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# Posttranslational modifications of the insulin-like growth factor-binding protein 3 in patients with type 2 diabetes mellitus assessed by affinity chromatography

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ABSTRACT

Structural and ligand-binding properties of the insulin-like growth factor-binding protein (IGFBP)-3 in patients with poorly controlled diabetes mellitus type 2 were investigated using boronic acid- and lectin-affinity chromatography. IGFBP-3 species separated by chromatography were analyzed by immunoblotting and surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS). Increased IGFBP-3 binding to boronic acid in patients was shown to be accompanied by the increased ligand-binding. Increased binding of IGFBP-3 forms to lectins from *Sambucus nigra* (SNA) and *Canavalia ensiformis* (ConA) in patients, on the other hand, was either not accompanied by altered ligand-binding (in the case of ConA) or it was reduced (in the case of SNA). Strong and opposite effects of glycation and additional sialylation on ligand binding qualify them as factors that may be involved in the regulation of the amount of free, physiologically active IGFs, and modulation of processes that accompany development and progression of diabetes. SELDI-TOF MS analysis revealed a fragment of 13.9 kDa as representative for the non-glycosylated form of IGFBP-3, whereas a fragment of 28.0 kDa profiled as typical for the glycosylated/glycated IGFBP-3 species. The same fragmentation pattern found in healthy persons and in patients indicates that the same degradation process predominantly occurs in both groups of individuals.

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## 1. Introduction

Diabetes mellitus (DM) type 2 is a metabolic disorder characterized by hyperglycemia and insufficient action of the secreted insulin. Many biomolecules are modified by non-enzymatic glycation and oxidation (glycoxidation) under conditions of hyperglycemia [1,2]. Glucose oxidation is believed to be the main source of free radicals [3], and proteins are the principal targets of these reactive species [4]. Numerous complications of DM result from structural and functional modifications of various proteins.

Saccharide moieties in glycoproteins are involved in their folding, interaction with ligands, cellular recognition, adhesion and so on. Degree and type of glycosylation depend on the (patho)physiological status of an organism, whereas protein glycation is a unique consequence of the increased glucose concentration. Modified (glyco)proteins play important role in altered response of cells. Insulin, a crucial regulator of glucose homeostasis, itself can be glycated [5]. Glycated insulin represents up to 27% of the total cellular insulin [6], it is up to 40% less effective than native insulin in stimulating glucose metabolism [7], and its clearance rate is reduced [8].

Homologous peptides, insulin-like growth factors (IGFs), besides having glucose-lowering potential, have been suggested to play an important role in the progression of diabetic complications [9]. In the circulation, IGFs are bound to the IGF-binding proteins (IGFBPs) and 1–5% is free and physiologically active [10]. The major binding protein, a key regulator of IGF availability, is IGFBP-3. IGFBP-3 is a glycoprotein having three potential N-glycosylation sites [11]. Naturally occurring isoforms of IGFBP-3 of approximately 40–45 kDa differ in the amount and the site of saccharide binding. In patients with DM a glycated IGFBP-3 was detected [9], and it was postulated to have an increased number of IGF-binding sites [12]. Increased proteolytic activity against IGFBP-3 was measured in these patients [13] and it seems to be related to glycosylation

*Abbreviations:* ADU, arbitrary densitometric units; BAC, boronic acid-affinity chromatography; BMI, body mass index; ConA, *Canavalia ensiformis* agglutinin; DM, diabetes mellitus; ECL, enhanced chemiluminescence; HbA1c, glycated hemoglobin; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IRMA, immunoradiometric assay; LAC, lectin-affinity chromatography; LCA, *Lens culinaris* agglutinin; NP, normal phase; OGTT, oral glucose tolerance test; PHA-E, *Phaseolus vulgaris* erythroagglutinin; PS, preactivated surface; RCA, *Ricinus communis* agglutinin; RIA, radioimmunoassay; SNA, *Sambucus nigra* agglutinin; SELDI-TOF MS, surface-enhanced laser desorption/ionization-time of flight mass spectrometry; WIB, Western immunoblotting.

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and/or glycation status [14]. Several enzymes may cleave IGFBP-3 causing formation of different fragments [15] and a fragmentation pattern can point to an enzyme principally responsible for IGFBP-3 degradation.

The aim of this study was to investigate structural and ligand-binding properties of IGFBP-3 molecules in patients with poorly controlled DM type 2. As structural modifications of IGFBP-3 may result both from non-enzymatic glycation and from enzyme-catalyzed glycosylation, potential alterations in the glycan structure of IGFBP-3 were investigated using boronic acid-affinity chromatography in combination with lectin-affinity chromatography [16,17]. Boronic acid is able to form high affinity reversible bonds with cis diols and is commonly used for the measurement of glycated hemoglobin (HbA1c). Under conditions of hyperglycemia, the content of cis diols in proteins increases due to formation of adducts between free amino groups of proteins and glucose. Lectins are carbohydrate-binding proteins that recognize specific saccharide moieties. Altered glycosylation of proteins results in altered reactivity with lectins. Plant lectins have been used for a long time in the analysis of animal glycoproteins. In this study we have employed lectins specific for the most common carbohydrates found in serum glycoproteins. IGFBP-3 species separated by boronic acid- or lectin-affinity chromatography were further analyzed by immunoblotting and surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS), searching for alterations in protein pattern characteristic for diabetes mellitus.

## 2. Materials and methods

### 2.1. Samples

Venous blood samples were obtained from 97 healthy non-pregnant adult persons (38 males and 59 females) and 78 non-pregnant adult patients with DM type 2 (35 males and 43 females). Healthy volunteers were subjected to oral glucose tolerance test (OGTT). They were given orally 75 g of glucose in 200 mL of water after the fasting blood sampling (0 h). The blood was collected after 1 h and 2 h. The concentration of HbA1c was measured in whole blood samples collected at 0 h, whereas the concentration of glucose was measured in serum samples obtained at all three time intervals. Volunteers were considered to have normal OGTT response if the concentration of their blood glucose increased at most to 7.8 mM and returned to the referent range after 2 h.

Diagnosis of DM type 2 in patients was made in Clinical Centers in Serbia, apart from this study. Criteria to establish DM type 2 were those recommended by the World Health Organization [18]. Patients were subjected to OGTT in Clinical Centers. The maximum concentration of their blood glucose was in the interval 11.3–24.2 mM and 3 h after the OGTT had started it was still above the initial concentration.

The aim of this study was to investigate alterations of IGFBP-3 molecules in patients with poorly controlled DM type 2 (where the greatest change is expected to occur), so patients with fasting blood glucose concentration above 10 mM were chosen for further analysis. There were 28 such patients (12 males and 16 females, age 42–72 years, BMI 28–36 kg/m$^2$). Subjects included in this study were non-smokers, neither alcoholics, nor vegetarians, they were not subjected to any extreme nutritional regime and none of them was involved in sports except moderate recreation. Several patients had retinopathy, but their kidney function was not severely impaired. Patients were treated with diet plus oral hypoglycemic agents.

Control subjects were selected to match patients in respect to age, sex and BMI ($n = 32$, 15 males and 17 females, age 46–69 years, BMI 26–33 kg/m$^2$). The study was approved by the Ethics committee in INEP and all subjects gave informed consent to participate.

### 2.2. Measurement of hematological and biochemical parameters

The concentration of HbA1c was measured in blood samples collected into anticoagulant solution (Pointe Scientific HbA1c Reagent set, Canton, USA), whereas concentrations of other parameters were measured in serum: glucose (Randox GOD-PAP reagent, Crumlin, UK), C-peptide (INEP IRMA kit, Belgrade, Serbia), IGF-I (INEP, RIA kit, Belgrade, Serbia) and IGFBP-3 (DSL-6600 IGFBP-3 IRMA kit, DSL, Webster, USA).

### 2.3. Boronic acid-affinity chromatography (BAC)

Serum samples (50 μL) were applied to a Sephadex G-25 column (0.8 cm × 10.0 cm) and eluted with 0.25 M ammonium acetate solution pH 8.0 supplemented with 0.05 M $MgCl_2$. This chromatographic step was used to remove small sugar molecules containing cis diols. The flow-through fractions were applied to the boronic acid-agarose (MP Biomedicals, Costa Mesa, USA) microcolumn (0.3 cm × 0.8 cm) and incubated for 15 min at room temperature using a rotator. Separation of the unbound proteins was performed by centrifugation for 30 s at 2000 × *g*, followed by washing the matrix three times with washing solution 0.25 M ammonium acetate/0.05 M $MgCl_2$ pH 8.0 (500 μL each). Bound proteins were eluted with 500 μL of 0.2 M mannitol in 0.1 M Tris–HCl pH 8.5, for 15 min at room temperature using rotator and separated from the gel by centrifugation. The elution step was repeated six times. The column was washed with destilled water (5× 500 μL) and washing buffer (2× 500 μL) prior to the next chromatographic cycle. Collected fractions were pooled, dialyzed against distilled water overnight at 4 °C and analyzed by immunoblotting and SELDI-TOF MS.

In a separate experiment serum samples (50 μL) were incubated with $^{125}$I-IGF-I (1.5 × 10$^5$ cpm) overnight at 4 °C before being applied to a Sephadex G-25 column. $^{125}$I-labeled IGF-I was prepared using the chloramine T method [19], in the same way as it is prepared for the commercial RIA IGF-I kit (INEP, Belgrade, Serbia), validated against reference material WHO 87/518. Native IGF-I and $^{125}$I-IGF-I were found to have equal binding potency in this RIA IGF-I assay. Chromatographic fractions were collected and their radioactivity (cpm) measured in a γ-counter.

Preliminary experiments were conducted to determine the IGFBP-3 elution profile: fractions obtained after chromatography without labeled ligand were subjected to immunoblotting (and densitometry) and those obtained after chromatography with $^{125}$I-IGF-I were measured in a γ-counter. All fractions containing immuno and ligand reactive proteins were pooled and the presence of IGFBP-3 calculated taking into account the effect of dilution. The recovery of IGFBP-3 ranged between 95 and 100%.

### 2.4. Lectin-affinity chromatography (LAC)

Five agarose-immobilized lectins (Vector Laboratories, Burlingame, USA) were packed into columns (0.5 cm × 3.0 cm): SNA (*Sambucus nigra* agglutinin, specific for terminal α-2,6-linked-sialic acid residues), ConA (lectin from *Canavalia ensiformis* which binds trimannosyl core from high-mannose, hybrid type and biantennary complex type N-glycans, but it fails to recognize highly branched saccharide structures), RCA-I (*Ricinus communis* agglutinin I, specific for galactose β-1–4-linked to N-acetyl glycosamine residue), PHA-E (*Phaseolus vulgaris* erythroagglutinin, specific for biantennary N-glycans that contain bisecting N-acetyl glycosamine residue) and LCA (*Lens culinaris* agglutinin which recognizes core-fucosylated, biantennary N-glycans with

terminal galactose residues at the non-reducing ends). All buffers and hapten sugar solutions were prepared following procedures recommended by the producer. An exact composition of each buffer was published elsewhere [20]. Serum samples (100 μL) diluted with 900 μL of the corresponding buffers were circulated through columns for 1 h. Unbound material was washed away with 20 mL of appropriate dilution buffers. The elution of bound glycoproteins was performed with 10 mL of hapten sugar solution (0.2–0.5 M, as suggested by the producer) in 0.1 M acetic acid pH 3.0. Elution of the PHA-E column was performed using only 0.1 M acetic acid. Collected fractions were immediately neutralized with 2 M Tris–HCl buffer pH 8.9, pooled, dialyzed against distilled water overnight at 4 °C and analyzed by immunoblotting and SELDI-TOF MS.

In a separate experiment serum samples (100 μL) were incubated with $^{125}$I-IGF-I ($3 \times 10^5$ cpm) overnight at 4 °C before being applied to the lectin columns. Fractions were collected and their radioactivity (cpm) measured in a γ-counter.

The recovery of IGFBP-3 was greater than 98% as assessed using the same methods as in BAC.

### 2.5. Western immunoblotting (WIB)

Protein fractions obtained after affinity chromatography (BAC and LAC) were subjected to SDS-PAGE on 10% gels under reducing conditions. Immunoblotting [21] was performed using an affinity purified goat polyclonal anti-IGFBP-3 antibody (DSL, Webster, USA) followed by an anti-goat IgG antibody coupled to horseradish peroxidase (Biosource, Camarillo, USA). Immunoreactive proteins were visualized with enhanced chemiluminescence (ECL) reagents (Pierce, Minneapolis, USA). Molecular mass markers were from Bio-Rad Laboratories (Hertfordshire, UK).

Densitometric evaluation of protein bands on immunoblots was performed using ImageMaster™ TotalLab Software (Amersham Biosciences, Newcastle, UK).

### 2.6. Surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS)

Protein fractions obtained after BAC and LAC were analyzed by SELDI-TOF MS (ProteinChip SELDI System, Series 4000, Bio-Rad Laboratories, Marnes-la-Coquette, France) using two protein chip arrays: a normal phase (NP20) and a preactivated surface-epoxide (PS20). PS20 chips were incubated with goat polyclonal anti-IGFBP-3 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) and samples following the manufacturer's Application guide. Spectra were calibrated using internal standards and analyzed with the corresponding ProteinChip software. A dilution buffer was used as a loading control.

### 2.7. Statistical analysis

Numerical data are presented as mean values ($X$) and standard deviation (SD), and analyzed using Mann–Whitney $U$-test for the significance of differences between the study groups.

## 3. Results

Concentrations of glucose, HbA1c and C-peptide in sera from healthy persons and patients with poorly controlled DM type 2 are shown in Table 1. Patients had much higher concentrations of glucose, HbA1c and C-peptide than healthy persons. The level of IGF-I did not differ between the two groups. The concentration of IGFBP-3 in patients was lower than in healthy individuals, but the difference was slightly above the limit of the statistical significance ($P = 0.07$).

**Table 1**
Concentrations of glucose, HbA1c, C-peptide, IGF-I and IGFBP-3 in healthy persons and patients with DM type 2 ($X \pm$ SD).

| Parameter | Healthy persons ($n = 32$) | Patients with DM type 2 ($n = 28$) |
|---|---|---|
| Glucose (mM) | 4.8 ± 0.61 | 12.8 ± 3.22[a] |
| HbA1c (%) | 4.8 ± 0.45 | 10.2 ± 1.00[a] |
| C-peptide (nM) | 0.5 ± 0.19 | 0.9 ± 0.27[a] |
| IGF-I (nM) | 22.0 ± 8.66 | 18.8 ± 6.74 |
| IGFBP-3 (nM) | 92.2 ± 11.11 | 75.2 ± 13.16 |

[a] Statistically significant differences between two groups ($P < 0.05$).

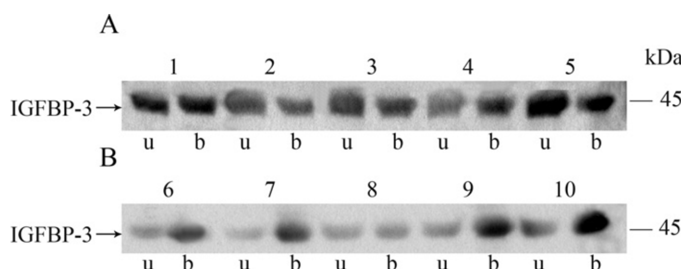


**Fig. 1.** WIB of IGFBP-3 species from the boronic acid-unbound (u) and bound (b) fractions from sera of (A) healthy persons (samples 1–5) and (B) patients with DM type 2 (samples 6–10), after SDS-PAGE under reducing conditions.

All sera were subjected to affinity chromatography BAC and LAC. Unbound and bound protein fractions were analyzed by WIB and SELDI-TOF MS.

Representative WIB profiles of monomer IGFBP-3 of healthy and DM type 2 persons after BAC are shown in Fig. 1. A 45 kDa isoform was the only one clearly visible. A 40 kDa band (generally present in lower amounts) could not be distinguished from the 45 kDa band, possibly due to greater degradation during chromatography and subsequent steps prior to electrophoresis. IGFBP-3 was detected in both boronic acid-unbound and bound fractions and their relative abundance was evaluated by densitometric analysis. Results were expressed in arbitrary densitometric units (ADU, delivered by the ImageMaster™ TotalLab Software) per nM IGFBP-3 and the data for the boronic acid-bound protein fractions are shown in Table 2. A significantly increased binding of IGFBP-3 to boronic acid was found in patients.

WIB of the lectin-bound protein fractions also demonstrated changes in the amount of lectin-reactive IGFBP-3 forms due to diabetes. Representative results are shown in Fig. 2, whereas results of the corresponding densitometric analysis are given in Table 2. Reactivity of IGFBP-3 with three of the tested lectins was found to be altered. The amount of SNA- and ConA-reactive IGFBP-3 was significantly greater and the amount of LCA-reactive IGFBP-3 lower in patients with DM. Knowing the carbohydrate specificities of these

**Table 2**
Relative abundance (in arbitrary densitometric units per nM IGFBP-3) of boronic acid- and lectin-bound IGFBP-3 forms in sera from healthy persons and patients with DM type 2 ($X \pm$ SD).

| Relative abundance of IGFBP-3 (ADU/nM) | Healthy persons ($n = 32$) | Patients with DM type 2 ($n = 28$) |
|---|---|---|
| Boronic acid-bound | 0.51 ± 0.087 | 0.72 ± 0.133[a] |
| SNA-bound | 0.09 ± 0.013 | 0.62 ± 0.106[a] |
| ConA-bound | 0.12 ± 0.023 | 0.40 ± 0.077[a] |
| RCA I-bound | 0.17 ± 0.023 | 0.15 ± 0.024 |
| PHA E-bound | 0.43 ± 0.066 | 0.54 ± 0.085 |
| LCA-bound | 0.67 ± 0.114 | 0.44 ± 0.077[a] |

[a] Statistically significant differences between two groups ($P < 0.05$).

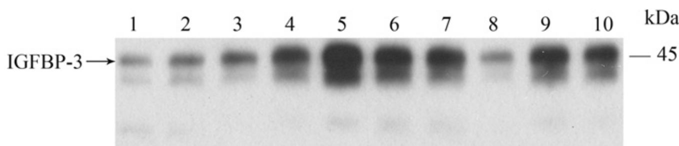


**Fig. 2.** WIB of IGFBP-3 species from sera of healthy persons (lanes 1–5) and patients with DM type 2 (6–10) bound to lectins SNA (lanes 1 and 6), ConA (2 and 7), RCA I (3 and 8), PHA E (4 and 9) and LCA (5 and 10), resolved by SDS-PAGE under reducing conditions.

lectins, it can be said that IGFBP-3 from patients with diabetes possesses additional α-2,6-linked sialic acids and increased content of complex type N-glycans.

In order to investigate the possible alteration in the reactivity of IGFBP-3 towards its ligand IGF-I due to diabetes, a separate set of experiments was performed introducing an incubation step of serum samples with $^{125}$I-IGF-I prior to affinity chromatography (BAC or LAC). The amount of bound radioactivity was an indicator of IGF-I-binding to IGFBP-3. Results of radioactivity measurements, expressed in cpm/nM IGFBP-3, are shown in Table 3.

Increased binding of IGFBP-3 forms to boronic acid in patients with diabetes was shown to be accompanied by the increased amount of the bound ligand $^{125}$I-IGF-I. In contrast, increased binding of IGFBP-3 to lectins SNA and ConA was either not accompanied by altered ligand-binding (in the case of ConA) or it was even reduced (in the case of SNA). According to these results, altered glycosylation of IGFBP-3 and its glycation in diabetic persons had opposite effects on ligand-binding properties.

Data were also analyzed according to gender of the subjects involved in the study. The measured parameters were not significantly different between females and males (data not shown).

SELDI-TOF MS analysis (using PS20 chip with the immobilized anti-IGFBP-3 antibody) of the boronic acid- and lectin-unbound and bound fractions was performed to analyze a fragmentation pattern of IGFBP-3 and investigate possible differences between samples from healthy persons and patients with DM type 2. Clear difference between the IGFBP-3 species was seen only in the spectra of proteins fractionated by BAC. Representative spectra are shown in Fig. 3.

Protein fragments were detected in the region of 5.0–30 kDa and each spectrum contained multiple bands. Two major differences in fragmentation pattern of proteins originating from boronic acid-unbound and bound fractions were found. An IGFBP-3 fragment of 13.9 kDa was present only in the boronic acid-unbound fraction, both in the sera of healthy persons and patients with diabetes, whereas a fragment of 28.0 kDa was seen predominantly among the boronic acid-bound proteins (also in both groups of samples).

**Table 3**
Radioactivity of $^{125}$I-IGF bound to boronic acid- and lectin-reactive IGFBP-3 forms (in cpm/nM IGFBP-3) from sera of healthy persons and patients with DM type 2 ($X \pm$ SD).

| Radioactivity of bound $^{125}$I-IGF-I (cpm/nM IGFBP-3) | Healthy persons ($n = 32$) | Patients with DM type 2 ($n = 28$) |
|---|---|---|
| Boronic acid-bound | 554 ± 49 | 782 ± 53[a] |
| SNA-bound | 390 ± 50 | 290 ± 48[a] |
| ConA-bound | 421 ± 59 | 483 ± 69 |
| RCA I-bound | 132 ± 30 | 151 ± 31 |
| PHA E-bound | 298 ± 53 | 335 ± 57 |
| LCA-bound | 244 ± 48 | 249 ± 44 |

[a] Statistically significant differences between two groups ($P < 0.05$).

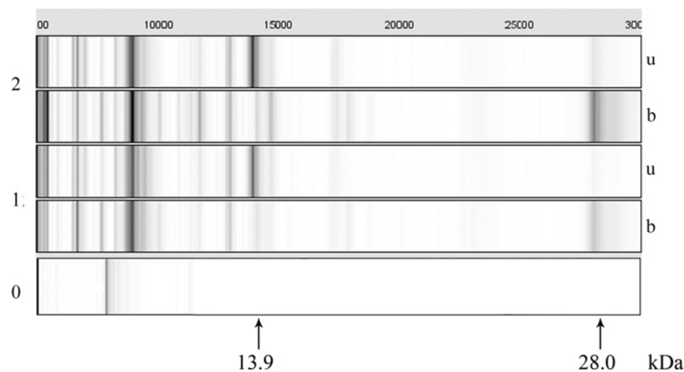


**Fig. 3.** SELDI-TOF MS analysis (region 5–30 kDa) of the boronic acid-unbound (u) and bound (b) IGFBP-3 forms from the sera of a healthy person (1) and a patient with DM type 2 (2); sample 0 is a loading control.

## 4. Discussion

This study has shown that in patients with DM type 2 both non-enzymatic and enzyme-catalyzed posttranslational modifications of IGFBP-3 occur. Changes were recorded at the level of glycation and glycosylation of IGFBP-3, and these changes coincided with altered ligand binding.

The concentration of IGFBP-3 was lower in patients than in healthy persons, although statistical analysis did not confirm the significance at $P < 0.05$. Yamada et al. [22] have reported decreased serum IGFBP-3 in patients with type 2 diabetes and studies on patients with type 1 diabetes also found the concentration of IGFBP-3 to be lower [23,24]. According to Cortizo et al., levels of IGF-I and IGFBP-3 were comparable in the type 2 diabetic patients and controls for the population ranging from 35 to 70 years [9]. The amount of intact IGFBP-3 depends on the rate of its synthesis, a degree of proteolysis, which increases in diabetes [14,25], and the severeness of diabetic nephropathy, which slowers the clearance rate of IGFBP-3 [26]. Clemmons et al. [14] have demonstrated that the non-glycosylated IGFBP-3 is degraded more rapidly than the glycosylated IGFBP-3. If methodological differences in IGFBP-3 assays are taken into account (some immunoassays measure only intact IGFBP-3, whereas others measure both intact and fragmented molecules) [27], the search for more accurate data on IGFBP-3 levels in diabetics is still actual.

In patients with diabetes the amount of total IGFBP-3 was lower than in control subjects, whereas the amount of boronic acid-reactive IGFBP-3 forms was greater. A relative increase of approximately 40% of the glycated IGFBP-3 per unit concentration of IGFBP-3 in patients was calculated. Cortizo et al. [9] have found that the amount of glycated IGFBP-3 in patients with type 2 diabetes was increased by about 30%. In experiments with $^{125}$I-IGF-I, the reactivity of IGFBP-3 with its ligand was assessed. An increase in the amount of complexes formed between boronic acid-reactive IGFBP-3 and $^{125}$I-IGF-I in patients was also approximately 40%. A correlation between decreased amount of total IGFBP-3 in serum, increased amount of glycated IGFBP-3 and increased $^{125}$I-IGF-I-binding in patients with diabetes can be explained by increased affinity of the glycated IGFBP-3 for IGF-I.

The differences in the glycosylation of IGFBP-3 in patients with DM type 2 were also detected. Glycosylation occurs as a part of glycoprotein synthesis [28] and diabetes mellitus, being a systemic metabolic disorder, affects numerous biochemical pathways. In this study, we have measured the relative abundance of IGFBP-3 forms that interact with lectins specific for carbohydrate structures and terminal sugar residues most commonly found in serum glycoproteins. The biantennary N-glycans, with or without core fucose

residues, are major type N-glycans attached to IGFBP-3 [20]. Both ConA and LCA recognize and bind biantennary N-glycans, but LCA requires the presence of core fucose for high affinity binding [29]. The increased amount of ConA-reactive, but decreased amount of LCA-reactive IGFBP-3 forms in patients with diabetes reflects the increased number of IGFBP-3 molecules bearing biantennary N-glycans without core fucose. The increased binding of IGFBP-3 from NIDDM patients to SNA suggests an increased α-2,6-sialylation of terminals of saccharide branches. In experiments with $^{125}$I-IGF-I, the reactivity of lectin-rective IGFBP-3 forms with the ligand was assessed. The only difference between two study groups was found in LAC with SNA. SNA-reactive IGFBP-3 molecules from patients with diabetes were found to bind approximately 30% less $^{125}$I-IGF-I compared to healthy subjects. Increased content of sialic acid residues in IGFBP-3 accompanied by decreased IGF-I-binding can be explained by decreased ligand affinity due to IGFBP-3 sialylation.

Increased levels of sialic acid were found in sera from diabetic persons [30] and, according to the results of this study, IGFBP-3 is subjected to additional sialylation in such patients. Sialylation of IGFBP-3 has opposite effect on ligand-binding compared to IGFBP-3 glycation. These two posttranslational modifications, thus, may be involved in the regulation of ligand-binding equilibrium in persons with diabetes. IGFs are less potent than insulin in stimulating intracellular transport of glucose, but since their blood concentration is much greater than that of insulin [31] and they are already available (stored) in the circulation, their potential contribution in glucose homeostasis is not insignificant. The majority of IGFs (7.5 kDa) in the circulation is bound in ternary complexes (150 kDa) with IGFBP-3 and acid-labile subunit [10], so these peptides may be “sheltered” from glycation and their activity preserved, in contrast to insulin which is directly subjected to glycation that reduces its effectiveness [7]. IGFs are also factors that accelerate tumor growth [32] and increased sialylation of IGFBP-3 in women with breast cancer was reported [33]. As diabetic patients are known to have higher risk of cancer [34], altered glycation/glycosylation of IGFBP-3 may be possibly involved in development of diabetic complications. Tumor cells express insulin and IGF receptors and degree of their expression correlates with tumor progression and prognosis [35]. Recent studies have shown that diabetic patients with low insulin, IGF-I and IGF-II levels are relatively protected from intensive cancer growth [34]. Thus, decreased affinity of IGFBP-3 for its ligands due to sialylation may be regarded as a potential tumor promoting factor.

SELDI-TOF MS analysis is commonly used to differentiate physiological samples of healthy individuals from those of diseased, searching for markers that can reliably detect protein transformations [36]. The pattern of protein fragmentation can be used in the assessment of its structure. In this study, anti-IGFBP-3 antibody was immobilized on the PS20 chip surface prior to the sample load, so the obtained spectrometric signals originated from IGFBP-3-immunoreactive forms. Spectral analysis demonstrated significant differences between spectra of boronic acid-unbound and bound IGFBP-3 species. A fragment of 13.9 kDa was distinguished as typical for the non-glycosylated form of IGFBP-3, whereas a fragment of 28.0 kDa profiled as representative for the glycosylated/glycated IGFBP-3. There were, however, no differences in SELDI-TOF MS signals between spectra from healthy persons and those from patients with diabetes. Fragmentation of IGFBP-3, assessed by SELDI-TOF MS analysis, does not seem to offer a biomarker that can differentiate diabetic from healthy individuals.

IGFBP-3 can be cleaved by plasmin, thrombin, cathepsin D, several matrix metaloproteases, prostate-specific antigen, nerve growth factor and other enzymes producing different fragments [15]. Specificity of these enzymes is related to the primary protein sequence, but also to the state of glycosylation of IGFBP-3. Characteristic IGFBP-3 fragments detected by SELDI-TOF MS analysis in our study correspond most closely to proteolytic fragments produced by the action of plasmin [15]. Plasmin is capable to cleave both non-glycosylated IGFBP-3 (producing a fragment of 15–16 kDa) and glycosylated forms (producing a fragment of 29–30 kDa). The same fragmentation pattern of IGFBP-3 found in healthy persons and patients with DM type 2 indicates that the same degradation process predominantly occurs in both groups of individuals. A difference may be found in the abundance of specific fragments reflecting the intensity of fragmentation (proteolysis) which is, as previously said, increased in patients with diabetes [13,25]. Preliminary data analysis suggested greater intensity of a 28 kDa fragment in some patients with DM type 2 compared to healthy persons, but population study and quantification of proteolytic activity are needed before making confident statements.

## 5. Conclusions

The most pronounced posttranslational modifications of IGFBP-3 in patients with diabetes mellitus type 2 are glycation and additional sialylation. Glycation increases the affinity of IGFBP-3 for IGF-I, whereas sialylation decreases it. Strong and opposite effects of these two modifications on ligand binding qualify them as factors that may be involved in the regulation of the amount of free, physiologically active IGFs, and modulation of processes that accompany development and progression of diabetes.

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