Clinica Chimica Acta 414 (2012) 58–64

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Disialo–trisialo bridging of transferrin is due to increased branching and fucosylation of the carbohydrate moiety

Eva Landberg [a,*], Eva Åström [b], Bertil Kågedal [c], Peter Påhlsson [b]

[a] Division of Clinical Chemistry, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, County Council of Östergötland, Linköping, Sweden
[b] Division of Cell Biology, Department of Clinical and Experimental Medicine, Linköping University, S-58185 Linköping, Sweden
[c] Division of Clinical Chemistry, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

ARTICLE INFO

Article history:
Received 27 April 2012
Received in revised form 31 July 2012
Accepted 31 July 2012
Available online 7 August 2012

Keywords:
Transferrin
CDT
Di–tri bridging
Glycosylation
Liver disease

ABSTRACT

Background: Carbohydrate deficient transferrin (CDT) is used for detection of alcohol abuse and follow-up. High performance liquid chromatography (HPLC) of transferrin glycoforms is highly specific for identification of alcohol abuse, but unresolved disialo- and trisialotransferrin glycoforms sometimes makes interpretation difficult. The cause of this phenomenon is unknown, cannot be explained by genetic variants of transferrin, but seems to be associated with liver disease.
Methods: Nineteen serum samples showing di–tri bridging when analyzed by HPLC were collected. Transferrin was purified by affinity chromatography, and N-linked oligosaccharides were released enzymatically. The N-glycans were further analyzed by high performance anion-exchange chromatography with pulsed amperometric detection and MALDI-TOF mass spectrometry.
Results: The HPLC-analysis showed three different types of glycoform patterns. The N-glycans from fifteen samples showed patterns with increased number of triantennary structures containing one or two fucose residues. One sample contained an increased amount of triantennary glycans without fucose. Three samples showed a glycosylation pattern similar to normal transferrin.
Conclusions: The di–tri bridging phenomenon was associated with alterations in transferrin glycosylation in the majority of cases. Transferrin contained a higher extent of triantennary and often fucosylated N-linked oligosaccharides. These results may be important in future diagnostic approaches to liver diseases.

1. Introduction

Human transferrin is a globular protein responsible for iron transport in plasma. It is composed of a single polypeptide chain of 679 amino acids arranged in two independent metal ion-binding globular domains. It has two main glycosylation sites at Asn-413 and Asn-611 [1]. The oligosaccharides found on these sites are mainly bi- and triantennary complex type glycans with terminal sialic acid [2–4]. Normal human serum typically contains a major transferrin glycoform containing two biantennary N-glycans with a total number of four sialic acid residues (tetrasialotransferrin), and minor isoforms with two (disialotransferrin), three (trisialotransferrin), and five (pentasialotransferrin) sialic acid residues.

Chronic heavy alcohol drinking alters the transferrin glycoform pattern so that the disialoisoform increases and asialoisoforms may appear [5–8]. These isoforms lack one or both of the carbohydrate chains [9,10] and are collectively denoted carbohydrate deficient transferrin (CDT). Among several laboratory tests routinely used in clinical and forensic medicine, the percentage of CDT of total transferrin (% CDT) is considered the most specific biochemical marker for detecting chronic alcohol abuse and for monitoring abstinence during treatment [6,11,12].

A number of methods have been applied for CDT quantification including immunometric, electrophoretic, chromatographic and mass-spectrometric methods. High performance liquid chromatography (HPLC) of transferrin glycoforms was recommended as the interim reference method to be applied in CDT confirmatory analysis [13]. To overcome the lack of standardization affecting CDT analysis, the Working Group on Standardization of CDT measurement (IFCC–WG–CDT) suggested to use disialotransferrin as the primary target molecule for CDT measurement, expressed as relative amount of total transferrin concentration [13,14]. Although there are great advantages of this method, in particular regarding analytical and clinical specificity, a number of atypical separation patterns, not reflecting alcohol abuse, have been identified. Atypical patterns are often attributed to genetic transferrin variants of the amino acid sequence such as BC or CD [15]. In addition, there are also cases with a chromatographic pattern where disialo- and trisialotransferrin are not adequately separated. This phenomenon is called disialo–trisialo bridging (di–tri bridging) [16]. Presence of di–tri bridging makes the interpretation of CDT difficult and increases the uncertainty of the measurement. It was recently established that the

* Corresponding author at: Division of Clinical Chemistry, University Hospital, S-581 85 Linköping, Sweden. Tel.: +46 10 1033226; fax: +46 10 1033206.
E-mail address: eva.landberg@lio.se (E. Landberg).

0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2012.07.026

di–tri bridging phenomenon could neither be explained by a genetic transferrin protein variant nor by an increased trisialotransferrin fraction [16]. It has been suggested that the di–tri bridging phenomenon may be prevalent in patients with liver cirrhosis or other severe liver abnormalities [16,17].

The aim of this study was to examine if altered glycosylation of transferrin may explain the occurrence of a di–tri bridging pattern on HPLC.

2. Material and methods

2.1. Patients and serum samples

During a period of 6 months, serum samples admitted to the laboratory for routine analysis of CDT were selected for further analysis if the chromatogram indicated presence of di–tri bridging. Data on age, gender and plasma liver enzymes (γGlutamyl transferase (γGT), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)) were collected from the laboratory data system. The patient identity of each sample was then removed (Table 1). CDT was analyzed by high-performance liquid chromatography as previously described [18]. The control samples consisted of one serum pool from 150 patients with normal CDT values and one pool from 150 patients with increased CDT (>3%). The controls were analyzed in each run. Nineteen plasma samples that showed di–tri bridging were identified and stored at -20 °C until further analysis.

2.2. Chemicals and reagents

Human transferrin from Sigma (Holo-transferrin, iron-saturated, T3400 Sigma-Aldrich) was used as reference (reference transferrin). All water solutions were prepared with Milli Q Water. All chemicals were from Merck except for glycine, Triton-X100, BSA, AGP and KH_2PO_4 (Sigma-Aldrich), SDS and β-mercaptoethanol (Bio-Rad Laboratories).

2.3. Purification of transferrin

Transferrin was isolated from serum samples using affinity chromatography. Transferrin specific antibodies (Rabbit anti-human transferrin, A0061, DAKO) were immobilized on a 5 ml HiTrap NHS-activated column (Amersham Biosciences) according to the manufacturer's instructions. The column was connected to an ÄKTA prime instrument equipped with a UV detector (Amersham Bioscience). Chromatography was performed at a flow rate of 1.0 ml/min. Serum was diluted twofold with phosphate buffered saline, pH 7.4 (PBS), and after equilibration of the column with 2 column volumes of PBS, the diluted serum sample was injected. Unbound proteins were eluted with 30 ml of PBS. The absorbance of the effluent was recorded at 280 nm. Transferrin was eluted using 0.2 M glycine–HCl buffer, pH 2.0, and collected in 1-ml fractions. The fractions were immediately neutralized with phosphate buffer, 0.5 M, pH 8, and fractions containing transferrin were pooled and stored at −20 °C.

Transferrin concentration in serum samples was determined by an immunonephelometric method on BN ProSpec (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The calibrator was traceable to CRM 470. Transferrin concentration in purified samples was determined using the bicinchoninic acid assay (BCA Protein Assay Reagent, Pierce Biotechnology) using reference transferrin (Sigma-Aldrich, see also 2.2) as a standard. According to the manufacturer this standard was 98% pure.

Table 1
Data on liver enzymes and transferrin glycoform pattern.

Age (years)	Gender (male/female)	P-γGT (μkat/l)	P-ALT (μkat/l)	P-AST (μkat/l)	Transferrin glycoform pattern
67	M	4.6[a]	0.65	1.9[a]	TGP-1
54	M	4.3[a]	0.36	0.50	TGP-1
36	F	14[a]	0.31	0.91[a]	TGP-3
67	M	15[a]	0.46	0.84[a]	TGP-1
22	M	0.31	0.25	0.32	TGP-2
67	M	5.6[a]	0.57	0.81[a]	TGP-1
48	M	2.8[a]	0.54	1.2[a]	TGP-1
51	F	7.1[a]	3.3[a]	4.6[a]	TGP-1
41	M	4.2[a]	2.2[a]	2.6[a]	TGP-1
74	F	6.9[a]	0.45	0.74[a]	TGP-1
49	F	10[a]	1.5[a]	3.2[a]	TGP-1
51	M	5.3[a]	2.4[a]	2.2[a]	TGP-1
39	M	1.6[a]	0.95	0.90[a]	TGP-1
56	M	3.9[a]	3.2[a]	2.6[a]	TGP-1
48	F	14[a]	1.9[a]	3.8[a]	TGP-1
38	M	0.34	0.27	0.44	TGP-2
61	M	2.6[a]	0.42	1.1[a]	TGP-1
55	M	3.7[a]	0.38	0.54	TGP-1
23	M	0.60	0.51	0.39	TGP-2

[a] Result above normal reference limit according to NORIP [38].

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Purified transferrin was analyzed by SDS-PAGE on 10% Tris–HCl gels (Bio-Rad) under denaturing conditions according to Laemmli [19]. Bands were visualized using Coomassie blue staining.

2.5. Release of glycans from transferrin

The transferrin samples were dialyzed against 3×2 l of water for 20 h at 4 °C (Spectra/Por Dialysis membrane, Spectrum Laboratories, MWCO: 12–14,000) and lyophilized.

From each sample 250 μg transferrin was dissolved in 180 μl of phosphate buffer (0.05 M, pH 7.5). Then 20 μl denaturizing solution (1% SDS, containing 1 M β-mercaptoethanol) was added. After boiling the samples for 5 min in a water bath and cooling to room temperature, 20 μl of 10% Triton X-100 (Sigma-Aldrich) and 2 μl (1 U) of Peptide-N-glycosidase F (PNGase F, Sigma-Aldrich) were added and the samples were incubated for 20 h at 37 °C.

After cooling of the samples to room temperature, 20 μl of 5% acetic acid was added and the samples were incubated for 2 h at room temperature.

2.6. Purification of released oligosaccharides

Sep-Pak C18 cartridges (Waters Corporation) were pre-conditioned with 10 ml methanol followed by 10 ml of water. The samples were applied, eluted with 1 ml of water and lyophilized. The lyophilized samples were dissolved in 100 μl of water and applied to columns containing Bio Gel P2 (2 ml, extra fine, Bio-Rad). Five hundred micro liters of water was applied to the column, before the desalted transferrin oligosaccharides were recovered by elution with 0.6 ml of water and lyophilized.

2.7. Analysis of oligosaccharides by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Analysis of released oligosaccharides was performed using a ISC-3000 HPAEC-PAD-system (Dionex) equipped with a CarboPac PA200 column at a flow rate of 0.5 ml/min [20]. Chromatography was performed using a constant concentration of 0.1 M NaOH and a two step gradient of sodium acetate (NaOAc) as indicated in Table S1 (Supplementary data). The lyophilized samples were dissolved in 100 μl of water. Injection volume was 20 μl.