Involvement of Hepcidin in the Anemia of Multiple Myeloma

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Abstract

Purpose: Hepcidin is a liver-produced peptide implicated in the anemia of inflammation. Because interleukin (IL)-6 is a potent inducer of hepcidin expression and its levels are elevated in multiple myeloma, we studied the role of hepcidin in the anemia of multiple myeloma.

Experimental Design: Urinary hepcidin and serum levels of IL-6, ferritin, C-reactive protein, tumor necrosis factor-α, and IL-1β were studied in newly diagnosed myeloma patients. *In vitro* hepcidin induction assay was assessed by real-time PCR assay.

Results: Pretreatment urinary hepcidin levels in 44 patients with stage III multiple myeloma were 3-fold greater than normal controls. In the subset of multiple myeloma patients without renal insufficiency (n = 27), a marked inverse correlation was seen between hemoglobin at diagnosis and urinary hepcidin level (P = 0.014) strongly supporting a causal relationship between up-regulated hepcidin expression and anemia. The urinary hepcidin also significantly ($P < 0.05$) correlated with serum ferritin and C-reactive protein, whereas its correlation with serum IL-6 levels was of borderline significance (P = 0.06). Sera from 14 multiple myeloma patients, with known elevated urinary hepcidin, significantly induced hepcidin mRNA in the Hep3B cells, whereas normal sera had no effect. For 10 patients, the ability of anti-IL-6 and anti-IL-6 receptor antibodies to prevent the serum-induced hepcidin RNA was tested. In 6 of these patients, hepcidin induction was abrogated by the anti-IL-6 antibodies, but in the other 4 patients, the neutralizing antibodies had no effect.

Conclusions: These results indicate hepcidin is up-regulated in multiple myeloma patients by both IL-6-dependent and IL-6-independent mechanisms and may play a role in the anemia of multiple myeloma.

Anemia occurs in up to 60% to 80% of untreated patients with active myeloma and is usually normochromic/normocytic and associated with reticulocytopenia and hypoferremia (1–3). Use of recombinant erythropoietin results in modest increases of hemoglobin levels in ~60% of patients and anemia often improves spontaneously when patients go into remission. The cause of anemia in myeloma is probably multifactorial, as some patients also have renal insufficiency with attendant low endogenous erythropoietin levels, some show an apoptotic effect of myeloma cells on RBC precursors, and in some cases there is inhibition of erythroid colony formation (4–6). However, most cases are ascribed to "impaired iron utilization" consistent with the anemia of inflammation also called "anemia of chronic disease."

Hepcidin is a liver-produced peptide hormone that is implicated in anemia of inflammation (7, 8). Hepcidin binds to the cell membrane iron exporter ferroportin and induces internalization and degradation, thus decreasing iron release from macrophages and enterocytes (9). Diminished iron flow into plasma results in hypoferremia, which restricts the delivery of iron to maturing erythrocytes and eventually causes anemia. Hepcidin expression is induced by inflammatory and infectious stimuli in part through stimulation by increased interleukin (IL)-6 (10). In addition, other cytokines, including IL-1, may also contribute to hepcidin increase during inflammation (11). As IL-6 is involved in the pathogenesis of multiple myeloma and serum IL-6 levels are often increased in myeloma patients (12, 13), we explored the hypothesis that elevated hepcidin expression contributes to the anemia characteristic of this disease.

Materials and Methods

Patient selection. Untreated Durie-Salmon (14) stage III myeloma patients and monoclonal gammopathy of unknown significance patients from VA hospitals in Los Angeles, Pittsburgh, and Chicago were enrolled in this study. All patients were newly diagnosed and were included in the study before receiving any cytotoxic chemotherapy. None of the patients had history of recent infection. All human studies were done in accordance with local regulations and the Declaration of

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Helsinki. Informed consent was obtained from all subjects. Laboratory tests were all done at diagnosis and serum IL-6 levels and tumor necrosis factor-α (TNF-α) were determined by ELISA as described previously (10). Serum IL-1β ELISA was done with an ELISA kit from R&D Systems.

Urinary hepcidin assay. Cationic peptides were extracted from patients' urine and hepcidin quantitated in the extract by immunodot assay as described before (10). Urinary creatinine was measured at the University of California-Los Angeles Clinical Laboratories. Hepcidin quantity in each sample was normalized using urinary creatinine concentrations and hepcidin urinary concentration was expressed as nanogram hepcidin per milligram creatinine.

***Effect of patient sera on hepcidin mRNA* in vitro.** Hep3B cells were cultured in DMEM with 10% fetal bovine serum. Sera from multiple myeloma patients or normal controls were added to these cells to a final concentration of 10%. As a positive control, some wells were treated with 100 unit/mL recombinant human IL-6. After 24 h, RNA was extracted with Qiagen mini RNA kit and reverse transcribed and quantitative real-time PCR was done for hepcidin and actin. The primer sequences are as follows: human hepcidin forward 5-CCTGAC-CAGTGGCTCTGTTT-3 and reverse 5-CACATCCCACACTTTGATCG-3 and human β-actin forward 5′-ATCGTGCGTGACATTAAG-3′ and reverse 5′-ATTGCCAATGGTGATGAC-3. Data are shown as fold induction of hepcidin standardized to β-actin control using the method of Pfaffl (15).

Neutralization with anti-IL-6 antibodies. To study the role of IL-6 in hepcidin induction, Hep3B cells were incubated with 10% multiple myeloma patient sera for 24 h in the presence of neutralizing antibodies against IL-6 (clone MQ2-13A5 from Bioscience at 10 ng/mL) and blocking antibodies against the IL-6 receptor (clone B-R6 from Biosource at 10 ng/mL). As a control, the same concentration of a nonspecific antibody of the same isotype was added.

Isolation of primary myeloma cells. Primary human myeloma cells were isolated from a bone marrow aspirate by RosettesSep Antibody cocktail method (Stem Cell Technologies). Briefly, the bone marrow aspirate was filtered with a cell strainer to remove particulate matter. The antibody cocktail was added and cells were incubated at room temperature for 20 min. A Ficoll-Hypaque isolation was then done and cells were collected at the interface. The harvested cells were >98% plasma cells.

Statistical analysis. The Student's *t* test was used to compare normally distributed data. Correlations between the various measured parameters were calculated by Pearson correlation.

Results

Hepcidin levels and anemia in myeloma patients. We studied 44 patients with Durie-Salmon stage III multiple myeloma and 8 monoclonal gammopathy of unknown significance patients and compared them with 105 previously analyzed normal controls. As a group, the myeloma patients showed a variable degree of anemia (mean, 10.14 g%; SD, 1.47). None of these patients had reticulocyte counts >2.5%. Furthermore, no patient had history of bleeding, iron deficiency, hemolysis, or active infection. The mean serum iron of the entire group was 67 mg/dL and the serum transferrin was 253 mg/dL. These levels are consistent with the anemia of chronic disease. The mean level of hepcidin in multiple myeloma patients was 186.6 ng/mg creatinine, which is significantly higher ($P < 0.0001$) than normal controls (56.5 ng/mg creatinine; Fig. 1). The mean hepcidin level in the monoclonal gammopathy of unknown significance patients was not significantly different from the normal controls.

Seventeen of the 44 patients had renal insufficiency at diagnosis (serum creatinine, >1.4; mean ± SD, 2.7 ± 1.89), whereas 27 had normal renal function (serum creatinine, <1.4; mean ± SD, 1.05 ± 0.3). Although the mean urinary hepcidin levels did not significantly differ between these two groups (two-tailed $P = 0.36$), renal insufficiency could independently affect the red cell mass through its effect on erythropoietin production. Thus, we attempted to correlate hepcidin levels with hemoglobin at diagnosis only in the cohort of patients with normal renal function ($n = 27$). Figure 2A shows a strong inverse correlation between hemoglobin level and urinary hepcidin (Pearson correlation r value is -0.46 with a two-tailed $P = 0.0014$) in these patients. When data were analyzed by arbitrarily dividing multiple myeloma patients in two groups (Fig. 2B), one with hepcidin <100 ng/mg and the other with hepcidin >100 ng/mg creatinine, the hemoglobin levels were significantly lower in the high hepcidin group (two-tailed $P = 0.027$). The inverse correlation between hepcidin and hemoglobin levels is even more striking, considering that hepcidin levels are suppressed during anemia because of increased erythropoietic drive (16). There was no significant difference in transferrin saturation when comparing the patients with hepcidin levels >100 ng/mg urinary creatinine with those with levels <100 ng/mg urinary creatinine.

Hepcidin correlation with IL-6 levels and inflammatory markers. Hepcidin is induced as an acute-phase reactant during infection and/or inflammation. To examine this effect in myeloma patients, we correlated hepcidin with serum ferritin and C-reactive protein, two other proteins induced in acute-phase reactions (Fig. 3A and B). The analysis shows significant correlations between hepcidin and ferritin ($P = 0.048$) and hepcidin and C-reactive protein ($P = 0.0012$). Since prior work identified IL-6 as an important inflammatory regulator of hepatic hepcidin, we also analyzed the correlations between serum IL-6 levels and urinary hepcidin in multiple myeloma patients. All patients were analyzed irrespective of their serum creatinine. Mean IL-6 level of all the multiple myeloma patients ($n = 27$) tested was 27.7 pg/mL with no statistically significant difference between patients with ($n = 14$) or without ($n = 13$) renal insufficiency (two-tailed $P = 0.35$). As shown in Fig. 3C,

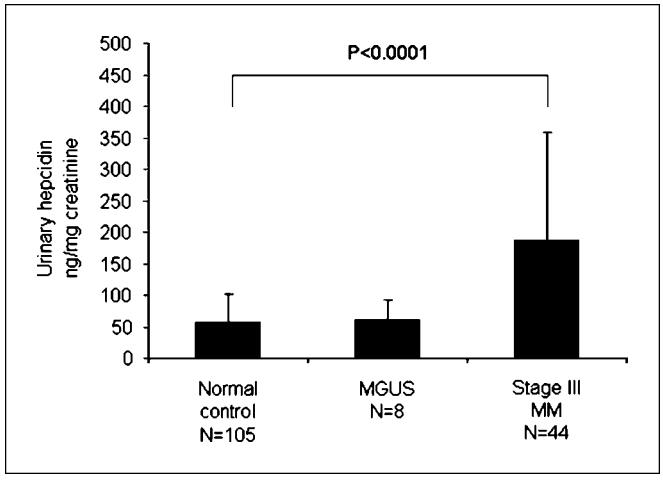

Fig. 1. Urinary hepcidin levels in myeloma patients. *Columns,* mean hepcidin levels; *bars,* SD. *NL,* hepcidin in controls; *MM,* monoclonal gammopathy of unknown significance patients and in myeloma patients. Mean hepcidin levels in multiple myeloma were significantly higher than in normal controls ($P < 0.0001$).

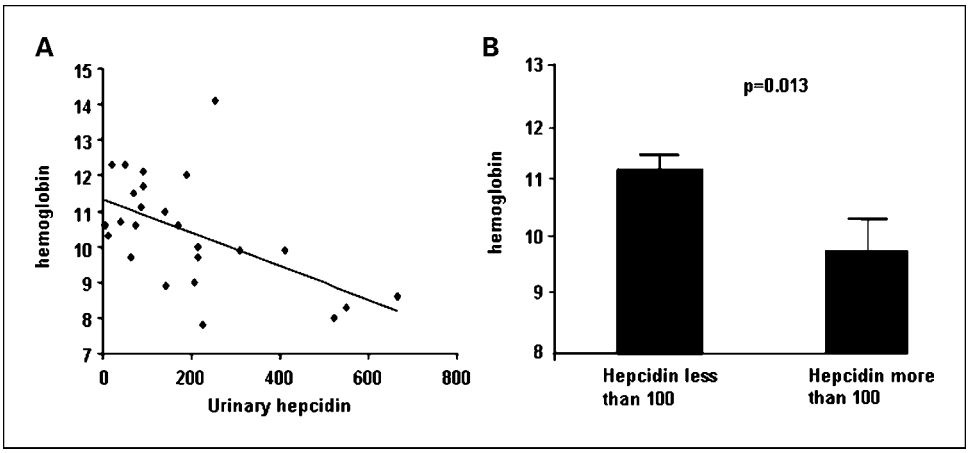

Fig. 2. Correlation between urinary hepcidin and hemoglobin in multiple myeloma patients. *A,* scatter plot with linear regression line showing significant inverse correlation between urinary hepcidin levels and hemoglobin levels in multiple myeloma patients with serum creatinine <1.4 mg/mL at the time of diagnosis ($P = 0.0014$). *B, columns,* mean hemoglobin levels in multiple myeloma patients with hepcidin <100 or >100; *bars,* SD.

the correlation between serum IL-6 and urinary hepcidin levels was of borderline significance with a *P* value of 0.06.

TNF-α and IL-1β levels may be also elevated in myeloma (17–19) and both have been implicated in regulating hepcidin expression (11, 20, 21). Thus, we also measured these cytokines in the sera of our patients. No significant correlation was observed between TNF-α or IL-1β and hepcidin levels arguing against any role for these cytokines in hepcidin up-regulation in myeloma patients [$P = 0.91$ for TNF- α (Fig. 3D) and $P = 0.99$ for IL-1β (Fig. 3E)]. IL-1α was also shown to up-regulate

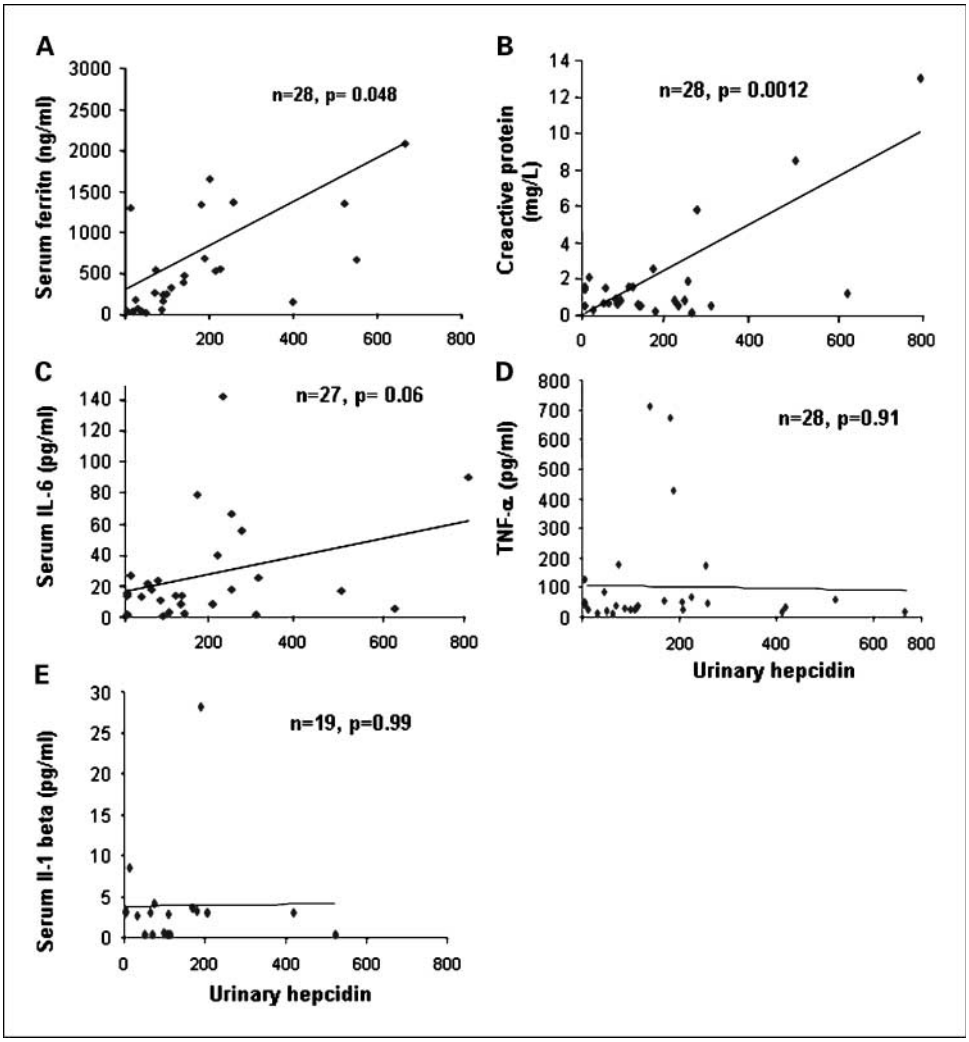

Fig. 3. Correlation of urinary hepcidin with serum ferritin, C-reactive protein, IL-6, TNF-α, and IL-1β Scatter plot with linear regression lines are shown with the number of multiple myeloma patients studied (*n*) and significance of correlation (*p*).

Table 1. Hepcidin induction, neutralization by anti-IL-6 and anti-IL-6R antibodies, and serum IL-6 levels in myeloma patients

Sample ID	Fold hepcidin induction in Hep3B cells	Hepcidin induction neutralized by anti-IL-6 antibodies	Serum IL-6
IL-6 (100 units/mL)	5.8	Yes	
Normal control (n = 7)	1.3 ± 0.3		
Patient 1	9.75	Yes	21.2
Patient 2	9.53	Yes	23.9
Patient 3	5	Yes	90
Patient 4	3.11	Yes	15.3
Patient 5	2.5	Yes	142
Patient 6	2.0	Yes	17.7
Patient 7	3.9	No	14
Patient 8	3.9	No	2.8
Patient 9	3.9	No	2.5
Patient 10	2.3	No	1.6
Patient 11	5.2	ND	
Patient 12	3.9	ND	
Patient 13	2.4	ND	
Patient 14	2.1	ND	
Patient 15	1.8		
Patient 16	1.7		
Patient 17	1.6		
Patient 18	1		
Patient 19	0.9		
Patient 20	0.4		

NOTE: Hep3B cells were treated with IL-6 (100 units/mL) as a positive control for hepcidin RNA induction as described in Materials and Methods. Hep3B cells were also exposed to sera from myeloma patients or normal controls in the presence or absence of anti-IL-6 antibody and anti-IL-6 receptor antibody. Patients 15 to 20 showed significant hepcidin induction (>mean ± 2 SD).
Abbreviation: ND, not done.

hepcidin expression (11), but there are no data to support that its levels are up-regulated in myeloma patients and therefore was not tested.

Induction of hepcidin by patient sera and the role of IL-6. We next tested the effects of patient sera on hepcidin expression in Hep3B cells, a human hepatoma cell line, relative to 10% fetal bovine serum as a reference. Hep3B cells were treated with sera from multiple myeloma patients, normal controls, or growth medium containing recombinant IL-6 (Table 1). We confirmed the expected induction of hepcidin expression with recombinant IL-6 (100 units/mL). Seven sera from normal donors did not show an appreciable induction (1.3 ± 0.3-fold compared with 10% fetal bovine serum reference, mean ± SD). Fold inductions above the mean ± 2 SD level observed in normal controls were considered significant inductions (>1.9-fold). Of the 20 multiple myeloma sera tested, 14 caused a significant induction.

To define the contribution of IL-6 in patient sera to hepcidin induction, we repeated the assay with the addition of neutralizing antibodies that would prevent IL-6 signaling. The effects of neutralizing antibodies against IL-6 and blocking antibodies to IL-6 receptor were compared with

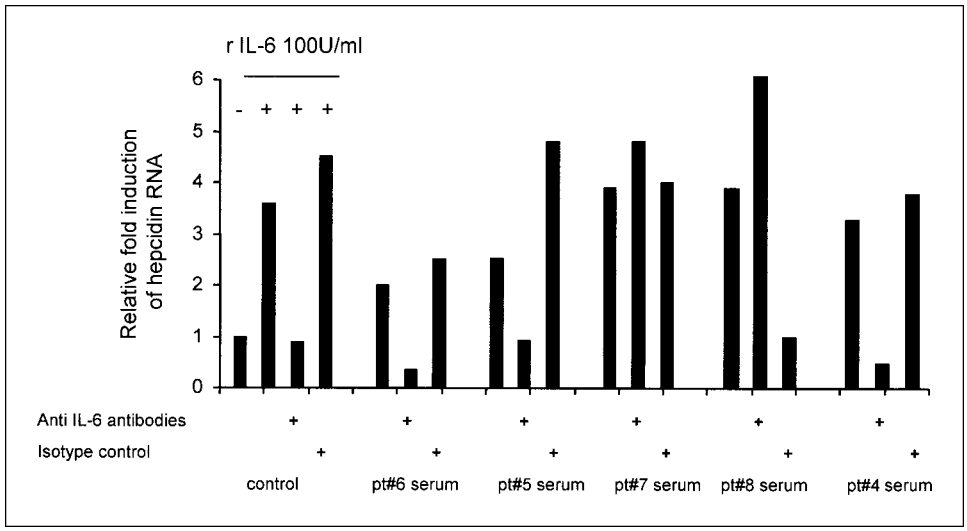

Fig. 4. Effect of IL-6 blockade on hepcidin induction by multiple myeloma sera *in vitro*. Hepcidin mRNA levels were determined by quantitative real-time PCR normalized to actin and displayed as a ratio to 10% fetal bovine serum control. In the control group, Hep3B cells were cultured with 10% fetal bovine serum and treated with IL-6. Sera from three patients 6, 5, and 4 are shown as an example where the addition of anti-IL-6 antibodies neutralized the hepcidin induction. Serum from patients 7 and 8 are shown as an example where the anti-IL-6 antibodies were unable to inhibit hepcidin mRNA induction.

those of isotype-identical control immunoglobulin. The blocking antibodies completely prevented hepcidin induction by recombinant IL-6 (Fig. 4). We had sufficient serum remaining in 10 multiple myeloma sera that have shown significant induction of hepcidin expression. In 6 of these 10 sera, the hepcidin induction could be completely blocked by the antibodies (Table 1), whereas no inhibitory effect was seen in the other four patients. The suppression of hepcidin induction by the antibodies was either complete (that is, 100% inhibited) or not at all. Several examples of this experiment are shown in Fig. 4. In this figure, hepcidin induction by sera from patients 6, 5, and 4 were completely inhibited by the antibodies, whereas sera from patients 7 and 8 were unaffected. The mean IL-6 levels in the former group (patients 1-6; Table 1) were 51.5 pg/mL and the latter group (patients 7-10) was only 5.25 pg/mL. However, as the number of sera tested were low, this difference was not statistically significant ($P = 0.1$). These results overall suggest a role of IL-6 in hepcidin induction in some multiple myeloma patients with other as yet unidentified factors playing a role in other patients.

Hepcidin expression in primary myeloma cells. To assess the possibility that myeloma cells may secrete sufficient hepcidin to increase systemic levels, we studied hepcidin expression by quantitative PCR in primary cells from two patients with and without treatment with IL-6 (100 units/mL). The hepcidin mRNA concentrations in myeloma cells were at least 3,000-fold lower than those measured in human primary hepatocytes (data not shown). Unlike in Hep3B cells, recombinant IL-6 did not induce hepcidin in myeloma cells (Fig. 5). To confirm the integrity of the IL-6 signaling pathway in the primary myeloma cells, a significant up-regulation of Mcl-1 RNA expression, an IL-6 target gene (22), was observed (data not shown).

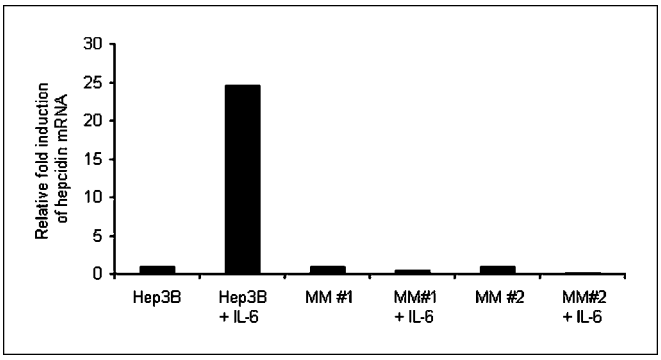

Fig. 5. Hepcidin expression in primary human myeloma cells. Quantitative real-time PCR was done on Hep3B cells and primary myeloma cells from two patients with and without treatment with IL-6. Data are relative fold induction of hepcidin mRNA compared with the untreated cells. IL-6 strongly induced hepcidin in Hep3B cells but not in multiple myeloma cells.

Discussion

The major findings of this study are increased urinary hepcidin concentrations in multiple myeloma patients and a striking inverse correlation between the severity of anemia at diagnosis and urinary hepcidin levels. The elevated hepcidin levels in our anemic patients are even more impressive considering that anemia per se is associated with decreased hepcidin levels due to the effects of increased erythropoietic drive (16). Chronic overexpression of hepcidin in mice results in severe anemia (23–25). Furthermore, the levels of elevated hepcidin in our anemic patients are comparable with those in patients with anemia of chronic inflammation (7). Collectively, these data support the hypothesis that the increased hepcidin levels play an etiologic role in the anemia of myeloma. It is obviously impossible to directly test this notion in individual human subjects and it is certainly possible that active myeloma independently results in hepcidin overproduction and anemia. However, the extremely strong inverse correlation between hepcidin level and hemoglobin concentration supports an etiologic relationship.

It could be argued that the anemia of myeloma actually causes the elevated hepcidin levels rather than the reverse. In the report by Pak et al. (16), inhibition of erythropoietic activity in chemotherapy-treated anemic mice led to increased hepcidin levels. However, an increased erythropoietic drive found in anemic states was found to suppress hepcidin levels (8). The fact that the majority of myeloma patients do respond to recombinant erythropoietin with a significant increase in hemoglobin levels (26) and that reticulocyte counts in myeloma patients are not zero (actually as high as 0.7%, corrected reticulocyte count; ref. 27) support the notion of increased erythropoietic activity at diagnosis in this disease. Because anemia associated with an increased erythropoietic drive results in decreased hepcidin levels instead of up-regulated levels, we believe that the anemia in myeloma patients is due to increased hepcidin levels and not the reverse.

The hepcidin levels correlated with serum ferritin and C-reactive protein, consistent with the notion that hepcidin expression is part of an acute-phase reaction. Because IL-6 is often up-regulated in myeloma patients (12, 13) and has the ability to induce a hepatic acute-phase reaction that includes increased hepcidin expression (7), we tested a possible connection between hepcidin levels and serum IL-6. In 27 patients in whom we could assay serum IL-6 concentrations, a trend was noted, which fell short of significance ($P = 0.06$). There was no correlation whatsoever between hepcidin and serum TNF-α concentrations or IL-1β.

To determine whether hepcidin-inducing molecules circulate in multiple myeloma plasma, we tested the ability of patients' sera to induce hepcidin mRNA in Hep3B cells. Unlike sera from normal donors, 14 of 20 patient sera at 10% concentration induced hepcidin above mean $\pm$ 2 SD of the normal control. Because there was a borderline correlation between IL-6 and hepcidin levels and because IL-6 is known to be potent inducer of hepcidin (10), we retested 10 of the 14 sera after blockade with anti-IL-6 and anti-IL-6 receptor antibodies. In 6 of the 10 sera, hepcidin RNA induction was abrogated. However, the same concentrations of antibodies had no effect on induction caused by the other four sera. Thus, the results support the notion that IL-6 contributes to hepcidin induction in some multiple myeloma patients, but in others, additional serum substances can also stimulate hepcidin expression. This is consistent with studies in IL-6 knockout mice (28) where inflammatory stimuli still increase hepcidin expression. Although assaying sera for TNF-α and IL-1β did not support a role for these cytokines, we did not test bone morphogenetic

protein (29, 30) and TGF-β (31). These molecules induce hepcidin expression *in vitro* and may be expressed at high level in myeloma patients (32, 33).

Myeloma cells themselves do not appear to contribute to systemic hepcidin levels as their hepcidin mRNA expression was very low compared with primary hepatocytes and was not inducible by IL-6. Thus, our results are consistent with the following scenario: growth of myeloma is associated with an increased expression of IL-6 and other hepcidin-inducing cytokines. These circulate systemically and induce hepcidin expression in liver cells. Elevated hepcidin levels then block iron release from hepatocytes, enterocytes, and macrophages resulting in iron restriction and eventually in anemia. This scenario provides a rationale for therapeutic targeting of hepcidin as a specific modality for managing anemia in myeloma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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