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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Biomarkers of early kidney cell dysfunction in patients with membranous nephropathy

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SUMMARY

Introduction/Objective An unfavorable prognosis of membranous nephropathy (MN) is determined by the presence of persistent proteinuria and extensive tubulointerstitial lesions at initial biopsy. Our study investigated the value of markers of renal cell dysfunction (glomerular filtration rate, urinary excretion of protein, ectoenzyme proximal tubular epithelial cells, and oxidative stress) in patients with MN, and points to the use of these markers in a possible therapeutic modification.

Methods The study included 28 patients with MN and 30 healthy individuals as control. In addition to the basic laboratory studies, enzyme [aminopeptidase N (APN), plasma cell glycoprotein-1 (PC-1), N-acetyl-β-D-glucosaminidase (NAGA), and dipeptidyl peptidase-4] activity was determined in serum and urine, as well as parameters of oxidative damage [thiobarbituric acid concentration of substance-responders (TBARS), malondialdehyde, and the concentration of the total sulfhydryl (SH) group].

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

INTRODUCTION

Membranous nephropathy (MN) is the most common glomerulonephritis that causes nephrotic syndrome in adults (over 80%). An unfavorable prognosis is 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, is a class II transmembrane glycoprotein, implicated in the pathogenesis of insulin resistance in obesity, diabetes, and uremia, since it inhibits insulin receptor signaling either at the level of the insulin receptor tyrosine kinase or downstream at a postreceptor site [1–5]. Urinary PC-1 was found to be mainly produced by the kidneys. Ectonucleotide pyrophosphatase has been found in the brush border of the proximal tubule; however, highly active phosphodiesterase-1 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, decreased excretion has been observed in type 1 diabetics with micro- or macroalbuminuria, in patients with primary glomerulonephritis, including those with renal failure, as well as in those without apparent kidney damage. The therapeutic modification of the PC-1 expression was demonstrated in insulin-resistant type 2 diabetics after a three-month metformin treatment [6].

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

N-acetyl-β-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 originates in renal proximal tubular cells and positively correlates 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,

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NAGA began to rise in the third year of diabetes, maintained a plateau between three and 10 years, and rapidly increased after the 10th year of the duration of this disease [7–12].

Dipeptidyl peptidase-4 (DPP-4) 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 things, 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 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, reactive oxygen species 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; it 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 of proximal tubular epithelial cells, and oxidative stress) in patients with MN point to a possible therapeutic modification of the expression as a useful treatment.

METHODS

Subjects

The present study was carried out at the Clinic for Nephrology, Faculty of Medicine, Niš, Serbia. The study included 28 patients with MN, 59.6 ± 7.4 years old. The control group consisted of 30 individuals, 48.7 + 11.6 years old, 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 institutional 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 using BioSystems reagents (BioSystems S.A., Costa Brava, Barcelona, Spain) using standardized protocols.

Urinary and serum enzyme activities

Phosphodiesterase activity of PC-1 was measured by hydrolysis of thymidine 5′-monophosphate p-nitrophenyl ester (Sigma Chemical Co., St. Louis, MO, USA). APN, N-acetyl-β-D-glucosaminidase, and DPP-4 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 MDA was determined by a 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 hydroxytoluene and TBA solution. After incubation at 1,000°C, the absorbance of the samples at 532 nm was measured. The concentration of thiobarbituric acid-reactive substances (TBARS) was calculated using 156,000 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 SigmaStat® for Windows Version 2.0 (Systat Software, Inc., San Jose, CA, USA). 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. 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 the MN group were significantly higher than those in the control group ($p < 0.05$). Also, urinary NAGA excretion was markedly

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

Parameter	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 (× 10^9/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]	109.9 ±16.41
T Proteins (g/l)	59.28 ±10.32[A]	74.43 ± 4.88
Albumins (g/l)	34.70 ± 8.3[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]	5.48 ± 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 cell count; CCr – creatinine clearance; T – total; CRP – C-reactive protein;
[A]p < 0.001 compared to the control group;
[B]p < 0.01 compared to the control group;
[C]p < 0.05 compared to the control group

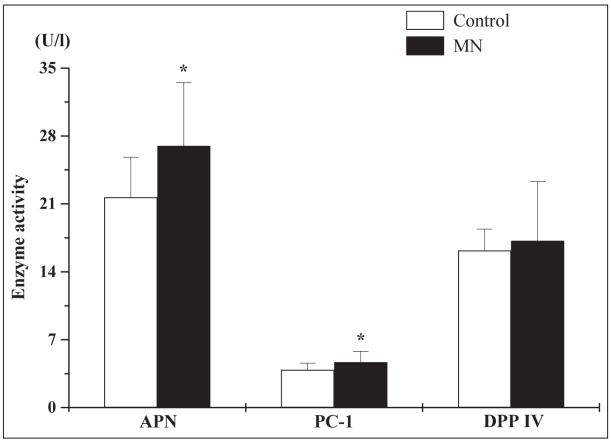

Figure 1. Enzyme activity in serum of patients with membranous nephropathy (MN) compared to the control group; APN – aminopeptidase N; PC-1 – plasma cell glycoprotein 1; DPP IV – dipeptidylpeptidase IV; *p < 0.01 compared to the control group

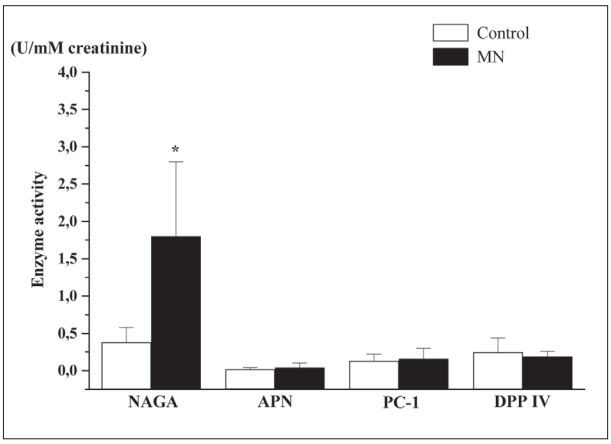

Figure 2. Enzyme activity in urine of patients with membranous nephropathy compared to control group; NAGA – N-acetyl-β-D-glucosaminidase; APN – aminopeptidase N; PC-1 – plasma cell glycoprotein 1; DPP IV – dipeptidylpeptidase IV; *p < 0.01 compared to the control group

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

Parameter	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-4 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-4 – dipeptidyl peptidase-4; APN – aminopeptidase N; CCr – creatinine clearance

Table 3. Oxidative stress parameters

Parameter	Membranous nephropathy	Control group
Serum MDA-S (μmol/l)	10 ± 1.55[C]	14.66 ± 2
Urine MDA (μmol/gCr)	0.6 ± 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 ± 36.4[B]	252.18 ± 24.02

MDA – malondialdehyde; TBARS – thiobarbituric acid-reactive substances; SH groups – sulphydryl groups

[A]p < 0.001 compared to the control group;
[B]p < 0.01 compared to the control group;
[C]p < 0.05 compared to the control group;
[D]p < 0.005 compared to the control group

(p < 0.01) higher in the MN group as compared to healthy controls. The results are given in Figures 1 and 2.

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 enzyme activities and creatinine clearance. The results are given in Table 2.

Analysis of oxidative stress parameters showed that urine and serum MDA was significantly lower in the MN group (p < 0.01, p < 0.001, respectively) than in the control group. Serum level of TBARS and TBARS urine excretion, as well as serum level of total SH group levels were significantly lower in patients with MN than in healthy controls. The 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 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].

A 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, the study also showed a significant correlation between proteinuria and urinary NAGA excretion in patients with MN. It is important to emphasize that, in the majority of our patients with MN, the disease manifested itself 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 ($p < 0.05$). Both may represent damage of brush border of the proximal tubule. Since no correlation between these findings and decline in renal function was found, increased PC-1 and increased APN serum activity might be considered as early markers of tubular dysfunction that appeared prior to interstitial fibrosis, and might have an important role in making a decision concerning the therapeutic approach. In contrast to the stated data, we have found significant correlation ($p < 0.05$) between decline of renal function and urinary DPP-4 activity. As an intrinsic membrane glycoprotein, DPP-4 localized on the proximal tubule brush border, as well as on glomerular visceral epithelial cells, and increased urinary excretion may represent adverse glomerular cell damage.

Intracellular communication plays a major role in the development of glomerulonephritis, particularly 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. Stefanović et al. [7] suggested that it is not only a brush border damage marker of the proximal tubule but may be a marker of cell differentiation, and may play a role in glomerular cell proliferation.

We found significant correlation ($p < 0.01$) between the decline of glomerular filtration rate, measured by creatinine clearance, and increasing urinary excretion of APN. This data suggests that urinary APN activity represents 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 a significant correlation to proteinuria in the 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 the importance of antioxidant therapy in preventing impairment as part of future therapies.

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Биомаркери ране дисфункције ћелија бубрега код болесника са мембранозном нефропатијом

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САЖЕТАК

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

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 болесника са МН и процени могућност примене ових маркера при избору терапије.

Методе Студијом је обухваћено 28 болесника са МН и 30 клинички здравих особа као контролна група. Поред основних лабораторијских анализа, одређена је активност ензима [аминопептидазе *N* (*APN*), ћелијски плазме гликопротеин 1 (*PC*-1), *N*-ацетил *SS-D*-глукозаминидазе (*NAG*) и дипептидилпептидазе IV (*DPP* IV)] у серуму и урину, као и параметри оксидативног оштећења [концентрација реактивних супстанци везаних за тиобарбитуричну киселину (*TBARS*), малондиалдехида (MDA) и укупних сулфхидрилних (СХ) група].

Резултати У групи болесника са МН активност *PC*-1 и *APN* у серуму, и уринарна екскреција *NAG* били су статистички знатно већи него у контролној групи. Уочена је и значајна корелација између *PC*-1 активности у серуму и екскреције *NAG* урином са дневном протеинуријом код болесника са МН. Концентрација *TBARS* у серуму и урину као и концентрација укупних СХ група знатно је нижа код болесника са МН у поређењу са контролном групом.

Закључак Оштећење бубрега у МН прати ослобађање тубулских ензима, са могућим 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