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SHORT COMMUNICATION

Haptoglobin glycoforms in a case of carbohydrate-deficient glycoprotein syndrome

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Alterations in haptoglobin (Hp) glycosylation were examined in the plasma of the first patient with carbohydrate-deficient glycoprotein syndrome (CDGS) who was described in Poland. Hp concentration in the CDGS patient plasma was low (240 mg/l) and the Hp phenotype was shown to be 2-2. Three glycoforms of the Hp β subunit were observed in SDS-PAGE in CDGS. The densitometric analysis and molecular weight determinations suggested that 50% of glycoforms were fully glycosylated; 30% contained three out of four and 20% only two out of four glycan units compared to those that are present in Hp derived from healthy people. Results with lectins (concanavalin A and *Sambucus nigra*, *Maackia amurensis* and *Alleuria aurantia* agglutinins) indicate that all three glycoforms of β subunit of CDGS-Hp contained biantennary complex glycans terminated with α2,6 bound sialic acid, but without fucose or α2,3 linked sialic acid. Hp glycosylation abnormalities described in this work suggest that this case was a type I carbohydrate-deficient glycoprotein syndrome.

Keywords: **carbohydrate-deficient glycoprotein syndrome, CDGS, haptoglobin, acute phase protein, N-glycosylation, glycoforms**

Abbreviations: **CDGS, carbohydrate-deficient glycoprotein syndrome; Hp, haptoglobin; CD-Hp, carbohydrate-deficient haptoglobin; HRP, horseradish peroxidase; ConA, concanavalin A; AAA, lectin from *Alleuria aurantia*; MAA, lectin from *Maackia amurensis*, SNA, lectin from *Sambucus nigra*; DIG, digoxigenin; TBS, Tris-buffered saline**

Introduction

During the last decade a lot of attention was paid to investigate clinical features, as well as the biochemical background of a new inherited metabolic disorder termed by Jaeken [1] as carbohydrate-deficient glycoprotein syndrome (CDGS). CDG syndrome is biochemically divided into five main types differing in their clinical symptoms and molecular background [2]. The main biochemical marker of CDGS has been established as a deficiency in sialic acid content in oligosaccharide moiety of serum transferrin (Tf) [2,3]. In CDGS type 1, which is the most common form, the tetrasialotransferrin is reduced to ~50% and replaced by disialo- and asialo-forms missing one or both entire glycans [2,4]. However, sialic acid deficient Tf does appear at low level, in normal serum [5], in hepatocellular carcinoma and nonalcoholic liver disease [6], or alcohol abuse [7].

Up to date only Tf glycan structure has been analysed in detail [4], although there are some reports that glycans of some other serum and membrane bound glycoproteins are affected in CDGS [8–11]. The first well documented case of CDGS in Poland was described in 1996 in two sisters. The diagnosis was based on clinical symptoms and Tf characteristics. The younger patient died at the age of eight years, and the other one is still being treated in the Białystok Medical Academy [12]. The aim of this study was to find out, if any changes existed in the level, electrophoretic pattern and glycan structures of haptoglobin (Hp), derived from plasma of this CDGS patient.

Materials and Methods

Clinical subject

The clinical subject was a girl born in 1983 in a first uncomplicated pregnancy. The delivery took place at term, the

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newborn weight was 3.1 kg and the development at the early infancy was reportedly normal. At the age of 5 months, a psychomotor retardation was observed. The results of routine haematology and coagulation examinations, as well as serum levels of electrolytes, were normal. Serum level of carbohydrate-deficient Tf was 8.5 mg/l, while in the sera of healthy people this underglycosylated form is present at the concentration of 1.2 ± 0.4 mg/l [12]. Patient's blood was taken using heparin as anticoagulant agent and blood plasma were separated by centrifugation. Sera were taken from healthy volunteers to be used in control experiments.

Haptoglobin quantification and purification

Hp concentration was determined by microguaiacol method as previously described [13] or by direct Hp-ELISA [14]. Hp was isolated by affinity chromatography on Sepharose 4B-coupled two mouse monoclonal anti-Hp antibodies (clones 2.36.71.41 and 7.60.65.55) according to procedure described earlier [14]. Hp was released from the matrix with 100 mmol/l acetate buffer, pH 3.8. Collected, neutralized, and dialyzed fractions were checked for the Hp purity using SDS-PAGE and kept at −80°C until use.

Electrophoretic analysis and Hp detection

SDS-PAGE, according to Laemmli [15] procedure, was performed in 12.5% gel under denaturing conditions. The Hp samples were heated for 5 min. at 100°C with 5% β mercaptoethanol. The gel was stained for protein using silver nitrate detection [16] or electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schüll) according to Towbin et al. [17]. Hp on blots was detected with polyclonal goat anti-Hp antibodies (produced as described in [14] and diluted 1:1000 in TBS-Tween 0.05%, 1h incubation at 37°C). The membranes were incubated with rabbit-anti-goat IgG HRP-conjugated (Sigma, diluted 1:20 000, 37°C for 1h) and the peroxidase reaction was developed with diaminobenzidine and H_2O_2. Alternatively, blots were incubated in TBS containing 1 mmol/l $MnCl_2$, $MgCl_2$ and $CaCl_2$ for 1 h at 37°C with DIG-labeled lectins (Boehringer Mannheim): ConA (1 µg/ml), AAA (2.5 µg/ml), MAA (5 µg/ml) and SNA (1 µg/ml). After incubation with anti-DIG-Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim, diluted 1:1000), the phosphatase reaction was developed with 5-bromo-4-chloro-3-indolilphosphate (Sigma Chemicals) and nitroblue tetrazolium chloride (Sigma Chemicals).

Enzymatic N-deglycosylation

Forty µg of Hp in 50 mmol/l Tris-HCl buffer, pH 8.5 was treated with 5 µl of N-glycanase (Genzyme) according to Mann's [18] procedure. The degree of deglycosylation was checked in 10 µl samples taken immediately after enzyme addition (t_0) and then in 15 min intervals. The samples were denatured by heating in 2.5% SDS and 5% βmercaptoethanol and separated by SDS-PAGE.

Desialylation procedure

To 20 µg of Hp 2-2 in acetate buffer (100 mmol/l, pH 5.0) 0.02 U of *Vibrio cholerae* neuraminidase (Boehringwerke AG) was added and the mixture was incubated at 37°C. Samples were taken after 15 min., 30 min., 5 h and 24 h and boiled with SDS and β-mercaptoethanol containing buffer to be analysed by SDS-PAGE.

Results

Haptoglobin level and heterogeneity of β subunit

Hp concentration in the plasma of CDGS patient was low (240 mg/l) compared to normal values (1100 ± 580 mg/l, [13]). CD-Hp was of the 2-2 type (pattern not shown).

In SDS-PAGE the reference Hp 2-2 preparation gave a main β-band of 40 kDa, an α^2 band of 17 kDa, and a low amount of a 37.2 kDa protein (Fig. 1 a). In the CD-Hp preparation, there was a 17 kDa band that corresponded to the α^2 subunit of Hp, and in the electrophoretic region of β Hp subunit, three protein bands of 40 kDa, 37.2 kDa and 34.7 kDa were observed (Fig. 1 b). All three bands of CD-β-Hp and two bands of normal -β-Hp bound goat polyclonal anti-Hp antibodies (Fig. 1 c,d), so it was assumed that they were isoforms of the β subunit. A fifth band of molecular weight of about 15 kDa, evident in the electrophoretic pattern of CD-Hp (Fig. 1 b), was red coloured before silver staining, did not bind anti-Hp antibodies, so it was probably haemoglobin subunits.

In order to investigate the heterogeneity of CD-Hp β subunits we examined the electrophoretic pattern of the 'healthy' control Hp after partial desialylation and deglycosylation. Exposing Hp to neuraminidase for 30 min and 5 h resulted in forms that were partially and completely desialylated. For the main 40 kDa band of the β Hp subunit, two isoforms were obtained. Their molecular masses were calculated as 39 kDa and 37.5 kDa, as shown in Figure 2 'A' b, c, respectively. The glycoform pattern obtained after 24 h neuraminidase treatment was identical to that shown in Figure 2 'A' c (data not shown), indicating that sialic acid release was complete. A molecular mass decrease was also observed for the second, 37.2 kDa, Hp isoform indicating that both isoforms (40 and 37.2 kDa) underwent desialylation.

N-glycanase digestion produced modified glycoforms of 'healthy' Hp (Fig. 2 'A' d–g), which were successively devoid of one to four glycan units. The molecular masses of these forms were calculated as 37.5 kDa, 35 kDa, 33 kDa, and 30 kDa and are marked with arrows in Figure 2. Comparison of the molecular masses of the deglycosylated forms of the 'healthy' Hp with the masses of the CD-Hp β-subunit isoforms suggest that the glycosylation defect of CD-Hp could be due to incomplete occupation of N-glycosylation sites.

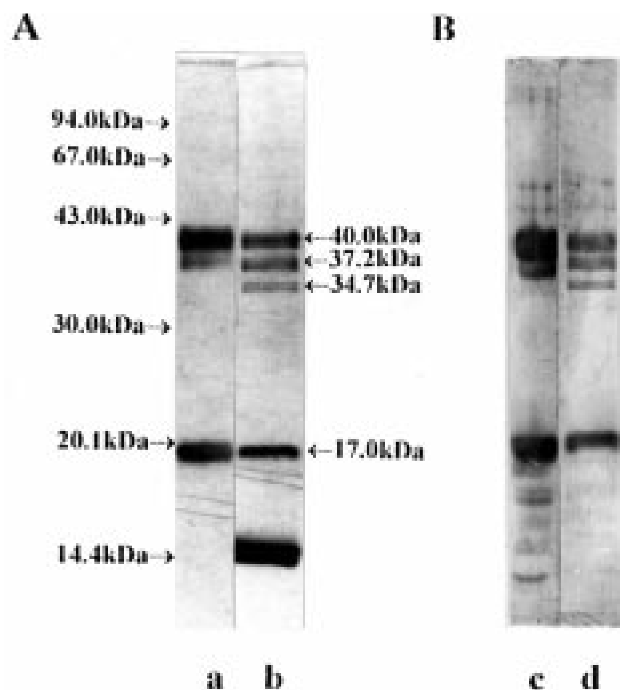

Figure 1. PAGE and Western-blotting analysis of CD-Hp. Lanes a and c represent a healthy control preparation of Hp 2-2, lanes b and d represent CD-Hp; a,b—silver stained, c,d—SDS-PAGE components that were transferred to nitrocellulose and treated with anti-Hp antibodies as described in Materials and Methods.

Part of the electrophoretic separation of CD-Hp in Fig. 1 b, enclosed in the area between 40 to 36 kDa, was submitted to densitometric analysis. The area under each peak was calculated by computer analysis and used to estimate the relative amount of the respective glycoforms. On this basis it was calculated, that about 50% of the Hp β chains of CDGS patient were fully glycosylated (containing four oligosaccharide chains), about 30% were lacking one chain, and about 20% were lacking two out of the four glycan chains.

Reactivity of CD-Hp glycans with lectins

The results of lectin blotting studies of CD- and control Hp are shown in Figure 2 'B'. The main 40 kDa isoform of β-subunit of 'healthy' control Hp 2-2 was strongly bound to Con A and SNA. However, the 37.2 kDa isoform of β subunit of 'healthy' Hp did not react with these two lectins. In contrast, all three glycoforms of β-chain of CD-Hp reacted with Con A and SNA. Both reference and CD-Hp glycans did not bind MAA and AAA even after a prolonged incubation time.

Discussion

In CDGS type I, the following glycoforms of transferrin were previously described: fully glycosylated, completely unglycosylated and one lacking one out of two N-glycans [4]. It was reported [3,19], that the lack of one sugar branch in

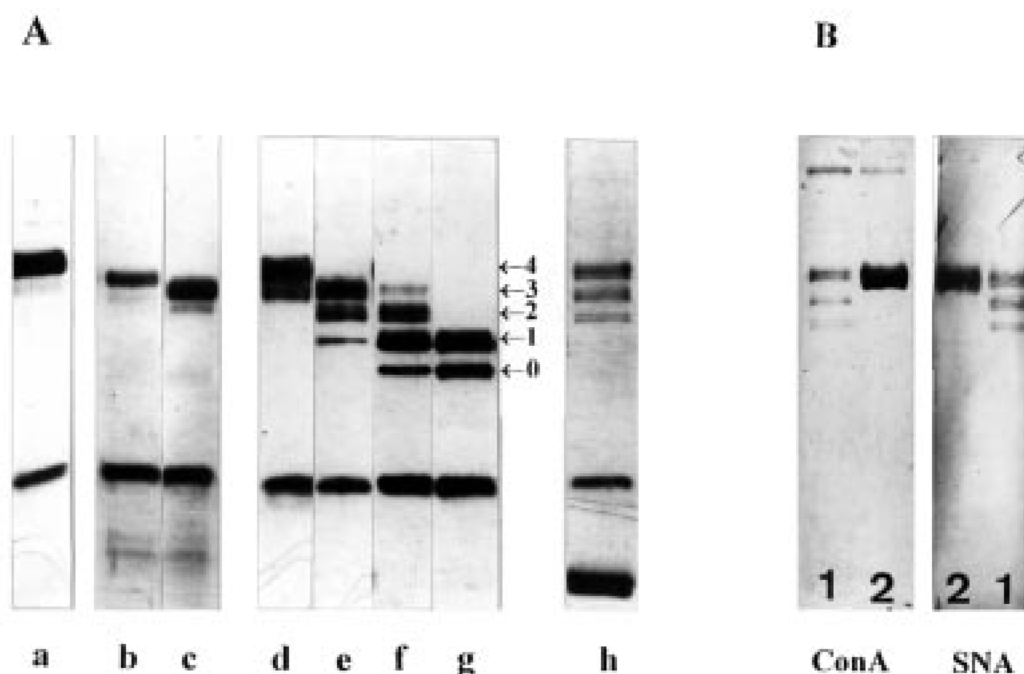

Figure 2. Carbohydrate analysis of Hp. 'A' The effect of desialylation and N-deglycosylation on electrophoretic pattern of β-subunit of Hp: Lane a—control Hp 2-2 preparation, lanes b–c—the same Hp after neuraminidase treatment for b: 30 min., c: 5 h; lanes d, e, f and g—control Hp 2-2 treated with N-glycanase as described in Materials and Methods for: d—0 min (the sample taken immediatly after enzyme addition), e—15 min., f—30 min., g—60 min.; lane h—CD-Hp. The gel after SDS-PAGE was silver stained. Arrows indicate the number of glycan units on the β-subunit. 'B' Reactivity of Hp with lectins: Hp was subjected to SDS-PAGE/Western blotting. Blots were incubated with DIG-labelled lectins: ConA, SNA and then with anti-DIG-Fab fragments conjugated with alkaline phosphatase. For details see Materials and Methods. On the each set of blots lane 1 represents CD-Hp and lane 2 "healthy" control Hp.

Tf/molecule results in a molecular weight shift of 2.2 kDa, and this value agrees with the calculated mass of a biantennary sialylated N-glycan type complex. In the case of Hp only its β subunit, which contains four occupied N-glycosylation sites, is glycosylated [20]. Our findings indicate, that only 50% of Hp prepared from CDGS patient's plasma are completely glycosylated, and that the remaining 50% of the Hp exists in forms containing only three (30%) or two N-glycans (20%), lacking one or two glycans, respectively. The molecular mass differences between bands of 2.8 kDa (Fig. 1) could indicate the lack of triantennary structures. The results of lectin blotting indicate that all glycoforms of Hp contained biantennary complex chains and at least some of them are terminated with sialic acid α2,6 linked to galactose. The lack of reactivity with AAA and with MAA may suggest, that there is no fucosylation, or sialic acid bound in α2,3 position, respectively. No further "underglycosylation" was apparent such as complete lack of N-glycans as observed in the case of CD-Tf. Further examination would be required to determine if the Hp glycoform pattern changes during the course of the disease and if observed changes occur in other patients with CDGS.

We observed that Hp preparation derived from the 'healthy' subjects consists of two isoforms detected by sensitive silver staining and/or by specific antibodies in the Western-blots. In addition to the main 40 kDa fraction, corresponding to fully glycosylated glycoform of β chain, there appeared to be small amounts of another glycoform having a molecular weight of 37.2 kDa. On the basis of molecular weight determination, this second glycoform may have only three out of four glycosylation sites occupied. This second band was observed by us in other Hp preparations obtained from healthy sera; and also in Hp derived from patients suffering from other diseases (ovarian cancer, lymphoma, nephrotic syndrome, unpublished observations). However, it should be underlined that this glycoform never normaly exceeded a few percent, whereas in CDGS Hp it was 30%. The third glycoform of CD-Hp (20%) with glycans probably attached to two sites was never observed in Hp derived from 'healthy' people and other diseases.

Early reports on CDG syndrome suggested mainly a deficiency of terminal sialic acid, but later more internal monosaccharides in the glycan chain (galactose and N-acetylglucosamine) were found to be lacking [1,4]. Later structural studies proved that the defect in Tf sugar structure is different in particular types of the syndrome, but in CDGS type I no changes in the remaining glycan units have been reported [4,21]. The glycans present in β-chain of CD-Hp are bi- or triantennary, terminated with sialic acid bound α2,6 but without fucose, similar to those present in healthy subjects. The microheterogeneity, observed in ConA and SNA reactivity suggest that some modifications in branch structures could also be possible and further examination of glycan structure is necessary. The electrophoretic pattern of Hp β-chain seems to well characterise the biochemical alterations that are typical of CDGS type I. In agreement with Seta et al. [10] we show that Hp glycoform analysis could be helpful in CDGS diagnosis, especially in differentiating particular types of syndrome.

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