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Hepcidin generated by hepatoma cells inhibits iron export from co-cultured THP1 monocytes☆

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***Background/Aims*: The antimicrobial peptide hepcidin is generated in the liver and released into the circulation in response to iron, oxygen and inflammatory signals. Hepcidin serves as a hormonal regulator of duodenal iron absorption and iron trafficking in the reticuloendothelial system. The aim of this study is to explore the effects of this regulatory peptide in macrophage iron metabolism.**

***Methods*: Hepcidin-mediated iron efflux and parameters of cellular iron homeostasis were studied in THP1 monocytic cells co-cultured with hepcidin-producing hepatic cells.**

***Results*: Stimulation of hepcidin expression in Huh7 cells with interleukin-6 promoted a significant ~30% decrease in ^{59}Fe efflux from THP1 cells, previously loaded with ^{59}Fe-transferrin. Similar results were obtained with HepG2 cells transfected with a hepcidin cDNA. Importantly, hepcidin expression from Huh7 cells elicited a decrease in the levels of the iron-sensitive post-transcriptional regulator IRP2 in THP1 cells, accompanied by de novo synthesis of the iron storage protein ferritin.**

***Conclusions*: Physiologically generated hepcidin inhibits iron efflux and promotes iron accumulation in monocytic cells, mimicking a pathophysiological response commonly observed in the anemia of inflammation. Our results highlight the crucial role of hepcidin in the control of macrophage iron homeostasis.**

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***Keywords*: Ferroportin; Hemochromatosis; Anemia of chronic disease; HFE; Hemojuvelin**

1. Introduction

Hepcidin, a conserved cysteine-rich peptide of 20–25 aminoacids, is produced in the liver and functions as the principal hormonal regulator of body iron homeostasis [1–3]. Hepcidin negatively regulates iron absorption in the duodenum and transport in reticuloendothelial cells by controlling the levels of the iron exporter ferroportin [4,5]. The expression of hepcidin is turned off in response to low body iron stores, anemia or hypoxia [6]. On the other hand, hepcidin is induced by iron overload [7] or by inflammatory signals via interleukins IL-1 or IL-6 [8,9]. Misregulation of hepcidin expression is associated with a broad spectrum of iron-related disorders.

Genetic mutations leading to complete silencing of hepcidin are etiologically linked to a rare form of juvenile hemochromatosis [10], an early onset disease of iron overload characterized by pathological iron absorption and deposition within parenchymal cells with a relative sparing of macrophages [11]. More common forms of hereditary hemochromatosis (caused by mutations in HFE, TfR2 or HJV) [11] correlate with various degrees of hepcidin deficiency [12–15]. Likewise, hepcidin is suppressed in patients with thalassemia syndromes but increases in patients with 'ferroportin disease' [16]. Similar phenotypes have been described in

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mouse models of hemochromatosis [17–23] and thalassemia [24]. Transgenic mice over-expressing hepcidin from a liver-specific promoter show profound defects in materno-foetal iron transport and display severe iron deficiency anemia [25].

Hepcidin levels are normally elevated following iron ingestion or infection [9]. The upregulation of hepcidin expression by inflammatory signals is tightly linked to the 'anemia of chronic disease' (ACD) or 'anemia of inflammation' (AI) [26,27]. This condition is characterized by hypoferremia due to iron retention within macrophages and decreased iron absorption. The withholding of iron may be protective against growing bacteria but eventually limits erythropoiesis. Even though the development of ACD depends on multiple factors [27], hepcidin is considered as the key mediator for the diversion of iron traffic by controlling the stability of ferroportin [4,5], which is unique in its capacity to export iron from macrophages and intestinal cells [28].

The experimental evidence supporting this mechanism was based on the direct binding of human hepcidin to transfected mouse ferroportin-GFP in HEK293 and HeLa cells, promoting its internalization and lysosomal degradation [4,5]. In a separate study, synthetic human hepcidin inhibited iron export and decreased the levels of transfected murine ferroportin in J774 macrophages [29]. These data are consistent with a function of hepcidin as a principal regulator of iron efflux from macrophages.

Considering that the effector (hepcidin) and the target (ferroportin) are expressed in different cell types, the above mechanism would require cell-to-cell communication between hepcidin-producing hepatocytes with macrophages. To further validate the molecular basis of hepcidin regulatory activity under physiologically relevant conditions, we establish here a co-culture model of Huh7 or HepG2 hepatoma and THP1 monocytic cells. We demonstrate that hepcidin generated by hepatoma cells is capable of regulating iron metabolism in neighboring monocytes.

2. Materials and methods

2.1. Materials

Hemin, IL-6 and ceruloplasmin were purchased from Sigma (St Louis, MI). Desferrioxamine (DFO) was from Novartis (Dorval, QC, Canada). High molecular weight desferrioxamine (HMW-DFO), a non-permeable hydroxyethyl starch conjugate [30], was obtained from Biomedical Frontiers (Minneapolis, MN).

2.2. Cell culture

Human Huh7 and HepG2 hepatoma cells and H1299 lung cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Media for Huh7 and HepG2 cells also contained 1% non-essential amino acids. Human THP1 monocytic cells were cultured in supplemented RPMI.

2.3. Co-culture experiments

THP1 suspension cells were inserted into a co-culture cartridge (BD Falcon), which was placed on top of 6-well dishes containing monolayers of 0.2×10^6 Huh7, HepG2 or H1299 cells. Co-culture experiments were performed in serum-free RPMI to avoid possible interference of serum transferrin on iron uptake and release.

2.4. Generation of HepG2 hepcidin transfectants

Human hepcidin cDNA (kindly provided by Dr M. Muckenthaler, University of Heidelberg, Germany) was digested out of the parent pDNR-LIB vector with EcoR1 and Xho1 and ligated into pcDNA3. The construct was transfected into HepG2 cells by Lipofectamine Plus (Invitrogen) and stable clones were selected and maintained in the presence of 500 μg/ml G418 (Invitrogen).

2.5. Iron release assays

^{59}Fe-labelled transferrin (Tf) was prepared as previously described [31]. THP1 suspension cells were loaded overnight with 5 μM ^{59}Fe–Tf in serum-free RPMI. Subsequently, the cells were harvested by centrifugation at 4 °C and washed 4× with ice-cold RPMI to remove traces of soluble ^{59}Fe–Tf. Under these conditions, less than ~0.1% of radioactivity could be extracted after the second wash, indicating complete removal of non-internalized transferrin. Aliquots of 2×10^6 cells were resuspended in pre-warmed serum-free RPMI containing 100 μM HMW-DFO and immediately placed into a CO_2 incubator at 37 °C either alone, or in co-culture with other cells. Radioactivity in the supernatant, corresponding to ^{59}Fe release, and in THP1 cells was monitored at specified time intervals on a γ-counter. To calculate the percentage of ^{59}Fe release, the amount of soluble radioactivity was divided by the total amount of radioactivity in media and THP1 cells.

2.6. Western blotting

Cells were lysed in cytoplasmic lysis buffer (1% Triton X-100, 300 mM NaCl and 50 mM Tris/HCl; pH 7.4). Cell debris was cleared by centrifugation and protein concentration was measured with the Bradford reagent (BioRad). Cell lysates (15 μg) were resolved by SDS/PAGE and proteins transferred onto nitrocellulose filters. The blots were saturated with 10% non-fat milk in Tris-buffered saline (TBS) and probed overnight at 4 °C with 1:500 diluted ferritin (DakoCytomation Inc.) or IRP2 [32] antibodies. After 3× washes with TBS containing 0.1% (v/v) Tween 20, the blots were further incubated for 2 h at room temperature with 1:5000 diluted goat anti-rabbit IgG (Sigma). Detection of the peroxidase-coupled secondary antibodies was performed with the ECL® method (Amersham). The blots were quantified by densitometry.

2.7. Metabolic labeling with ^{35}S-methionine/cysteine and immunoprecipitation of ferritin

THP1 cells were metabolically labeled during co-culture with (50 μCi/ml) *trans*-[^{35}S]label, a mixture of 70:30 ^{35}S-methionine/cysteine (ICN). Cytoplasmic lysates (160 μg) were subjected to quantitative immunoprecipitation with 5 μl ferritin antibody (Roche). Immunoprecipitated proteins were analysed by SDS/PAGE and visualized by autoradiography. Radioactive bands were quantified by phosphorimaging.

2.8. Northern blotting

Cells were lysed with the Trizol reagent (Invitrogen) and RNA was prepared according to the manufacturer's recommendations. Total cellular RNA (10 μg) was electrophoretically resolved on a denaturing agarose gel, transferred onto nylon membranes, and hybridized to ^{32}P-labeled human hepcidin or β-actin cDNA probes. Autoradiograms were quantified by phosphorimaging.

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