Epidermal hepcidin is required for neutrophil response to bacterial infection

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Introduction

Necrotizing fasciitis (NF) is an infection characterized by widespread necrosis of the skin, subcutaneous tissues, and fascia that was first described by Hippocrates in the 5th century (1). The standard treatment of NF consists of broad-spectrum antibiotics, extensive surgical debridement, and supportive care. However, even with current state-of-the-art treatment, NF frequently takes a fulminant course and is still associated with high mortality rates up to 35% (1). Group A Streptococcus (GAS) is considered the most common cause of NF (2). Despite treatment with systemic antibiotics and radical debridement of necrotic tissue, lethality remains high. The key iron regulatory hormone hepcidin was originally identified as a cationic antimicrobial peptide (AMP), but its putative expression and role in the skin, a major site of AMP production, have never been investigated. We report here that hepcidin production is induced in the skin of patients with group A Streptococcus (GAS) NF. In a GAS-induced NF model, mice lacking hepcidin in keratinocytes failed to restrict systemic spread of infection from an initial tissue focus. Unexpectedly, this effect was due to its ability to promote production of the CXCL1 chemokine by keratinocytes, resulting in neutrophil recruitment. Unlike CXCL1, hepcidin is resistant to degradation by major GAS proteases and could therefore serve as a reservoir to maintain steady-state levels of CXCL1 in infected tissue. Finally, injection of synthetic hepcidin at the site of infection can limit or completely prevent systemic spread of GAS infection, suggesting that hepcidin agonists could have a therapeutic role in NF.

Results and Discussion

We examined hepcidin expression on skin biopsies derived from patients suffering from GAS NF (detailed in Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI126645DS1). Hepcidin staining of...
human liver tissue sections was used as a positive control (Supplemental Figure 1). Hepcidin expression was higher and more widespread in the skin of NF patients than in the skin of a healthy subject, especially in keratinocytes, the predominant cell type in the epidermis (Figure 1A). Hepcidin mRNA expression was induced (Figure 1B) in a human 3D organotypic skin model (Supplemental Figure 2) as a direct consequence of GAS infection.

To investigate the role of hepcidin in the development of NF, we used an established model of necrotizing soft tissue infection (12, 13) where a strain of GAS, isolated from a patient with NF (14), is introduced subcutaneously into a shaved area on the flank of a mouse. Compared with skin biopsies of healthy mice, hepcidin expression was induced in the skin of infected mice (Figure 1C) and clearly detected in the keratinocytes, as visualized by keratin 14 (K14) staining (Figure 1D).

To probe the functional significance of keratinocyte-derived hepcidin in vivo, we developed a mouse model of keratinocyte-specific hepcidin deficiency (Hamp1Δker) by crossing Hamp1lox/lox mice with K14cre+ mice (Figure 1E). We observed an efficient truncation of the floxed Hamp1 allele in the epidermis of the Hamp1Δker mice, but not in the Hamp1lox/lox mice or K14cre+ mice (Supplemental Figure 3; see complete unedited blots in the supplemental material). Systemic iron parameters were unchanged between Hamp1lox/lox and Hamp1Δker mice (Figure 1F), in agreement with our previous study.

**Figure 1. Keratinocyte hepcidin prevents bacterial systemic spread.** IHC with or without primary antibody detecting (A) hepcidin (in brown) on sections of cutaneous human biopsies of GAS NF patients and healthy control using PerkinElmer’s Lamina multilabel slide scanner Panoramic Viewer software. (B) Real-time reverse transcription PCR (qPCR) for hepcidin from GAS-infected human 3D organotypic skin equivalent model; n= 4 per group. (C) qPCR for hepcidin in murine GAS-infected skin; n ≥ 3 per group. (D) Hepcidin (in blue) and K14 (in brown) IHC on cutaneous biopsies of WT mice challenged or not with GAS. Scale bars: 100 μm. Leica DMI3000B microscope, Leica DFC310FX camera, S/0.4; Leica LAS Core software. (E) Generation of Hamp1Δker mice. (F) Plasma iron, ferritin, transferrin, and skin iron levels in Hamp1lox/lox and Hamp1Δker mice; n≥ 4 per group. (G) Bacterial count in skin, blood, and spleen of Hamp1lox/lox and Hamp1Δker mice 4 days after infection with GAS; n≥ 10 per group. (H) Weight variation of Hamp1lox/lox and Hamp1Δker mice during infection; n= 10 per group. Statistical analysis was performed using a Mann Whitney test (B, C, F, and G) or a 2-way ANOVA followed by Tukey’s test for weight kinetics (H). *P < 0.05; **P < 0.01; ***P < 0.001.
demonstrated bacteriostatic activities against GAS (Figure 2A), hepcidin had neither bactericidal (Figure 2A) nor bacteriostatic activities (Figure 2B). Moreover, primary keratinocytes derived from Hamp1lox/lox and Hamp1Δker mice displayed the same bactericidal activity against this pathogen (Figure 2C). We therefore ruled out a direct antimicrobial effect of hepcidin on these bacteria.

AMPs have been reported to have pleiotropic effects and influence a host’s inflammatory responses during infection (15). We therefore asked whether hepcidin could have an immunomodulatory role in keratinocytes. For this purpose, we performed a cytoplex on the supernatant of murine primary keratinocytes incubated with 0.36 μM and 3.6 μM synthetic hepcidin. Interestingly, hepcidin induced a dose-dependent increase of the key neutrophil chemokine CXCL1 but not of the other inflammatory cytokines we tested (Figure 2D). The capacity of mouse hepcidin to induce CXCL1 in primary keratinocytes was confirmed by ELISA (Supplemental Figure 5), as was the capacity of human hepcidin to induce the production of IL-8, the human functional homolog of CXCL1, in the human HaCat keratinocyte cell line and in a human 3D organotypic skin model (Figure 2E).

To investigate the mechanisms by which hepcidin protected against the spread of GAS infection, we first determined the putative bacteriostatic and bactericidal effects of hepcidin against GAS in vitro. While the well-known antimicrobial peptide LL-37 demonstrated bacteriostatic activities against GAS (Figure 2A), hepcidin had neither bactericidal (Figure 2A) nor bacteriostatic activities (Figure 2B). Moreover, primary keratinocytes derived from Hamp1lox/lox and Hamp1Δker mice displayed the same bactericidal activity against this pathogen (Figure 2C). We therefore ruled out a direct antimicrobial effect of hepcidin on these bacteria.

**Figure 2. Hepcidin promotes CXCL1 production by keratinocytes.** (A) GAS killing kinetics with 32 μM of LL-37 and hepcidin; n = 3 per group. Representative of 2 independent experiments. (B) GAS growth curve in the presence of penicillin G, LL-37, hepcidin, or PBS; n = 3 per group. Representative of 2 independent experiments. (C) Bacterial recovering at 1 hour and 3 hours following incubation of log-phase GAS with murine primary keratinocytes (KC) from Hamp1lox/lox and Hamp1Δker mice. Data are representative of 2 independent experiments performed in triplicate. (D) Cytokines measured with the V-PLEX Proinflammatory Panel1 kit in the culture supernatant of murine primary keratinocytes stimulated for 1 or 3 hours with hepcidin or PBS; n = 3 per group. Representative of 3 independent experiments. (E) IL-8 ELISA on the culture supernatant of HaCat or a human 3D skin equivalent model stimulated with 3.6 μM hepcidin; n ≥ 3 per group. Representative of 3 independent experiments. (F) CXCL1 levels measured by ELISA on the culture supernatant of murine primary keratinocytes stimulated for 3 hours with 3.6 μM hepcidin in the presence of PBS or 100 μM FPN inhibitor (2D-014); n ≥ 3 per group. Representative of 3 independent experiments. Statistical analysis was performed using a 2-way ANOVA followed by Tukey’s test (A, B, and D), unpaired Student’s t test (E and G), or a 1-way ANOVA followed by Tukey’s test (C and F). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
The cognate receptor of hepcidin is the iron exporter FPN, questioning the role of FPN/iron in the induction of CXCL1 by hepcidin. The stimulatory effect of hepcidin on CXCL1 was reduced by the addition of a drug preventing the interaction of hepcidin with the iron exporter FPN (16) (Figure 2F). These data suggest that hepcidin, in primary keratinocytes, induces CXCL1 through a FPN-dependent pathway. Binding of hepcidin to FPN is well known to induce its internalization and degradation, resulting in an increase of intracellular iron (5). In corroboration with the action of hepcidin on FPN, incubation of primary keratinocytes with iron stimulated CXCL1 production (Figure 2G).

In agreement with the in vitro results showing that hepcidin stimulates CXCL1 production in primary keratinocytes, the in vivo keratinocyte CXCL1 production in response to GAS infection was lower in Hamp1Δker mice than in Hampflox/lox littermates, as shown by IHC (Figure 3A) and ELISA (Figure 3B) on skin biopsies. As a consequence of the lower CXCL1 production, less neutrophil recruitment was observed in the skin of Hamp1Δker mice compared with that of control littermates, as shown by IHC (Figure 3C) and by cytometry analysis (Figure 3D). This defect in the ability of keratinocyte-derived hepcidin to recruit neutrophils at the site of infection translated into a decrease in the necrotic skin lesion size of the Hamp1Δker mice as compared with controls (Figure 3E). Subcutaneous injection of CXCL1 into GAS-infected Hamp1Δker mice (Figure 3F) significantly decreased the number of bacteria to even below that found in the lesions of Hampflox/lox mice (Figure 3G). These results strongly suggest that the lack of CXCL1 production in Hamp1Δker mice was responsible for their susceptibility to GAS infection. Altogether, these results suggest that hepcidin is critical for regulating CXCL1 production in keratinocytes and that it may tune the magnitude of the neutrophil recruitment in the immune response.
Genetic interactions coevolve, we could speculate that whereas GAS has already evolved to counteract the activity of CXCL1, it has not yet developed a virulence factor able to neutralize the activity of hepcidin. Because of hepcidin resistance to bacterial protease activities such as SpyCEP or SpeB, and in view of its unanticipated immunomodulatory role, we also asked whether local hepcidin injection could have a therapeutic effect on the systemic spread of bacteria in a NF model. Twenty-four hours after GAS infection, 1 μg synthetic hepcidin or PBS was subcutaneously injected at the bacterial inoculation site, followed by 2 injections of 500 ng hepcidin or PBS for 2 consecutive days (Figure 4B). As expected, hepcidin-treated mice showed an increase in neutrophil recruitment (Figure 4C). In contrast to the PBS-treated mice, which exhibited systemic signs of infection including weight loss (Figure 4D), rough hair coat, and hunched posture (data not shown), hepcidin-treated mice did not present any signs of systemic disease and accordingly recovered their initial weight. Remarkably, whereas all the control mice presented with systemic bacterial dissemination (as shown by the number of bacteria in the spleen), 7 of the 9 hepcidin-treated mice showed absolutely no bacterial dissemination during 4 days. Statistical analysis was performed using a Student’s t test (C), a 2-way ANOVA followed by Tukey’s test (D), or a Mann Whitney test (E). * P < 0.05.

Figure 4. Hepcidin is resistant to SpyCEP cleavage and has a therapeutic role in NF. (A) Mass spectroscopy analysis of CXCL1 or hepcidin incubated overnight with SpyCEP or PBS. Electrospray ionization generated a series of multiply charged ions (indicated as m/z; mass-to-charge ratio) from which the average molecular mass (m) of each was deduced. The blue arrows indicate uncleaved peptide peaks at 7.8 kDa (CXCL1) and 2.7 kDa (hepcidin). Red arrows show the cleavage products of CXCL1 with a small (1.3 kDa) and a big (5.9 kDa) fragment. (B) Therapeutic protocol. (C) Neutrophil count (3 measurements per individual mouse were averaged); n = 6 per group. (D) Weight variation and (E) bacterial count in spleen of WT infected mice treated with PBS or hepcidin (n = 9, red square) or PBS (n = 9, black square) during 4 days. Statistical analysis was performed using a Student’s t test (C), a 2-way ANOVA followed by Tukey’s test (D), or a Mann Whitney test (E). * P < 0.05. 
Author contributions
MM, SL, SC, SMS, and JRRM designed the experiments, carried out experiments, and performed data analysis. AF, ASZ, CH, CG, NO, and JWD provided essential reagents and scientific advice. JRRM and CP designed the experiments and supervised the project. CP wrote the manuscript.

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Methods
See Supplemental Methods.

Study approval
For human studies, written informed consent to the protocol was obtained for all subjects and was approved by the Institutional Review Board and the regional ethics committee Paris IV (IRB 2016/40NICB and IRB 00003835). The collection of personal data was approved by the “Commission Nationale de l’Informatique et des Libertés.”

The animal studies described here were reviewed and approved (agreement no. CEEA34.CP.003.13) by the “Président du Comité d’Étude pour l’Expérimentation Animale Paris Descartes” and are in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals (Council of Europe, ETS 123, 1991).

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