URINARY BIOMARKERS IN THE EARLY DIAGNOSIS OF RENAL DAMAGE IN DIABETES MELLITUS PATIENTS

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ABSTRACT

PURPOSE: The aim of this study was to determine the prevalence of tubular enzymes such as N-acetyl-β-D-glucosaminidase (NAG), alanine aminopeptidase (AAP) and gamma glutamyl transferase (γ-GT), α1-microglobulin, β2-microglobulin (β2-M) as well as microalbuminuria among patients with diabetes mellitus.

MATERIAL AND METHODS: Biomarkers were evaluated in 285 type 1 diabetes mellitus patients and 30 healthy volunteers as a control group. Urinary levels of tubular enzymes such as NAG, AAP and γ-GT as well as microalbuminuria, α1-microglobulin and β2-M were determined by routine methods. Diabetes mellitus patients were divided into two groups - with normoalbuminuria (3-30 mg/L) and microalbuminuria (31-300 mg/L).

RESULTS: Compared with healthy individuals, diabetes mellitus patients with normoalbuminuria excreted significantly higher levels of urinary NAG (p<0,05). In patients with microalbuminuria, the most significant changes of urinary NAG, AAP and g-GT, serum cystatin C and β2-M (p<0,001) were established. There was no significant difference between the patients and controls in respect to serum/urine creatinine. Urinary α1-microglobulin level was higher in the patients with poor glucose control (HbA1c >8,5%) and directly related to albuminuria. A significantly decrease of creatinine clearance in these patients was found out, too.

CONCLUSION: Measuring the urinary NAG excretion, α1-microglobulin and cystatin C could be useful for the assessment of renal failure in diabetes mellitus patients. The activity of AAP and γ-GT is a less sensitive indicator of tubular lesions. The levels of β2-M and cystatin C which diagnostic accuracies are superior to those of serum creatinine represent early indicators of incipient diabetic nephropathy. Therefore, urinary biomarkers are useful in early detection of tubular and glomerular lesions in type 1 diabetes mellitus patients.

Key words: *diabetes mellitus, urinary enzymes, urinary biomarkers, α1-microgobulin, β2-microglobulin, cystatin C*

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Received: February 1, 2013
Accepted: July 21, 2013

INTRODUCTION

Diabetes mellitus is a world health problem, affecting any age groups. It is defined as a state of chronic hyperglycemia, i.e. an excessive blood glucose concentration (3). Renal damage is a severe microvascular diabetic complication in patients with insulin-dependent diabetes mellitus (IDDM) leading to their death. Approximately 30-40% of such patients

Scripta Scientifica Medica, vol. 45, No 3, 2013, pp. 58-64

develop end-stage renal disease (11) and require either dialysis, or renal transplantation for survival (29). Epidemiological studies demonstrate that 20-30% of all type 1 diabetes mellitus patients with disease duration of 15-25 years develop a clinically significant renal disease. Thus, diagnosis of renal complication at an earlier stage would be critical and help reducing the morbidity and mortality rates. Diabetic nephropathy is the most common cause of end-stage renal failure in both IDDM and non-insulin dependent diabetes mellitus (NIDDM) (22). At the early stages, there are no clinical signs or symptoms of renal disease. The first clinical evidence of nephropathy is microalbuminuria (i. e. the appearance of low, but abnormal, albumin levels in the urine >30 mg/24 h). Without specific interventions, approximately 80% of IDDM patients present with increased urinary albumin excretion at a rate of 10-20% per year to the stage of overt nephropathy or macroalbuminuria (>300 mg/24 h or clinical albuminuria) over 10-15 years, developing hypertension along the way. Approximately 30% of type 2 diabetes mellitus individuals present with microalbuminuria or macroalbuminuria (overt nephropathy) shortly after the diagnosis of their illness as diabetes actually exists since many years onwards (22).

Serum and urinary biomarkers, both glomerular and tubular, in such patients play an important role in the early detection of renal damage (11). Urine as a diagnostic medium allows for non-invasive detection of biomarkers, including some of them that are associated with both types of diabetes and its complications. Detection of renal tubular proteins and enzymes in the urine demonstrates a tubular involvement that leads to renal complications of diabetes mellitus (1,23). The markers of tubular lesions include some urinary enzymes such as β-N-acetyl-β-D-glucosaminidase (β-NAG), γ-GT and AAP as well as tubular low molecular weight (LMW) proteins - α1-microglobulin and β2-microglobulin. Assessment of urinary enzymes is used in clinical diagnostic practice concerning the renal parenchymal tubular impairment (6,7). NAG is one of the most important and frequently evaluated urinary enzymes. It is a high-molecular weight hydrolytic lysosomal enzyme. Principally, it originates in proximal tubules and normally can't pass through the glomerular filtration (12). It is a very sensitive and reliable marker of renal failure (22).

Gamma glutamyl transferase (γ-GT) and alanine aminopeptidase (AAP) in urine originate from the surface of brush border of epithelial cell membrane in the proximal tubular lumen (28). Increased urinary γ-GT and AAP concentrations are observed in the patients with diabetes mellitus without signs of renal functional disorders or even microalbuminuria. Urinary enzyme excretion relates to the duration, regulation (HbA1c level) and degree of renal functional damage. LMW proteins, α1-microglobulin and β2-microglobulin (β2-M), in unbound form freely filtered through the renal glomerular basement membrane are reabsorbed by the proximal tubular cells (10). Hence, any proximal tubular cell dysfunction results in increased quantities of these proteins in urine (19). Urinary α1-microglobulin is higher in both IDDM (24) and NIDDM subjects than that in normal control ones and is present even without any clinical nephropathy. In NIDDM subjects, α1-microglobulin excretion directly correlates with albuminuria (12) and HbA1c levels (18) and decrease with improved glycemic control (9). Cystatin C is another LMW protein freely filtered by the glomerulus (5,8). The serum concentration of cystatin C is less dependent on extrarenal factors than in the case of creatinine (13,15). Early detection of renal function impairment in microalbuminuric diabetes mellitus patients would be of great value allowing the accurate treatment, adjustment of drug dose, and prevention of more severe renal damage (17,28). Previous studies demonstrate the superiority of serum cystatin C compared to creatinine in the evaluation of glomerular filtration rate (GFR) (11,20,25), especially when there is a minor GFR reduction (24,28).

This objective of the present study was to evaluate the clinical usefulness of sensitive urinary biomarkers such as urinary enzymes and LMW proteins in predicting the renal impairment in normoalbuminuric and microalbuminuric diabetes mellitus patients and the association between albuminuria and serum creatinine and cystatin C.

MATERIAL AND METHODS

The study covered of 285 randomly selected IDDM patients. They were on human insulin from the time of diagnosis and presented with a normal serum creatinine and without any signs of clinical nephropathy. A total number of 30 healthy subjects (25 men and 15 women aged 33,0±6,5 years) were randomly selected as a control group without any inflammatory conditions, illness and abnormalities in carbohydrate metabolism. The Ethics Committee approved the study protocol and every participant signed a fully informed consent to take part in the study. Venous blood specimens were collected from the subjects of both groups after a fasting state into two test tubes (one without anticoagulants and another with K3EDTA as anticoagulant). A 24-hour urine was collected from patients and controls for measuring the urinary creatinine, creatinine clearance, proteins and enzymes.

Glycemic control was assessed by measuring the glucose and glycohemoglobin in HbA1c used test kits from Roche Diagnostic. Serum/urine creatinine and urea were determined with standardized test applied in Cobas Integra analyzer. Spot urine samples were used to measure the excretion of the following urinary LMW proteins: α1-microglobulin, β2-microglobulin, serum cystatin C and microalbuminuria by means of immunoturbidimetric methods applied in DAKO tests. Normoalbuminuria and microalbuminuria were defined as below 30 mg/24 h and 31-300 mg/24 h, respectively. Urinary enzyme activity of NAG, AAP, and γ-GT was determined with the methods of Jung (18). The activity was expressed in U/mmol creatinine. Creatinine clearance (CCr mL/min) was calculated with reference to age, sex, serum and urine creatinine concentrations using the standard Cockrofts formula.

A nonparametric statistical analysis was used. Any data were presented as mean±standard error of the mean (SEM) and analyzed using an IBM personal computer. The differences between the two groups were calculated with Student's unpaired t-test. Values of $p < 0,05$ were considered statistically significant. Linear correlations between different variables (urinary enzymes and proteins) were estimated.

RESULTS

Table 1 shows the demographic data of both groups concerning age and sex. There are

Table 1. Biochemical and clinical characteristics of the patients and controls

	Controls (n=30)	Normoalbuminuric patients (n=170)	Microalbuminuric patients (n=115)
gender (males/females)	20/10	96/74	65/50
age (years)	33±6,5	50,0±3,2	57,3±4,9
fasting glucose (mmol/L)	4,8±1,4	6,7±1,3*	10,7±1,5**
serum creatinine μmol/L	86,0±12,0	89,0±9,0	94,0±10,2
urinary creatinine (mmol/L)	12,6±5,3	11,3±6,0	13,9±3,5
creatinine clearance (mL/min)	90,2±4,6	82,5±5,9	67,3±8,0**
cystain C (mg/L)	0,9±0,40	0,98±0,5	1,38±0,12*
HbA1c (%)	4,2±0,15	7,1±0,6**	8,9±0,75**
microalbuminuria (mg/L)	12,3±3,9	24,6±3,9*	82,5±20,6**
disease duration (years)	-	12,6±4,2	15.3±2,9

*Data are presented as mean±SEM; *p<0,01; **p<0,001*

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no significant differences between the patients and controls. In the control group, no significant differences are observed in the values of urinary enzymes and LMW proteins between males and females (p>0,05). There is a statistically significant increase of fasting blood glucose and HbA1c in diabetes mellitus patients. There are no statistically significant changes of serum and urine creatinine concentrations between the examined groups. There is a significant reduction of creatinine clearance in diabetes mellitus patients with microalbuminuria than of that in the control group (p<0,01). However, there are significant differences between the mean values of urinary microproteins in the control and the normoalbuminuric patients (p<0,01).

In diabetes mellitus patients with normoabuminuria and good metabolic control (HbA1c <8,0%), there is a statistically significant increase of urinary NAG and α1-microglobulin (p<0,05) (Table 2).

AAP, γ-GT and β2-M show no statistically reliable changes among the patients compared with those of the control group. However, in the patients with microalbuminuria and poor metabolic control (HbA1c >8,0%) our results demonstrate a significantly higher excretion of NAG and α1-microglobulin (p<0,001) than of the AAP, γ-GT and β2-M (p<0,01).

The correlation coefficient between the different urinary biomarkers is displayed on Table 3. There is a significant positive correlation in normoalbuminuric diabetes mellitus patients between the urinary enzymes and albuminuria (p<0,01). LMW protein, α1-microglobulin, correlates well with microproteins,

Table 2. Urinary biomarkers of the patients and controls

	Controls (n=30)	Normoalbuminuric (n=170)	Microialbuminuric (n=115)
NAG (U/mmol creatinine)	0,75±0,5	2,05±0,9*	6,8±0,7**
AAP (U/mmol creatinine)	1,1±0,3	1,7±0,6	2,4±0,8*
γ-GT (U/mmol creatinine)	1,42±0,4	1,55±0,7	2,75±0,95*
α1-microglobulin (mg/L)	8,0±4,0	11,6±2,1*	16,8±4,3**
β2-M (mg/L)	0,105±0,7	0,111±0,5	0,269±0,15*

*Data are presented as mean±SEM; *p<0,01; **p<0,001*

Table 3. Correlation between urinary enzymes, LMW proteins and albuminuria

	Biomarker	Normoalbuminuric	Microalbuminuric
urinary NAG	albuminuria	n. c.	0,570**
urinary AAP	urinary NAG	0,418*	0,650**
	albuminuria	n. c.	0,512*
urinary γ-GT	urinary NAG	0,512*	0,615**
	albuminuria	n. c.	0,538**
urinary α1-microglobulin	albuminuria	0,580**	0,708**
	urinary NAG	n. c.	0,590**
urinary β2-M	albuminuria	n. c.	0,412*

*n. c.= no correlation; *p<0,01; **p<0,001*

too, however, β2-M correlation coefficient is small. Urinary microproteins don't possess any positive correlation with the enzyme activities between the controls and the patients with normoalbuminuria However, in cases with microalbuminuria, there is a very strong positive correlation between urinary enzymes such as NAG, AAP and γ-GT and microalbumins (p<0,001). The concentration of α1-microglobulin during the development of albuminuria shows a very strong positive correlation (r=0,708; p<0,001). On the other hand, β2-M concentration possesses a smaller correlation coefficient.

There is no positive correlation between urinary enzymes and microproteins in diabetes mellitus patients with normoalbuminuria. Brush border enzymes such as AAP and γ-GT show a good correlation with NAG (p<0,01) in such patients.

A group of patients is separated at a late stage of development of diabetic nephropathy, where besides pathoanatomical and morphological alteartions of glomerular basal membrane, there are significant changes of the activity of examined tubular enzymes, too. It should be noted that the urinary β2-M concentration is significantly altered, which suggests changes in the structure of the glomerular basal membrane (Table 4).

DISCUSSION

There is no reliable therapy of diabetes mellitus complications in most patients, therefore preventing or delaying these complications contributes to reducing the morbidity and mortality rates (3). Several known risk factors such as age, age of disease onset, abnormal metabolic environment, hyperglycemia, arterial hypertension, protein overload, disease duration, and tobacco smoking are important in diabetic complications (3). Arterial hypertension and poor glycaemic control are causal risk factors for microalbuminuria. Recent data suggest that the kidney function in IDDM does not depend only on the duration of the disease, but also on other factors such as presence of microalbuminuria and proteinuria. We do not reveal any significant differences between diabetes mellitus patients and controls concerning age and sex. Mean disease duration is longer in microalbuminuric patients (15,3±2,9 years). This finding is similar to other results (23) and indicates a higher risk of microlabuminuria in the patients with longer disease duration.

Urinary microprotein excretion is elevated in the patients with a poor metabolic control (HbA1c >8,0%) compared to those with a good one (p<0,001). There are no significant differences of the serum and urine creatinine levels and creatinine clearance between the control group and normoalbuminuric diabetes mellitus patients. We prove that these urinary enzymes and LMW proteins are very sensitive parameters for early diagnosis of tubular cell lesions. Urinary NAG excretion is statistically significantly higher in the patients with microalbuminuria than

Table 4. Biomarkers in patients during the stages of diabetic nephropathy

	Controls	Patients with diabetic nephropathy
UNAG (U/mmol creatinine)	0,75±0,5	8,3±0,9**
UAAP (U/mmol creatinine)	1,1±0,3	3,6±0,9*
Uγ-GT (U/mmol creatinine)	1,42±0,4	4,8±1,05**
Uα1-microglobulin (mg/L)	8,0±4,0	29,3±5,6**
Uβ2-M (mg/L)	0,105±0,7	1,35±0,25**
serum creatinine (μmol/L)	86,0±12,0	118,0±15,5*
cystatin C (mg/L)	0,9±0,40	1,95±0,23**

*Data are presented as mean±SEM; *p<0,01; **p<0,001*

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in the controls (p<0,001) and there exists a positive correlation with AAP and γ-GT. The quantitative analysis of AAP and γ-GT demonstrates that they are less sensitive biomarkers (p<0,01) of renal tubular damage than NAG. Our data confirm other results (27) that excretion of urinary tubular enzymes is influenced by the some pathological conditions such as diabetes mellitus. Urinary excretions of renal tubular enzymes and LMW proteins are recommended as useful markers for detection of minor changes in proximal tubular function long before elevation of other markers such as proteinuria and serum creatinine increase (14).

Our data are similar to other findings that urinary NAG excretion is significantly higher in IDDM patients than in control subjects (p<0,001) and is significantly associated with disease duration (4,11,27). Urinary α1-microglobulin concentration and albumin directly relate to duration, severity and control of diabetes mellitus. Our results confirm other reports (21) about patients with IDDM duration longer than 10 years. In addition to albuminuria measuring glomerular dysfunction, urinary α1-microglobulin estimating proximal tubular dysfunction is useful for the early detection of nephropathy in diabetes mellitus. The higher urinary β2-M excretion in microalbuminuria (p<0,01) indicates an increased glomerular permeability and/or decreased reabsorption of proximal tubules. This is a sensitive biomarker of increased glomerular filtration and proximal renal tubular function and thus an early indicator of diabetic nephropathy (2). Elevated cystatin C concentration as an early marker of GFR changes among the diabetes mellitus patients with microalbuminuria >300 mg/24 h is due to poor metabolic control, hypertensive status and disease duration. There is a direct link between proteinuria and tubulointerstitial injury in the progression of glomerular disease (16,26).

CONCLUSION

Our results suggest that urinary NAG and α1-microglobulin excretion and cystatin C are the most sensitive parameters for early diagnosis of tubular cell damages. NAG activity increases before microalbuminuria appearance and its values are highest in such diabetes mellitus patients. The activities of AAP and γ-GT are less sensitive biomarkers of tubular damage than that of β-NAG. Urinary β2-M and serum cystatin C levels are sensitive markers for increased GFR and early indicators for advanced diabetic nephropathy. Site-specific urinary biochemical markers provide valuable information about early renal proximal tubular lesion that ultimately may precede glomerular permeability disorders in diabetes mellitus.

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