Serum N-Acetyl- β -D-Glucosaminidase Profiles in Type 1 Diabetes Secondary Complications: Causes of Changes and Significance of Determination

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The connection between changes in the activity of serum N-acetyl- β -D-glucosaminidase (NAG, E.C.3.2.1.30) and iso-enzymes and degree of secondary complications was analyzed in four groups of type 1 diabetic patients ($n=69$): without complications ($n=22$); with retinopathy ($n=16$); with retinopathy and polyneuropathy ($n=13$), and with retinopathy, neuropathy, and nephropathy ($n=18$). In all groups statistically significant higher ($P<0.001$) percent fraction of A form (83.84 ± 6.09 , 84.37 ± 5.74 , 81.76 ± 6.02 , $76.37\pm7.38\%$, resp.) and lower ($P<0.001$, $P<0.01$) fraction of B form (15.87 ± 5.65 , 15.66 ± 5.74 , 18.33 ± 5.98 , 23.63 ± 7.38 , resp.) in total NAG compared with the control ($A=69.38\pm4.79\%$, $B=30.61\pm4.78\%$) were found. The differences in A as well as B forms between diabetic groups were not statistically significant. Significant

strong positive correlations between total NAG and glycemia (0.494–0.623), total NAG and A form (0.934–0.966), and A form and glycemia (0.512–0.638) were found in all groups. No correlation was found between the fractions of B and A forms, except in the fourth group. The A form of diabetic patients in the fourth group was more acidic compared with the control and other diabetic groups. It was concluded that the changes in serum NAG and iso-enzymic profiles in diabetes are the consequence of its increased exocytose, especially of the A form, in hyperglycemia and posttranslational modifications of iso-enzymes. The total activity of serum NAG and iso-enzymic profiles cannot be used for monitoring the development and distinction of type 1 diabetes secondary complications. J. Clin. Lab. Anal. 22:307–313, 2008. ©2008 Wiley-Liss, Inc.

Key words: serum N-acetyl- β -D-glucosaminidase; iso-enzymes A and B; type 1 diabetes; diabetic complications; retinopathy; neuropathy; nephropathy

INTRODUCTION

Increased activity of N-acetyl- β -D-glucosaminidase (NAG, EC 3.2.1.30) in type 1 diabetic patients has been described by many authors (1–3). Poor glycemia control (4), long-term disruption of metabolic equilibrium in diabetes conditions with alleviated release of lysosomal enzymes in the extracellular liquid, which interfere with the mechanisms controlling the half-life of enzymes (5,6), the activation of NAG by glycomaterial accumulated on the walls of blood vessels, i.e., the induction of NAG owing to the degradation of mucopolysaccharides (7,8), were proposed as possible causes of changes in the activity of serum NAG. The proposed causes of the NAG activity changes have not yet been fully explained and proven.

The pathogenesis of diabetic microangiopathy (retinopathy—R, neuropathy—P, and nephropathy—N) is highly complex and not elucidated so far. Diabetic

Abbreviations: NAG, N-acetyl- β -D-glucosaminidase; R, retinopathy; P, neuropathy; N, nephropathy; DBP, diastolic blood pressure; SBP, systolic blood pressure; DEAE, diethylaminoethyl.

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microvascular disease still presents problems of early diagnosis and treatment. Some authors found significant increase of the NAG activity in the secondary diabetic complications (9–11), whereas others (12,13) did not find any difference in total NAG activity between diabetic patients with or without microangiopathy. Changes in iso-enzymic NAG profiles in diabetes (3,9,11) indicated the potential importance of determining the activity of each iso-enzyme in monitoring the development of secondary complications.

The aim of this study was to investigate the relationship between the changes of total serum NAG activity, iso-enzyme forms, degree of secondary complications (R, P, and N) development, and glycemia in type 1 diabetes mellitus. Understanding the causes of changes in the activity of NAG would also contribute to better comprehend the significance of NAG determination in clinical practice.

SUBJECTS, MATERIALS, AND METHODS

Subjects

Sixty-nine type 1 diabetic patients, who were hospitalized owing to poor metabolic control, were selected for this study. The diabetic patients were divided into four groups according to secondary complications. There were 22 patients without complications in the first group. The second group consisted of patients ($n = 16$) with retinopathy. In the third group there were 13 patients with retinopathy and neuropathy. The fourth group consisted of patients ($n = 18$) with retinopathy, neuropathy, and nephropathy. The existence of vascular complications was evaluated by one or more of the following parameters: retinopathy by ophthalmoscopic evidence and fluorescent angiography of microaneurysms, hemorrhages, exudates, new vessels; nephropathy by the determination of microalbuminuria—persistent microalbuminuria was defined as urinary albumin excretion rates between 30 and 300 mg/24 hr, in at least two of the three consecutive samples; neuropathy by evaluation of the motor nerve conduction velocity of the peroneal nerve (electroneurography). Hypertension was diagnosed, and antihypertensive medication was prescribed if the mean of the last three measurements was 135/85 mmHg. Patients with ascertained or suspected diseases other than diabetes and the diabetic patients with macroangiopathy complications were not included in the study.

A control group consisted of 25 healthy volunteers of appropriate age and sex.

Informed consent was obtained from all persons participating in this study. The investigations were performed in accordance with the principles of the Declaration of Helsinki.

Chemicals and Instrumentation

Synthetic substrate 2-methoxy-4-(2'-nitrovinyl)-phenyl-*N*-acetyl- β -D-glucosaminide (MNP-NAGlc) was used to determine NAG activity (14). The required chemicals (sodium dihydrogen phosphate monohydrate, sodium hydrogen phosphate dihydrate, sodium chloride, sodium carbonate, sodium bicarbonate, sodium hydroxide, citric acid) were purchased from Merck GmbH (Darmstadt, Germany), whereas diethylaminoethyl (DEAE) cellulose was obtained from Sigma Aldrich, Chemie GmbH (Steinheim, Germany). Spectrophotometric measurements were performed using a Beckman DU-50 spectrophotometer (Beckman, Fullerton, CA).

Serum Samples

Blood samples of healthy subjects and diabetic patients for biochemical analysis and determination of the total NAG activity, and its iso-enzyme profiles, were collected after an overnight fast between 07:00 and 07:30 hr. The determination of NAG activity in fresh samples was performed in triplicate.

Methods

The activity of NAG was assayed by adding 100 μ l of serum sample to 1,000 μ l of 1.5 mol/l substrate solution and 500 μ l of 0.1 mol/l citrate buffer, pH 4.5. After 30 min at 37°C the enzyme reaction was stopped by adding 500 μ l of 0.1 mol/l carbonate buffer, pH 9.5, and the absorbance read at 505 nm. Reagent blanks were performed for each determination. The calibration curve was recorded with 2-methoxy-4-(2'-nitrovinyl)-phenol as the standard in the concentration range 0.025–0.200 mmol/l ($y = 1.275x - 0.00089$, $r = 0.9996$). The unit of enzymatic activity (U) is defined as the amount of enzymes that hydrolyzes 1 μ mol of substrate in one minute at 37°C ($U/l = \mu\text{mol}/\text{min}/l$).

Fasting plasma glucose was measured by a glucose oxidase method (test purchased from ELITECH Diagnostics, Sees, France). Tests for determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from HUMAN (Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) and ELITECH Diagnostics, respectively. Serum AST and ALT were within the normal range in all patients. This allows us to exclude that serum NAG, which increases in liver diseases, was elevated in diabetic patients because of liver involvement. Glycated hemoglobin (HbA_{1c}) was measured by fast performance liquid chromatography (15).

Ion-exchange chromatography on DEAE cellulose was applied to separate the iso-enzyme forms of NAG in

the control group and all diabetic patients. The flow through the column was 80 ml per hour, and the eluted fractions from the column were 4 ml each. The volume of serum used for separation was 4–6 ml; before that serum was dialyzed for 24 hr against 0.01 mol/l phosphate buffer, pH 7.0, at 4°C. The B form was eluted with 0.01 mol/l phosphate buffer of pH 7.0, whereas a linear concentration gradient (0–0.3 mol/l) of sodium chloride in the same buffer was used for the A form. The activity of NAG in the fractions was determined by MNP-NAGlc as the substrate (calibration curve: $y = 0.854x + 0.00499$, $r = 0.999$).

Statistical Analysis

Statistical analysis was performed by Student's *t*-test for unpaired and paired values. Linear regression analysis and Pearson's correlation coefficient were used to compare the biochemical variables. The results were expressed as the means $\pm$ standard deviation. Only values of $P < 0.05$ were regarded as significant.

RESULTS

The clinical and biochemical characteristics of the control group and the four diabetic groups are presented in Table 1. In all diabetic groups there was a statistically relevant increase ($P < 0.001$) of the total NAG activity compared with the control group. If the values of the mean NAG activity of the diabetic groups are compared, it may be noticed that the diabetic patients with complications had higher values of the NAG activity compared with the diabetic patients without complications. In the case of patients with retinopathy there was a statistically significant increase of NAG activity

compared with the diabetic patients without complications ($P < 0.05$) and compared with the fourth group ($P < 0.05$). In the third group of diabetic patients with retinopathy and neuropathy, the values of the total NAG activity were slightly decreased compared with the second group and slightly increased compared with the fourth group (5.43 ± 1.07 , 5.74 ± 1.16 , 5.1 ± 2.17 , resp.). High values of the glucose and HbA_{1c} content were characteristic for all the diabetic groups compared with the control group ($P < 0.001$), which indicated their poor metabolic control. Significantly shorter duration of diabetes was present in patients without complications as compared with the patients with complications ($P < 0.001$). No differences between diabetic groups were found in age and diastolic blood pressure, whereas patients with complications had significantly higher systolic blood pressure compared with the patients without complications and with the control group ($P < 0.05$, $P < 0.001$, resp.).

To better understand the causes of the significant changes in total serum NAG, the iso-enzyme profiles of diabetic and the control groups were analyzed. Typical elution profiles are presented in Figure 1. Two peaks are clearly defined in chromatograms: the B iso-enzyme of NAG was eluted with 0.01 mol/l phosphate buffer of pH 7.0, whereas the A form was eluted with a linear concentration gradient (0–0.3 mol/l) of sodium chloride in the same buffer. Intermediary forms were not eluted under the mentioned eluting conditions.

Analysis of iso-enzyme profiles in type 1 diabetic patients indicated that they changed considerably compared with the control. The percent fractions of A and B iso-enzymes in total NAG activity in control and diabetic groups are given in Table 2. In all diabetic

TABLE 1. Clinical and Biochemical Characteristics of the Control and Diabetic Groups

Characteristics	Control group	Diabetic patients			
		First group	Second group	Third group	Fourth group
<i>n</i> (M/F)	25 (11/14)	22 (10/12)	16 (6/10)	13 (8/5)	18 (8/10)
Age (yr)	30.2 $\pm$ 8.7	24.4 $\pm$ 7.3	26.4 $\pm$ 2.4	34.9 $\pm$ 15	31.2 $\pm$ 10.1
Diabetes duration (yr)	—	6.5 $\pm$ 5.5	14.4 $\pm$ 5.6 ^d	15 $\pm$ 11 ^d	18.4 $\pm$ 9.6 ^d
SBP (mmHg)	109 $\pm$ 9	114 $\pm$ 8 ^c	120 $\pm$ 4 ^{a,c}	121 $\pm$ 8 ^{b,c}	132 $\pm$ 8 ^{a,d,g}
DBP (mmHg)	83 $\pm$ 6	76 $\pm$ 7	83 $\pm$ 6	78 $\pm$ 7	88 $\pm$ 6
Glucose (mmol/l)	4.8 $\pm$ 0.5	11.6 $\pm$ 3 ^a	14.2 $\pm$ 2.8 ^{a,e,j}	9 $\pm$ 3.4 ^a	12.9 $\pm$ 3.3 ^{a,k}
HbA _{1c} (%)	4.9 $\pm$ 0.5	9 $\pm$ 0.9 ^a	11 $\pm$ 0.8 ^a	9.7 $\pm$ 1.3 ^a	10.1 $\pm$ 0.46 ^a
NAG (IU/l)	3.18 $\pm$ 0.52	4.55 $\pm$ 1.23 ^a	5.74 $\pm$ 1.16 ^{a,e}	5.43 $\pm$ 1.07 ^a	5.1 $\pm$ 2.17 ^{a,h}
Album. (mg/24 hr)	13.34 $\pm$ 6.07	18.44 $\pm$ 5.49	15.47 $\pm$ 7.24	9.24 $\pm$ 3.64	194.84 $\pm$ 104.4 ^{a,d,f,i}
Proteinuria (g/l)					1.12 $\pm$ 0.75

First group without complications. Second group with retinopathy. Third group with retinopathy and polyneuropathy. Fourth group with retinopathy, polyneuropathy, and nephropathy. Values are expressed as the mean $\pm$ the standard deviation (SD). ^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$ compared with control group. ^d $P < 0.001$, ^e $P < 0.05$ compared with first group. ^f $P < 0.001$, ^g $P < 0.01$, ^h $P < 0.05$ compared with second group. ⁱ $P < 0.001$, ^j $P < 0.01$, ^k $P < 0.05$ compared with third group. SBP, systolic blood pressure; DBP, diastolic blood pressure; NAG, *N*-acetyl- β -D-glucosaminidase.

Fig. 1. Typical elution profiles of the iso-enzymes of serum NAG in the control group (white circles) and diabetic patients (black squares) obtained by chromatography on DEAE cellulose. NAG, *N*-acetyl- β -D-glucosaminidase; DEAE, diethylaminoethyl.

TABLE 2. The Amounts of A and B Forms of Serum NAG (Expressed as Percentage of Total Activity) in the Control Group and in the Diabetic Groups

Group	B (%)	A (%)
Control group	30.61 $\pm$ 4.78	69.38 $\pm$ 4.79
First—patients without complications	15.87 $\pm$ 5.65 ^a	83.84 $\pm$ 6.09 ^a
Second—patients with R	15.66 $\pm$ 5.74 ^a	84.37 $\pm$ 5.74 ^a
Third—patients with R and P	18.33 $\pm$ 5.98 ^a	81.76 $\pm$ 6.02 ^a
Fourth—patients with R, P, and N	23.63 $\pm$ 7.38 ^{a,b,c}	76.37 $\pm$ 7.38 ^{a,b,c}

R, retinopathy; P, polyneuropathy; N, nephropathy; NAG, *N*-acetyl- β -D-glucosaminidase. Values are expressed as the mean $\pm$ the standard deviation (SD). ^a P < 0.001 compared with control group. ^b P < 0.01 compared with first group. ^c P < 0.05 compared with second group.

groups, with and without complications, the percent fraction of the most abundant A form was statistically significantly higher (P < 0.001) compared with the control group (69.38 $\pm$ 4.79%). Comparing diabetic groups the lowest amount of the A form was found in the fourth group of patients (with R, P, and N; 76.37 $\pm$ 7.38%) and the highest amount in the second group of patients with retinopathy (84.37 $\pm$ 5.74%). In the fourth group the contribution of A form was statistically significantly lower compared with the first and second groups (P < 0.01, P < 0.05, resp.).

The fraction of the B form in the total NAG activity was statistically significantly (P < 0.001) lower in all four diabetic groups compared with the control group (30.61 $\pm$ 4.78%). The lowest amount of the B form was found in the second group of patients with retinopathy (15.66 $\pm$ 5.74%), and the highest amount in the fourth group (with R, P, and N; 23.63 $\pm$ 7.38%). A statistically

significant higher percent of the B form was found in the fourth group compared with the first and second ones (P < 0.01, P < 0.05, resp.).

In all diabetic groups statistically significant (P < 0.001) very strong positive correlations (0.934–0.966) between total serum NAG and the A form and good positive correlations (0.497–0.780) between total NAG and B form were found (Table 3). Only in the fourth diabetic group a good correlation between A and B forms was found (r = 0.594, P < 0.05). In all diabetic groups statistically significant good correlations (0.512–0.638) between the A iso-enzyme and glycemia were found, whereas the correlation (r = 0.614) between the B form and glycemia was significant (P < 0.01) only in the first group of diabetic patients. Weak positive relationships between total NAG as well as A form and HbA_{1c} were found.

In order to check whether the posttranslational modification of serum NAG, which appeared in hyperglycemia, might be responsible for the changes in the NAG activity, the binding strength of A iso-enzyme to DEAE cellulose, e.g., the concentration of sodium chloride used to elute of A form of diabetic and control groups was investigated (Fig. 1). The A iso-enzyme in the control group was eluted with a 6.37×10^{-2} mol/l sodium chloride solution, whereas in the case of the diabetic patients it ranged from 6.35×10^{-2} to 6.95×10^{-2} mol/l. The differences between salt concentrations obtained for control and diabetic groups were not statistically significant.

DISCUSSION

The increased values of the total activity of serum NAG in diabetic patients with and without complications, obtained in this investigation, were in agreement with previous studies, which indicated that the NAG activity is increased in type 1 diabetes (1–3). Several explanations have been put forward when analyzing the causes of the changes in NAG activity. As NAG can participate in the degradation of glycosaminoglycans (16) and glycoconjugates, substances that have been accumulated on blood vessels in microvascular diabetic complications (7), mechanism of activation by glyco-material is one of the considered causes of its increase. Comparing the results obtained for all diabetic groups, it was found that diabetic patients with complications (and longer duration of diabetes) have higher values of NAG activity compared with the group without complications. It could be concluded that development of secondary complications and durations of diabetes were responsible for further increase of the NAG activity. However, the highest statistically significant (P < 0.05) increase of NAG activity, compared with the

TABLE 3. Correlations Between Total NAG and its Iso-Enzymes and Degree of Metabolic Control (Glycemia and HbA_{1c}) in Diabetic Groups

Correlation between	Diabetic patients			
	First group <i>r</i>	Second group <i>r</i>	Third group <i>r</i>	Fourth group <i>r</i>
Total NAG-A	0.945 ^a	0.943 ^a	0.934 ^a	0.966 ^a
Total NAG-B	0.584 ^b	0.635 ^b	0.497	0.780 ^a
A-B	0.294	0.342	0.155	0.594 ^c
Total NAG-glycemia	0.623 ^b	0.568 ^c	0.559 ^c	0.494 ^c
A-glycemia	0.512 ^c	0.555 ^c	0.638 ^c	0.568 ^c
B-glycemia	0.614 ^b	0.314	0.004	0.167
Total NAG-HbA _{1c}	0.417	0.405	0.235	0.225
A-HbA _{1c}	0.477	0.299	0.217	0.258
B-HbA _{1c}	0.042	0.479	0.112	0.073

^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$. NAG, *N*-acetyl- β -D-glucosaminidase.

group without complications, was found in the second group of patients who only had retinopathy. Complications combined with neuropathy (third group, R+P) do not lead to further NAG increase (the value is even lower). In the fourth diabetic group (R+P+N) approx. 50% have proteinuria ranging from 0.85 to 2.66 g/l, whereas seven patients have microalbuminuria—from 35 to 311.3 mg/24 hr (Table 1). The decreased total NAG activity in this group can therefore be the consequence of serum NAG influx into urine (17,18). The results presented indicate that glycomaterial depositing on the blood vessels' walls lead to further NAG increase in diabetic complications (the protection of blood vessels against excessive deposition of glyco-compounds) compared with the patients without complications. However, it is not a crucial factor that causes changes in the NAG activity. The total NAG activity cannot be used as a parameter for monitoring the development (only R, R+P, R+P+N), i.e., differentiation of complications.

Besides NAG, high values of HbA_{1c} and glucose were also found in all groups of diabetic patients. The influence of the glycemia (19,20) and the poor metabolic control (HbA_{1c}) (13,21,22) on the changes of total NAG activity was earlier described. In this study a good correlation was found between total NAG activity and glycemia in all groups (the highest value in the group without complications, 0.623). The mentioned correlation and the fact that in the group with retinopathy the glucose content is highest ($P < 0.05$ compared with the first group and $P < 0.01$ compared with the third group) as well as NAG activity indicate the direct relationship between those two parameters. One of the possible explanations for this relation could be that the insufficient influx of glucose into cells owing to the lack of insulin led to the decreased synthesis of adenosine triphosphate (ATP). In laboratory models a transient

decrease in ATP was accompanied by the apoptosis of macrophage and fibroblast, i.e., by the release of acidic hydrolases into the serum. Recent studies have demonstrated that disturbed metabolism of glycosaminoglycans (16), the excess of posttranslational *O*-glycosylation of proteins by *O*-linked β -*N*-acetylglucosamine (*O*-GlcNAc), as well as single nucleotide polymorphism in gene encoding *O*-GlcNAc-selective NAG may be involved in the pathogenesis of diabetes and late diabetic complications (23). Posttranslational modification of proteins is a dynamic and regulated process including the coordinated action of *O*-GlcNAc transferase (attaching *O*-GlcNAc residue) and *O*-GlcNAc-selective NAG (removal of *O*-GlcNAc). In hyperglycemia extracellular glucose passes through metabolic ways independent of insulin. There occurs *O*-GlcNAc flux increase, i.e., increased intracellular posttranslational protein modification (24,25), thereby activation and release of NAG for removing *O*-GlcNAc. Clark et al. (26) have demonstrated that removal of *O*-GlcNAc residues either enzymatically or by introducing virally transmitted *O*-GlcNAc-selective NAG is sufficient to normalize cell function despite continued exposure to elevated extracellular glucose. Long-lasting hyperglycemia (increased HbA_{1c}) in all groups can also lead in all of them to partial inactivation of NAG by reaction of nonenzymatic glycosylation (27). The ratio of NAG activation and inhibition determines the amount of glycoprotein and glycosaminoglycan that deposit in the basal membranes of the retina, conjunctiva, renal tubules, and renal glomeruli and lead to their thickening (8).

The analysis of iso-enzyme profiles evidenced that percent of the most abundant A form in the total NAG activity in all groups is statistically significantly increased ($P < 0.001$) compared with control. The contribution of the B form to total activity is statistically

significantly decreased ($P < 0.001$) compared with control. Very high positive correlation (0.934–0.945; $P < 0.001$) between A form and total NAG and that slightly lower between B form and total NAG indicated that increase in the total activity of serum NAG in diabetic patients is mostly owing to the specific increase in iso-enzyme A activity. Statistically significant positive correlation (0.512–0.638; $P < 0.05$) between A form and glycemia in all diabetic groups indicates that specific increase of the serum A form is the consequence of its increased exocytosis in hyperglycemia.

No difference was found in abundance of A and B forms, respectively, in the first (without complications), second (R), and third (R+P) diabetic groups. Consequently, iso-enzyme profiles of the serum NAG cannot be used as parameters for prediction and distinction of secondary complications.

Some authors find that the posttranslational modification that appeared in hyperglycemia might be responsible for changes in the NAG profiles (3,9). It was proposed that some differences between the iso-enzymes can result from perturbation of protein α and β subunits during posttranslational modifications of protein chains (28). In order to check the prospective changes of iso-enzyme charge owing to posttranslational modifications, the concentration of sodium chloride used to elute the serum A form of diabetic and control groups from DEAE cellulose was investigated. Statistically significant difference in the bonding strength of the serum A form to DEAE cellulose in control and diabetic groups (Fig. 1), as well as between diabetic groups, was not found. However, the A iso-enzyme of patients with retinopathy, neuropathy, and nephropathy (group 4) was eluted with slightly higher concentration of sodium chloride compared with the values of the other three groups of diabetic patients. As it was strongly bound to DEAE cellulose column, it is more acidic than the A form of the other diabetic groups. This slight change in the behavior of the A form might be the consequence of posttranslational modifications that appeared in hyperglycemia. Nonenzymatic glycation of NAG (27), i.e., formation of Schiff base, between protein amino group and glucose can lead to acidity increase. Wiese (29) and Barone (30) found that the carbohydrate part of glycoproteins changed in diabetes. This change could lead to different distribution of enzymes inside and outside the cell. A more detailed characterization of the iso-enzyme carbohydrate parts could explain the changes found.

On the basis of all the results, it was concluded that the increase in the activity of serum NAG and the changes of the iso-enzyme profiles in type 1 diabetes were the consequence of poor metabolic control, increased exocytosis of the A form and posttranslational

modifications, and in hyperglycemia. The serum NAG total activity and iso-enzyme profiles cannot be used to monitor the development and the distinction of secondary complications in type 1 diabetes mellitus.

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