidin levels showed increased hemosiderin presence in bone marrow macrophages when compared to healthy donors (Figure 2).

Hepcidin Levels Correlate With IL-6 and CRP in WM Patients

Since IL-6 and BMP6 have been implicated in hepcidin induction, we next measured their levels in patients with WM, whose hepcidin levels were determined. In addition, we determined levels of CRP, an acute phase reactant which is induced by IL-6, and serves as a de facto marker for serum IL-6 levels. Serum IL-6 [$P < .001$; Spearman's rho = 0.481] and CRP levels [$P = .033$, Spearman's rho = .312] were directly correlated with hepcidin levels. In contrast, no association between bone morphogenetic protein 6 and hepcidin was observed (data not shown).

Hepcidin Transcripts and Protein Are Produced by WM Cells

Since hepcidin levels positively correlated with bone marrow disease involvement, we next investigated whether WM cells pro-

duce hepcidin. Both by gene expression profiling, and confirmatory RT-PCR, transcripts for hepcidin were detected in CD19+ bone marrow lymphoplasmacytic cells isolated from WM patients. Hepcidin was expressed in CD19+ cells in 15/21 WM patients versus 8/8 healthy donors. By gene expression profiling, hepcidin expression levels were similar for WM versus healthy donor cells (median expression ratio for WM/healthy donor 1.04; $P = .693$). By semi-quantitative RT-PCR analysis, when hepcidin expression in bone marrow CD19+ cells was normalized against the TATA box housekeeping protein, the median expression level for WM patients ($n = 19$) was 1.29-fold higher versus healthy donors ($n = 7$). Importantly as shown in Figure 3, western blot analysis demonstrated production of hepcidin protein in primary WM cells, and the BCWM.1 cell line.

Discussion

To our knowledge, this is the first study addressing a role for hepcidin in WM. We show that hepcidin levels are elevated in WM patients, correlate positively with bone marrow involvement and serum B2M, and inversely with hemoglobin levels. Importantly, we show a strong correlation between levels of hepcidin and IL-6, and the IL-6 surrogate marker CRP. Similar to us, Hohaus et al¹² reported a direct correlation between hepcidin and IL-6 levels in Hodgkin patients. IL-6 is a powerful inducer of hepcidin production whose levels are elevated in WM patients.¹³⁻¹⁵ These studies also demonstrate that WM cells are capable of producing hepcidin themselves. While the production of hepcidin by WM cells may explain the direct correlation observed with bone marrow involvement in this study, the elaboration of IL-6 by tumor cells or by bone marrow stromal cells upon WM cell binding could also lead to augmented hepcidin by other tissues such as liver and/or monocytes or macrophages. An interesting observation in this study was also the association of hepcidin with serum B2M levels, a powerful prognostic marker in WM disease which may herald hepcidin as a prognostic tool in WM.¹ The association of higher hepcidin levels with poor prognostic indices was also noted in the study by Hohaus et al in Hodgkin patients.¹² Further studies to address the role of hepcidin as a prognostic marker should be considered.

In contrast to our expectations, hepcidin levels did not stratify with serum iron levels, TIBC, iron saturation, or ferritin levels. In 8 WM patients who exhibited high hepcidin levels, examination of the bone marrow by Perls Prussian blue iron stain showed increased iron sequestration by bone marrow macrophages. These findings are consistent with an iron re-utilization defect in WM patients as a con-

sequence of hepcidin overproduction.^{16,17} These studies also suggest that examination of iron distribution in the context of the cellular elements of the bone marrow (ie, erythroid cells vs. macrophages) may be helpful in clarifying an iron re-utilization defect in WM patients. One important outcome of hepcidin related studies are initiatives directed at the development of therapeutics targeting hepcidin and its signaling pathways.¹⁸ The development of such therapeutics may be particularly of benefit to patients with WM, and defer the necessity for immediate chemotherapeutic intervention, or improve outcomes in combination with WM directed chemotherapy.

In summary, the results of this study implicate hepcidin as a contributor to anemia in WM, and suggest that an iron re-utilization defect accompanies overproduction of hepcidin leading to its sequestration in WM patients.

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