Serum Level of Sialic Acid (SA) and Carbohydrate-Deficient Transferrin (CDT) in Type 2 Diabetes Mellitus With Microvascular Complications

Bogdan Cylwik,^{1*} Lech Chrostek,¹ Barbara Jakimiuk,¹ Anna Popławska,² and Maciej Szmitkowski¹

¹Department of Biochemical Diagnostics, Medical University, Białystok, Poland

²Department of Endocrinology, Medical University, Białystok, Poland

Sialic acid (SA) is responsible for the composition of different isoforms of transferrin and is reported to be a marker of microvascular complications in type 2 diabetes mellitus. Therefore, we explored the serum concentration of SA, and the less sialylated isoforms of transferrin, termed carbohydrate-deficient transferrin (CDT), in relation to the presence of microvascular complications in type 2 diabetes mellitus. We studied 21 patients with type 2 diabetes with microangiopathy and 22 patients without complications who were hospitalized at a diabetic clinic. The prevalence of microvascular complications was based on clinical history, funduscopy, and laboratory tests. Blood samples were taken for measurements of SA, CDT, total transferrin, glucose, HbA_{1c}, fibrinogen, C-reactive pro-

tein (CRP), and indicators of renal dysfunction (i.e., creatinine, urea, albumin excretion rate (AER), and glomerular filtration rate (GFR)). A rise in serum SA and a decrease in CDT concentrations were observed in both diabetic groups with and without complications, and there were no differences between the two groups of patients. There was a statistically significant correlation between serum SA and CDT in diabetic subjects with microvascular complications, but not in patients without such complications. This proves that the serum changes in CDT and SA levels in the course of type 2 diabetes mellitus are associated with each other in the presence of microangiopathy. *J. Clin. Lab. Anal.* 20:68–73, 2006. © 2006 Wiley-Liss, Inc.

Key words: carbohydrate-deficient transferrin; sialic acid; diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus is a chronic disease that is associated with the development of long-term complications. These complications generally are divided into two categories: macrovascular (e.g., cardiovascular disease) and microvascular (e.g., diabetic retinopathy, nephropathy, and neuropathy). One of the main metabolic factors that contributes to the development of microangiopathy is hyperglycemia, which leads to increased nonenzymatic glycosylation (glycation) of many proteins and the formation of advanced glycation end products (AGEs). Nonenzymatic glycosylation alters the properties or functions of several proteins (1). The second type of glycosylation is a physiological enzymatic process in which other carbohydrates (i.e., galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid (SA)) take part (2). Changes of glycosylation of various

proteins in diabetic patients have been observed by many authors (3,4). The majority of glycosylated proteins (glycoproteins) have attached SA residues in the carbohydrate chain (2). One of these glycoproteins is transferrin, the main iron transporter in human serum (5). Transferrin is also a negative acute-phase response protein. The isoforms of transferrin without SA and with one or two SA residues are known as desialylated transferrin, or carbohydrate-deficient transferrin (CDT) (6).

Serum SA has been reported to be elevated in the course of various diseases, such as inflammatory

*Correspondence to: B. Cylwik, Department of Biochemical Diagnostics, Medical University, Białystok, Washingtona 15A, 15-269 Białystok, Poland. E-mail: zdb@amb.edu.pl

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disorders, cardiovascular diseases, tumors, alcohol abuse, and diabetes mellitus (7–11). Raised serum SA concentrations have been observed in both type 1 (12,13) and type 2 diabetes mellitus (14,15). In one study, in type 1 the serum SA was higher in the group with microalbuminuria compared to that with normal albuminuria, and higher in the group with clinical proteinuria compared to that with microalbuminuria (12). Elevated serum SA levels precede the onset of microalbuminuria in patients with type 1 diabetes mellitus (16). In a multiple regression analysis, plasma SA was independently related to the urinary albumin excretion rate (AER) (13). Serum and urinary SA is also elevated in patients with type 2 diabetes mellitus (15). Elevated levels of SA are associated with markers of inflammation such as C-reactive protein (CRP), fibrinogen, and orosomucoid (8). Chen and co-workers (14) showed a progressive rise in serum SA with increasing AER in type 2 diabetes mellitus, but not with retinopathy or neuropathy.

SA is reported to be a marker of microvascular complications in type 2 diabetic patients (17) and is responsible for the composition of transferrin. The aim of this study, therefore, was to explore the levels of carbohydrate-deficient isoforms of transferrin (CDT) and SA in the serum of diabetic patients in relation to the presence of microvascular complications.

MATERIALS AND METHODS

The study was carried out in 43 patients (32 females and 11 males) with type 2 diabetes mellitus who were hospitalized at a diabetic clinic, and 26 normal individuals (18 females and eight males). The diabetic patients were divided into two groups: 21 patients

(16 females and five males) with microangiopathy, and 22 patients (16 females and six males) without any microvascular complications. Among the patients with microangiopathy, eight had retinopathy, nine had nephropathy, and four had both complications. In this group, 10 patients had a normal AER (normal albuminuria), 10 patients had microalbuminuria, and one patient had macroalbuminuria. The median age of the diabetic patients with microangiopathy was 61 years (range = 29–85 years), compared to 56 years (range = 40–80 years) for the group without complications and 43 years (range = 28–60 years) for the normal subjects. The median duration of diabetes mellitus was 15 years (range = 2–33 years). The patients were treated with oral antidiabetic agents, insulin, diet, and combined therapies. The characteristics of these subjects are presented in Table 1. Diabetes mellitus was diagnosed according to the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (18). Retinopathy was assessed by fundoscopy. Renal function was evaluated by AER, glomerular filtration rate (GFR), serum creatinine, and urea concentrations. The diabetic and control patients did not have any history of heavy alcohol abuse, and self-reported an intake of less than 50 g ethanol per week.

Blood samples for biochemical investigations were obtained from a peripheral vein after the subjects fasted for 12 hr. The serum was separated by centrifugation at $1,500 \times g$ for 10 min at room temperature, collected, and then stored at -86°C until it was assayed. Besides serum, a portion of each blood sample was collected into tubes containing anticoagulants (sodium citrate) for measurement of fibrinogen and ethylene-diamine tetraacetic acid (EDTA-2K) for determination of glycated hemoglobin (HbA_{1c}).

TABLE 1. Clinical characteristics of control and diabetic subjects with and without microvascular complications[†]

Variable	Control subjects	Diabetic patients without microvascular complications	Diabetic patients with microvascular complications (retinopathy, nephropathy)
No. of patients	26	22	21
Gender (F/M)	18/8	16/6	16/5
Age (years)	43 (28–60)	56 (40–80)	61 (29–85)
Diabetic duration (years)	–	2 (1–13)	15 (2–33)
Treatment			
Diet	–	8	0
Oral antidiabetic agents	–	12	4
Insulin	–	2	11
Combined therapy	–	0	6
Glucose (mmol/L)	4.95 (3.63–5.67)	6.22 (3.13–14.36)	8.06* (4.65–17.02)
HbA _{1c} (%)	5.4 (4.9–6.4)	7.8* (5.8–11.8)	9.0* (4.8–11.3)

[†]All results are presented as median and ranges.

* $P < 0.05$ when compared to the control subjects (Mann-Whitney U test).

CDT (as %CDT) was measured by immunoturbidimetric assay (Tina-quant %CDT second-generation test, Roche, Mannheim, Germany) on a Hitachi 912 analyzer (Roche) after a separation step by anion-exchange chromatography. Before column separation was performed, the total transferrin was determined. This test evaluated the percentage of CDT relative to the total transferrin concentration according to the following equation: $\%CDT = 325 \times CDT \text{ (mg/L)} / \text{total transferrin (mg/L)} - 0.1$. The measurement range of transferrin was 1.0–24 mg/L. The expected values of %CDT in the social-drinking control population were <2.6 (95th percentile).

Total plasma SA was assayed using an enzymatic method adapted for use on the Konelab 30i analyzer (Thermo Clinical Labsystems, Vantaa, Finland) with commercial kits (Sialic Acid Test-Combination, Roche, Mannheim, Germany). The reaction includes hydrolysis of glycoprotein by neuraminidase, cleavage of SA to pyruvate by N-acetyl neuraminic acid aldolase, oxidation pyruvate by pyruvate oxidase, and peroxidation of 4-aminoantipyrine to a red dye. The normal range is 0.58–0.79 g/L, and the mean value is 0.69 g/L.

Glycated hemoglobin HbA_{1c} was assayed using a turbidimetric immunoinhibition method with an automatic biochemistry analyzer (Synchron CX 4 Delta; Beckman-Coulter, Fullerton, CA) with commercial kits (Hemoglobin A_{1c} reagent; Beckman-Coulter, Fullerton, CA). In this reaction, hemoglobin A_{1c} antibodies combine with hemoglobin A_{1c} from the sample (hemolysate) and form soluble antigen-antibody complexes. Polyhaptenes from the reagent then bind with the excess antibodies and the resulting agglutinated complex is measured turbidimetrically at 340 nm. The final result was expressed as %HbA_{1c} using the special formula. Expected values are in the range of 4.4–6.4%. The measurement range is 2–16%. The within-run precision is (%CV) 1.1–1.4, and the between-run precision is (%CV) 1.6–1.8.

AER was determined by an immunoturbidimetric method using goat anti-human albumin antiserum (albumin/microalbuminuria kit; Thermo Clinical Labsystems, Vantaa, Finland) on a timed 24-hr urine collection on the Konelab 30i analyzer. Microalbuminuria was defined as a urinary AER of 20–200 µg/min (30–300 mg/24 hr). Normalalbuminuria was defined as a urinary protein excretion rate of 7–20 µg/min (10–30 mg/24 hr). Macroalbuminuria was defined as AER above 200 µg/min (>300 mg/24 hr). The measurement range is 8–152 mg/L. The within-run precision is (%CV) 1.7 and the between-run precision is (%CV) 5.0.

The other biochemical tests were performed using the following routine methods from BioMerieux (Mercy l'Étoile, France): glucose ((glucose oxidase/peroxidase,

glucose RTU kit, expected values: 4.1–6.1 mmol/L, measurement range: 0.07–22.2 mmol/L, within-run precision (%CV): 0.75–0.92, between-run precision (%CV): 1.54–2.44), urea (urease/glutamate dehydrogenase, Uree Cinetique UV kit, expected values: 1.70–8.33 mmol/L, measurement range: up to 50 mmol/L, within-run precision (%CV): 2.14–3.97, between-run precision (%CV): 2.27–3.34, creatinine (Jaffe kinetic method, Creatinine Cinetique kit, expected values: women: 53–80 µmol/L, men: 62–97 µmol/L, measurement range: 10–800 µmol/L, within-run precision (%CV): 1.1–1.9, between-run precision (%CV): 1.4–2.5) on the Synchron CX 4 Delta analyzer (Beckman-Coulter). The GFR was calculated from the standard $U \times V/P$ formula, where V = urine flow rate, U = urine creatinine concentration, and P = plasma creatinine concentration. The expected values for GFR are 80–200 mL/min.

The quantitative determination of fibrinogen was carried out in citrated plasma with a modified clot-rate assay using the STA Fibrinogen kit (Roche, Mannheim, Germany) and Diagnostica STAGO ST4 analyzer (Roche, Mannheim, Germany). Expected values for adult patients are in the range of 2.0–4.0 g/L. The measurement range on STAGO analyzers is 1.5–8.0 g/L. The within-run precision is (%CV) 2.3–3.3 and the between-run precision is (%CV) 2.0–3.8.

CRP was measured using the commercial kit from Beckman Coulter GmbH (Krefeld, Germany; expected values: <10.0 mg/L; measurement range from value of calibrator 1: 3.0 to the value of calibrator 5: 199.3, within-run precision: %CV: 5.0) in a Synchron CX 4 Delta analyzer (Beckman-Coulter, Fullerton, CA). The CRP status was divided into three groups according to the following concentrations: group I <3 mg/L, group II 3–10 mg/L, and group III >10 mg/L. The categorical variable (CRP) was analyzed using a χ^2 test.

The results are expressed as the median and ranges. Differences between diabetic groups and control subjects were evaluated using the Mann-Whitney U-test, and differences between the diabetic patients with and without complications were assessed by an analysis of variance (ANOVA) rank Kruskal-Wallis test. Spearman's rank correlation coefficient was used to calculate the correlation between different variables. The differences were considered statistically significant at $P < 0.05$.

RESULTS

The median serum SA level was significantly higher in type 2 diabetic patients with microvascular complications (retinopathy, nephropathy) compared to the control subjects ($P = 0.036$; Table 2). The level of SA in diabetic patients without complications was also significantly higher in comparison to the control subjects

TABLE 2. Carbohydrate-deficient transferrin (CDT), sialic acid (SA), and indicators of kidney dysfunction in relation to presence or absence of microvascular complications in type 2 diabetic patients[†]

Patients	%CDT	SA (g/L)	Transferrin (g/L)	Fibrinogen (g/L)	Creatinine (μmol/L)	Urea (mmol/L)	GFR (mL/min)	AER (mg/24 hr)
Diabetic patients with microvascular complications (retinopathy, nephropathy)	1.59* 0.68–3.88	0.69* 0.32–1.15	2.46* 1.74–3.36	3.91* 2.73–6.62	90.26* 58.40–223.88	8.83* 4.50–36.65	69.4* 20.7–221.4	30.0 3.5–610.5
Diabetic patients without microvascular complications	1.51* 1.05–2.22	0.70* 0.53–0.91	2.52* 2.16–3.54	4.07* 1.96–6.83	79.64 61.06–129.19	6.16 3.33–8.33	80.5* 49.5–100	25.3 8.0–65.8
Control Subjects	1.82 1.31–2.52	0.60 0.42–0.79	2.94 2.28–3.60	3.15 2.36–3.93	79.64 57.52–116.81	5.50 3.49–7.83	96.5 80.3–148.7	10.5 7.0–27.0

[†]All results are presented as medians and ranges.

* $P < 0.05$ when compared to the control subjects (Mann-Whitney U test).

($P = 0.004$). When SA concentrations were compared between the diabetic patients with and without microangiopathy, there was no significant difference ($P = 0.858$).

The median serum %CDT was significantly lower in type 2 diabetic patients with microvascular complications compared to the control subjects ($P = 0.018$). The median serum %CDT in diabetic patients without complications was also significantly different from that of the control individuals ($P = 0.005$), but no significant differences were found between the diabetic patients with and without microangiopathy ($P = 0.721$).

The level of transferrin was significantly lower in diabetes with ($P < 0.001$) and without ($P = 0.036$) complications when compared with the control subjects. There was no significant difference in transferrin concentrations between the diabetic patients with and without microangiopathy ($P = 0.331$). Of the indicators of kidney dysfunction, only GFR levels were significantly decreased in diabetic patients with and without complications ($P = 0.022$, $P = 0.002$, respectively) compared to control subjects. The concentration of serum urea was significantly increased in diabetic patients with complications ($P = 0.000$) compared to controls. Statistically significant differences in urea levels between two diabetic groups were observed ($P = 0.005$). No significant differences were found in serum creatinine levels among all tested groups. The urinary excretion rate was significantly higher in diabetic patients with and without complications ($P = 0.003$, $P = 0.046$, respectively) compared to controls. The levels of fibrinogen were significantly increased in two diabetic groups ($P = 0.000$, $P = 0.003$, respectively). Higher values of CRP were found in both groups of diabetic

patients compared to controls ($P < 0.05$), but there were no significant differences between diabetic groups ($P = 0.672$).

The glucose concentration was significantly higher in diabetic patients with microangiopathy than in the control subjects ($P = 0.000$), but did not differ in comparison to the individuals without complications ($P = 0.117$). There were no significant differences in serum glucose levels for patients with uncomplicated diabetes mellitus vs. controls ($P = 0.107$). HbA_{1c} concentrations were significantly higher in both diabetic groups ($P = 0.000$) compared to controls, but there were no significant differences between the diabetic groups ($P = 0.258$).

The serum %CDT negatively correlated with the level of SA ($R = -0.449$) in diabetic patients with microvascular complications (Table 3). There were no statistically significant correlations between serum %CDT and glucose, HbA_{1c}, and the indicators of kidney dysfunction (creatinine, urea, and GFR) in patients with complications. The serum %CDT also did not correlate with the serum fibrinogen and urinary albumin excretion in diabetic patients with microangiopathy. The serum SA concentration significantly positively correlated with the serum fibrinogen level ($R = 0.735$) in diabetic subjects with complications. The significantly positive correlation was observed between serum SA and HbA_{1c} concentrations in diabetic patients without microvascular complications ($R = 0.642$).

In diabetic subjects with complications, serum total transferrin positively correlated with glucose ($R = 0.470$) and HbA_{1c} ($R = 0.454$).

TABLE 3. Correlation of carbohydrate-deficient transferrin (CDT) and sialic acid (SA) with biochemical variables in type 2 diabetes mellitus with and without microvascular complications

	SA	Transferrin	Fibrinogen	Creatinine	Urea	GFR	AER	Glucose	HbA _{1c}	Duration
CDT										
MIC+	-0.449*	0.542*	-0.337	0.013	-0.021	0.302	-0.184	0.394	0.340	-0.377
MIC-	0.083	0.164	0.454	0.445	-0.054	0.200	-0.009	0.259	0.223	-0.498
SA										
MIC+		0.109	0.735*	0.234	0.418	-0.125	-0.079	-0.143	0.173	0.245
MIC-		0.132	0.509	-0.118	-0.164	0.500	0.248	0.278	0.642*	-0.048

* $P < 0.05$.

MIC+, presence of microvascular complications; MIC-, absence of microvascular complications.

DISCUSSION

The purpose of this study was to explore the levels of SA and CDT in the serum of diabetic patients in relation to the presence of microvascular complications. Previous studies have reported a structural and metabolic association between CDT and SA (5), and that both are markers of acute-phase response (10).

We have shown that the serum SA level was significantly elevated in the diabetic patients with and without microvascular complications. No significant difference was found in the serum SA level between the two diabetic groups. A progressive rise in the serum SA level has been observed with increasing urinary AER in patients with normalalbuminuria, microalbuminuria, and diabetic nephropathy (14). There are several possible mechanisms that might explain these results. One is the increased sialylation of total blood glycoproteins, and another is the increased amount of positive acute-phase proteins (e.g., α_1 -acid glycoprotein, α_1 -antitrypsin, haptoglobin, and fibrinogen). In this study we found that serum fibrinogen and CRP concentrations were significantly elevated in both diabetic groups (with and without microvascular complications) compared to controls. This is in agreement with previous results (8,19). In our study the serum fibrinogen concentration showed a significant positive correlation ($R = 0.735$) with the serum SA level in diabetic patients with microangiopathy ($P < 0.001$). A strong connection between SA and fibrinogen has also been reported by other authors ($R = 0.800$) (8,20), and SA has been shown to be a good marker for acute-phase response (21). The level of serum fibrinogen did not differ between diabetic groups. The second positive acute-phase protein determined in this study was CRP. The increase of this inflammation marker did not influence the SA value because it is a nonsialylated acute-phase protein. Another protein, transferrin (a negative acute-phase reactant), showed a significantly decreased serum concentration in the diabetic patients (with or without

microangiopathy) compared to controls. The serum total transferrin did not differ between the groups with and without complications.

The second tested parameter in this study was CDT. This test is widely used to diagnose chronic alcohol consumption (6). In our study the serum %CDT was significantly decreased in the diabetic subjects compared to the controls ($P < 0.05$). There was no significant difference in serum %CDT between the patients with and without complications. Thus, we conclude that decreased serum levels of %CDT are not associated with the presence of microangiopathy in type 2 diabetic patients. The decrease in serum %CDT was accompanied by an increase in serum SA concentrations (confirmed by the correlation study). We also found that the serum SA concentration was significantly associated with the parameter of glycemic control (HbA_{1c}) in diabetic subjects without microangiopathy, but was not significantly related to diabetes mellitus duration. In the group of diabetic patients with microangiopathy, the common indicators of kidney failure (e.g., urinary AER, GFR, serum creatinine, and urea concentration) were significantly changed compared to those of control subjects. There was no significant relationship between serum SA and the indicators of kidney failure in patients with microangiopathy. Previous studies have shown a progressive rise in serum SA with increasing urinary AER in type 2 diabetic patients (15). The reduction of GFR may also lead to an increase of serum SA levels.

The changes in desialylated transferrin concentrations may be caused by the difference in the activities of enzymes operating in processes of sialylation (glycosyltransferase) and desialylation (sialidase) of glycoproteins in alcohol abuse (22). In animal studies of diabetes, decreased sialyltransferase and increased sialidase activity were observed (23,24). It has been reported that advanced glycosylation end products (AGEs) may affect the activities of enzymes involved in galactose, fucose, and SA metabolism (i.e., glycosyltransferases (synthesis)

and glycosidases (catabolism)) and modify retinal microvascular cell glycoproteins in vitro and in vivo conditions (25). It is likely that disturbances in the sialylation of serum transferrin are partly related to the concentration of AGEs. Evidence for disturbances in the processes of sialylation was obtained in the analysis of correlation between serum total transferrin and glucose status (actual and retrospective). In diabetes mellitus with microvascular complications, the serum total transferrin positively correlated with the actual ($R = 0.470$) and retrospective ($R = 0.454$) glycemia. However, there was no relation between glycemic status and serum isoforms of transferrin (CDT).

In conclusion, the serum changes in %CDT and SA levels did not differ between the groups of diabetic patients with and without microvascular complications. The association between serum CDT and SA concentrations existed only in the group with diabetes mellitus with microangiopathy. The presence of long-term complications may affect the processes of sialylation and desialylation of transferrin, and consequently affect the serum levels of CDT and SA.

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