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Hepcidin Expression by Human Monocytes in Response to Adhesion and Pro-Inflammatory Cytokines

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Abstract

Background—A previous urine proteomic analysis from our laboratory suggested that hepcidin may be a biomarker for lupus nephritis flare. Immunohistochemical staining of kidney biopsies from lupus patients showed that hepcidin was expressed by infiltrating renal leukocytes. Here we investigated whether inflammatory cytokines relevant to the pathogenesis of lupus nephritis and other glomerular diseases regulate hepcidin expression by human monocytes.

Methods—Human CD14⁺ monocytes were incubated with interferon alpha (IFN α), interferon gamma (IFN γ), interleukin-6 (IL6), interleukin-1 beta (IL1 β), monocyte chemotactic factor-1 (MCP1), or tumor necrosis factor alpha (TNF α). Hepcidin expression was examined by real-time PCR and enzyme immunoassay.

Results—Monocyte hepcidin mRNA increased during adherence to the tissue culture wells, reaching a level 150-fold higher than baseline within 12 hours of plating. After accounting for the effects of adhesion, monocytes showed time and dose-dependent up-regulation of hepcidin mRNA upon treatment with IFN α or IL6. One hour of incubation with IFN α or IL6 increased hepcidin mRNA 20 and 80-fold respectively; by 24 hours the mRNA remained 5 and 2.4-fold higher than baseline. IL1 β , IFN γ , and MCP-1 did not affect monocyte hepcidin expression. TNF α inhibited hepcidin induction by IL6 in monocytes by 44%. After 24 hours of treatment with IFN α or IL6, immunoreactive hepcidin production by monocytes increased 3 and 2.6-fold respectively.

Conclusion—Human monocytes produce hepcidin in response to adhesion and the pro-inflammatory cytokines IFN α and IL6.

General Significance—The appearance of hepcidin in the kidneys or urine during glomerular diseases may be from infiltrating monocytes induced to express hepcidin by adherence and exposure to pro-inflammatory cytokines found in the renal milieu.

Keywords

Hepcidin; Interferon Alpha; Human Monocytes; Nephritis

1. Introduction

Hepcidin was initially identified as an antimicrobial peptide in ultrafiltrates of human blood [1] and urine [2], although it is now better known for its role in iron homeostasis. Mature

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hepcidin is a cysteine-rich 25 amino acid cationic peptide with four intramolecular disulfide bonds. It is predominantly expressed by the liver, although various tissues can express hepcidin at low levels, such as heart, brain and monocytes [3,4]. Hecpidin binds to ferroportin on cell surfaces, causing its internalization and subsequent degradation [5,6]. The loss of ferroportin inhibits cellular iron efflux from macrophages and hepatocytes, and decreases intestinal iron absorption, leading to a fall in plasma iron.

Hepcidin expression is increased by iron loading and inflammation, whereas iron deficiency, blood loss, erythropoiesis and hypoxia reduce hepcidin expression [7–10]. Although the molecular mechanisms of hepcidin regulation are complex, BMP-SMAD signaling appears to have a central regulatory role [7–10]. Recently endoplasmic reticulum (ER) stress was shown to transcriptionally activate the hepcidin gene promoter through the cyclic AMP response element-binding protein H (CREBH) [11]. Inflammatory cytokines such as IL6 and LPS activate hepcidin transcription through janus kinase and signal transducers and activators of transcription (JAK/STAT) signaling in hepatoma and monocyte cell lines [7,12], however this pathway also requires BMP-SMAD [12]. Interferon- γ (IFN γ) alone is a poor activator of hepcidin expression, but acts synergistically with mycobacteria to induce hepcidin in mouse macrophages [13]. Excluding IL6 [14,15] and LPS [16,17] cytokine regulation of hepcidin has not been fully investigated, especially in primary human monocytes.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple organs, including the kidneys in 40–60% of patients. In SLE, various cytokines play important roles in recruiting monocytes, T-lymphocytes and neutrophils to mediate local tissue injury [18,19]. Type I interferons (IFN α and IFN β), IL6, and TNF α have all been implicated in the pathogenesis of lupus and lupus nephritis [20–22]. IFN α can modulate immune responses by facilitating dendritic cell maturation from monocytes, promoting long-term antibody and chemokine production, enhancing responses to IL6 and IFN γ , and suppressing responses to TNF α . IL6 plays a critical role in B cell hyperactivity and mesangial cell proliferation. The role of TNF α in LN is complex. Some studies suggest TNF α helps maintain tolerance in SLE, delaying disease appearance [23]. However after SLE is initiated, TNF α may be proinflammatory and TNF α expression is especially associated with LN [23–25]. All of these cellular and systemic effects of IFN α , TNF α , and IL6 can mediate tissue damage in the kidneys of SLE patients.

A urine proteomic evaluation of lupus nephritis from our laboratory demonstrated that hepcidin may be a biomarker for SLE renal flare [26]. The urine levels of hepcidin-25 and hepcidin-20 changed according to lupus nephritis activity, and leukocytes (probably monocytes) infiltrating the renal interstitium of patients with SLE and active nephritis expressed hepcidin. These findings suggested that hepcidin from infiltrating renal monocytes may contribute to urinary hepcidin expression in lupus nephritis. A limited amount of work has been done on hepcidin regulation in monocytes and macrophages. Most studies have used the human monocyte cell line THP-1, or murine peritoneal macrophages, and the stimuli were IL6 or infection [13,27, 28]. Work from Weiss, et. al., demonstrated that both IL6 and LPS induced hepcidin in a human monocyte cell line, but other cytokines were not explored [4]. The present study was undertaken to characterize the regulation of hepcidin expression in primary human monocytes by inflammatory cytokines relevant to lupus and lupus nephritis (LN).

2. Materials and Methods

2.1 Human monocyte isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from American Red Cross source leukocytes (product code 20301) using Cellgro Lymphocyte Separation Medium (Mediatech Herndon, VA). These cells were from multiple donors and replicate experiments were done

with cells from different donors. CD14⁺ monocytes were then further purified by MACS CD14 MicroBeads (Miltenyi Biotech, Auburn, CA). Approximately 4 million CD14⁺ monocytes were plated in each well of 12-well plates in RPMI 1640 containing 2% fetal bovine serum (FBS), and incubated with interleukin-1 β (IL1 β), interleukin-6 (IL6), tumor necrosis factor alpha (TNF α), monocyte chemotactic protein 1 (MCP1), interferon alpha (IFN α) or interferon gamma (IFN γ) from R&D Systems (Minneapolis, MN) at the indicated concentrations and for the indicated durations. HepG2 cells from ATCC (Manassas, VA) cells were grown in DMEM/F12 medium with FBS in parallel to human monocytes as a positive hepcidin control. For experiments using Surface Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF MS) to detect hepcidin in cell culture supernatants or cell lysates, cells were incubated in serum free medium to reduce interference from serum proteins.

2.2 Real-Time RT-PCR

Total RNA from CD14⁺ monocytes and HepG2 cells cultured in vitro was isolated using Invitrogen TRIzol Reagent (Carlsbad, CA), and hepcidin mRNA levels were assessed by real-time RT-PCR using Invitrogen SuperScript III Platinum SYBR Green One-Step qRT-PCR kit. The primer set for human hepcidin was designed using Integrated DNA Technologies (IDT, Coralville, IA) software: Forward-TTTCCCACAACAGACGGGACAACCT; Reverse-GGGCAGCAGGAATAAATAAGGAAGGG. A GAPDH internal control primer set was made as described [13]: Forward-GAAGGTGAAGGTCTGGAGTC and Reverse-GAAGATGGTGATGGGATTTC. Relative hepcidin expression was calculated using GAPDH as a reference [29] and the Δ Ct method as described previously [30].

2.3 Hepcidin-25 peptide enzyme immunoassay (EIA)

The hepcidin-25 peptide EIA kit S-1337 was purchased from Bachem Group (Torrance, CA). Cell culture supernatants, lysates, or hepcidin-25 standards in serial dilutions brought to 50 μ l were added to the antiserum pre-coated plate for competitive immunoassay according to kit instructions. Hepcidin concentrations of 0.031 to 4.0 ng/ml were used for standard curves, and the R-squared statistic was 0.976. The coefficient of variation (CV) using a hepcidin-25 concentration of 1.56 ng/ml was 3.49% intra-assay and 3.43% inter-assay. The hepcidin-25 standard Liver-Expressed Antimicrobial Peptide 1 (LEAP1) from Peptides International Inc (Louisville, KY) was used for validation of this EIA kit. The R-squared was 0.997 for LEAP1 from 0–50 ng/ml using a sigmoid regression curve fitting algorithm.

2.4 SELDI-TOF MS

Human monocyte and HepG2 culture medium or cell lysates were analyzed by SELDI-TOF MS for hepcidin expression. Each cell lysate was prepared at 4°C using M-PER protein extraction reagent (Pierce Biotechnology, Rockford IL). Briefly, 20 μ l of cell culture medium or 10 μ l of cell lysate was added to each spot of CM10 ProteinChips (providing a weak cation exchange chromatography surface) using the same binding protocol published previously [26]. HEPC71P, a hepcidin-20 peptide from Alpha Diagnostic International (San Antonio, TX), and LEAP1, a hepcidin-25 peptide from Peptides International (Louisville, KY) were spiked into cell culture medium as a positive controls for SELDI analysis. To minimize the protein chip spot variance, each sample was done in duplicate and the spectra were normalized to the total ion current within duplicates. External calibration of the Protein Chip Reader was performed using the Ciphergen Biosystem All-in-1 peptide standard C100-0005, and the calibration equation was applied to each spectrum before analysis to ensure peak accuracy.

2.5 Data Analysis

All statistics were performed using Minitab 15.1.2 statistical software (Minitab Inc, State College, PA). Data were tested for normal distribution and then analyzed by ANOVA followed

by post-hoc testing where $p < 0.05$ was considered significant. Hepcidin mRNA and peptide levels were expressed as mean $\pm$ standard error.

3. Results and Discussion

3.1 Monocyte hepcidin mRNA increases during adhesion

After immunohistochemistry indicated that renal interstitial leukocytes in the kidneys of patients with LN produced hepcidin [26], we postulated that cytokines present in the kidney during LN could regulate monocyte hepcidin expression. To test this hypothesis, hepcidin mRNA was first measured in monocytes derived from PBMC after they were allowed to adhere to tissue culture wells for different lengths of time. As shown in Figure 1A, CD14⁺ monocyte hepcidin mRNA began to increase after 1 hour in vitro, and continued to increase over time, reaching a maximum of 150-fold baseline about 12 hours after plating. Although hepatocytes are the main source of hepcidin in vivo, after adherence in vitro monocyte hepcidin mRNA was 2.6-fold higher than that of unstimulated HepG2 cells, after normalizing to GAPDH mRNA (Fig. 1B). Because hepcidin is anti-microbial, it is conceivable that hepcidin up-regulation as monocytes arrive at a focus of infection is consistent with this host-defense function. Importantly, hepcidin mRNA in freshly isolated human monocytes measured before plating into tissue culture was very low, and by comparison was about 55-fold less than the constitutive hepcidin mRNA level of unstimulated HepG2 cells (Fig. 1B). Expressed on a per cell basis, unstimulated HepG2 cells produced 4.4 times more hepcidin mRNA than adherent monocytes (Fig. 1C). Nonetheless, these data suggest that infiltrating renal monocytes in LN may secrete hepcidin.

3.2 Interferon alpha and IL6 up-regulate monocyte hepcidin mRNA

Cytokine regulation of hepcidin has mainly been studied in hepatocytes. IL6 was the first cytokine shown to upregulate hepcidin in human primary hepatocytes, and appears to be the major factor linking hepcidin expression to inflammation [14]. IL-6 was also found to induce hepcidin in monocytes/macrophages [14,15]. Because of the potentially important role of IFN α in the pathogenesis of lupus and LN, we examined monocyte hepcidin expression in response to treatment with IFN α . HepG2 cells were used for comparison, and both monocytes and HepG2 cells treated with IL6 served as positive controls. IL6 and IFN α were used in concentrations similar to that of other studies of hepcidin production [14], and were similar to those found in the serum of patients with active LN [31–33]. Given the spontaneous increase in hepcidin expression with time in culture, untreated monocyte controls were used at each time point, and analyses for cytokine induction were mostly done within 6 hours of monocyte isolation. Hepcidin mRNA levels in CD14⁺ monocytes and HepG2 cells increased in a time- and dose-dependent fashion after exposure to IFN α and IL6 (Fig. 2). Monocytes responded to these cytokines more rapidly and more robustly than HepG2 cells (compare Fig. 2A, 2B and 2C). The time to peak induction of hepcidin mRNA in monocytes for both IFN α (250 iu/ml) and IL6 (50 ng/ml) was around 1 hour, compared to 3–6 hours for HepG2 cells. The increase in monocyte hepcidin mRNA in response to IFN α and IL6 was 20- and 80-fold respectively, approximately 10 times higher than the 2- and 8-fold changes observed in HepG2 cells (Fig. 2A and 2C). Although IL6 caused a higher and more rapid increase of monocyte hepcidin, IFN α exposure resulted in a more sustained increase in hepcidin expression, and after 24-hour in culture it was double that of IL6-treated cells (Fig 2B). Monocyte hepcidin mRNA increased in a dose-dependent fashion after treatment with IFN α for 1 hour (Fig. 2D). The RT-PCR amplification of monocyte hepcidin mRNA is shown in Figure 2E.

3.3 TNF α inhibits hepcidin mRNA induction by IL6 in monocytes

The cytokines IL1, MCP1 and IFN γ were also tested to determine their effect on hepcidin expression, because they have significant roles in monocyte recruitment and activation of

kidney inflammation in lupus nephritis. Interestingly, none of these cytokines showed any significant induction of hepcidin in either monocytes or HepG2 cells (data not shown).

Contrary to the pro-inflammatory mediators IL6 and endotoxin, TNF α inhibited hepcidin expression in the Hep3B hepatocarcinoma cell line [15]. The effect of TNF α on the regulation of hepcidin expression in monocytes and HepG2 cells was therefore tested. The TNF α concentration used was of the same order of magnitude as TNF α serum levels during active LN [34]. TNF α had no effect on basal hepcidin mRNA levels, but caused a 44% inhibition of IL6-induced hepcidin mRNA expression in monocytes (Fig. 3), and a 27% inhibition of IL6-induced hepcidin expression in HepG2 cells (data not shown). Although TNF α showed the same tendency to inhibit the induction of hepcidin expression by INF α in monocytes, this did not reach significance.

3.4 Immunoreactive hepcidin in monocytes is increased by interferon alpha

To verify that monocytes also produce hepcidin peptide, immunoreactive hepcidin was measured in cell culture supernatants and cell lysates by a hepcidin EIA. HepG2 cells were used as a positive control. As shown in Figure 4A the basal level of hepcidin secreted by HepG2 cells was 431 ± 88 pg/million cells, and increased to 1194 ± 131 pg/million cells after 24 hours of incubation with IL6. There was no significant change in hepcidin secretion by HepG2 cells secretion after incubation with IFN α . Immunoreactive hepcidin in monocyte-conditioned cell culture medium dose-dependently increased after treatment with IFN α and IL6 (Fig. 4). IFN α (1000 U/ml) for 24 hours increased hepcidin 2.97-fold over baseline to 89.47 ± 16.84 pg/million cells, while IL6 (20 ng/ml) increased hepcidin 2.64 times to 60.38 ± 15.76 pg/million cells (Fig 4B). Hepcidin peptides in monocyte lysates displayed the same magnitude of increase compared to basal levels (data not shown).

To further verify that hepcidin is secreted by human monocytes, we used a SELDI approach to identify the peptide in culture medium. As shown in Figure 5B, conditioned medium from human monocytes in culture for 24 hours (control) had a peptide ion at 2802.9 m/z, similar to the LEAP1 standard. The appearance of LEAP1 as 2 ions (2785 and 2802.9 m/z) is most likely due to oxidation of its methionine to methionine sulfoxide, which causes a shift in molecular mass of 16. Although SELDI is only semi-quantitative, the increased height of the hepcidin-25 peaks in the treated cells compared to control is consistent with a cytokine-stimulated increase in monocyte hepcidin mRNA and peptide EIA expression. Hepcidin-20, another hepcidin isoform, was also observed in the cell culture medium, but at a much lower intensity than hepcidin-25, and there were no apparent differences in hepcidin-20 expression between cytokine-treated and untreated monocytes (Fig 5A).

In summary, although PBMC-derived monocytes may not totally recapitulate the phenotype of intra-renal monocytes/macrophages, this investigation shows that adherent monocytes do express and secrete hepcidin, and that IFN α and IL6 can upregulate hepcidin production further. The more robust stimulation of monocyte versus HepG2 hepcidin by IFN α might indicate tissue-specific regulation. That is, IFN α may play a more important role in monocyte hepcidin regulation, while IL6 is more important for liver expression. Despite the finding that monocytes/macrophages produce less hepcidin than hepatocytes in vitro, concentrations may be higher in an inflammatory micro-environment like the kidney interstitium during immune-mediated injury. This may account in part for the presence of hepcidin in the urine of patients with lupus nephritis flare, although we cannot presently exclude a contribution from filtered hepcidin of hepatic origin stimulated by inflammatory cytokines expressed systemically during SLE. While the role of hepcidin in lupus nephritis and other forms of kidney disease remains to be determined, its regulation by pro-inflammatory cytokines suggests that hepcidin could reflect the intra-renal cytokine milieu during active disease. It is interesting to note that other iron-regulatory proteins such as transferrin and neutrophil gelatinase-associated lipocalin have

also been identified as potential urine biomarkers of lupus nephritis flare [35]. The prospective measurement of urine and serum hepcidin and other iron-regulatory proteins during kidney diseases may help determine the origin of these urinary proteins, and contribute novel information to the understanding of the pathogenesis of renal injury.

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Figure 1.

Hepcidin mRNA Expression in Human Monocytes and HepG2 cells. Four million human monocytes and 2 million HepG2 cells were cultured for the indicated times. A. Hepcidin mRNA expression in human monocytes over time in vitro. RNA expression was analyzed by real-time RT-PCR in duplicate and hepcidin mRNA levels were normalized to GAPDH mRNA. B. Relative hepcidin mRNA expression by human monocytes and HepG2 cells normalized to GAPDH. C. Relative hepcidin mRNA expression by human monocytes and HepG2 cells normalized to cell numbers. Hepcidin mRNA was expressed as fold-change over freshly isolated cells (before plating) for both monocytes and HepG2 cells. Data are presented as mean $\pm$ standard error with $n=5$ for each experiment.

Figure 2.

Hepcidin mRNA expression after cytokine treatment. Human monocytes (A) and HepG2 cells (C) were treated with 50 ng/ml IL6 or 250 IU/ml IFN α for time-course studies. B. Monocyte hepcidin mRNA regulation by IL6 and IFN α after 24 hours of incubation. D. Monocyte hepcidin mRNA dose-response to IFN α after 1 hour of treatment. E. Real time RT-PCR products after 1 hour of IFN α treatment of human monocytes. The hepcidin mRNA levels were normalized to GAPDH mRNA and then expressed as fold-change over non-treated cells at each time point for monocytes. Data are presented as mean $\pm$ standard error with $n=5$ for each experiment.

Figure 3.

TNF α inhibits hepcidin mRNA expression by human monocytes. Fifty ng/ml of TNF α was added to monocytes in the presence or absence of IL6 or IFN α , and total RNA was isolated after 1 hour of incubation for real time RT-PCR. Hepcidin mRNA levels were normalized to GAPDH mRNA and then expressed as fold-change of TNF α -treated over non-TNF α -treated cells in each of the IL6 or IFN α groups. The data are presented as mean $\pm$ standard error with n=3 for each experiment. The * indicates a significant difference compared to control (P<0.05).

Figure 4.

HepG2 and monocytes secrete immunoreactive hepcidin. A. Time-dependent appearance of hepcidin in HepG2-conditioned culture medium. HepG2 cells were treated with 50 ng/ml IL6 or 250 iu/ml IFN α for different times and hepcidin was measured by EIA. B. Dose-dependent levels of hepcidin in CD14⁺ monocyte-conditioned cell culture medium at 24 hours. CD14⁺ monocytes were cultured with 10–20 ng/ml IL6 or 250–1000 IU/ml IFN α for 24 hours and hepcidin was measured by EIA. Data in A and B are expressed as mean $\pm$ SE (n=6).

Figure 5. SELDI-TOF-MS identification of hepcidin isoforms produced by human monocytes. Monocytes were untreated, or treated with 50 ng/ml IL6 or 250 IU/ml IFN α for 24 hours. Monocyte-conditioned medium was analyzed on CM10 protein chips and hepcidin-20 and 25 standards were spiked into the culture medium at the indicated concentration. A. Representative ions at the position of hepcidin-20. B. Representative ions at the position of hepcidin-25. These spectra are representatives of two independent experiments.