Immunoassay for human serum hemojuvelin

Carole Brasse-Lagnel,[1,3] Maura Poli,[2] Céline Lesueur,[1] Bernard Grandchamp,[3] Alain Lavoinne,[1] Carole Beaumont,[3] and Soumeya Bekri[1]

[1]Laboratoire de Biochimie Médicale, Hôpital Universitaire de Rouen; EA4309 "Endothélium Microvasculaire et Lésions Cérébrales Néonatales"; Université de Rouen, Rouen, France; [2]Dipartimento Materno Infantile Tecnologie Biomediche, University of Brescia, Brescia, Italy, and [3]INSERM U773, Centre de Recherche Biomédicale Bichat Beaujon, CRB3, Université Paris Diderot, Site Bichat, Paris, France

ABSTRACT

Background
Hemojuvelin, a critical regulator of iron homeostasis, is involved in the regulation of hepcidin expression and iron homeostasis. It is expressed both as a membrane-bound form and as a soluble one. Serum hemojuvelin can be produced by secretion following furin cleavage or by proteolytic cleavage of the membrane-bound form by matriptase 2 (TMPRSS6). These forms contribute to down-regulation of hepcidin expression upon iron deficiency or hypoxia. This study describes the development and validation of the first enzyme-linked immunosorbent assay for hemojuvelin in human serum.

Design and Methods
This assay is based on the use of a recombinant human repulsive guidance molecule-c peptide and a polyclonal antibody against hemojuvelin able to recognize the recombinant peptide and the native soluble hemojuvelin by immunoprecipitation.

Results
The enzyme-linked immunosorbent assay was validated and appeared to be a robust method with intra- and inter-coefficients of variance ranging from 2.6% to 15%. The assay was able to quantify hemojuvelin levels in a control population within a range from 0.88 to 1.14 mg/L. Patients with iron-refractory iron-deficiency anemia with a mutation in the *TMPRSS6* gene were found to have lower levels of circulating hemojuvelin than those in healthy patients. The enzyme-linked immunosorbent assay also showed that soluble hemojuvelin levels were significantly higher in patients with anemia of chronic disease than in control individuals.

Conclusions
This enzyme-linked immunosorbent assay has a good specificity and sensitivity for the quantification of soluble hemojuvelin in human serum and could be a valuable aid to understanding the physiological role of this protein.

Key words: iron metabolism, soluble hemojuvelin, ELISA, rhRGM-c, anemia, serum.

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Correspondence: Carole Brasse-Lagnel, NeoVasc, EA 4309, Faculté de Médecine-Pharmacie, 22 boulevard Gambetta, 76183 Rouen cedex, France. E-mail: carole.lagnel@univ-rouen.fr

The online version of this article has a Supplementary Appendix.

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Introduction

Hemojuvelin is a member of the repulsive guidance molecule (RGM) family, a family of glycosylphosphatidylinositol-anchored neuronal and muscle membrane glycoproteins. Hemojuvelin (also called RGM-c) is mainly expressed in skeletal muscle, heart and liver, and plays a major role in regulating hepcidin expression, the negative regulator of intestinal iron absorption and macrophage heme iron recycling. Patients with *HJV* mutations develop early-onset, severe iron overload, called juvenile hemochromatosis, due to a complete lack of hepcidin expression.[1] Hemojuvelin can be expressed both as a membrane-bound form and as a soluble form which have opposite effects on hepcidin gene expression. Cell-associated hemojuvelin acts as a co-receptor of bone morphogenic proteins to enhance hepcidin expression through the Smad pathway.[2,3] In contrast, soluble hemojuvelin, by binding bone morphogenic proteins, acts as a competitive antagonist of membrane-bound hemojuvelin, leading to decreased hepcidin expression.[2-4] Thus, chronic soluble hemojuvelin injection in mice causes iron overload.[5]

Several isoforms of the hemojuvelin protein are generated by multiple proteolytic cleavages (Figure 1). Membrane-bound hemojuvelin protein can be represented by both single- and two-chain species. The two-chain species is produced from the single chain by an intra-molecular cleavage and the two chains can remain bound to the plasma membrane through the formation of a disulfide bridge.[6,7] Soluble hemojuvelin can be produced by cleavage, mediated by a furin-like protease, at the C-terminus of the protein occurring in the endoplasmic reticulum.[8,9] This cleavage leads to the release of two soluble components (42 and 33 kDa) into extracellular fluids or into blood. These two fragments are probably produced from single- and two-chain species of hemojuvelin, as suggested by the authors. Nevertheless, the fact that the 42 and 33 kDa fragments originating from furin cleavage are released into the blood stream has not been confirmed. The furin-mediated release of soluble hemojuvelin can be enhanced by hypoxia and iron deficiency to reduce rapidly the amount of membrane-bound hemojuvelin and to inhibit hepcidin production. Recently, it has been demonstrated that membrane-bound hemojuvelin, the glycosylphosphatidylinositol-anchored form, is cleaved by matriptase 2, a transmembrane serine protease encoded by the *TMPRSS6* gene, mostly expressed in the liver. Co-transfection experiments with vectors expressing hemojuvelin and matriptase 2 have shown that this serine protease can cleave the membrane-bound hemojuvelin to generate several smaller fragments (25-35 kDa)[10] but these fragments have not yet been identified in serum samples. The physiological role of these soluble hemojuvelin fragments and their presence in human serum remain to be investigated. However, mutational inactivation of matriptase 2 causes iron-refractory iron-deficient anemia in mice[11] and humans,[10,12-14] by inappropriately high levels of hepcidin expression. In summary, circulating hemojuvelin is represented by several soluble forms that can be produced by the action of at least two proteolytic enzymes, furin and matriptase 2.

The quantification of hemojuvelin in human blood may provide further insights into the pathogenesis of iron homeostasis disorders. Measurement of soluble hemojuvelin in biological fluids can improve our understanding of iron diseases and be a useful tool for their diagnosis and clinical management. This study describes a specific competitive immunoassay for soluble hemojuvelin quantification in human sera.

Design and Methods

Anti-hemojuvelin antibody and recombinant human repulsive guidance molecule-c

The hemojuvelin antibody was generated against a recombinant hemojuvelin protein (recombinant human repulsive guidance molecule-c; rhRGM-c) encompassing amino acids 226 to 402 as previously described.[15] The hemojuvelin antibody was purified and conjugated to horseradish peroxidase following the manufacturer's recommendation (Interchim, Montluçon, France). rhRGM-c is produced by R&D system (Wiesbaden, Germany) as a fusion protein consisting in the human CD33 fused to the C-terminal polyhistidine-tagged mature human RGM-c (Gln 36 - Asp 400; Accession # Q6ZVN8) expressed in a mouse myeloma cell line. Based on N-terminal sequencing, the rhRGM-c preparation contains a mixture of the mature protein (Gln 36 – Asp 400)-His, the N-Teminus chain (Gln 36 – Asp 172) and the C-terminus chain (Pro 173 – Asp 400)-His with molecular masses of 40 kDa, 14 kDa and 26 kDa, respectively.

Serum and tissue collection

The study population comprised 48 healthy individuals, 4 patients with iron-refractory iron-deficiency anemia and *TMPRSS6* mutations, and 36 patients with anemia of chronic disease. The patients with anemia of chronic disease were selected from the Intensive Care Unit of Bichat hospital, on the basis of their having a C-reactive protein level greater than 50 mg/L, a hemoglobin concentration below 10 g/dL and no known iron disorder. Each patient included in this study provided signed informed consent. The experimental protocol was performed according to French legislation on ethics and human research. Serum samples were immediately frozen and stored at -80°C. Serum from a patient with homozygous D149fsX245 *HJV* mutation was a kind gift from Laura Silvestri and Clara Camaschella (Milan, Italy). Normal muscle sample was used as a control for western blotting studies.

Serum hepcidin assay

Briefly, hepcidin concentrations were measured in duplicate using a competitive enzyme-linked immunosorbent assay (ELISA; Intrinsic LifeSciences, La Jolla, CA, USA). High performance liquid chromatography-purified, synthetic, bioactive hepcidin (Bachem Biosciences Inc, King of Prussia, PA, USA) was used as reference material for the construction of duplicate 12-point standard curves, used to convert sample absorbance readings to hepcidin concentrations.[16]

Immunoprecipitation and western blot

For immunoprecipitation, 500 μg of serum proteins were incubated overnight at 4°C with 4 μL of anti-HJV antibody in 200 μL of phosphate-buffered saline. A 20 μL aliquot of protein G PLUS-agarose was then added for 2 h at 4°C. The mixture was centrifuged and the pellet was washed twice in phosphate-buffered saline. Prior to electrophoresis, sera were treated when indicated using the Aurum serum protein kit (Bio-Rad laboratories) to simultaneously remove both albumin and immunoglobulin G. Tissue samples were homogenised in extraction buffer contain-

ing 25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, supplemented with protease inhibitor cocktail (P8340) (Sigma Chemical Co.). rhRGM-c (25 ng), proteins from tissue extracts (100 μg), serum (100 μg) or immunoprecipitated proteins were suspended in western sample buffer (100 mM Hepes, pH 6.8, 10% β-mercaptoethanol, 20% sodium dodecylsulfate), boiled, subjected to electrophoresis on 10 or 12% sodium dodecylsulfate polyacrylamide gel, as indicated and then transferred onto nitrocellulose membranes. After blocking for 1 h with Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) non-fat dry milk, membranes were incubated with anti-hemojuvelin antibody (dilution 1:1,000) or with horseradish peroxidase-anti-hemojuvelin (dilution 1:300) in blocking buffer overnight at 4°C, washed and incubated with the anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (True blot, CliniSciences, Montrouge, France) at room temperature for 1 h. Peroxidase labeling was detected using an electrochemical luminescence western blotting detection system (Amersham Biosciences).

Enzyme-linked immunosorbent assay procedure

A competitive ELISA technique was developed for the measurement of serum hemojuvelin. Ninety-six-well microtiter plates (Costar, Corning, NY, USA) were coated with 200 ng per well of rhRGM-c protein (R&D systems, Wiesbaden, Germany) diluted with 200 μL of coating buffer [15 mM sodium carbonate (Sigma-Aldrich, St Louis, MO, USA) and 35 mM sodium bicarbonate carbonate, pH 9.6 (Sigma-Aldrich)]. The plates were wrapped in plastic foil and stored overnight at 4°C. In parallel, serum (10 μL) or calibrator was incubated overnight at 4°C with anti-hemojuvelin antibody (dilution 1:25,000) in 100 μL of dilution buffer containing 1% bovine serum albumin (w/v) in phosphate-buffered saline to form immunocomplexes. rhRGM-c protein, diluted in phosphate-buffered saline (5, 10, 20, 25, 50, 75, 100 and 200 ng) was used as the calibrator. Subsequently, the plates were washed with phosphate-buffered saline and blocked with 100 μL of 1% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature. The 50 μL mix containing the immunocomplexes or 50 μL of 1% bovine serum albumin in phosphate-buffered saline (negative controls) was then added to the coated wells in duplicate and incubated for 1 h at 37°C. After three washes with phosphate-buffered saline containing 0.05% Tween 20, the plates were incubated with a secondary anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase (Sigma-Aldrich) diluted at 1/1,000 in blocking buffer for 1 h at room temperature. The plates were washed as before and the signal was visualized after addition of 3,3',5,5' tetramethylbenzidine (Pierce, Rockford, IL, USA) for 5 min at room temperature. The reaction was stopped by the addition of stop solution (Cell Signaling Technology, Danver, USA) and color development was measured photometrically at 450 nm with a microplate reader (Σ960 photometer, Metertech). Each serum sample was tested in duplicate and the optical density was calculated by subtracting the average optical density of negative controls from the sample average optical density. Serum hemojuvelin concentrations were expressed in milligrams per liter by using a standard curve. The standard curve was constructed using MicrosoftOffice Excel.

Statistical analysis

Statistical significance was calculated using the non-parametric Mann-Whitney test and correlations were analyzed using the Spearman's rank correlation test; *P* values less than 0.05 were considered statistically significant. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for the statistical evaluations.

Results

Identification of the electrophoretic pattern of serum hemojuvelin

Western blot analysis showed that the recombinant hemojuvelin protein (rhRGM-c) was detected by the polyclonal antibody against hemojuvelin (Figure 2A). Several bands were observed; from the indications given by the manufacturer, it can be inferred that these correspond to the full-length membrane-bound hemojuvelin (from Gln36 to Asp400, MW 50-55 kDa) and to the C-terminal fragment (Pro173 to Asp400, MW 37 kDa), generated by intra-molecular cleavage at the conserved consensus Gly-Asp-Pro-His site (Figure 1).[7] The N-terminal fragment (Gln36 to Asp172, 20 kDa) is not recognized by the antibody since it was generated against a recombinant protein from amino acids 226 to 402. The presence of two additional bands for each fragment probably reflects differences in glycosylation pattern as reported by Maxson *et al.*[17] In muscle extracts, only two bands are seen which may correspond to full-length membrane-bound hemojuvelin and to the C-terminal glycosylated fragment as previously described.[6] In serum samples, albumin (~60–70% of the total serum protein), and immunoglobulin G (~10–20% of total serum protein) are major components that can mask the presence of many underlying smaller and similar size proteins, limiting the amount of serum that can be resolved on western blotting. We, therefore, used the Aurum serum protein mini kit specifically designed to eliminate albumin and immunoglobulin G. As shown in Figure 2B (line 1), a significant amount of these proteins was eliminated and the identification of serum hemojuvelin bands was greatly improved as compared to that possible in total serum (line 2). In these conditions, two specific bands were identified, of 42 kDa and 33 kDa, which could correspond to the previously described fragments produced by furin cleavage (Figure 1). These bands were absent in the serum of a patient with the homozygous D149fsX245 mutation in *HJV* gene (Figure 2B, line 4).[18] This one base pair deletion in exon 3 creates a frame shift and premature stop codon, 96 amino acids downstream of Asp149. Even if this shorter form of hemojuvelin is produced, it will not be recognized by the polyclonal antibody generated against a recombinant protein from amino acids 226 to 402. Western blot analysis showed an additional band around 40 kDa that appears to be either related to a different glycosylation pattern or to a non-specific signal (Figure 2B). However, its absence in the serum from the patient with the *HJV* mutation suggests that this band is specifically recognized by the antibody. Finally, in order to confirm the specific binding of our antibody to hemojuvelin we performed western blotting using serum proteins immunoprecipitated with hemojuvelin antibody (Figure 2C). Serum immunoprecipitation yields a hemojuvelin-depleted supernatant (Figure 2C,a) and a high amount of hemojuvelin in the corresponding precipitate (Figure 2C,b). When the hemojuvelin antibody was omitted from the western blot (Figure 2C,c), the remaining bands corresponded to the immunoglobulins of hemojuvelin antibody used for the immunoprecipitation. Besides, in order to ascertain the antibody specificity, we used hemojuvelin antibody conjugated to horseradish peroxidase which enables the addition of secondary antibody to be avoided and reveals the specific bands corresponding to

soluble hemojuvelin fragments (Figure 2C,d). We can conclude that immunoglobulin bands are revealed by the secondary antibody while the hemojuvelin antibody binds solely to hemojuvelin.

Development and characterization of an enzyme-linked immunosorbent assay for serum hemojuvelin measurement

The recombinant peptide (rhRGM-c) and the polyclonal antibody against hemojuvelin were used to develop an immunological assay able to quantify circulating hemojuvelin in human serum. In a preliminary experiment we determined the optimal dilution of antibody and the optimal concentration of antigen according to Crowther.[19] A curve was obtained by adding serial dilutions of the antibody in wells coated with 200 ng recombinant hemojuvelin. Figure 3A shows that the optimal signal and lowest background for the ELISA were obtained with a 1:25,000 dilution for the antibody.

A typical calibration curve obtained with the rhRGM-c protein as calibrator is shown in Figure 3B and 3C (black triangles). In order to test the matrix effect, calibration was repeated using serum from which the soluble hemojuvelin had been depleted (Figure 3B) or hemojuvelin-deficiency serum (Figure 3C). The curve of the hemojuvelin-depleted serum was shifted compared to the curve obtained with bovine serum albumin because of the presence of endogenous soluble hemojuvelin as observed in Figure 2C. Similar profiles were obtained in phosphate-buffered saline and in hemojuvelin-deficiency serum (Figure 3C). Several serum sample volumes (5-30 μL) were then tested and the adequate volume was estimated to be 10 μL (Figure 3D).

A low (10 ng i.e. 1 mg/L) and a high concentration (100 ng i.e. 10 mg/L) of calibrator were used for statistical analysis of the reproducibility, linearity and recovery of the soluble hemojuvelin ELISA assay. The intra-assay coefficients of variance were 9.1 and 2.6 for low and high concentrations, respectively, as evaluated by assaying five replicates of each sample in a single assay (Table 1). The inter-assay coefficients of variance were 4.1-5.8% as evaluated by four subsequent measurements of the test samples. Intra- and inter-assay coefficients of variance for serum samples were 15% and 9.5%, respectively (Table 1).

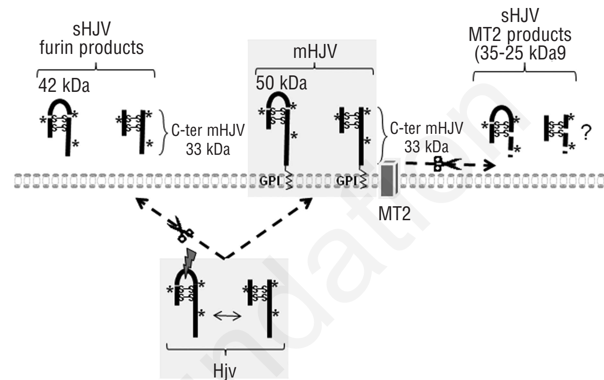

Figure 1. Schematic representation of hemojuvelin (HJV) protein on the cell surface, and of the secreted form. Membrane HJV (mHJV) and soluble HJV (sHJV) appear to be represented by both single- and two-chain species that can remain bound through the formation of disulfide bridge. The longest isoform is a 426-amino-acid protein. Locations of asparagine-linked glycosylation sites are indicated by asterisks, while the polybasic RNRR site (a site of intra-molecular proteolytic cleavage to generate two-chains) is indicated by a squiggle. Membrane HJV is the glycosylphosphatidylinositol anchored form. Soluble HJV (42 kDa) and its shorter fragment of 33 kDa are found in extracellular fluids and in blood,[4,7,9] potentially through the action of a furin-like protease in the endoplasmic reticulum. Other shorter soluble forms of HJV (25-35 kDa fragments) can be released from membrane-bound HJV by the action of the transmembrane serine protease matriptase 2 (TMPRSS6).

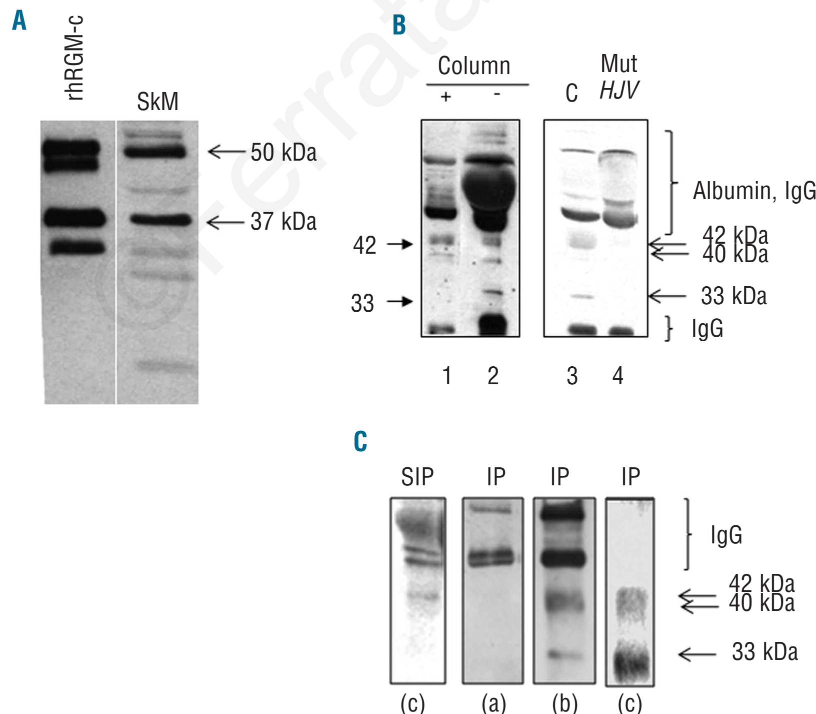

Figure 2. Specificity of the polyclonal antibody against hemojuvelin (HJV) (A) Western blot analysis of rhRGM-c and protein extracts from muscle (SkM) of healthy individuals. Twenty five nanograms of rhRGM-c and 100 μg of proteins from tissue extracts were subjected to 10% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot analysis using the polyclonal antibody against HJV. (B) Western blot analysis of soluble HJV from serum proteins: 2 μL of serum were subjected to 12 % SDS-PAGE, followed by western blot using the polyclonal antibody against HJV. Lines 1 and 2: albumin and Immunoglobulin (a IgG) from serum of a healthy patient were removed (line 1) or not (line 2) by using the Aurum serum protein mini kit. Line 3: serum from a healthy individual. Line 4: serum from a patient homozygous for the D149fsX245 *HJV* mutation (Mut *HJV*). (C) The pellet (IP) and the supernatant (SIP) of the immunoprecipitate were subjected to western blot by omitting the primary antibody (a) or by the HJV antibody (b and c) and with HJV antibody which was coupled with horseradish peroxidase (d). Black arrows indicate the full-length soluble HJV and the processed form found in serum.

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Soluble hemojuvelin concentration in healthy volunteers

Circulating hemojuvelin concentration was determined in serum samples from 48 healthy volunteers (control group C1; 18 men, 30 women). Hematologic parameters and iron indices of these healthy individuals are shown in Table 2. The 5% to 95% range of hemojuvelin concentrations varied from 0.88 to 1.14 mg/L (mean: 1.01±0.06 mg/L; n=48) with no significant difference between men (0.92 to 1.30 mg/L) and women (0.78 to 1.13 mg/L).

Soluble hemojuvelin concentration in iron-refractory iron-deficient anemia and anemia of chronic disease

Serum samples from patients with iron disorders (Table 2) were tested and compared with those of the control group. We found a significant decrease in serum hemojuvelin concentration (mean=0.47 mg/L; n=4) in the patients with iron-refractory iron-deficient anemia (Figure 4A) when compared to that in the control group of adult individuals (group C1; P=0.028) and that in an age-matched control group (group C2; P=0.006). The mean soluble hemojuvelin concentration in the group of patients with anemia of chronic disease was significantly higher (2.30 mg/L ± 0.27 mg/L, P<0.0001; n=37) than in the control group. The 5% to 95% range of hemojuvelin concentrations in the group with anemia of chronic disease varied from 1.73 to 2.85 mg/L with a maximum of 7.33 mg/L. Western blot analysis of serum from two patients with iron-refractory iron-deficient anemia confirmed the reduced level of both the 42 kDa and the 33 kDa hemojuvelin bands as compared to those in one control serum sample (Figure 4B) whereas the intensity of these two bands was increased in three sera from patients with anemia of chronic disease as compared to the intensity in serum samples from three controls (Figure 4B). These results are consistent with those of the ELISA.

Interestingly, the concentration of soluble hemojuvelin was inversely correlated with the serum iron concentration (r=-0.56; P<0.0001) (Figure 4C,a). Moreover, a correlation was found between soluble hemojuvelin and ferritin (r=0.62; P<0.0001) (Figure 4C,b) and C-reactive protein (r=0.62; P<0.0001) (Figure 4C,c). However, soluble hemojuvelin concentration was not correlated with hepcidin concentration in 28 patients in the group with anemia of

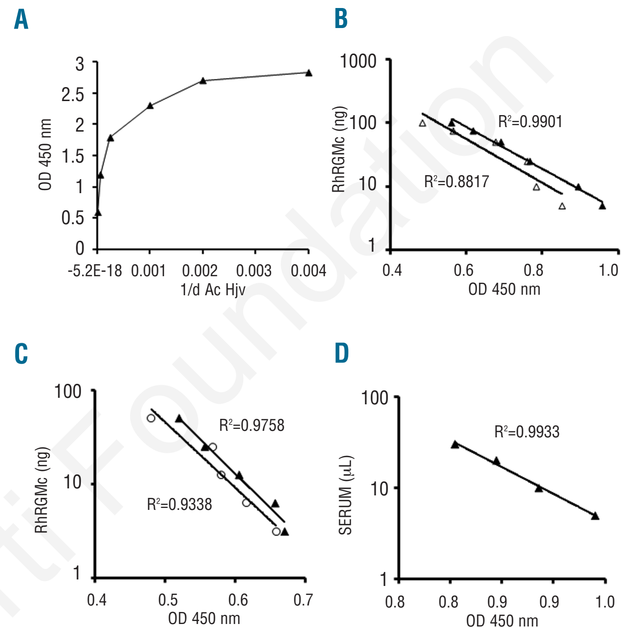

Figure 3. Characterization of an ELISA assay for serum hemojuvelin (HJV) measurement. (A) Determination of the optimal concentration of antibody against HJV. The curve was obtained by binding of serial dilutions of the polyclonal HJV antibody to a microtiter plate coated with 200 ng of recombinant HJV. The range of dilution is 250-64000. (B) Representative calibration for rhRGM-c obtained by diluting the hrRGM-C in the dilution buffer of the ELISA (black triangles) or in the HJV-free serum obtained by immunoprecipitation (white triangles). The range of the assay is 5-200 ng (0.5-20 mg/L). (C) Representative calibration for rhRGM-c obtained by diluting the hrRGM-C in phosphate-buffered saline (black triangles) or in HJV deficiency serum (white circles). The range of the assay is 3-50 ng (0.3-5 mg/L). (D) Determination of the optimal concentration of serum protein (5-30 µL of serum).

Table 1. Intra- and inter-assay precision of the hemojuvelin (HJV)-ELISA.

Intra Assay	n	HJV concentration	CV	Inter Assay	n	HJV concentration	CV
Calibrator 1	5	1 mg/L	9.1	Calibrator 1	4	1 mg/L	4.1
Calibrator 2	5	10 mg/L	2.6	Calibrator 2	4	10 mg/L	5.8
Serum 1	5	1.65 mg/L	14.9	Serum 3	3	2.2 mg/L	9.5
Serum 2	6	3.34 mg/L	11.4	Serum 4	4	7.3 mg/L	9.6

CV: coefficient of variance.

Table 2. Characteristics of group subjects.

Patients' characteristics [Normal range]	Controls - group 1 Median (95 % confidence interval)	Controls - group 2 Median (95 % confidence interval)	IRIDA Median (95 % confidence interval)	ACD Median (95 % confidence interval)
Number	48	7	4	36
Females/Males	13/8	4/3	2/2	17/19
Age (years)	34.5 (33-41)	13 (3-17)	9 (2-15)	52 (44-59)
Iron (µmol/L) [10-27]	17.2 (15.07-19.04)	17 (9-20)	3 (2.4-4.6)	5 (1.5-14.5)
Hemoglobin (g/dL) [13.5-17.5]	13.8 (13.7-14.8)	12.7 (11.6-14.6)	10.3 (8.6-10.5)	8.1 (6.3-10.1)
Ferritin (µg/L) [25-250]	86 (76-139)	40 (20-257)	165 (30-858)	621 (32-3664)
C-reactive protein (mg/L) [<5]	<5	<5	<5	123 (30-340)

IRIDA: iron-refractory iron-deficient anemia; ACD: anemia of chronic disease.

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chronic disease for whom serum hepcidin values were available (r=0.22; P=0.26) (*data not shown*). Finally, an ELISA was done to measure hemojuvelin in the serum from the patient with a HJV mutation that was used for western blotting in Figure 2B. The hemojuvelin concentration in this serum sample was 0.3 mg/L which is below the normal range (0.88 to 1.14 mg/L).

Discussion

The present report describes the first immunological assay for hemojuvelin quantification in human serum, based on the use of a rhRGM-c peptide and a polyclonal anti-hemojuvelin antibody. The western blot analysis of serum hemojuvelin performed to confirm the specificity of the antibody revealed two major components corresponding to the 33 kDa C-terminal fragment and the 42 kDa soluble form of hemojuvelin. Indeed, several isoforms of circulating hemojuvelin may exist. The shorter fragments resulting from matriptase 2 cleavage may be either contained in the 33 kDa band or be of too limited abundance to be detected by western blotting. In order to quantify all these circulating hemojuvelin forms, we chose to set up a competitive ELISA technique using an antibody against hemojuvelin able to recognize rhRGM-c peptide and the native serum forms of hemojuvelin. Thereby, we achieved intra- and inter-assay coefficients of variance ranging from 2.6 to 15%, attesting to the reproducibility of the method.

As a preliminary step in evaluating the usefulness of this assay in clinical situations, we assessed the concentrations of circulating hemojuvelin in a control group and in a group of patients with iron disorders (iron-refractory iron-deficient anemia with a mutation in the *TMPRSS6* gene or anemia of chronic diseases). In healthy controls, serum hemojuvelin values ranged from 0.88 to 1.14 mg/L with no difference between men and women.

In patients with iron-refractory iron-deficient anemia with *TMPRSS6* mutations that induce failure in matriptase 2 activity[13] we found significantly lower levels of serum hemojuvelin than in healthy patients. These patients were reported to have hypochromic microcytic anemia and high hepcidin levels;[13,14] however, the concentration of hemojuvelin was not assessed. Our findings suggest that the matriptase 2 mutation may induce a decrease of circulating forms of hemojuvelin but the link between this mutation and serum hemojuvelin production remains to be investigated. Moreover, the potential physiological role of soluble hemojuvelin fragments produced by the matriptase 2 has not yet been characterized.

In a group of patients with anemia of chronic disease, we found that serum hemojuvelin concentrations were significantly higher than in the controls. The soluble form of hemojuvelin is thought to be a negative regulator of hepcidin expression but its role in the regulation of hepcidin following an inflammatory stimulus remains unclear. The production of hemojuvelin plays a key role in dietary iron sensing.[20] Besides, several studies have shown that *Hjv* expression is repressed in mice at a transcriptional level by a pro-inflammatory stimulus.[21,22] Moreover, Babitt *et al*. showed that injections of high doses of recombinant HJV revert the induction of hepcidin mRNA by interleukin-6.[5] In the light of those studies and of the present results, we can hypothesize that under inflammatory conditions, hepatic *HJV* expression is repressed and the level of the cell membrane form of the protein is reduced to inhibit hepcidin liver expression; besides, an increase of the concentration of serum hemojuvelin (originating from non-hepatic tissues such as muscle or from the hepatic membrane form through cleavage) may contribute to hepatic regulation of hepcidin expression. This hypothesis could be supported by the findings of this study of an inverse correlation between measured serum hemojuvelin and circulating iron concentrations and of a correlation with C-reactive protein, an index of inflammation. The inverse correlation between soluble hemojuvelin and serum iron is in accordance with the effect observed *in vitro*, in which treatment of cells with iron resulted in decreased release of soluble hemojuvelin.[4,23] However, we

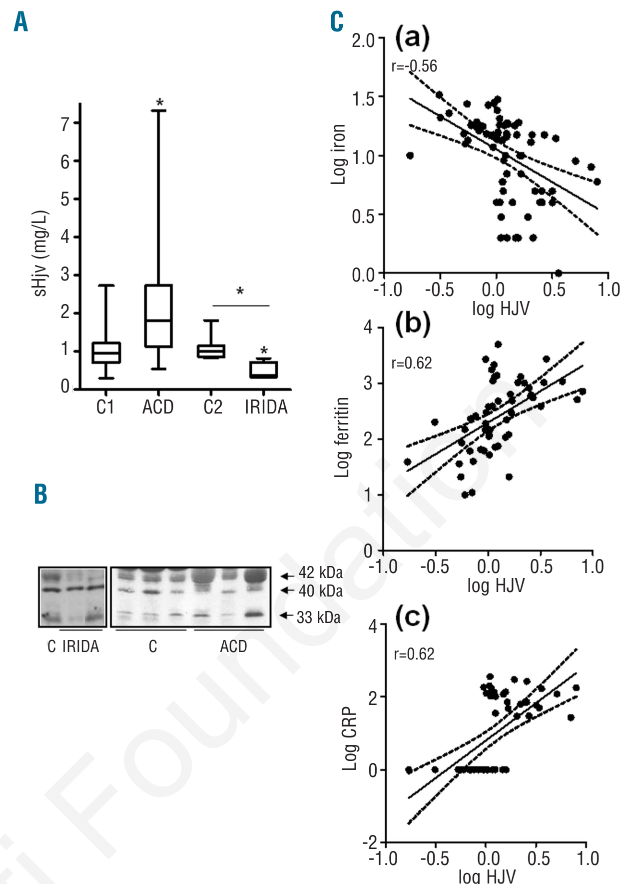

Figure 4. Soluble hemojuvelin (HJV) concentration in iron disorders. (A) Serum HJV concentrations in healthy adult controls (group C1, n=48), in a control group of age-matched patients with iron-refractory iron-deficient anemia (IRIDA) (group C2, n=7), in IRIDA patients with a mutation in the *TMPRSS6* gene (IRIDA; n=4) and in patients with anemia of chronic disease (ACD) (n=36). *; significantly different from control group C1 or C2 as indicated; P<0.05 by using the non-parametric Mann-Whitney test. (B) Western blot analysis of serum from one control subject and from two patients with a mutation in the *TMPRSS6* gene (IRIDA), and from three control subjects and three patients with ACD. Serum proteins (100 μg) were subjected to electrophoresis on 12% sodium dodecyl sulphate polyacrylamide gel, followed by Western blot using the polyclonal antibody against HJV. (C) Correlations were assessed by using the Spearman rank correlation test (r) between serum protein HJV concentration and serum iron (a), or ferritin (b), or C-reactive (c) concentrations respectively, in serum from patients belonging to the control and to the ACD groups.

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did not observe any correlation between hepcidin and hemojuvelin levels in the serum of patients with anemia of chronic disease. Nevertheless, the nature of hemojuvelin inducers (inflammatory conditions or iron deficiency) as well as the source tissue remain to be demonstrated. Indeed, large-scale human studies will be required to clarify the putative functional link between hepcidin and circulating hemojuvelin, as well as to identify the mechanisms responsible for the changes in hemojuvelin levels.

Altogether, these preliminary observations suggest that the ELISA we describe could be useful for improving our understanding of the regulation of serum hemojuvelin production and its pathogenic role in various iron disorders. They also indicate that circulating hemojuvelin could be a potential new treatment for anemia associated with excess hepcidin as in patients with iron-refractory iron-deficient anemia with mutations in the *TMPRSS6* gene or in inflammatory anemia.

Authorship and Disclosures

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