SERBIAN ARCHIVES OF MEDICINE Est.1872

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Paper Accepted* **ISSN Online 2406-0895**

Original Article / Оригинални рад

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Biomarkers of early kidney cells dysfunction in patients with membranous nephropathy

Биомаркери ране дисфункције ћелија бубрега код пацијената са мембранозном нефропатијом

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Received: February 21, 2017
Accepted: February 22, 2017
Online First: March 14, 2017
DOI: 10.2298/SARH170221072M

* **Accepted papers** are articles in press that have gone through due peer review process and have been accepted for publication by the Editorial Board of the *Serbian Archives of Medicine*. They have not yet been copy edited and/or formatted in the publication house style, and the text may be changed before the final publication.
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SUMMARY

Introduction/Objective Worse prognosis of membranous nephropathy (MN) is determined by the presence of persistent proteinuria, and extensive tubulointerstitial lesions at initial biopsy.

Study investigated the value markers of renal cell dysfunction (glomerular filtration rate, urinary excretion of protein, ectoenzymes proximal tubular epithelial cells, and oxidative stress) in patients with MN, and point to the use of these markers in possible therapeutic modification.

Methods The study included 28 patients with MN and 30 healthy individuals as control. Addition to basic laboratory studies, the enzyme (aminopeptidase N-APN, plasma cell glycoprotein 1 -PC-1, N-acetyl-ß-D-glucosaminidase-NAG and dipeptidylpeptidase IV-DPP IV) activity was determined in serum and urine, as well as parameters of oxidative damage (thiobarbituric acid concentration of substance-responders TBARS, malondialdehyde-MDA and the concentration of total sulfhydryl-SH-group).

Results In patients with MN serum activity of PC-1 and APN, and urinary excretion of NAG was significantly higher than in the control group. Also, significant correlation between daily proteinuria and serum PC-1 activity and urinary excretion of NAG was found in patients with MN. Serum and urine levels of TBARS, as also total sulfhydryl-SH-group levels were significantly lower in patients with MN as compared with healthy controls.

Conclusion Kidney damage in MN is accompanied by the release of several tubular enzymes, with potential diagnostic and prognostic significance. The study suggests a possible role of oxidative stress in pathogenesis of MN and the use of antioxidants in preventing impairment as part of future therapy.

Keywords: membranous nephropathy; ectoenzyme; oxidative stress

САЖЕТАК

Увод/Циљ Неповољна прогноза мембранозне нефропатије (МН), одређена је перзистентном протеинуријом и опсежним тубулоинтерстицијским лезијама доказаним иницијалном биопсијом.

Циљ рада био је да се испита значај маркера дисфункције ћелија бубрега (јачине гломерулске филтрације, уринарне екскреције протеина, ектоензима епителних ћелија проксималних тубула, и оксидативни стрес) код пацијената са МН и процени могућност примене ових маркера при избору терапије.

Методе Студијом је обухваћено 28 пацијената са МН и 30 клинички здравих особа као контролна група. Поред основних лабораторијских анализа, одређена је активност ензима (аминопептидазе Н-АПН, ћелијски плазме гликопротеин 1 – ПГ-1, Н-ацетил СС-Д-глукозаминидазе–НАГ и дипептидилпептидазе IV–ДПП IV) у серуму и урину, као и параметри оксидативног оштећења (концентрација реактивних супстанци везаних за тиобарбитуричну киселину –ТБАРС, малондиалдехида–МДА и укупних сулфхидрил-СХ-групе).

Резултати У групи болесника са МН активност ПГ-1 и АПН у серуму, и уринарна екскреција НАГ су били статистички значајно већи него у контролној групи. Уочена је и значајна корелација између ПГ-1 активности у серуму и екскреције НАГ урином са дневном протеинуријом код пацијената са МН. Концентрација ТБАРС у серуму и урину као и концентрација укупних сулфхидрилних-СХ-група значајно је нижа код болесника са МН у поређењу са контролном групом.

Закључак Оштећење бубрега у МН прати ослобађање тубулских ензима, са потенцијалним дијагностичким и прогностичким значајем. Студија указује и на могућу улогу оксидативног стреса и значај примене антиоксидативне терапије у спречавању прогресивног тока болести.

Кључне речи: мембранозна нефропатија; ектоензими; оксидативни стрес

INTRODUCTION

Membranous nephropathy (MN) is the most common glomerulonephritis that cause nephrotic syndrome in adults (over 80%). Worse prognosis determined by the presence of persistent proteinuria, and extensive tubulointerstitial lesions at initial biopsy. In different morphological forms of MN enzymes of proximal tubular epithelial cell markers are valuable in the assessment of tubular damage, even in patients with normal renal function and normal urinary albumin excretion rate. Parameters of oxidative stress, as the primary mediators in glomerulonephritis, may represent non-invasive, early

biological markers of renal damage. However, none of these markers has been recognized as a marker offering the possibility to modify therapy in order to slow down the progression of the disease.

Plasma cell glycoprotein 1 (PC-1), known as ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), is a class II transmembrane glycoprotein, implicated in the pathogenesis of insulin resistance in obesity, diabetes and uremia [1], since it inhibited insulin receptor signaling [2, 3] either at the level of the insulin receptor tyrosine kinase [4] or downstream at a postreceptor cite [5]. Urinary PC-1 was found to be mainly produced by the kidney. An ectonucleotide pyrophosphatase has been found in the brush border of the proximal tubule, however, a highly active phosphodiesterase I was demonstrated in glomerular epithelial and mesangial cells . Its increased urinary excretion has been observed in newly diagnosed type 1 diabetic patients with poor glycemic control, however, the decreased excretion in type 1 diabetics with micro- or macroalbuminuria, in patients with primary glomerulonephritis, including those with renal failure, as well as in those without an apparent kidney damage. The therapeutic modification of the PC-1 expression was demonstrated in insulin resistant type 2 diabetics after a 3-month metformin treatment [6].

Aminopeptidase N (APN) is an ectopeptidase with a wide substrate specificity, widely expressed in numerous human cells and tissues [7,8]. However, its urinary excretion is an established marker of the damage of a brush border of the proximal tubule.

N-acetyl-beta-D-glucosaminidase (NAGA) is a lysosomal enzyme, clearly indicated as a valuable measure to evaluate tubular damage and metabolic control in kidney disease patients, even in the early stages, because urinary NAGA originated in renal proximal tubular cells and positively correlated with microalbuminuria. It was found to be abnormally raised in 60% of type 1 diabetics before any increase in albumin excretion rate. However, in type 2 diabetics, NAGA began to rise in the third year of diabetes, maintained a plateau between 3 and 10 years, and rapidly increased after the tenth year of the duration of this disease [7-12].

Dipeptydilpeptidase IV (DPP IV) is an intrinsic membrane glycoprotein, localized on glomerular visceral epithelial cells, endothelial cells and the proximal tubule brush border [11].

There is an increase in oxidative stress in chronic renal insufficiency. Overproduction of superoxide and other related reactive oxygen species resulting in oxidative stress reduces the biological effects of nitric oxide. Among other, nitric oxide, as a potent endogenous vasodilatator, regulates systemic blood pressure and renal functions. The bioactivity of nitric oxide is reduced by superoxide, a major reactive oxygen species. Though both of these highly reactive species have distinct roles in other pathways, their interaction is emerging as a major regulatory factor in normal and pathological renal function [13].

Reactive oxygen species (ROS) play an important role in the pathophysiology of kidney disease and are designated as primary mediators of glomerulonephritis, responsible for a modification of the glomerular permeability to proteins, the development of morphological lesions and impaired glomerular hemodynamics (reduction in glomerular blood flow and glomerular filtration rate). In

glomeruli, ROS are generated by both infiltrating cells (neutrophils, monocytes) and resident glomerular cells (mesangial and endothelial cells and podocytes) [13,14].

A large increase in plasma levels of malondialdehyde MDA was found in patients with focal segmental glomerulosclerosis, occurs early and could play an important role in the pathogenesis of glomerulosclerosis

Attenuation of antioxidant system is also present in patients with nephrotic syndrome, lupus nephritis, IgA nephropathy, and other glomerular diseases [13-17].

The aim of the study was to investigate whether markers of renal cell dysfunction (glomerular filtration rate, urinary excretion of protein, ectoenzymes proximal tubular epithelial cells, and oxidative stress) in patients with MN, and point to possible therapeutic modification of the expression as a useful treatment.

METHODS

Subjects

The present study was carried out at the Clinic of Nephrology, Faculty of Medicine, Nis, Serbia. The study included 28 patients with MN age 59.6 ± 7.4 years. The control group consisted of 30 individuals, age 48.7 +11.6 years, clinically healthy, with no personal history or first degree relatives with kidney diseases or abnormal laboratory test results of clinical significance. The study was approved by the local Research Ethics Committee and informed consent was obtained from all participants enrolled in the study.

Baseline assessments. Blood samples and urine were taken after an overnight fast of 12 hours and baseline biochemical analyses were performed on BioSystems S.A. (Costa Brava, Barcelona, Spain) using standardized protocols.

Urinary and serum enzyme activities. Phosphodiesterase activity of plasma cell membrane glycoprotein 1 (PC-1) was measured by the hydrolysis of thymidine-5`-monophosphate p-nitrophenyl ester (Sigma Chemical Co., St. Louis, MO, USA) . Aminopeptidase N (APN), N-acetyl-β-D-glucosaminidase (NAGA) and dipeptidylpeptidase IV (DPP IV) activities were determined by the spectrophotometric method, using alanine-p-nitroanilide, N-acetyl-β-D-glucosaminide and p-nitroanilide as substrates, respectively [6, 8, 10]. Urinary enzyme activities were expressed as enzyme-to-creatinine ratios.

Oxidative stress parameters. Plasma malondialdehyde (MDA) was determined by modified thiobarbituric acid (TBA) method and the products of the reaction were measured at 535 nm after $FeSO_4$ administration. In order to determine urinary MDA, urine was combined with 5% butylated hydroxitoluene (BHT) and thiobarbituric acid (TBA) solution. After incubation at 1000ºC, the absorbance of samples at 532 nm was measured. The concentration of thiobarbituric acid-reactive substances (TBARS) was calculated using 156000 as the molar extinction coefficient. The quantity of TBARS is proportionate to the amount of MDA, a lipid peroxidation product generated by the

oxidation of membrane lipids by ROS. MDA reacts with TBA to form a 1:2 MDA-TBA adduct. Reduced glutathione was determined by the modification of the method of Ellman , based on the formation of the colored product, monitored at 412 nm after Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid) was added.

Statistical analysis

Data were analyzed using statistical software Jandel SigmaStat® for Windows Version 2.0. Student`s t-test and non-parametric Mann-Whitney Rank Sum Test were used when appropriate and data were expressed as means ± SD, medians ± SD, or medians with range in parentheses. Parameters were correlated using simple linear regression test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Baseline anthropometric and biochemical characteristics are given in Table 1.

Mean serum PC-1 and APN activities in MN group were significantly higher than those in control group ($p<0.05$). Also, urinary NAGA excretion was markedly ($p<0.01$) higher in MN group as compared to healthy controls. Results are given in Figure 1and 2 .

Table 1. Baseline anthropometric and biochemical characteristics of patients with membranous nephropathy and healthy controls

	Membranous nephropathy	Control group
n (M:F)	28 (6:4)	30 (15:15)
Age (years)	59.6±7.4 [B]	47.7±11.6
Hemoglobin (g/dl)	12.33±2.07 [B]	13.99±1.06
WBC (x10^9/ml)	6.81±2.11	5.97±1.34
Creatinine (μmol/l)	122.93±92.57 [C]	74.04±11.47
CCr (ml/min)	70.33±31.27 [B]	109.90±16.41
T Proteins (g/l)	59.28±10.32 [A]	74.43±4.88
Albumins (g/l)	34.70±8.30 [B]	41.85±3.87
T Cholesterol (mmol/l)	8.23±3.07 [C]	5.87±0.96
Triglycerides (mmol/l)	2.61±1.09 [C]	1.67±1.12
Glucose (mmol/l)	5.09±0.57 [C]	5.4±0.45
CRP (mg/dl)	5.43±1.07 [A]	1.43±1.01
Fibrinogen (g/l)	4.77±2.61	3.91±1.22

Results are given as means±SD.
n(M:F)–number (male:female); WBC–white blood cells; CCr–creatinine clearence; T–total; CRP–C reactive protein.
[A] $P<0.001$ compared to control group;
[B] $P<0.01$ compared to control group;
[C] $P<0.05$ compared to control group.

Significant correlation between daily proteinuria and serum PC-1 activity and urinary excretion of NAG was found in patients with MN ($p<0.01$). Significant correlation was also found between urinary enzame activities and creatinine clearance. Results are given in Table 2.

Analysis of oxidative stress parameters showed that urine and serum MDA was significantly lower in MN group ($p<0.01$, $p<0.001$, respectively) than in control group. Serum level of TBARS and TBARS urine excretion, as well as serum level of total sulfhydryl-SH-group levels were significantly lower in patients with MN than in healthy controls. Results are given in Table 3.

DISCUSSION

Previous studies have suggested that proteinuria resulting from glomerular disease has a direct role in activating the cascade initiated by epithelial cell injury. High absorption rates of proteins may lead to striking changes in tubular morphology, including dramatic enlargement of protein absorption

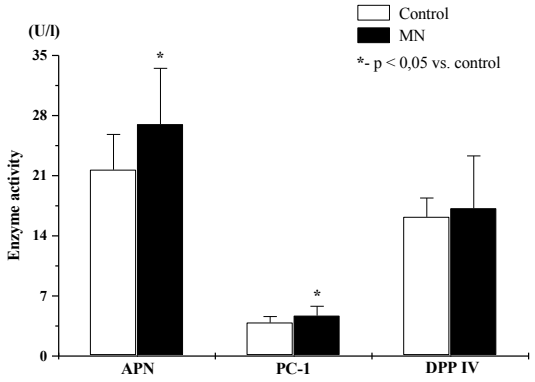

Figure 1. Enzyme activity in serum of patients with membranous nephropathy compared to control group. APN, aminopeptidase N; PC-1, plasma cell glycoprotein 1; DPP IV, dipeptidylpeptidase IV.

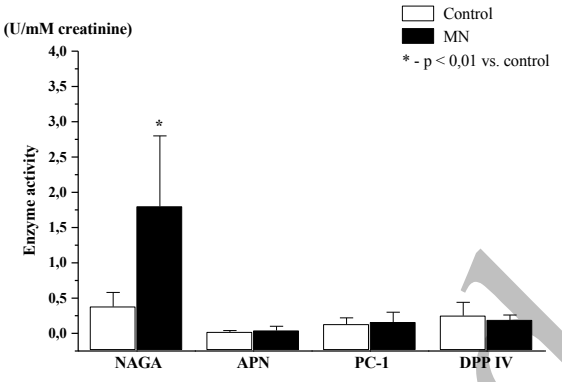

Figure 2. Enzyme activity in urine of patients with membranous nephropathy compared to control group. NAGA, N-acetyl-β-D-glucosaminidase; APN, aminopeptidase N; PC-1, plasma cell glycoprotein 1; DPP IV, dipeptidylpeptidase IV; *$p < 0.01$ compared control group

Table 2. The correlation between serum enzyme activity and proteinuria, and creatinine clearance.

	Urine-protein g/24 h		CCr ml/min	
	R	*p*	R	*p*
SerumPC1, U/l	0.77	0.82		
Urine NAGA, U/mmol creatinine	0.82	0.82		
Urine DPP IV urine, U/mmol creatinine			0.71	< 0.05
Urine APN urine, U/mmol creatinine			0.75	< 0.01
Urine PC-1 urine, U/mmol creatinine			0.39	< 0.05
Urine NAGA urine, U/mmol creatinine			0.38	< 0.05

PC-1 – plasma cell glycoprotein 1; NAGA – N-acetyl-β-D-glucosaminidase; DPP IV – dipeptidylpeptidase IV; APN – aminopeptidase N; CCr–creatinine clearance.

Table 3. Oxidative stress parameters.

	Membranous nephropathy	Control group
Serum-MDA-S (μmol/l)	10.00±1.55 [C]	14.66±2.00
Urine-MDA (μmol/gCr)	0.60±0.24 [A]	1.33±0.63
Serum-TBARS,	0,58±0,24[B]	1,39±0,73
Urine-TBARS	10,19±1,58 [D]	13,94±2,86
SH-groups (μmol/l)	181.41±36.40 [B]	252.18±24.02

MDA – malondialdehyd; TBARS – thiobarbituric acid-reactive substances; SH-groups, sulphydril groups.
[A] p<0.001 compared to control group
[B] p<0.01 compared to control group
[C] p<0.05 vs. control group
[D] p<0.005 compared to control group

droplets and loss of brush border structure, suggesting pathologic injury. In the case of lysosome, as the concentration of absorbed protein increases, there is concomitant increase in the activity of cathepsin D, a powerful protease, which leads to a compensatory increase in the rate of lysozyme hydrolysis within these cell organelles [18,19, 20]

Recent study demonstrated the highest increase urinary NAGA activity in patients with primary glomerulonephritis [7]. From our data it is evident that urinary NAGA excretion was significantly (p<0.01) increased in MN patients as compared to that of controls. Furthermore, study also showed significant correlation between proteinuria and urinary NAGA excretion in patients with MN. It is important to emphasis that, in majority of our patients with MN, the disease has manifested by nephrotic range of proteinuria. This data suggests that urinary NAGA activity may be indicative of tubular damage with lysosomal cell injury. Our results showed an increased serum PC-1 activity in MN patients, as well as

serum APN activity compared to controls ($p<0.05$). Bought may represent damage of brush border of the proximal tubule. Since no correlation between these findings and decline in renal function was found, these increased PC-1 and APN increased serum activity might be considered as early markers of tubular dysfunction that appeared prior to interstitial fibrosis, and might have important role in making decision of therapeutic approach. Opposite of above maintained data, we have found significant correlation ($p<0.05$) between decline of renal function and urinary DPP IV activity. As an intrinsic membrane glycoprotein, DPP IV localized on the proximal tubule brush border, as well as on glomerular viscelar epithellial cells, and increased urinary excretion may represent adverse glomerular cells damage.

Intracellular communication play a major role in the development of glomerulonephritis, particulary including mesangial cells, which are the source and the target of a variety of autacoids. The role of APN activity in glomerular mesangial cells is still unknown. Stefanovic et al. [9] suggested that it is not only a marker of damage of brush border of the proximal tubule but may be a marker of cell differentiation, and may play role in glomerular cell proliferation.

We found significant correlation ($p< 0.01$) between decline of glomerular filtration rate, measured by creatinine clearence, and increasing urinary excretion of APN. This data suggests that urinary APN activity represent severe renal injury and adverse outcome.

CONCLUSION

Kidney damage in membranous nephropathy is accompanied by the release of several tubular enzymes, with potential diagnostic and prognostic significance. Urinary NAGA activity showed significantly correlation to proteinuria in examined group with MN, without correlation to renal function, and may play a direct role in establishing early tubular damage, important to therapeutic approach. The study also suggests a possible role of oxidative stress and antioxidant therapy importance in preventing impairment as part of future therapies.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Science and Environmental Protection of Serbia No. 175092

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