Glycoforms of Six Serum Glycoproteins in a Patient with Congenital Disorder of Glycosylation Type I

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Abstract. In this paper the occurrence and relative content of defectively glycosylated serum glycoforms in transferrin (Tf), α₁-acid glycoprotein (AGP), haptoglobin (Hp), α₁-antitrypsin (α₁-AT), α₂-macroglobulin (α₂-MG) and ceruloplasmin (Cpl) in the serum of a patient with congenital disorder of glycosylation type I are reported. Blood samples were taken when the patient was 14 years old and then after a one-year interval. The patterns of glycoforms in both samples were compared. In 4 out of 6 examined glycoproteins, glycoforms lacking one and two oligosaccharide chains occurred. “Underglycosylated” glycoforms of α₂-MG and Cpl were not clearly detectable. Tf was shown to be affected with this defect to a higher extent than other glycoproteins, containing only 30% properly glycosylated molecules and also as much as 30% of the molecules lacking two glycan units. In Hp and α₁-AT the proportions of properly and defectively glycosylated forms were similar. This properly glycosylated form comprised 47% of the Hp and 51–55% of the α₁-AT molecules. As in AGP and Tf, about 30% of the molecules lacked one glycan unit. Twenty-one percent of the Hp molecules were devoid of two glycans, and this amount slightly increased in the course of the year. In α₁-AT, 19 and 17% of the molecules lacked two glycans in both samples, respectively. Only in AGP we did find a substantial difference between the two blood samples. In the course of the year, the amount of the form lacking 2-chains decreased from 12 to 3%, resulting in a simultaneous increase in the forms lacking one chain and the properly glycosylated. Our work also indicates, that applying a simple method of biochemical analysis such as SDS-PAGE/Western-blotting could be helpful in preliminary diagnosis and could improve the identification of congenital disorders of glycosylation.

Key words: glycosylation; congenital disorders of glycosylation; α₁-acid glycoprotein; α₁-antitrypsin; haptoglobin; transferrin; α₂-macroglobulin; ceruloplasmin.

Introduction

Inherited disorders in the glycosylation pathway were reported relatively recently¹², ¹³, ¹⁵. The disease was called carbohydrate-deficient glycoprotein (CDG) syndrome and was diagnosed on the basis of deficiencies in the sugar content of transferrin (Tf) as well as some other serum glycoproteins⁸, ¹¹, ²¹, ²⁷. The reported frequency of the disease in European populations is estimated at 1:40 000 to 1:80 000 newborns²⁵. As the

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amount of information concerning these disorders rapidly increased, it was possible to set down new criteria for differentiating their subtypes and also to improve terminology. Last year the disease was renamed congenital disorders of glycosylation, with the same abbreviation as earlier (CDG). It was also recommended to diagnose the disease identifying mutations causing deficiencies of particular glycosylation enzymes. On the other hand, it was reported recently that most mutations followed by enzyme deficiencies were multiallelic. Moreover, Schollen et al. established that the frequency of the most common mutation causing phosphomannomutase-2 deficiency in CDG-Ia is about 10 times higher than the frequency of the disease, clearly showing a lack of Hardy-Weinberg equilibrium. Genetic tests are still hardly accessible in many health service centers. It seems that, taking into account the current status of the data on CDG-genetics, a biochemical diagnosis of CDG, based on underglycosylation of glycoproteins, should not be neglected.

Another problem under discussion is that, apart from the almost complete deficiency of the key enzyme participating in the common glycosylation pathway, the affected glycoproteins in CDG are still glycosylated to a relatively high extent. It was also found that underglycosylated isoforms of some glycoproteins were found with different frequencies and that they were more frequent in younger than in older patients. The mechanism of such “bypassing” of the defect, although the object of some speculation, is still poorly documented.

In Poland, CDG was diagnosed for the first time in two related patients (siblings) at the Medical Academy in Bialystok. Previously, we examined the glycosylation pattern of haptoglobin (Hp) in one of these patients in detail. The glycosylation of glycoporin was also studied. Although one more case of CDG-I was reported in our country, comparison with the suggested frequency of the disease clearly indicates that congenital disorders of glycosylation are probably still under-diagnosed.

This report focuses on a comparative analysis of the content of underglycosylated isoforms in six serum glycoproteins (Tf, α1-acid glycoprotein (AGP), Hp, α1-antitrypsin (α1-AT), α2-macroglobulin (α2-MG) and ceruloplasmin (Cpl)) from two blood samples of a CDG-I patient taken one year apart. Another point of interest was to estimate whether this pattern changed with the patient’s age. We also hope that the simple method of Western-blotting analysis can find easy application and provide clear interpretation of glycosylation patterns in the preliminary diagnostics of CDG.

### Materials and Methods

**Serum samples.** Two serum samples were taken from a girl suffering from the CDG-I. The patient was 14 years old at the moment of the first blood collection. The second sample was taken one year later.

Control sera were obtained from healthy volunteers with the normal range of typical blood assays.

The serum samples were stored at -20°C before analysis.

**Estimation of serum glycoprotein concentrations.** The concentrations of the serum glycoproteins were estimated by Laurell’s rocket immunoelectrophoresis method, using monospecific polyclonal antibodies. The assay was performed in 1% agarose gel containing commercial (Sigma Biochemicals) goat antibodies against α2-MG, Cpl, Tf and α1-AT. Goat antibodies against Hp and AGP were obtained by us in collaboration with Dr T. Stefaníak of the Department of Prevention and Veterinary Immunology, Agricultural Academy of Wrocław.

**SDS-PAGE and Western-blotting.** Polyacrylamide gel electrophoresis was performed according to the Laemmli method. The gel contained 0.1% SDS. Gel concentrations were adapted to the molecular sizes of the analyzed glycoproteins and were as follows: 12.5% for AGP and Hp, 10% for α1-AT and Tf, and 5% for α2-MG and Cpl. Serum samples were denaturated by 5 min heating at 100°C with 2.5% of SDS and 5% of β-mercaptoethanol. An LMW calibration kit (Sigma) was used for the molecular weight determinations of AGP, Hp, α1-AT and Tf. The molecular masses of α2-MG and Cpl were compared with pure commercial glycoproteins (Sigma). Gels were transferred after electrophoresis onto nitrocellulose membranes according to Towbin et al. Transfer was carried out for 1.5 h at 120 mA.

**Immunodetection.** After transfer, the nitrocellulose sheets were blocked overnight with 0.5% Tween 20 in 25 mmol/l Tris-buffered saline (TBS), pH 7.4. After washing out the excess blocking reagent with TBS containing 0.1% Tween 20 (TBS-T-0.1%), the membranes were incubated with goat antibodies appropriate for the detection of the particular glycoproteins for 1 h at 37°C. The dilutions of the commercial antibodies were as follows; anti-α1-AT 1:4000, anti-Tf 1:3000, anti-α2-MG 1:1000 and anti-Cpl 1:1000. After incubation, the nitrocellulose membranes were washed thoroughly with TBS-T-0.1%, and soaked with anti-goat-peroxidase-labeled conjugate (Sigma) diluted 1:10 000 with the same buffer. After one hour of incubation at 37°C and washing out the reagents, the peroxidase reaction
was developed using H₂O₂ and dianaminobenzidine as the substrates (12 μl of 30% solution and 6 mg, respectively, per 10 ml of 100 mmol/l citrate buffer, pH 6.0).

**Densitometric analysis.** Blots were scanned by videocamera (BioRad GelDoc). GelScan (Kucharczyk Electrophoretical Techniques) software was applied for densitometric analysis and molecular mass determination. BioRad GelDoc software was used for the calculation of the relative content of particular glycoforms. The volume analysis option was applied to calculate the content of protein on the basis of staining intensity and the area of the band.

**Results**

**Concentrations of serum glycoproteins**

The concentrations of the examined glycoproteins in the serum samples of the CDG patient are shown in Table 1. The concentrations of α1-AT and α2-MG in both samples of patient serum were, though low, within the range of the respective norm values for adult persons. The level of Cpl was slightly below the minimum values considered as normal. The concentrations of 3 out of 6 glycoproteins examined, i.e. AGP, Hp and Tf, were prominently decreased; for Tf it was about 4 times lower than the average value accepted for healthy people. Except of AGP, there were no substantial differences in the levels of the analyzed glycoproteins after one year from the first examination. The concentration of AGP increased during the year from 0.39 to 0.66 g/l, approaching normal values.

**Electrophoretic patterns**

The electrophoretic patterns of all the examined glycoproteins in the serum of the CDG patient and the control sera as well as their densitograms are shown in Fig. 1. The glycoprotein patterns of the patient differed markedly from those of the control samples. All the glycoproteins in normal serum showed the typical pattern of a single band when stained with specific antibodies. Altered patterns were observed in glycoproteins derived from the patient, i.e. in Tf, AGP and Hp, three distinct glycoforms were observed in both CDG serum samples, differing in molecular mass. This pattern was clearly reflected in the three distinct peaks in their densitograms (Fig. 1A, B, D). In α1-AT, three bands were also observed, although they were not distinctly separated from each other (Fig. 1C). In the high molecular mass glycoproteins, α2-MG and Cpl, additional glycoforms were not observed, although the bands were smeared over a larger area (Fig. 1E, F), suggesting that fractions of lower molecular mass could also be present in both of the glycoproteins derived from the CDG-I patient.

**Quantitative analysis of the glycoform content**

The differences between the molecular masses calculated for normal and disease-related fractions of glycoproteins varied from 2.4 to 3.5 kDa, suggesting the lack of one, probably bi- or triantennary glycan.

The relative contents of the particular glycoforms were calculated by the volume analysis option of GelDoc BioRad System. The results are shown in Table 2.

**Table 1. The concentrations of glycoproteins in CDG patient serum**

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Normal range [g/l]¹²</th>
<th>CDG(1) [g/l]</th>
<th>CDG(2) [g/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Acid glycoprotein</td>
<td>0.70–1.30</td>
<td>0.39</td>
<td>0.66</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>0.90–2.10</td>
<td>1.26</td>
<td>1.18</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>0.95–1.95</td>
<td>0.36</td>
<td>0.42</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>1.70–4.10*</td>
<td>1.72</td>
<td>1.75</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>0.15–0.55</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2.00–3.00</td>
<td>0.70</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Women.

**Table 2. The relative content of glycoforms in glycoproteins in CDG patient serum**

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>AGP</th>
<th>Hp</th>
<th>α1-AT</th>
<th>Tf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>CDG(1)</td>
<td>CDG(2)</td>
<td>CDG(1)</td>
<td>CDG(2)</td>
</tr>
<tr>
<td>Lacking 1</td>
<td>50</td>
<td>62</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Lacking 2 chains</td>
<td>37</td>
<td>35</td>
<td>32</td>
<td>26</td>
</tr>
<tr>
<td>Lacking 3 chains</td>
<td>12</td>
<td>3</td>
<td>21</td>
<td>27</td>
</tr>
</tbody>
</table>

1⁰ Women.
The amount of the completely glycosylated form comprised about 50% for Hp, α1-AT and the first sample of AGP. Transferrin seemed to be affected to a higher extent, as only in 30–35% of molecules the occupation of glycosylation sites was complete. The amount of forms lacking two oligosaccharide chains was also greater in this glycoprotein and reached 34–38%, versus 17–19% in α1-AT, 21–27% in Hp and 12% in the first sample of AGP. In all four glycoproteins the content of the second isoform varied from 26 to 37%.

Only in AGP the difference between both samples was clearly visible. The amount of the fully glycosylated form in the serum sample taken after the one-year interval increased from 50 to above 60%, mainly at the cost of the most affected, lacking 2 glycans form which decreased to 3%. In the three remaining glycoproteins the differences in the content of the particular glycoforms between both samples did not exceed a few percent.

Discussion

In congenital disorders of glycosylation, detailed analysis of the glycan structures have, mainly concerned transferrin, as this glycoprotein is used for the biochemical diagnosis of the disease. Ohno et al. were the first authors who detected glycoforms lacking sialic acid in a glycoprotein other than Tf, namely α1-AT. Moreover, abnormal electrophoretical patterns of AGP, Hp and α1-AT were shown by Seta et al. These authors focused their interest on the possibility of Western-blot application for CDG diagnosis, but they did not compare the glycosylation abnormalities of the glycoproteins analyzed. Our earlier reports concerning glycosylation defects in Hp isolated from CDG patient serum and they represented the first attempt to quantify the relative contents of incompletely glycosylated forms and the structures of glycans in a glycoprotein other than Tf.

In this work we have focused on the comparative analysis of the relative contents of disease-related glycoforms in six glycoproteins over a one-year interval. Our approach enabled quantification of the percentage of the particular glycoforms lacking one and two glycan chains. The glycoproteins chosen for analysis differed in molecular mass (from 40 kDa for the Hp β-subunit to 179 kDa for the α2-MG subunit) as well as in the relative content of carbohydrate: from 8% in α2-MG to over 40% in AGP. The number of glycan units attached to one polypeptide chain varied from 2 in Tf to 5 in AGP. We aimed to ascertain if the different glycoproteins are affected with carbohydrate deficiency to the same extent. Such data should help to answer the question if the “bypassing” glycosylation mechanisms are in any way related to the properties of the protein or the accessibility of its glycosylation sites.

Though the glycoproteins examined in this study the differed in their number of glycosylation sites, no glycoforms lacking more than two oligosaccharide chains were detected. In 3 out of 4 glycoproteins, half of their glycoforms were properly glycosylated. Some differences in the degree of underglycosylation could be clearly observed in these glycoproteins. Our data confirm that Tf in the serum of the CDG patient is defective to a higher extent than the other glycoproteins studied. In Tf, the lowest percentage of properly glycosylated molecules was present as well as the highest amount of the form devoid of both glycan units. On the contrary, AGP, the glycoprotein with the highest relative content of carbohydrates and also the number of glycans, showed the lowest content of the form lacking two glycans, so it seemed to be affected rather weakly. It is also worth noting, that the electrophoretic pattern of Hp and α1-AT of our patient differed slightly from those presented by Seta et al.. These authors have shown only two glycoforms present in α1-AT versus the three detected by us. Moreover, in AGP analyzed by Seta et al. the glycoform devoid of one glycan seemed to be predominant, whereas it comprises only 35–37% in the serum of our patient. These differences suggest that the level of underglycosylation varies not only among particular glycoproteins, but also among patients. A question arises as to whether this feature may also be reflected in the variable pattern of clinical symptoms.

The first comparative analysis of the content of underglycosylated glycoforms in serum glycoproteins of CDG patients was reported by Stibler et al.. The authors reported on the frequencies of the occurrence of glycan-deficient forms in glycoproteins of 48 patients. Apart from Tf, other glycoproteins were examined: α1-AT, antithrombin and tyroline-binding glycoprotein. According to Stibler et al., glycoforms devoid of one or two glycan units were found in all patients, and the occurrence of isoforms affected to a higher extent was substantially less frequent. In any case, asialo-tyroxine binding protein and asialo-antithrombin occurred in almost 20% of patients. Although that report may suggest some kind of correlation between the level of underglycosylation and patient age, so far there has been no direct observation of glycosylation changes in the serum of a particular patient over time. Such an attempt was a goal in our work. AGP
was found to be the only glycoprotein in which the pattern of glycoforms after a one-year interval was altered, approaching normal, parallel with an increase in the serum concentration of the glycoprotein. According to Stibler at al.26, the concentrations of some glycoproteins in the sera of children suffering from CDG were decreased, but in the patients over 15 years of age they became normal. In our patient, 14 and 15 years old at the times of blood collection, the concentrations of the examined glycoproteins were either below or in the lower range of the values considered as normal. Apart from AGP, we did not find this difference in glycoprotein concentrations in both samples, so the alterations in the pattern as well as concentration of this glycoprotein suggest that it could probably be applied for monitoring glycosylation defect in the course of the disease.

In conclusion, we can underline that the electrophoretic patterns observed in 4 out of 6 proteins examined undoubtedly showed defective glycosylation. Moreover, for the glycoproteins of lower molecular mass, i.e., Hp and AGP, this pattern was unequivocally indicative of CDG-I. This resulted from the fact that the ratio of the molecular mass of deleted glycans versus the molecular mass of the whole glycoprotein is higher and is reflected in the larger shift of the band. In the glycoproteins of large molecules, i.e., α-MG and Cpl, the mass ratio is low, resulting in a worse separation of the underglycosylated forms.

In spite of the development of genetic examinations, analysis of underglycosylated glycoforms of serum glycoproteins is still being applied for CDG diagnosis with good results2. As reported earlier by Seta et al.25, our data also indicate that Western-blotting is a very helpful technique for the preliminary diagnostics of CDG-I. Moreover, taking into consideration that the disease still seems to be underdiagnosed, such analysis is worth wider application. In this case, lower molecular biochemical markers, such as Hp or AGP, could be applied apart from Tf.

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