DETERMINATION OF SERUM FERRITIN GLYCOSYLATION IN HYPERFERRITINEMIA ASSOCIATED TO IRON OVERLOAD AND INFLAMMATION.

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Abstract

Background: Serum ferritin is a commonly used clinical biochemical parameter and hyperferritinemia is used as a surrogate marker for iron overload, acute or chronic inflammation, malignancy or cell death. The aim of the present study was to develop purification strategies of ferritin from sera to determine if micro-heterogeneity of serum ferritin can be used to differentiate the underlying cause of the hyperferritinemia.

Patients, Materials and Methods: Sera from patients with hemochromatosis, rheumatologic diseases, aceruloplasminemia, ferroportin disease or iron loading anemia have been collected and stored and ferritin purified by negative affinity followed by ion exchange and size exclusion chromatography. Purified serum ferritin was analyzed by western blotting and MALDI TOF mass spectrometry and the spectra compared with the results from ferritin isolated from human liver, spleen and placenta.

Results: By Western blotting a major band of 19kD has been found in most sera, suggesting that the L-ferritin is the predominant isoform present in serum regardless of the cause of hyperferritinemia. Multistep chromatography can be used for significant enrichment and purification of ferritin from serum, which can be further analyzed by MALDI TOF MS. Tryptic digestion and peptide mass finger-printing by MALDI TOF MS of ferritin purified from human tissues shows differential spectra.

Discussion and conclusions: Analysis of ferritin micro-heterogeneity by MALDI TOF allows determination of the tissue origin of ferritin, which could be applied in the differential diagnostic workup of hyperferritinemia.

Key words: MALDI TOF, micro-heterogeneity, purification, mass-spectrometry

INTRODUCTION

Serum ferritin is a surrogate for body iron stores, acute phase reactions and tumors and is determined as part of routine blood investigations in patients with suspected iron overload or deficiency, suspected inflammation or for tumor surveillance (1, 8). Intracellular ferritin is a multimeric iron storage protein that is expressed in all tissues and is transcriptionally and translationally regulated in response to cellular iron availability (7). Two genes encode ferritin H- and L-chains and their relative contribution to hetero-multimeric aggregates varies in different tissues(5). In addition, several ferritin pseudo-genes exist and the exact origin of serum ferritin is matter of debate and may vary in different clinical conditions (3).

MICRO-HETEROGENEITY OF FERRITIN

Serum ferritin is a biochemically and structurally heterogeneous macro-molecule; glycosylation, iron content and isoelectric point of serum ferritin has been found to vary. Its glycane structure is determined by the tissue source of ferritin and thus on the underlying cause of hyperferritinemia (2, 6, 10). The intracellular storage protein ferritin is

usually translated by free intracellular ribosomes, but hepatocytes have also been found to actively secrete ferritin (4, 9). As the intracellular concentration of ferritin is ~ 1000-fold higher than the extracellular concentration, cell lysis can cause marked hyperferritinemia. However, in vitro studies have shown that iron loading causes increased secretion of glycosylated ferritin (4). As glycosylated ferritin can be isolated from sera of patients with iron overload, but not from patients with inflammation, we sought to exploit the micro-heterogeneity of ferritin for the differential diagnosis of hyperferritinemia.

Our aim was to develop a micro-scale preparation technique to isolate ferritin from a few milliliters of human serum and analyze glycosylation patterns of ferritin by mass spectrometry. This will form the basis for development of a refined serum ferritin assay that allows differentiation of the source of ferritin and facilitates differential diagnosis.

PATIENTS, MATERIALS AND METHODS.

PATIENT SAMPLES.
Remaining aliquots of serum from patients with hyperferritinemia that have been collected and stored were used for ferritin purification. Pathologic conditions of interest included hemochromatosis (Type 1), rheumatologic diseases, ferroportin disease and iron loading anemia. In addition, serum samples from a family with a suspected aceruloplasminemia case have been also included for this study. Patient's sera were used after informed consent had been obtained and all procedures have been carried out in compliance with the Declaration of Helsinki ethical principles for medical research involving human subjects.

MATERIALS.
As standards, ferritin purified from human heart, liver, spleen and placenta (Fitzgerald Industries International, MA) were used.

ISOLATION AND PURIFICATIONS TECHNIQUES.

HEAT TREATMENT.
Acidification, followed by heat denaturation is known to produce a significantly enriched ferritin fraction, in which neither the relative distribution of ferritin, nor the iron content is disturbed.

Six mL of serum were diluted with an equal volume of 50mM sodium acetate and after acidification to pH 4.8 with 1M acetic acid, rapidly heated to 70°C and this temperature was maintained for 10 min. This resulted in denaturation of a large proportion of serum proteins but ferritin was heat stable and remained in the supernatant. After centrifugation at 15,000g for 30 min, the pH was adjusted to 5.2 with NaOH at 4°C and kept at this temperature during the remaining isolation steps.

Ferritin was precipitated from 4mL of supernatant by drop-wise addition of ammonium sulfate to 50% saturation under carefully control of pH and temperature.

The pellet was resuspended in 3mL of 20mM sodium phosphate pH 7.0 and stored at 4°C for further investigations.

CHROMATOGRAPHY TECHNIQUES.
For further purification of ferritin from the heat treatment precipitation procedure, the pellet was resuspended in 20mM sodium phosphate buffer at pH 7 to do preparative chromatography on an ÄKTA purifier system. To remove the most abundant serum proteins negative affinity chromatography on a HiTrap 1mL Blue HP column (GE Healthcare Biosciences AB, Uppsala, Sweden) was carried out followed by another negative affinity step on a 1mL HiTrap Protein G HP column. Flow through fractions were colleted and analyzed for the presence of ferritin by western blotting. Ferritin containing fractions were pooled and ammonium sulfate slowly added to achieve 30% saturation at 4°C. After an over night incubation at 4°C, the sample was cleared from any precipitate by centrifugation at $15,000 \times g$ for 15 min.

Ferritin was then further purified from the supernatant of approximately 2 mL by hydrophobic interaction chromatography in a HiTrap ^{TM} 1mL Phenyl HP column (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Ferritin typically eluted from the Phenyl column at ~ 15% ammonium sulphate.

Alternatively ferritin was purified by molecular size exclusion chromatography, where a Superdex Peptide 10/300 GL Tricorn high performance column was used (Amersham Biosciences, Uppsala, Sweden). To see if ferritin isolated in a the peak corresponding to a M_r of ~ 400,000 Da could be denatured into ferritin monomers, denaturing size exclusion chromatography was carried out on a Superdex peptide 10/300 HR Tricorn column using 6M urea and 50mM DTT in phosphate buffered saline (PBS) as a running buffer.

IMMUNOLOGICAL TESTS.

To follow ferritin during the purification, samples were analyzed by Western blotting using a 1:2000 polyclonal rabbit anti-ferritin antibody (Abcam, Cambridge, UK.). As a secondary antibody, polyclonal goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (Dako, Glostrup, Denmark) at a dilution of 1:2000 was used and the immune complexes visualized with chemiluminescence using the ECL western blotting kit (GE Healthcare, Buckingham, UK) and a Chemidoc XRS CCD-camera (Biorad, Hercules, MA).

RESULTS

Ferritin isolated from sera of patients with various underlying conditions shows a major band of 19kD upon Western blotting, which suggests that L-ferritin is the predominant isoform present in serum regardless of the cause of hyperferritinemia.

To confirm this result and further determine the protein sequence as well as the glycan structure of serum ferritin, ferritin was purified from human serum. Purification of ferritin by heat denaturation and precipitation, was carried out. Subsequently negative affinity, anion exchange and size exclusion techniques were applied to further enrich serum ferritin. As shown by Western blotting, ferritin appears to be quantitatively recovered from the negative affinity steps with affi blue and protein G chromatography **(Figure 1A).** Although serum ferritin can be further purified by anion exchange chromatography, poor recovery limits the application of this preparative step **(Figure 1B).**

Purification of ferritin based on its hydrophobicity is possible on phenyl columns, but the high salt concentration required for elution of ferritin interferes with subsequent mass spectrometry and SDS PAGE. Ferritin was therefore buffer exchanged into low and volatile salt buffers (25mM ammonium bicarbonate) by ultrafiltration and size exclusion chromatography.
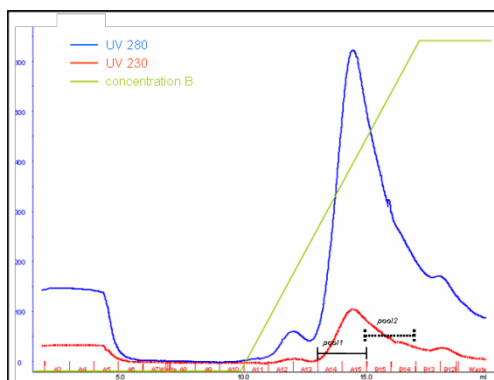
FIGURE 1A: *Hydrophobic interaction chromatography(HIC).*

Serum ferritin partially purified by heat denaturation and negative affinity steps. Six mL of serum was partially purified by heat denaturation and depleted of albumin and immunoglobulins using Affi Blue and Protein G sepharose. After addition of saturated ammonium sulphate to 30%, the supernatant was loaded on a HiTrap ᵀᴹ Phenyl HP 1mL column. When a gradient from 30% ammonium sulphate to 10mM NaCl in MES pH 6.8 was applied, ferritin is enriched in a broad elution peak at ~15% ammonium sulphate.
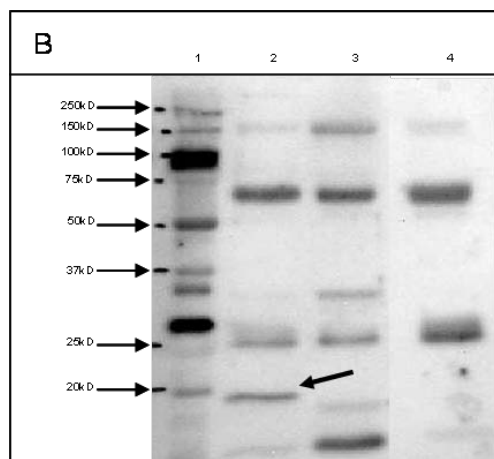

**FIGURE 1B:** *Detection of ferritin during multistep chromatography.*

30µL of eluate fractions from HiTrap Protein G columns was loaded on lane 2, where a 19kDa band demonstrates the presence of L-ferritin. Lanes 3 and 4 correspond to elution fractions from the HIC column. To reduce interference of high salt concentrations upon western blotting, desalting was carried out by ultra filtration, which causes significant losses of ferritin.

Under native condition ferritin self assembles into a multimeric protein of ~440 kD. This property of ferritin was exploited for buffer exchange into a volatile buffer system. Ferritin appears to be quantitatively retained by a 3 kD MWCO ultrafiltration membrane.

Although ferritin can be purified from human serum by multistep chromatography, peptide mass fingerprinting of serum ferritin was prohibited by low recovery. However, tryptic digestion of isolated tissue ferritins after purification by SDS PAGE (Figure 1A) has shown that differential mass spectra can be obtained and peptide mass fingerprints of different tissue ferritins are shown in Figure 2B
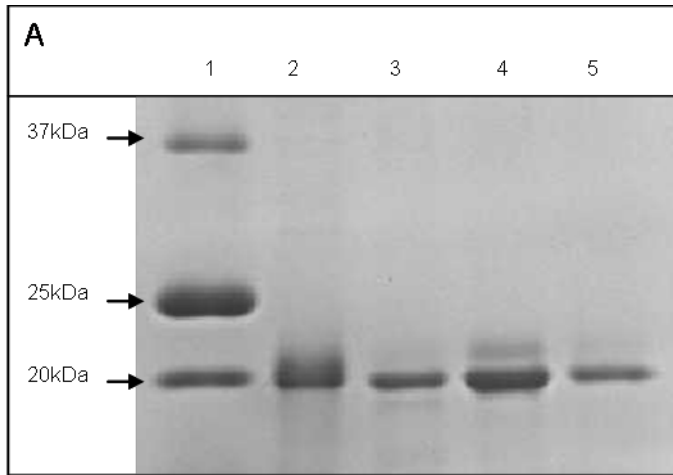
FIGURE 2A: Coomassie Blue stained SDS poliacrylamide gel of ferritins isolated from: (2) Heart (3) Liver (4) Spleen and (5) Placenta tissues.
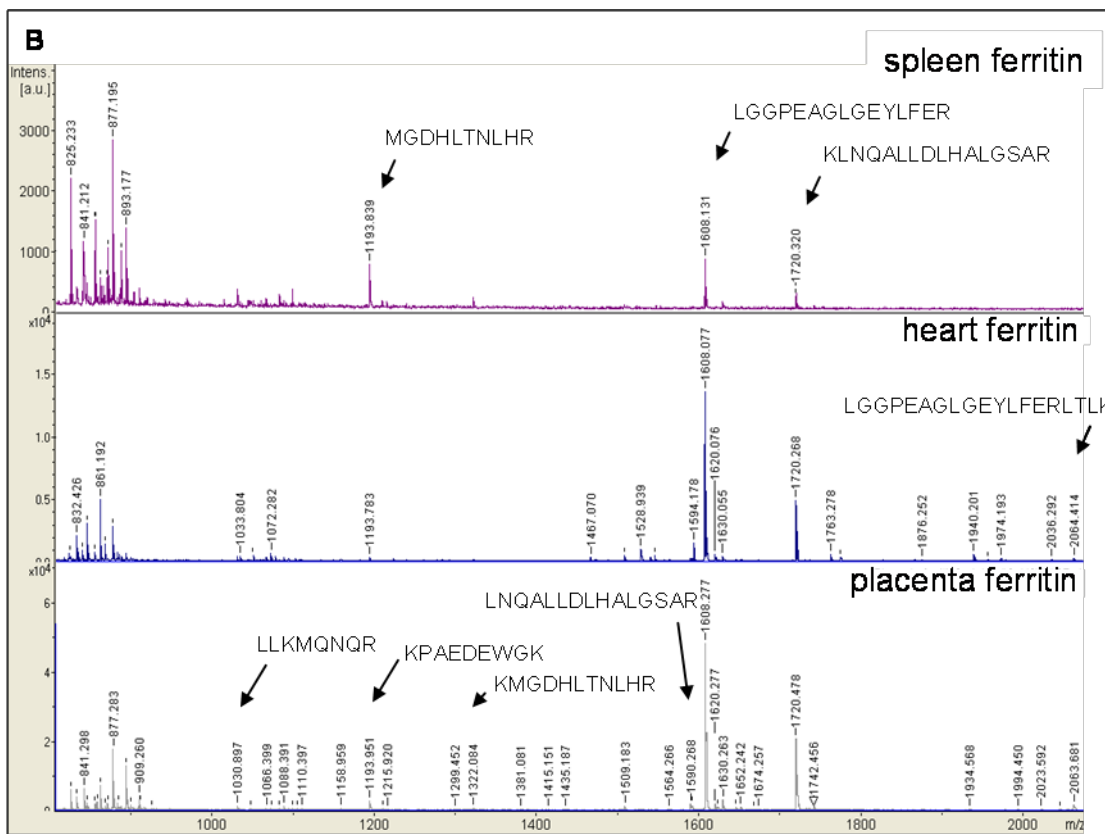
FIGURE 2B: *Representative MALDI TOF mass spectra of tryptic digests of ferritins isolated from spleen, liver and placenta.* Ferritin characteristic mass species are highlighted with an arrow and the corresponding peptide sequence of human ferritin light chain is listed.

DISCUSSION

Here we show that ferritin can be isolated from human serum using multistep chromatography and that peptide mass fingerprints are specific for ferritins isolated from different tissues. In all diseases studied, L-ferritin is the predominant

ferrtin isoform in serum and differentiation between H- and L-chains using immunological methods are unlikely to help in determining the underlying cause of hyperferritinemia.

As the origin of ferritin in serum is unknown, our observations indicate that mass spectrometry could elucidate the pathogenesis of hyperferritinemia in different diseases. The yields of the current purification method is too low to analyze ferritin by MALDI TOF MS, but purification to homogeneity is required to exclude interference from mass peaks that arise from contaminating proteins.

The differences in mass spectra from tryptic digests of ferritins isolated from different tissues are thought to arise from differences in the glycosylation pattern. Comparison of mass spectra before and after treatment with glycosidases will help to resolve this intriguing observation.

As one of our aims was to exploit the glycan structure for determination of the tissue origin of serum ferritin, spectra fro tissue ferritins will serve as a reference for future work.

References

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