Detection of biomarkers for lysosomal storage disorders using novel technologies
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Chapter Four

Limitations in quantitation of the biomarker CCL18 in Gaucher disease blood samples by Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

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Abstract

SELDI-TOF MS assisted the discovery of the chemokine CCL18 as plasma biomarker for pathological storage cells in Gaucher disease patients. Prognostic elevation of CCL18 in blood of Gaucher patients has been confirmed by ELISA. Given its low molecular mass, positive charge, and relatively high abundance, CCL18 seems a particular attractive protein for SELDI-TOF based quantitation. Therefore, we determined CCL18 levels in plasma using SELDI-TOF MS and ELISA, in parallel. CCL18 levels in some blood samples were significantly underestimated when determined by SELDI-TOF MS. Spiking of recombinant CCL18 indicated that its detection by SELDI-TOF MS is strongly determined by the nature of the sample, even markedly varying between samples obtained from one donor at different time points. Independent of the total CCL18 concentration in blood only 1-10% of the chemokine bound to the ProteinChip® Array. Even when comparable amounts of CCL18 from distinct samples were bound to the ProteinChip® Array, diverse peak intensities could be observed. Thus, limited binding capacity and sample-dependent suppression of CCL18 ionization contribute significantly to the final peak intensity. In conclusion, SELDI-TOF MS offers no reliable procedure to quantitatively monitor CCL18 levels in blood and thus cannot be applied in evaluation of disease status of Gaucher patients.
Introduction

The non-neuronopathic type I variant of Gaucher disease (GD) is one of the most frequently encountered lysosomal storage disorders. Type I GD is caused by a deficiency in the lysosomal hydrolase glucocerebrosidase (EC 3.2.1.45), resulting in massive storage of the glycosphingolipid glucosylceramide in lysosomes of tissue macrophages [1]. The characteristic lipid-laden macrophages, so-called Gaucher cells, are viable cells that secrete diverse factors involved in local tissue damage and formation of more storage cells. The pathological cells are mainly found in liver, spleen, and bone marrow. The accumulation of Gaucher cells leads to marked hepatosplenomegaly and bone marrow abnormalities resulting in pancytopenia. Gaucher cells also seem to trigger skeletal disease [1].

Accurate prediction of the clinical course of type I GD on the basis of glucocerebrosidase genotype is not feasible. Most strikingly in this respect, discordant phenotypes among identical twins have been reported [2]. Successful therapeutic interventions have been developed for type I GD. Widely applied is already the so-called enzyme replacement therapy (ERT) based on chronic intravenous administration of recombinant glucocerebrosidase [3]. ERT results in marked improvements of the major symptoms in Gaucher patients. More recently, beneficial responses have also been documented for treatment of Gaucher patients with Zavesca (N-butyldeoxynojirimycin), an inhibitor of glucosylceramide biosynthesis [4]. This so-called substrate reduction therapy (SRT) also results in major clinical improvements in moderately to mildly affected type I Gaucher patients.

Very high costs are associated with ERT and SRT of type I Gaucher patients. The poor predictability of clinical responses of individual Gaucher patients to ERT or SRT, has prompted the search for biomarkers that may assist clinicians in decision making regarding initiation of therapy and optimizing dosing regimens for individual patients [5]. Biomarkers are specific biomolecules found in an increased/decreased amount in body fluids and/or tissues and are indicators of the progress of a disease or responses to therapeutic intervention. Ideally, biomarkers are produced by the storage cells themselves, Gaucher cells in the case of GD, and detectable in easily accessible material such as blood or urine.

In 1994 a truly specific marker for the pathological Gaucher cells was already discovered [6]. Chitotriosidase (EC 3.2.1.14), a human analogue of chitinases from lower animals, showed a thousand fold increased activity in serum of symptomatic Gaucher patients. Chitotriosidase activity does not reflect one particular clinical symptom of GD, but is an indicator of total storage burden (Gaucher cell marker) [6]. Nowadays monitoring of this biomarker is commonly used for decision making regarding initiation and optimization of costly therapeutic interventions (ERT or SRT). Because about 6% of the population is completely chitotriosidase-deficient due to a mutation of the gene [7], not every Gaucher patient can be monitored by measuring chitotriosidase activity in plasma. This has stimulated research for novel plasma biomarkers for Gaucher cells. For this purpose
surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) was employed [8].

SELDI-TOF MS is a relatively novel application of mass spectrometry that combines absorption chromatography with time-of-flight mass spectrometric detection. Using different kind of arrays, with a wide variety of chromatographic (for protein profiling and peptide mapping applications) or pre-activated surfaces (for immunoassay, receptor-ligand binding, and DNA-binding protein applications), groups of proteins are selectively bound to the chip. Subsequently, chips are washed several times to remove unbound molecules and other interfering substances. As in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, molecules are co-crystallized with matrix molecules and ionized using a laser. The advantage of SELDI-TOF MS over conventional techniques is the possibility to apply complex biological samples such as serum or plasma directly due to specific retention of target proteins.

SELDI-TOF mass spectrometric analysis of blood from Gaucher patients using negatively charged CM10 ProteinChip® Arrays (weak cation exchange arrays) assisted the discovery of another valuable biomarker for GD [8]. A peptide of 7.8 kDa was prominent in plasma of an untreated symptomatic Gaucher patient but was nearly absent in control samples [8]. The protein was subsequently identified as CCL18, a member of the human C-C chemokine family. mRNA of CCL18 (also known as PARC: pulmonary and activation-regulated chemokine) was previously noted to be up-regulated in the spleen of a symptomatic Gaucher patient [9]. Subsequent analysis with an ELISA revealed that plasma CCL18 is elevated 10 to 50-fold in symptomatic Gaucher patients, with no overlap between values in symptomatic patients and controls. The median plasma CCL18 level in controls is 33 ng/mL (equalling 4.2 nM), ranging from 10 to 72 ng/mL (1.3-9.2 nM). In Gaucher patients the median plasma CCL18 level is 948 ng/mL (121 nM), ranging from 237 to 2285 ng/mL (30-291 nM) [8]. Gaucher cells are responsible for the massive overproduction and secretion of CCL18. The levels of CCL18 measured by ELISA correspond nicely to severity of disease manifestation and reductions in the serum chemokine occur during effective therapeutic correction of the disorder. Changes in plasma CCL18 mimic those observed in chitotriosidase activity, the established biomarker for GD [8]. The demonstration of massive production of CCL18 mRNA and protein by pathological storage macrophages in Gaucher patients further confirmed its value as biomarker in monitoring this disorder. Measurement of plasma CCL18 has been reported by several investigators to be a useful tool to monitor changes in total storage burden, especially for chitotriosidase deficient individuals [8,10,11].

Given CCL18’s favourable physico-chemical characteristics for mass spectrometrical detection (low molecular mass, positive charge, and relatively high abundance), we investigated whether SELDI-TOF based quantitation of CCL18 in blood can be used to quickly monitor the disease status of Gaucher patients.
Materials & methods

Reagents
Recombinant human CCL18 (rhCCL18) was purchased from PeproTech Inc. (Rocky Hill, New Jersey, USA) and R&D Systems Inc. (Minneapolis, Minnesota, USA). DTT, Tris, and Urea were purchased from Sigma Chemical Company (St Louis, Missouri, USA). Acetonitrile and Hydrochloric Acid were purchased from Merck (Darmstadt, Germany). Triton X-100 was purchased from BHD Laboratory Supplies (Poole, Dorset, UK). CHAPS, Sinapinic Acid, and TFA were purchased from Fluka Biochemika (Buchs, Switzerland). All-in-1 Peptide Standard was purchased from Ciphergen Biosystems Inc. (Fremont, California, USA). The pre-mixed peptide standard includes Arg⁸-Vasopressin, 1084 Da; Somatostatin, 1637 Da; Porcine Dynorphin, 2147 Da; Human Adrenocorticotropin Hormone (1-24), 2933 Da; Bovine Insulin β-chain, 3495 Da; Human Insulin, 5807 Da, and Hirudin BHVK, 7033 Da.

ProteinChip® Arrays
CM10 ProteinChip® Arrays (weak cation exchange arrays with carboxylate functionality, formerly known as WCX2 ProteinChip® Arrays) were purchased from Ciphergen Biosystems Inc. (Fremont, California, USA).

Patients
Of the 8 Gaucher patients (all type I), 5 received ERT (algulcerease, imiglucerease, [individualized dosing], Genzyme, Cambridge, Massachusetts, USA) and 3 patients were not treated. All patients with GD studied were known by referral to the Academic Medical Center. EDTA plasma and serum samples were obtained before and after several years of therapeutic intervention. Controls consisted of male and female healthy volunteers. Approval was obtained from the Ethic Committee. Informed consent was provided according to the Declaration of Helsinki.

SELDI-TOF MS
Experiments with EDTA plasma and serum
Blood samples were surveyed for basic proteins with SELDI-TOF MS making use of the anionic surface of CM10 ProteinChip® Arrays. First 10 μL of EDTA plasma/serum was mixed with 90 μL of denaturation solution (9 M Urea, 2% CHAPS, and 1% DTT). After incubation for 1 hour at room temperature (RT), 10 μL of this solution was mixed with 90 μL binding buffer (50 mM Tris + 0.1% Triton X-100, adjusted to pH 8 with hydrochloric acid). Before application of the sample to a CM10 ProteinChip® Array, all spots were equilibrated. To equilibrate the CM10 ProteinChip® Array, spots were washed twice with 200 μL of binding buffer for 5 minutes on a platform shaker and using a Ciphergen Biosystems 96-well bioprocessor. After equilibration, buffer was removed and samples were added. The samples were allowed to bind to the anionic surface for 40 minutes at RT on a platform shaker. Subsequently the ProteinChip® Arrays were washed twice with 200
μL binding buffer for 5 minutes on a platform shaker. Next the ProteinChip® Arrays were washed twice with 200 μL binding buffer without Triton X-100 for 5 minutes on a platform shaker. To remove salts ProteinChip® Arrays were briefly washed with deionized water. After air-drying, 0.5 μL of sinapinic acid (10 mg/mL) dissolved in 50% aqueous acetonitrile and 1% TFA was added to each spot twice. After co-crystallization of the proteins with the matrix molecules, a pulsed nitrogen laser was used for ionization of the samples. ProteinChip® Arrays were analyzed using a PBSIIc ProteinChip® Reader (Ciphergen Biosystems Inc. Fremont, California, USA), a linear laser desorption/ionization time-of-flight mass spectrometer equipped with time-lag focusing. The result is a mass spectrum composed of the mass to charge ratios and intensities of the desorbed (poly)peptide ions. All spectra were acquired in the positive-ion mode. Spectra were externally calibrated against a mixture of known peptides (All-in-1 Peptide Standard, Ciphergen Biosystems Inc. Fremont, California, USA). Spectra were normalized to the total ion current in the mass range from 1000 to 10,000 Dalton.

Experiments with rhCCL18

Experiments with rhCCL18 were performed using CM10 ProteinChip® Arrays. After incubation of rhCCL18 with denaturation solution for 1 hour at RT, 10 μL of this solution was incubated with 90 μL binding buffer (50 mM Tris + 0.1% Triton X-100, adjusted to pH 8 (or 9) with hydrochloric acid). Samples were allowed to bind to the anionic surface for 40 minutes at RT on a platform shaker. For equilibration of CM10 ProteinChip® Arrays, washing steps, matrix application and MS analysis see “Experiments with EDTA plasma and serum”.

Spike experiments with rhCCL18 in EDTA plasma and serum

In these experiments equal amounts of rhCCL18 were added to EDTA plasma/serum samples before denaturing with 9 M Urea, 1% DTT, and 2% CHAPS at RT for 1 hour. Samples were spiked in such a way that up to 1000 femtomoles of CCL18 ends up in a final volume of 100 microliter (equalling 10 nM). Again CM10 ProteinChip® Arrays were used to specifically bind basic proteins. 10 μL of denatured EDTA plasma/serum spiked with rhCCL18 was mixed with 90 μL binding buffer and allowed to bind to the anionic surface for 40 minutes at RT on a platform shaker. For sample preparation, equilibration of CM10 ProteinChip® Arrays, washing steps, matrix application and MS analysis see “Experiments with EDTA plasma and serum”.

ELISA

CCL18 levels in EDTA plasma were measured by a sandwich ELISA using a commercially available CytoSet (Biosource International, Camarillo, California, USA), consisting of a capture antibody, a biotinylated detection antibody, recombinant CCL18 standard, and streptavidin-horseradish peroxidase conjugate. Assay conditions were exactly as described by the manufacturer.
Results

CCL18 levels in EDTA plasma of eight type I Gaucher patients, before and after several years of therapeutic intervention, were determined using SELDI-TOF MS and in parallel using ELISA (see Fig. 1). CCL18 plasma levels as determined by semi-quantitative SELDI-TOF mass spectrometric measurements did not correlate with CCL18 levels as measured by ELISA. In some samples none to very little CCL18 was detected by SELDI-TOF analysis, despite the presence of large amounts of chemokine as indicated by ELISA. Vice versa, in other samples low CCL18 concentrations as determined with ELISA and SELDI MS more closely mimicked each other. The determination of CCL18 by ELISA is highly reliable [9]. This is also demonstrated by the consistent finding that in patients receiving ERT the blood levels of CCL18 and those of chitotriosidase, the well established biomarker for GD, go down proportionally [8]. The mass spectrometric detection of CCL18 in specific EDTA plasma samples proved to be itself reproducible (not shown). However, in serial serum samples of Gaucher patients upon enzyme replacement therapy SELDI-TOF analysis revealed often no consistent reduction in CCL18 levels, sharply contrasting to the results with ELISA measurements. In light of the strong discrepancy between plasma CCL18 levels as determined by SELDI-TOF MS and ELISA, further investigations were performed.

![Figure 1. CCL18 levels in EDTA plasma of eight Gaucher patients, before and after therapy. CCL18 levels were determined in duplicate (mean values are shown) using SELDI-TOF MS (Y-axis: peak intensity in arbitrary units, A.U.) and in parallel using a validated ELISA (X-axis: ng/mL plasma). Each patient is represented by a different symbol, closed and open symbols representing samples taken before and after therapy, respectively.](image)

First we examined whether the lack of correlation between CCL18 levels as determined by SELDI-TOF analysis and ELISA was due to intrinsic problems with respect to the linearity of quantitative SELDI-TOF analysis. To investigate this we made titration curves with rhCCL18. Stepwise addition of increasing amounts of rhCCL18 resulted in proportionally increased peak intensities although complete proportionality was not
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retained (Fig. 2A). The titration experiment indicates that the SELDI method allows quantitative detection of CCL18 over a considerable range, at least with relatively low concentrations of pure rhCCL18 in a strictly defined buffer solution.

Our next objective was to determine whether the nature of the applied sample plays a role in the problems surrounding quantitation of CCL18 with SELDI-TOF MS. To test this we titrated rhCCL18 in control serum. As in the previous experiment, similar amounts of rhCCL18 were applied to the ProteinChip® Array, only this time in the presence of serum. Overall, much lower peak intensities were observed. The addition of 1000 femtomole rhCCL18 alone resulted in a peak intensity of 36 arbitrary units. In the presence of plasma, however, peak intensity was reduced to 16% of the original signal. Importantly, a stepwise increase of rhCCL18 did not consistently lead to higher peak intensities in the protein profile (Figs. 2B and 2C). These data demonstrate that detection of CCL18 in the protein profile obtained by SELDI-TOF MS is markedly affected by the concomitant presence of blood components.

Next, we examined whether the type of serum affected the detection of CCL18 with the SELDI-TOF procedure. We spiked a fixed amount of rhCCL18 into plasma of 8 different

Figure 2. Titration of rhCCL18 in buffer and serum. Increasing amounts of rhCCL18 (0-1000 fmol per spot) were applied to the surface of a weak cation exchange (CM10) ProteinChip® Array in the presence or absence of control serum. A.U.: Arbitrary Units (A) Titration curve of increasing amounts of rhCCL18 in a buffered solution. (B) Titration curve of increasing amounts of rhCCL18 in control serum. (C) SELDI protein profile of a titration of rhCCL18 in control serum. Mass range: 7000 to 9000 m/z (mass-to-charge ratio).
controls and examined the resulting mass spectra. With the use of constant amounts of rhCCL18 added to serum, comparable peak intensities should result. In contrast, highly variable peak intensities were found (Fig. 3A). These data show that detection of CCL18 by SELDI-TOF MS is affected differently in various blood samples. Finally, we spiked a fixed amount of rhCCL18 in EDTA plasma of two controls obtained at four different time points over a period of two years. Variable peak intensities of CCL18 were observed despite the fact that constant amounts of rhCCL18 were added to plasma samples of the same control (Fig. 3B).

Altogether, these spiking studies with rhCCL18 demonstrate that detection of CCL18 by SELDI-TOF MS is strongly determined by the nature of the sample, even markedly varying between samples obtained from the same donor at different time points.

Finally, we tested the capacity of CM10 ProteinChip® Arrays to bind CCL18 from EDTA plasma samples. For this purpose a variety of EDTA plasma samples with different levels of CCL18 were used. Basic proteins were captured as described before and CCL18 levels were measured by ELISA before and after binding of the sample to the ProteinChip® Array. In all cases we noted that just 1-10% (mean: 5.1%) of all the administered CCL18 actually bound to the ProteinChip® Array. Even when similar amounts of CCL18 from different

**Figure 3.** Addition of rhCCL18 to blood samples. A fixed amount of rhCCL18 (equal to 250 fmol per spot) was added to the samples. (A and C) Samples were applied to the surface of a weak cation exchange (CM10) ProteinChip® Array. CCL18 levels were determined using SELDI-TOF MS. A.U.: Arbitrary Units (B and D) In parallel CCL18 levels were determined using a validated ELISA. (A and B) Spiked rhCCL18 in serum of eight different controls. (C and D) Spiked rhCCL18 in EDTA plasma of two controls (circles and triangles) obtained at four different time points (a-d) over a period of two years.
samples were bound, significantly different peak intensities were detected with SELDI-TOF analysis. For example, in the case of two EDTA plasma samples with almost similar concentrations of CCL18 (472 and 461 ng/mL, respectively), similar amounts were retained on the surface (as concluded from ELISA analysis of binding of CCL18 to the ProteinChip) but peak intensities were found to be 2.3 and 14.1 arbitrary units.

**Discussion**

Biomarkers are useful as a diagnostic tool for (early) diagnosis of lysosomal storage disorders. Additionally, they are of great use in decision-making regarding initiation and optimization of expensive therapy (ERT or SRT) and monitoring efficacy of therapeutic intervention. To search for novel biomarkers, protein profiles are often generated. In principle, SELDI-TOF MS is an attractive technique to rapidly provide profiles of proteins and peptides from different specimens given its sensitivity, simplicity, ease-of-use and low sample requirements. A high-throughput approach such as SELDI-TOF technology is, in theory, a useful platform to search for new biomarkers or monitor established biomarkers for clinical management of patients.

In this report we show that SELDI-TOF protein profiling, while initially worthwhile in biomarker discovery [8], is not an ideal approach to quantitatively monitor CCL18 levels in Gaucher patients and can therefore not be applied in evaluation of disease status. The outcome of the study is particularly disappointing since CCL18 seems to be an ideal protein for this type of MS analysis. It is a small, relatively abundant protein (in the nM range) with a positive charge and without glycans. These properties should allow CCL18 to bind strongly to the surface of a CM10 ProteinChip® Array and ionize (relatively) easy too. This is indeed observed when using a pure rhCCL18 preparation. However, the complexity of blood-derived samples negatively influences the detection of CCL18 with the ProteinChip® Array.

Our study thus revealed a serious intrinsic problem associated with SELDI-TOF MS protein profiles obtained from blood samples. The observed peak intensity of a particular protein may not reflect the actual amount present in the sample but rather result from the amount of protein actually bound to the ProteinChip® Array and its efficiency of ionization, both potentially being influenced by other components present in the sample. Phenomena such as binding competition and suppression of ionization obviously play a role here. Because ProteinChip® Arrays have a limited binding capacity (estimated: 2-5 picomole for CM10 ProteinChip® Array), components present in blood compete for binding on the surface of the ProteinChip® Array. Bound proteins compete for laser energy, transferred via energy absorbing matrix molecules, to become ionized and eventually reach the detector of the mass spectrometer. Some components present in blood clearly suppress the signal of other proteins, like the chemokine CCL18, by competing for binding places and/or ionization energy. Even little alterations in blood composition may affect binding competition/ionization suppression and thus influence the outcome of the experiment.

We conclude from our investigation that SELDI-TOF based detection of CCL18 in blood
samples presently offers no reliable procedure to rapidly quantify actual chemokine levels in Gaucher patients and can therefore not be employed in reliable assessment of disease status. The use of an ELISA-based detection of CCL18 has to be recommended for this purpose. It seems likely that, apart from CCL18, other proteins in blood samples will encounter similar problems when analyzed by SELDI-TOF MS. Our observations with CCL18 clearly illustrate that semi-quantitative monitoring of disease-specific biomarkers in complex fluids with SELDI-TOF protein profiling experiments remains a tremendous challenge.

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