Competitive regulation of hepcidin mRNA by soluble and cell-associated hemojuvelin

Lan Lin, Y. Paul Goldberg, and Tomas Ganz

Mutations in a recently identified gene *HJV* (also called *HFE2,* or repulsive guidance molecule C, *RgmC*) are the major cause of juvenile hemochromatosis (JH). The protein product of *HJV*, hemojuvelin, contains a C-terminal glycosylphosphatidylinositol anchor, suggesting that it can be present in either a soluble or a cell-associated form. Patients with *HJV* hemochromatosis have low urinary levels of hepcidin, the principal iron-regulatory hormone secreted by the liver. However, neither the specific role of hemojuvelin in maintaining iron homeostasis nor its relationship to hepcidin has been experimentally established. In this study we used hemojuvelin-specific siRNAs to vary hemojuvelin mRNA concentration and showed that cellular hemojuvelin positively regulated hepcidin mRNA expression, independently of the interleukin 6 pathway. We also showed that recombinant soluble hemojuvelin (rs-hemojuvelin) suppressed hepcidin mRNA expression in primary human hepatocytes in a log-linear dose-dependent manner, suggesting binding competition between soluble and cell-associated hemojuvelin. Soluble hemojuvelin was found in human sera at concentrations similar to those required to suppress hepcidin mRNA in vitro. In cells engineered to express hemojuvelin, soluble hemojuvelin release was progressively inhibited by increasing iron concentrations. We propose that soluble and cell-associated hemojuvelin reciprocally regulate hepcidin expression in response to changes in extracellular iron concentration. (Blood. 2005;106:2884-2889)

Introduction

Juvenile hemochromatosis (JH) is an early-onset inherited disorder of iron overload. Two phenotypically very similar forms have been recently characterized, one due to the homozygous disruption of the *HJV* gene encoding a protein named hemojuvelin,[1] and the other due to the homozygous disruption of the *HAMP* gene encoding hepcidin. Hepcidin is a key iron-regulatory peptide hormone that controls extracellular iron concentration by regulating the major iron flows into plasma and normally constrains intestinal iron absorption.[2] The 2 forms of JH result in the same downstream defect because patients with homozygous *HJV* mutations, like those with homozygous *HAMP* mutations, have low levels of hepcidin.[1] This suggests that hemojuvelin could be a principal regulator of hepcidin. However, the specific role of hemojuvelin in iron homeostasis or its functional relationship to hepcidin has not been established.

The *HJV* gene produces multiple alternatively spliced mRNA isoforms. The longest isoform of hemojuvelin mRNA encodes a 426–amino acid protein, which contains a C-terminal putative transmembrane domain characteristic of a glycosylphosphatidylinositol-linked membrane anchor (GPI-anchor).[3,4] Removal of the GPI-anchor or proteolysis would be expected to generate a soluble form of hemojuvelin.

In this study, we explored the expression and regulatory roles of both cell-associated and soluble forms of hemojuvelin. On the basis of our observations, we propose a new model of extracellular iron homeostasis in which soluble and cell-associated forms of hemojuvelin act as opposing regulators of hepcidin, presumably by competing for an as yet unknown ligand of hemojuvelin.

Material and methods

Cell culture

Hep3B human hepatocarcinoma cells and HEK293T/17 cells (HEK293) were maintained in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Human hepatocytes (Liver Tissue Procurement and Distribution System, Minneapolis, MN) were cultured in human hepatocyte maintenance medium (Clonetics, San Diego, CA) at 37°C in 5% humidified CO_2. Hepatocytes were treated with purified recombinant soluble hemojuvelin (rs-hemojuvelin) for 24 hours before harvesting. Human recombinant interleukin 6 (IL-6; R&D Systems, Minneapolis, MN) was used at 20-ng/mL concentration.

Human specimens

Human serum and plasma were obtained from volunteer donors under a protocol approved by the Institutional Review Board (IRB) of UCLA. Frozen normal human liver tissue was obtained from the UCLA Human Tissue Resource Center (Los Angeles, CA) under an IRB-approved protocol.

Construction of siRNA

Four siRNA duplexes targeting human hemojuvelin mRNA and one siRNA-negative control were constructed using the Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions.

From the David Geffen School of Medicine at UCLA, Departments of Medicine and Pathology, Los Angeles, CA; and Xenon Pharmaceuticals, Inc, Burnaby, BC, Canada.

Submitted May 6, 2005; accepted June 20, 2005. Prepublished online as *Blood* First Edition Paper, July 5, 2005; DOI 10.1182/blood-2005-05-1845.

Supported by the Will Rogers Fund (T.G.) and Genome Canada grant (Y.P.G.).

Y.P.G. and T.G. have declared a financial interest in a company whose potential product was studied in the present work.

The online version of this article contains a data supplement.

Reprints: Tomas Ganz, CHS 37-055, Dept of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1690; e-mail: tganz@mednet.ucla.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

HJV siRNA targets included: HJVsi1, 5′-AACTCTAAGCACTCTCACTCT-3′; HJVsi2, 5′-AACCATTGATACTGCCAGACG-3′; HJVsi3, 5′-AAGTT-TAGAGGTCATGAAGGT-3′; HJVsi4, 5′-AAAGCTACAAATTCTTCA-CAC-3′; and negative control NCsi1 target, 5′-GCGCGCTTTGTAGGAT-TCG-3′ (Dharmacon, Lafayette, CO). Two additional siRNA-negative control duplexes were purchased: NCsi2, 5′-AATTCTCCGAACGTGT-CACGT-3′ (Qiagen, Valencia, CA), and NCsi3, Silencer Negative Control no. 2 siRNA (Ambion).

Transfections

In all siRNA treatment experiments, Hep3B cells were seeded at 10% confluence 24 hours before siRNA transfection. Hep3B cells were transfected with 20 nM siRNA duplexes using Oligofectamine Transfection Reagent (Invitrogen) according to the manufacturer's protocol for 24 hours, followed by 24-hour treatment with 20 ng/mL human recombinant IL-6 (R&D Systems) or its solvent. In hemojuvelin expression experiments, 24 hours before transfection, Hep3B cells were seeded at 50% confluence and HEK293 cells were seeded at 10% confluence. pcDNA-HJV was generated by cloning full-length human HJV cDNA into vector pcDNA3.1(+) plasmid (Invitrogen). The pcDNA-HJV or the control plasmid vector pcDNA3.1(+) were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol for 24 hours prior to further treatment.

rs-Hemojuvelin production and purification

To express human rs-hemojuvelin, a cDNA of human hemojuvelin truncated by 72 nucleotides at the 3′ end to remove the transmembrane segment and with an added stop codon was cloned into the BaculoDirect baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Culture medium from infected Hi5 insect cell culture was purified by cation exchange chromatography (CM Prep; Bio-Rad, Richmond, CA), followed by high-performance liquid chromatography on a C4 reverse-phase column (Vydac, 214TP54, Grace-Vydac, Hesperia, CA) eluted with an acetonitrile gradient.

RNA isolation, mRNA assay, and microarray analysis

RNA from Hep3B cells and primary human hepatocytes was prepared using TRIzol (Invitrogen) according to the manufacturer's instructions. Single-pass cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad). Human hepcidin and hemojuvelin mRNA concentrations were normalized to human glyceraldehyde 3-phosphate dehydrogenase (G3PD) or human β-actin. Human CCAAT/enhancer-binding protein δ (C/EBPδ) was used for IL-6 response-positive control. The following primers were used in qRT-PCR: hepcidin: forward, 5′-CACAACAGACGGGACAACTT -3′ and reverse, 5′-CGCAGCA-GAAAATGCAGATG-3′; hemojuvelin: forward, 5′-CTCTTAGCTCCACTC-CTTTCTG -3′ and reverse, 5′-GCCCTGCTTCCTTTAATGATTC -3′; G3PD: forward, 5′-TGGTATCGTGGAAGGACTC-3′ and reverse, 5′-AGTAGAG-GCAGGGATGATG-3′; β-actin: forward, 5′-ATCGTGCGTGACAT-TAAG-3′ and reverse, 5′-ATTGCCAATGGTGATGAC-3′; and C/EBPδ: forward, 5′-CAACGACCCATACCTCAG -3′ and reverse, 5′-GGTAAGTC-CAGGCTGTAG-3′. Affymetrix HG-U133 Plus2 (Affymetrix, Santa Clara, CA) was used for microarray analysis according to the manufacturer's protocol.

Western blot analysis and antibody

Cellular protein was extracted with 150 mM NaCl, 10 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.4, 1% Triton X-100 (NETT) and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Frozen normal human liver fragments were pulverized in liquid nitrogen with a mortar and pestle. Approximately 50 mg tissue was homogenized in 700 μL NETT buffer, and approximately 150 μg total protein extract was analyzed. Human sera and plasma samples were loaded directly at 1 or 2 μL/lane. Cell culture media were further processed before Western blot analysis. Serum-free conditioned cell culture media were concentrated by 5-kDa molecular weight cut-off ultrafiltration with Amicon Ultra-4 Centrifugal Filter Units (Millipore, Bedford, MA). Conditioned cell culture media that contained 30 μM apotransferrin and holotransferrin were extracted with the weak cation exchange matrix CM Macroprep (Bio-Rad), the matrix was eluted with 500 mM sodium chloride in 25 mM ammonium acetate buffer (pH 6.5), and the eluate was concentrated by ultrafiltration. Conditioned cell culture media that contained 10% FBS were partially purified by cation exchange chromatography before concentration. Protein samples were separated on 4% to 20% iGels (sodium dodecyl sulfate [SDS]–Tris-glycine; Gradipore, Hawthorne, NY) with dithiothreitol (DTT) if not mentioned specifically otherwise, and silver-stained or transferred on immobilon-P membrane (Millipore). Three different anti–hemojuvelin polyclonal antibodies were prepared by immunizing rabbits with peptide antigens: anti–G3pep2-2 and anti–G3pep2-3, target sequence, N-CRGDLAFHSAVHGIED-C, 1:1000, and Ab112, target sequence, N-CDYEGRFSRLHGRPPG-C 1:5000. Western blots were visualized by chemiluminescence.

Results

Hepcidin mRNA expression positively correlates with hemojuvelin mRNA expression

In principle, the deficiency of hepcidin in patients with homozygous *HJV* mutations could be due to a developmental defect in hepatocyte function or due to the involvement of hemojuvelin in hepcidin regulation. To establish whether hemojuvelin controlled hepcidin synthesis, we used a human hepatoma cell line Hep3B as a model for in vitro studies. Hep3B cells spontaneously produce hemojuvelin mRNA at a similar concentration as in primary human hepatocytes (data not shown). We used 4 different siRNA sequences to target the coding and noncoding 3′ untranslated (3′-UTR) regions of hemojuvelin mRNA. These siRNAs showed a wide range of efficiency (30%-90%) in suppressing hemojuvelin mRNA level 48 hours after transfection. The decrease in hepcidin mRNA correlated with decreased hemojuvelin mRNA levels ($R = 0.64$; Figure 1A). No significant suppression of hepcidin mRNA was observed when hemojuvelin mRNA concentration was above 50% of untreated control. This is consistent with the observation that individuals with only one copy of disrupted *HJV* do not develop iron overload.[1] Three different siRNA negative controls showed slight suppression or induction of either hemojuvelin or hepcidin mRNA, but no significant correlation or specificity was observed (Figure 1B).

Hemojuvelin and IL-6 independently regulate hepcidin mRNA

We next examined whether hemojuvelin is necessary for the inflammatory induction of hepcidin. IL-6 is a well-defined inducer of hepcidin during anemia of inflammation.[5] Hep3B cells were pretreated with hemojuvelin siRNA or diluent for 24 hours, followed by 24 hours of treatment with 20 ng/mL human IL-6 to induce hepcidin (Figure 2). Suppression of hemojuvelin to as low as 10% to 20% of the control (cells not treated with siRNA or IL-6) caused a maximum of 2-fold reduction of hepcidin baseline expression but did not interfere with its inducibility by IL-6 (a similar 4-fold induction of hepcidin mRNA level in both hemojuvelin siRNA treated and control cells). An IL-6–specific acute-phase protein C/EBPδ[6,7] was used as a positive control for IL-6 induction as well as a negative control for hemojuvelin siRNA specificity. The mRNA levels of C/EBPδ were unaffected by hemojuvelin siRNA treatment but were induced by approximately 4-fold with 20 ng/mL IL-6 in both hemojuvelin siRNA-treated and control cells. These data showed that IL-6 and hemojuvelin act independently to regulate hepcidin mRNA levels.

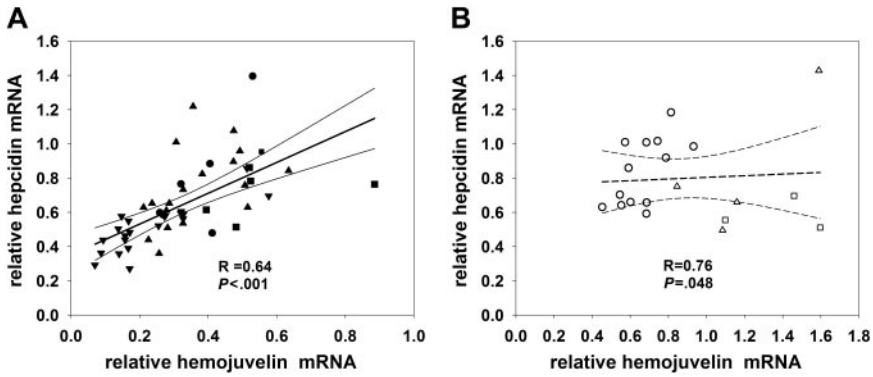

Figure 1. Suppression of hemojuvelin mRNA results in the suppression of hepcidin mRNA. Each individual point represents an experiment in which Hep3B cells were treated with one of the siRNA preparations overnight and then incubated for an additional 24 hours before mRNA extraction. In each experiment, hemojuvelin and hepcidin mRNA were quantified by qRT-PCR and normalized to the housekeeping gene *G3PD*. Control cells were treated only with transfection reagents and their hemojuvelin/G3PD and hepcidin/G3PD ratios were set as baseline at 1. (A) Closed symbols represent hemojuvelin siRNAs experiments (■ indicates HJVsi1; ●, HJVsi2; ▼, HJVsi3; ▲, HJVsi4). The regression line (all HJV siRNAs experiments, $R = 0.64$, with 95% confidence limit) indicates that as the HJV/G3PD ratio decreases, there is a corresponding decrease in the hepcidin mRNA/G3PD ratio. (B) Open symbols represent siRNA control experiments (△ indicates NCsi1; ○, NCsi2; □, NCsi3). No consistent effect on hepcidin is seen with control siRNAs. Note the larger horizontal scale compared to panel A.

Hemojuvelin protein is detected as both cell-associated and soluble forms

Total protein extract from human liver was analyzed on reducing SDS–polyacrylamide gel electrophoresis (PAGE) and the corresponding blot was probed with the polyclonal anti–hemojuvelin antibody anti–G3pep2-3 targeted to the N-terminus of hemojuvelin. One predominant protein band of about 46 kDa was detected in human liver from 2 different donors (Figure 3 lanes 1 and 2). Lysate of Hep3B cells (with endogenous hemojuvelin mRNA expression) was also analyzed by Western blot, but no signal was detected using any of the available antibodies (data not shown).

To confirm the specificity of antibody detection of the 46-kDa protein band in human liver, we cloned full-length hemojuvelin cDNA into pcDNA3.1(+) vector to generate the pcDNA-HJV construct and used it to transfect the Hep3B and HEK293T/17 (HEK293) cell lines (the latter with undetectable endogenous hemojuvelin mRNA) as positive controls for cellular expression of hemojuvelin. We compared hemojuvelin expression in vector (pcDNA3.1(+)) alone or construct (pcDNA-HJV)–treated cells. Cell lysate and conditioned medium were analyzed by Western blot with anti–G3pep2-3. In cell lysate of HEK293 cells, a unique protein band of approximately 46 kDa, identical in size to the band seen in human liver protein extract, was identified in pcDNA-HJV–treated cells but not in cells treated with control vector (Figure 3 lanes 3 and 4). No hemojuvelin-specific band was detected in the cell lysate of Hep3B cells transfected with pcDNA-HJV or pcDNA3.1(+) (data not shown). This could be due to a low transfection efficiency in Hep3B cells (generally 10% compared to over 90% in HEL293T/17 cells, estimated by green fluorescence) and low detection sensitivity of anti–G3pep2-3 antibody.

We next explored whether hemojuvelin was present in the media derived from cells expressing hemojuvelin. In the conditioned culture medium of HEK293 cells transfected with pcDNA-HJV, but not with vector pcDNA3.1(+), one unique prominent protein band of approximately 44 kDa was detected in Western blot using anti–G3pep2-3 (Figure 3). We obtained a similar result with

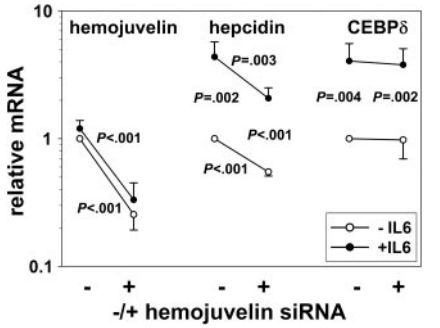

Figure 2. Hemojuvelin suppression decreased hepcidin expression but did not affect its inducibility by IL-6. Hep3B cells were first treated with hemojuvelin siRNA HJVsi3 (+) or diluent (−), followed by 20 ng/mL IL-6 (-●-) to induce hepcidin for 24 hours (n = 6 separate experiments). Hepcidin mRNAs were assayed by qRT-PCR and normalized to G3PD. In each experiment, expression of each target/G3PD ratio in control cells (not treated with IL-6 or siRNA) was set as the baseline of 1. Treatment with hemojuvelin siRNA significantly decreased both hemojuvelin and hepcidin mRNA levels in the presence and absence of IL-6, but did not affect mRNA expression of C/EBPδ. Significant differences as judged by the paired Student *t* test are indicated by their *P* values. Regardless of hemojuvelin siRNA treatment, IL-6 produced a similar fold induction of hepcidin and C/EBPδ mRNA expression, indicating that the IL-6 effect is not modulated by hemojuvelin expression.

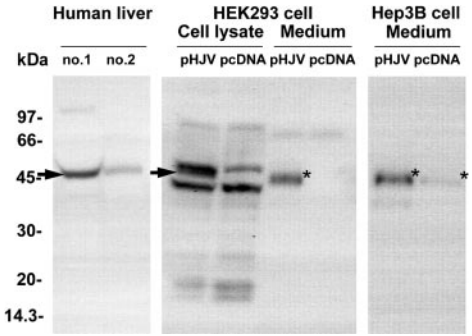

Figure 3. Hemojuvelin protein exists in both cell-associated and soluble forms. HEK293 and Hep3B cells were transfected with pcDNA3.1(+) or pcDNA-HJV in 6-well tissue culture plates and incubated overnight, followed by a 24-hour incubation in serum-free medium (2 mL/well). Whole-cell lysates were collected in 150 μL NETT buffer per well and 30 μL cleared total protein solution was analyzed. Conditioned cell culture media (2 mL/sample) were filter concentrated (5-kDa cut-off) and concentrates equivalent to 800 μL starting material were analyzed. Western blots after reducing SDS-PAGE were probed with anti–G3pep2-3 antibody. Arrows indicate cell-associated hemojuvelin (apparent molecular weight, 46 kDa) in both human liver protein extracts (no. 1 and no. 2) and whole-cell lysate of HEK293 cells transfected with pcDNA-HJV (pHJV), but not in HEK293 cells treated with control vector (pcDNA). Soluble hemojuvelin (apparent molecular weight, 44 kDa) is indicated by the asterisk and seen in conditioned cell culture media from HEK293 and Hep3B cells transfected with pcDNA-HJV (pHJV), as well as in conditioned media from Hep3B cells transfected with control vector (pcDNA) but not in media from HEK293 cells treated with control vector (pcDNA).

the conditioned culture medium of Hep3B cell transfected with both vectors (Figure 3). The detection of soluble hemojuvelin in vector-treated Hep3B but not HEK293 cells is consistent with the endogenous hemojuvelin mRNA expression in Hep3B cells.

An alternative antibody, Ab112, targeting a region 35 amino acids downstream from the region used to generate anti–G3pep2-3, detected both cell-associated and soluble hemojuvelin in transfected Hep3B and HEK293 cells, but not in human liver. Using Ab112, under reducing conditions, an additional 16-kDa reactive protein band was detected in both cell types but only one reactive protein band appeared under nonreducing conditions, 46 kDa for cell-associated form, and 44 kDa for soluble form (data not shown). There was an approximate 2-kDa difference between the size of the cell-associated and soluble hemojuvelin (46 kDa vs 44 kDa), indicating that a cleavage near the C-terminus of the cell-associated form caused the release of the soluble form.

Thus, we showed that hemojuvelin protein can be detected as a cell-associated form in human liver and in cultured cell lines engineered to express hemojuvelin. Moreover, soluble hemojuvelin can also be detected in the media derived from cultured cell lines with either endogenous or recombinant hemojuvelin expression.

Production of rs-hemojuvelin

We produced human rs-hemojuvelin in a baculovirus/insect cell expression system. Purified rs-hemojuvelin migrated as a single band in Western blots of nonreducing SDS-PAGE, but formed 2 bands in blots of reducing SDS-PAGE, reactive with anti–hemojuvelin antibody Ab112 (Figure S1, lanes 1 and 3; see the Supplemental Figures link at the top of the online article, at the *Blood* website) but not with preimmune serum (data not shown). The purified rs-hemojuvelin was similar in size to soluble hemojuvelin partially purified from HEK293 cell culture engineered to express hemojuvelin (Figure S1, lanes 2 and 4). The nonreducing SDS-PAGE gel staining indicated over 95% purity for rs-hemojuvelin (Figure S2, lane 2). In addition to the full-length rs-hemojuvelin (apparent molecular weight of 44 kDa), we also observed 2 additional bands of 29 kDa and 16 kDa (apparent molecular weight) on reducing SDS-PAGE (Figure S2, lane 4). These 2 bands were not observed on a nonreducing SDS-PAGE (Figure S2, lane 2), suggesting that they were the proteolytic cleavage products of rs-hemojuvelin linked together by disulfide bonds. We used Edman degradation to sequence the N-terminus of the 2 reduced fragments and nonreduced rs-hemojuvelin. Undetectable signal indicated a characteristically blocked N-terminal glutamine at the start of the N-terminal fragment (amino acid 36Q). The C-terminal fragment generated the sequence PHVR. . . indicating that it was generated from an Asp-Pro cleavage site after amino acid 172D (FGD ↓ PHVR). Nonreduced rs-hemojuvelin was also N-terminally blocked but generated a sequence suggestive of the exposure of a second N-terminus (PHVR) by cleavage. These results agree with previous report of 3 mouse repulsive guidance molecules (RGMs; a, b, and c) and chicken RGM, which all showed identical cleavage sites (FGD ↓ PH V/L R).[3,4] The conserved Asp-Pro bond is known to be unusually labile,[8] and can undergo hydrolysis in acidic cellular compartments or after treatment with mild acids. Our observation that soluble hemojuvelin forms a disulfide-linked 2-chain structure with one blocked N-terminus explains the inconsistency between the apparent molecular weight and sequencing results previously interpreted as glycosylation and removal of the N-terminal fragment in native RGMs.[3,4]

The unmodified hemojuvelin precursor protein (45.1 kDa) could be subject to a series of posttranslational modifications, due to the presence of an N-terminal signal peptide (3.57 kDa), a C-terminal transmembrane motif characteristic for GPI-anchor (2.46 kDa), and multiple putative glycosylation and protease cleavage sites. After the removal of the signal peptide and C-terminal transmembrane domain, the soluble hemojuvelin has a predicted molecular weight of 39.1 kDa. Mass spectrometry (matrix-assisted laser desorption ionization-time of flight [MALDI-TOF) of rs-hemojuvelin (apparent molecular weight of 44 kDa on SDS-PAGE) yielded a mass of about 41.5 kDa with multiple peaks at about 160-Da intervals, indicating a typical glycosylation pattern.

Soluble hemojuvelin can be detected in human plasma and serum

The release of soluble hemojuvelin into cell culture led us to consider the possibility that soluble hemojuvelin exists in vivo and has a physiologic function. We separated 2 μL human serum on a reducing SDS-PAGE and detected a single prominent protein band of 30 kDa reactive with Ab112 (Figure 4 left panel). Anti–G3pep2-2 antibody detected another specific protein band of 16 kDa in the same samples (Figure 4 middle panel, bottom bands). To confirm that the 30-kDa protein band is specific for hemojuvelin, we neutralized Ab112 with 50-fold excess of rs-hemojuvelin (antigen–specific IgG ratio) and performed a Western blot of human serum. The competition from excessive rs-hemojuvelin completely abolished the 30-kDa protein band in human serum (Figure S3, lanes 2 and 5), as well as the bands corresponding to rs-hemojuvelin (Figure S3, lanes 1 and 4, rs-) and soluble hemojuvelin from engineered HEK293 cells (Figure S3, Lane 3 and 6, s-). In multiple serum samples, the relative signal intensity of the 30-kDa band correlated well with the signal intensity of the 16-kDa band, suggesting that they were both components of soluble hemojuvelin in human serum.

To rule out the possibility that the cleavage of soluble human hemojuvelin in serum might be an artifact of the clotting process, we analyzed 1 μL human serum and plasma from the same donor on reducing SDS-PAGE probed with Ab112. The identical 30-kDa protein band was detected in both human serum and plasma (Figure 4 right panel), indicating the cleaved product is present in human blood.

The patterns of antibody reactivity of plasma hemojuvelin as compared to rs-hemojuvelin (Figure S3) indicated that the plasma hemojuvelin is cleaved between the 2 antigenic epitopes used for antibody generation rather than at the 172D ↓ P cleavage site of rs-hemojuvelin downstream of the epitope region for Ab112.

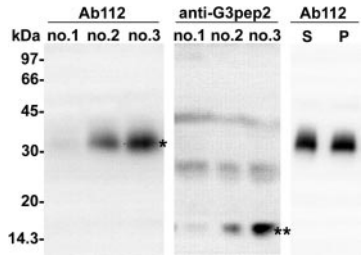

Figure 4. Soluble hemojuvelin is present in human serum and plasma. Serum and plasma samples were separated on reducing SDS-PAGE. In Western blot analysis of all serum samples, Ab112 detected a protein band (*) of 30 kDa (3 different donors nos. 1-3, left panel), and anti–G3pep2-2 antibody detected a protein band (**) of 16 kDa (middle panel). Pretreatment of Ab112 with excess rs-hemojuvelin abolished the 30-kDa Western blot signal (Figure S3). Blood plasma (1 μL, P, right panel) probed with Ab112 contained bands identical to those of serum from the same donor (1 μL, S, right panel) indicating that the hemojuvelin cleavage was not caused by the clotting reaction.

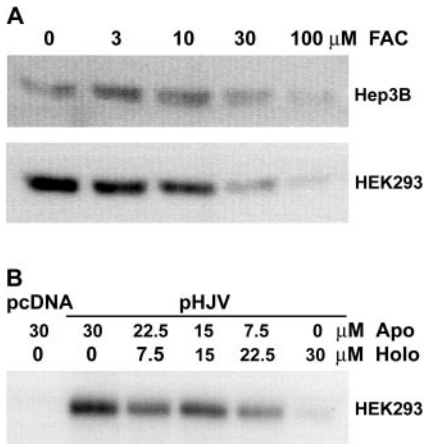

Figure 5. Iron loading reduces soluble hemojuvelin release into cell culture medium. Each panel is representative of at least 3 independent experiments. HEK293 and Hep3B cells were transfected with hemojuvelin vector (pcDNA-HJV) in 6-well tissue culture plates and incubated overnight, followed by a 24-hour incubation in serum-free medium (2 mL/well) with (A) FAC concentrations ranging from 0 to 100 μM. Conditioned cell culture medium (2 mL/sample) was filter-concentrated (5-kDa cut-off) and analyzed on a reducing SDS-PAGE/Western blot probed with anti–G3pep2-3. In both Hep3B (top panel) and HEK293 cells (bottom panel), the amount of soluble hemojuvelin decreased progressively with increasing FAC concentrations. (B) Holotransferrin and apotransferrin were added to pcDNA-HJV–transfected HEK293 cells at various ratios to reach a constant total transferrin concentration of 30 μM. Conditioned cell culture medium (2 mL/sample) was extracted by cation exchange and filter-concentrated (5-kDa cut-off) before being analyzed on a nonreducing SDS-PAGE/Western blot probed with Ab112. Lane 1 shows conditioned cell culture medium from pcDNA3.1(+) vector-transfected HEK293 cells as a negative control. The amount of soluble hemojuvelin decreased progressively with increasing iron saturation of transferrin.

Although the 2 sites are relatively close, the functional impact (if any) of the difference in cleavage sites remains to be established.

The strong signal detected in human serum by Western blot analysis indicates a substantial amount of soluble hemojuvelin in human blood, estimated to be in the microgram per milliliter range. Both the liver and the large mass of skeletal muscle could be the source of soluble hemojuvelin because both contain hemojuvelin mRNA at very high concentrations.[1]

Iron treatment reduces the amount of soluble hemojuvelin released into cell culture medium

To determine whether hemojuvelin protein expression or the release of soluble form is regulated by iron, we added ferric ammonium citrate (FAC) or apotransferrin/holotransferrin into cell cultures of both HEK293 and Hep3B cell lines transfected with either pcDNA-HJV or vector alone. Western blot probed with anti–G3pep2-3 or Ab112 was used to analyze both whole cell lysate and conditioned cell culture medium. No significant change in cell-associated hemojuvelin could be detected (data not shown). However, soluble hemojuvelin in cell culture media from both cell lines progressively decreased with increasing FAC concentration from 3 to 100 μM (Figure 5A). Similar results were also observed when treating hemojuvelin-transfected HEK293 cells with increasingly iron-saturated transferrin at a constant total transferrin concentration of 30 μM (Figure 5B).

rs-Hemojuvelin suppresses hepcidin mRNA in a dose-dependent manner in cultured primary human hepatocytes

According to previous reports, the mRNA concentrations of hepatic *RgmC* (the *HJV* homolog in mice) were not affected by iron feeding.[9] The inverse correlation of iron loading and soluble hemojuvelin concentration in vitro led us to hypothesize that soluble hemojuvelin is a negative regulator of hepcidin mRNA concentration.

Considering the amount of rs-hemojuvelin detectable on Western blot, we estimated the soluble hemojuvelin protein level to be less than 5 ng/mL in hepatocyte culture medium after 24 hours of incubation. We treated primary human hepatocytes for 24 hours with higher concentrations of rs-hemojuvelin (20-3000 ng/mL), similar to the concentrations detected in human sera, and observed that hepcidin mRNA concentrations decreased in a dose-dependent manner. No cytotoxicity was observed as judged by β-actin mRNA expression and cell morphology. The decrease in hepcidin mRNA level showed a striking log-linear anticorrelation with rs-hemojuvelin concentration ($R^2 > 0.9$ in each individual experiment, data not shown), and this log-linear anticorrelation was consistent in hepatocyte cultures from 4 different donors and 2 independent preparations of rs-hemojuvelin (Figure 6), indicating a possible competition for a hemojuvelin ligand.

We also observed a similar dose-dependent fractional suppression of hepcidin mRNA by rs-hemojuvelin in the presence of 20 ng/mL human IL-6 (Figure S4A). This result indicated that the suppression of hepcidin mRNA expression was IL-6 independent, consistent with our observation from the hemojuvelin siRNA treatment that cell-associated hemojuvelin regulated hepcidin mRNA expression in an IL-6–independent manner. Nevertheless, treatment with high-dose rs-hemojuvelin (1-3 μg/mL) largely reversed the 6- to 16-fold induction of hepcidin mRNA by 20 ng/mL IL-6 (Figure S4B).

The suppression of hepcidin mRNA by rs-hemojuvelin was highly selective. Using the Affymetrix HG-U133 Plus2 microarray, we compared the global gene expression pattern in primary human hepatocytes treated with rs-hemojuvelin (3 μg/mL) versus those treated with diluent (Figure S5). Hepcidin mRNA decreased approximately 5-fold after treatment with rs-hemojuvelin, the largest change of any transcript that was present in both treated and mock-treated hepatocytes. This decrease was significant at P less than .001 using the statistics (at default settings) of the Affymetrix GeneChip Operating Software version 1.2.

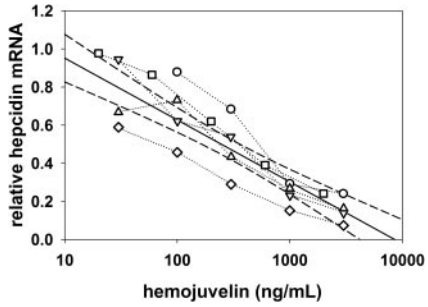

Figure 6. Dose-dependent suppression of hepcidin mRNA by rs-hemojuvelin in primary human hepatocyte culture. Primary human hepatocyte cultures (n = 5) from 4 different donors were treated for 24 hours with purified rs-hemojuvelin from 2 different preparations. Hepcidin mRNA was quantified by qRT-PCR and normalized to the housekeeping gene β-actin. For each experiment, the hepcidin–β-actin ratio of untreated cells was considered as a baseline of 1. Individual experiments (open symbols, dotted lines) and the regression line with 95% confidence intervals (solid line and dashed lines) are shown. Hepcidin mRNA expression showed a significant log-linear anticorrelation ($R = -0.88$, $P < .001$) with added rs-hemojuvelin concentration.

Discussion

The genetic linkage between JH due to *HJV* mutations and nearly absent hepcidin excretion in the affected individuals left open the possibility that hemojuvelin, like its congener RgmA,[10] is a developmental factor. If hemojuvelin, in analogy with RgmA, were necessary for the establishment of normal intercellular connections in the liver, its effects on hepcidin synthesis could be secondary to disrupted communications between 2 or more hepatic cell types. However, our studies of the proportional effects of cellular hemojuvelin suppression on hepcidin mRNA concentration show that cellular hemojuvelin is a regulator of hepcidin synthesis by hepatocytes. This conclusion is further supported by the highly selective nature of the suppressive effects of soluble hemojuvelin on hepcidin mRNA.

RgmA and hemojuvelin are associated with cell membranes,[3] but both lack cytoplasmic tails and contain consensus sequences indicating that they are GPI-linked proteins. RgmA is involved in neural development through binding to a protein ligand neogenin, a transmembrane receptor.[10] We propose that the GPI-linked cell-associated hemojuvelin (GPI-hemojuvelin) also interacts with an as yet unknown ligand, most likely a transmembrane receptor, to stimulate the production of hepcidin, as reflected in hepcidin mRNA levels. In hepatocyte culture, rs-hemojuvelin in the medium competes with membrane-bound GPI-hemojuvelin for this ligand and decreases the stimulatory signal for hepcidin mRNA expression. In vivo, the large amount of soluble hemojuvelin in human blood could function as a negative modulator of hepcidin synthesis. The resulting hepcidin concentration would be dependent on the ratio of soluble hemojuvelin to GPI-hemojuvelin. Iron loading of hemojuvelin-producing tissues would affect this ratio by decreasing the release of soluble hemojuvelin.

Based on the distribution of hemojuvelin mRNA in organs, there are 2 major sources of hemojuvelin. GPI-hemojuvelin of hepatic origin could transduce a signal from sensors of dietary iron uptake in the portal regions of the liver to mediate real-time regulation of hepcidin mRNA concentrations. On the other hand, skeletal muscle with its large mass and high concentration of hemojuvelin mRNA could be the major source of soluble hemojuvelin in the body. Soluble hemojuvelin may inversely reflect systemic iron load or signal the iron requirements for myoglobin synthesis. If so, soluble hemojuvelin could be a useful biomarker in disorders of iron metabolism.

In primary human hepatocytes, treatment with high-dose rs-hemojuvelin (1-3 μg/mL) effectively reversed the induction of hepcidin mRNA by 20 ng/mL IL-6 (Figure S4B). These observations suggest that rs-hemojuvelin or its active fragments could be used to treat anemia of inflammation, by opposing the cytokine-induced increase in hepcidin synthesis. Normalization of hepcidin concentrations in blood would release macrophage-sequestered iron and would be expected to provide adequate iron for erythropoiesis.

The molecular mechanisms of the activity of soluble and membrane-associated hemojuvelin are not yet known. The nature of the putative hemojuvelin ligand and the relationship of hemojuvelin to other genetically established regulators of hepcidin, including transferrin receptor 2 and HFE, are important subjects for further investigation.

Acknowledgments

We thank Erika Valore, Dharma Thapa, and Chun-Ling Jung for technical advice and assistance and Elizabeta Nemeth and Seth Rivera for discussions. Primary human hepatocytes were obtained through the Liver Tissue Procurement and Distribution System, Pittsburgh, PA, which was funded by National Institutes of Health contract no. N01-DK-9-2310. Gene array hybridizations were performed by the UCLA DNA Microarray Core, mass spectrometry was done at the UCLA Pasarow Mass Spectrometry Laboratory, and amino acid sequencing was performed by Dr Jan Pohl at the Microchemical Facility at Emory University, Atlanta, GA.

References

1. Papanikolaou G, Samuels ME, Ludwig EH, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet. 2004;36:77-82.
2. Ganz T. Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. Best Pract Res Clin Haematol. 2005;18:171-182.
3 Niederkofler V, Salie R, Sigrist M, Arber S. Repulsive guidance molecule (RGM) gene function is required for neural tube closure but not retinal topography in the mouse visual system. J Neurosci. 2004;24:808-818.
4. Monnier PP, Sierra A, Macchi P, et al. RGM is a repulsive guidance molecule for retinal axons. Nature. 2002;419:392-395.
5. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 2004;113:1271-1276.
6. Ramji DP, Vitelli A, Tronche F, Cortese R, Ciliberto G. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBP delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. Nucl Acids Res. 1993;21:289-294.
7. Alam T, An MR, Papaconstantinou J. Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. J Biol Chem. 1992;267:5021-5024.
8. Lidell ME, Johansson MEV, Hansson GC. An autocatalytic cleavage in the C terminus of the human MUC2 mucin occurs at the low pH of the late secretory pathway. J Biol Chem. 2003;278:13944-13951.
9. Krijt J, Vokurka M, Chang KT, Necas E. Expression of Rgmc, the murine ortholog of hemojuvelin gene, is modulated by development and inflammation, but not by iron status or erythropoietin. Blood. 2004;104:4308-4310.
10. Rajagopalan S, Deitinghoff L, Davis D, et al. Neogenin mediates the action of repulsive guidance molecule. Nat Cell Biol. 2004;6:756-762.