Activation of complement system by mannan pathway and *Mbl2* genotypes in patients with type 2 diabetes and nephropathy

Aktivacija komplementnega sistema po mananski poti in genotipi gena *Mbl2* pri bolnikih s sladkorno boleznijo tipa 2 in ledvično okvaro

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Ključne besede:
nefropatija; sladkorna bolezen; komplement; lektin; manan

Key words:
nephropathy; diabetes; complement; mannan-binding lectin

Citirajte kot/Cite as:
Zdrav Vestn 2014; 83: 661–9

Prispelo: 10. okt. 2013,
Sprejeto: 3. apr. 2014

Izvleček

V raziskavi smo predstavili možnost povezanosti proženja mananske (lektinske) poti aktivacije komplementnega sistema z nastankom sprememb pri diabetični nefropatiji. Zato smo zbrali krvne in urinske vzorce 20 bolnikov s potrjeno sladkorno boleznijo tipa 2 in ledvično okvaro ter dvajsetih zdravih prostovoljcev iz raziskovalnega okolja. Pri vseh smo izvedli laboratorijske preiskave, ki jih izvajamo za ugotavljanje vpletenosti komplementnega sistema v patogenezo bolezni. V vzorcih krvi smo določali: CH50, APH50, C3, C4, C3d/dg, C4d, SC5b-9, MBL in izvedli genotipizacijo gena *Mbl2*; v urinu pa: C3d/dg, C4d, MBL in SC5b-9. Pri bolnikih z diabetesom tipa 2 je bil komplementni sistem, za razliko od zdravih prostovoljcev, očitno spodbujen, kar so potrjevali rezultati analiz v plazmi in urinu. Ugotovili smo tudi očitno razliko v genski konstituciji zdravih prostovoljcev in bolnikov z diabetesom ter ledvično okvaro glede gena *Mbl2*. Zdravi preiskovanci so bili v 45 % tipa A0, ki kodira za srednje in nizke koncentracije proteina MBL, medtem ko so bili bolniki v 75 % tipa AA in so proizvajali velike količine proteina MBL, ki je potreben za aktivacijo mananske (lektinske) poti komplementne kaskade. Iz prikazanih rezultatov raziskave moremo sklepati, da velika količina beljakovine MBL v krvi skupaj s polimorfizmom gena *Mbl2*, ki kodira za veliko proizvodnjo beljakovine MBL, z veliko verjetnostjo vodi do okvare ledvic pri diabetikih z boleznijo tipa 2.

Abstract

To add new evidence that complement activation by the mannan (lectin) pathway is involved in the pathogenesis of nephropathy in patients with type 2 diabetes, we collected blood and urine samples from 20 patients with type 2 diabetes and nephropathy and 20 apparently healthy individuals from the general population. We performed tests for complement activation analysis (CH50, APH50, C3, C4, C3d/dg, C4d, SC5b-9, MBL and genotyping of *Mbl2* gene) in blood and (C3d/dg, C4d, MBL, and SC5b-9) in urine. We found significant signs of complement activation in patients with type 2 diabetes, and nephropathy in blood and in urine. We also observed significant genetic differences between healthy individuals and patients with type 2 diabetes and nephropathy in terms of the *Mbl2* gene. Healthy individuals were apparently more heterogeneous (45 % A0 type) in presentation of the structural *Mbl2* genotype, giving intermediate and low levels of MBL protein, compared to patients with type 2 diabetes and nephropathy, who had a very homogenous *Mbl2* genome (75 % AA type) giving a high level of MBL production. From our data we can conclude that patients with type 2 diabetes and nephropathy presents more frequently with *Mbl2* gene polymorphism giving high production of the MBL protein, which is very likely connected with acquired renal injury.

Grant No. P3-0083 (2009-2013) from the Slovene Research Agency.

1. Introduction

Homeostasis of glucose in the kidney is altered in patients with type 2 diabetes. Complex changes in gluconeogenesis, glucose metabolism, filtration, reabsorption, and consumption were described in patients with this disease.[1] Interesting fact for our investigation is that endogenous bio-transformation of glucose, employing fructose 6-phosphate pathway, also regulates the mannose level in blood. [2] Quoted contribute to the production of molecules able to activate complement by the mannan pathway. [2-5] Mannan pathway of complement activation is triggered by the binding of MBL protein to mannan residues.[6] Patients with type 2 diabetes with nephropathy present with an increased percentage of cases with genes coding for high serum levels of mannan-binding lectin (MBL). [6-10] In this report, we present data in support of the belief that mannan pathway activation of the complement system may be important for the pathogenesis of nephropathy in patients with type 2 diabetes.

2. Materials and Methods

2.1. Study population

Twenty patients with type 2 diabetes and overt nephropathy from the outpatient clinic of the Clinical Department of Endocrinology, Diabetes and Metabolic Diseases in Ljubljana, with elevated serum creatinine values and urinary albumin excretion rate U-albumin/creatinine in a morning spot urinary sample, and 20 healthy volunteers from the general population without any history of nephropathy or diabetes. All of them provided written informed consent. The protocol was in accordance with the Declaration of Helsinki and was approved by the Slovenian Medical Ethics Committee, 19 Oct. 2010, No. 73/10/10.

2.2. Laboratory and genetic analyses:

Complement analysis was carried out on samples of EDTA plasma and EDTA urine. All samples were prepared and treated in accordance to the methodology described in detail in the paper "Demonstration of apoptosis-associated cleavage products of DNA, complement activation products SC5b-9 and C3d/dg, and immune complexes CIC--C3d, CIC-IgA, and CIC-IgG in the urine of patients with membranous glomerulonephritis".[11] Samples were stored at -20°C until tested.

Screening of the classical pathway of activation (CH50 %) was performed as described by Mayer.[12] Sheep erythrocytes were coated with anti-sheep erythrocyte antibodies (haemolysin) and incubated together with different dilutions of EDTA plasma. A calibration curve was constructed and at 50 % of haemolysis for each of the samples, the concentration of the complement indicating classical activation was read out.

Screening of *alternative pathway* activity (APH50 %) was performed as described by Joiner et al..[13] Rabbit erythrocytes were incubated with different dilutions of EDTA plasma and the concentration of the complement indicating alternative activation was read out at 50 % of haemolysis for each sample.

The concentration of SC5b-9 in plasma and urine was determined as described by Accardo-Palumbo et al..[14] Microtiter plates (Nunc, Wiesbaden, Germany) were coated with mouse monoclonal antibodies against SC5b-9 (Diatec AS, Oslo, Norway). Appropriate dilutions of samples were then pipetted into micro-wells and incubated for 60 minutes. Bound SC5b-9 was detected by rabbit anti-C5 antibodies (Dako, Glostrup, Denmark). The reaction was visualized by the addition of horseradish peroxidase-conjugated goat anti-rabbit antibody (Dako) and 1,2-phenylendiamine dihydrochloride as substrate.

C3 and C4 were determined using commercially available radial-immunodiffusions tests (The Binding Site, Birmingham, UK).

C3d/dg was determined by double-decker rocket immunoelectrophoresis as described by Brandslund et al..[15] C3d specificities of C3 split products were determined in agar gel using anti-C3c (Dako, Hamburg, Germany)

in the lower gel and anti-C3d (Dako) in the upper gel.

C4d split product of C4 was determined using a commercially available MicroVue C4d Fragment EIA Kit (Quidel, San Diego, USA).

MBL protein concentration was determined using a commercially available Human MBL Immunoassay Kit (R&D Systems Europe Ltd., Abingdon, UK).

2.3. Molecular genetic analysis

Molecular genetic analysis was carried out on genomic DNA extracted from EDTA anticoagulated venous blood using an EZ1 DNA Blood 350 µl Kit performed on a BioRobot EZ1 Workstation (QIAGEN GmbH, Hilden Germany), according to the manufacturer's instructions.

Primers were designed using PyrosequencingTM Assay Design Software (Biotage, Uppsala, Sweden) and purchased from TIB MOLBIOL, Germany.

Specific fragments including SNPs were amplified using primers listed in Table 1. The reaction mix contained 1 x PCR buffer (Applied Biosystems, Corfornia, USA), 0.2 mM dNTP (Applied Biosystems, Corfornia, USA), 2.5 mM $MgCl_2$ (Applied Biosystems, Corfornia, USA), 0.2 µM of specific primers and 1.25 U AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Corfornia, USA). Amplification conditions were: denaturation for 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C, 15 s at 72 °C and a final elongation period for 5 min at 72 °C. PCR products were visualized prior to pyrosequencing using agarose gel electrophoresis.

SNPs were determined using pyrosequencing on a PyroMark Q96 ID (Biotage, Uppsala, Sweden). The manufacturer's instructions were followed for preparation and analysis of samples.

2.4. Statistical analysis

Statistical analysis was done using SPSS v.20 software (IBM, USA). Comparison of laboratory data for plasma and urine of patients with type 2 diabetes and nephropathy and the healthy volunteers was performed by the Mann-Whitney test.

Table 1: Composition of primers used in the determination of *Mbl2* genotype.

Gene	Primer name	Primer sequence 5' – 3'
Exon-1 codons 52, 54, 57	MBL2 F	CAg TgA TTg CCT gTA gCT CTC CA
	MBL 2 R-BIO	BIOTEG – ggC AgT TTC CTC Tgg AAg gTA AAg
	S6-MBL	TTC CCA ggC AAA gAT
Promoter+4	MBL+4 F	TTC CAA ATC CCC AgC TAg Agg C
	MBL+4 R	BIOTEG – gCT gCC ACC ATA CTC Agg AgA Ag
	MBL+4 S	gTA ggA CAg Agg gCA T
Promoter-70	MBL–70 F	BIOTEG – TCC CCA CTg CTC ATC ATA gTg C
	MBL–70 R	ATg ACC CAT CCC Tgg CCT CTA
	MBL–70 S	TCC CTg gCC TCT AgC
Promoter-221	MBL–221 F	CCC gAA gAg gAC ATg gAg AgA
	MBL–221 R	BIOTEG – CTg gCg TTg CTg CTg gAA
	MBL–221 S	ggC AAT gCA Cgg TCC
Promoter-550	MBL–550 F	ATT gCC AgT ggT TTT TgA CTC ACA
	MBL–550 R	BIOTEG – AgC CCA gAA TTA ACT ggA gTT TgC
	MBL–550 S	TTC CAg AgA AAA TgC TTA

3. Results

The laboratory characteristics of patients with type 2 diabetes and nephropathy participating in the study are listed in Table 2. Patients with type 2 diabetes participating in this study had typically elevated blood pressure, an increased level of S-creatinine and U-albumin/creatinine index. Proteinuria was moderately to severely increased on average, and ranged from 0.62 to 4.31 g/24 hours.

In Table 3 genetic characteristics of patients and healthy volunteers are presented. Structural genotypes AA are linked to medium or high MBL plasma concentration, and genotypes A0 to low MBL plasma concentration. The most abundant genotype was HYPA/LXPA (8/40; 20 %). The carriers of diplotype YA/YA had the highest plasma MBL concentrations (median 932.98 µg/l). In 75 % of patients with type 2 diabetes and renal disease, AA *Mbl2* gene structural genotype coding for normal or increased levels of MBL was expressed, and A0 variant coding for low MBL production only in 25 %. In comparison, healthy volunteers presented with the AA structural genotype only in 55 % and with the variant (A0) codons characteristic of low expression of MBL in as high as 45 %.

The median plasma concentration of MBL was higher in the group of patients with type 2 diabetes and kidney disease than in the general population (590.52 vs. 1353.44 µg/l; $p = 0.000$). Median urine concentrations of MBL also were higher in the group of patients with type 2 diabetes and kidney disease (4.42 vs. < 0.16; $p = 0.000$) (Table 4).

The median serum concentration of MBL was higher in the group of patients with type 2 diabetes and kidney disease than in the healthy population. Healthy individuals presented predominantly with *Mbl2* structural genes giving intermediate and low levels of MBL protein (45 %), in contrast to patients with type 2 diabetes who presented with genotypes giving a high level of MBL production (75 %).

4. Discussion

Increased plasma MBL level has been implicated in the pathogenesis of renal manifestations in patients with type 1 diabetes.[16-20] Significantly elevated MBL has been found in patients with rheumatic heart diseases.[21,22] The same was also observed in this study for patients with type 2 diabetes and nephropathy.

It has been suggested that complement activation via the MBL pathway plays a role in the pathogenesis of diabetic complications.[6,23,24] Since protein glycation is increased in diabetes, it is possible that some of the metabolic products of glucose to mannose transformation become a target for MBL binding. The glycation product fructoselysine is a candidate ligand for MBL and its interaction with this protein may initiate complement activation and provide a pathophysiological link between enhanced glycation and complement activation in diabetes.[6] Hovind et al. found micro- or macro-albuminuria in 41 % of patients with MBL levels above the median as compared to 26 % in patients with low levels of MBL.[10] It was assumed that high levels of MBL early in the course of type 1 diabetes are involved in the pathogenesis of diabetic micro-vascular complications.[4]

The kidney is an important site of glucose homeostasis.[1] It is the site at which processes of gluconeogenesis, glucose filtrati-

Table 2: Some characteristics of patients with type 2 diabetes and nephropathy participating in the study.

		BMI (kg/m²)	RRsist (mmHg)	RRdiast (mmHg)	Serum creatinine (µmol/l)	Serum urea (mmol/l)	Urine albumin/ creatinine (g/mol)	HbA1c (%)
Patients with type 2 diabetes	MEDIAN	32.70	150.00	81.00	170.00	14.20	211.00	8.20
	MIN	21.50	119.00	64.00	97.00	7.00	61.80	6.20
	MAX	47.60	170.00	100.00	334.00	30.30	430.90	12.00

on, glucose reabsorption and consumption take place. At least some of these activities are changed in patients with type 2 diabetes and end in an elevated level of MBL activators. It would therefore be reasonable to verify the link between high levels of blood glucose, fructose and mannan-like centers for activation of the MBL pathway in the kidneys of patients with type 2 diabetes. Since a close positive correlation between hyperglycaemia, plasma mannose and urinary protein concentrations has been found in metabolic syndrome associated with glomerulonephritis, regular measuring of glucose transformation products in blood would be a useful practice.[5,8]

It is interesting that laboratory determination of serum mannose in patients with type 2 diabetes is not used at all, though appropriate methods are available.[2,9] Mori at al. reported the clinical significance of plasma mannose concentrations in healthy and diabetic dogs and stated that circulating levels of monosaccharides can act as a reflection of systemic glucose metabolism. They reported that plasma mannose positively correlated with plasma glucose and fructosamine.[25] This finding is important for linking the presence of fructosamines, as one of the important MBL pathway activators, and damage to local or systemic complement activation in diabetes.[6] Activation of the complement may contribute the inflammatory component in the pathogenesis of diabetic renal injury.

The mannan pathway of complement activation is triggered by the binding of MBL to mannan, but also to other sugar residues. An association between levels of monosaccharides in blood, MBL genotypes and

Table 3: Distinct *Mbl2* genotypes and MBL concentration in patients with type 2 diabetes and renal disease and in healthy controls.

Structural genotype	Genotype	Promoter	MBL diplotypes	Number		MBL concentration (µg/l) – serum; range[a]	MBL concentration (µg/l) – urine; range[a]
				Patients (20)	Healthy volunteers (20)		
A/A	LXPA/LYPA	LX /LY	XA/XA	0	1	698	<0.16
A/A	LXPA/LXPA	LX/LX	XA/XA	0	2	332–400	<0.16
A/A	LXPA/LXQA	LX/LX	XA/XA	3	0	900–1640	<0.16–1.94
A/0	LXPA/HYPD	LX/HY	XA/YO	0	1	388	<0.16
A/0	LXPA/LYPB	LX/LY	XA/YO	2	2	77–1120	<0.16–9.46
A/A	HYPA/LXPA	HY/LX	YA/XA	3	5	554–1680	<0,16–3.47
A/A	HYPA/HYPA	HY/HY	YA/YA	3	0	1769–3600	<0.16–2.72
A/A	HYPA/LYPA	HY/LY	YA/YA	1	0	1760	<0.16
A/A	HYPA/LYQA	HY/LY	YA/YA	4	1	1353–3680	<0.16–0.92
A/A	LYQA/LYQA	LY/LY	YA/YA	1	1	1302–1314	<0.16–4.90
A/0	HYPA/HYPD	HY/HY	YA/YO	0	1	1185	<0.16
A/0	HYPA/LYPB	HY/LY	YA/YO	1	1	573–1160	0.16–13.56
A/0	HYPA/LYQC	HY/LY	YA/YO	0	2	148–552	<0.16
A/0	LYQA/HYPD	HY/LY	YA/YO	0	1	757	<0.16
A/0	LYPA/LYPB	LY/LY	YA/YO	1	0	560	<0.16
A/0	LYQA/LYPB	LY/LY	YA/YO	0	2	523–819	<0.16
0/0	HYPD/HYPD	HY/HY	YO/YO	1	0	1400	4.42

[a] *Data on concentration of MBL apply exclusively to this study!*

Table 4: Descriptive statistics for the group of healthy volunteers and the group of patients with type 2 diabetes and nephropathy.

		MBL (µg/l) plasma	MBL (µg/l) urine	SC5b-9 (µg/l) plasma	SC5b-9 (µg/l) urine	C4d (mg/l) plasma	C4d (mg/l) urine	CH50 (%) plasma	APH50 (E) plasma	C3 (mg/l) plasma	C4 (mg/l) plasma	C3d (mU/l) plasma
		(85–3077)	(< 0.200)	(300–350)	(< 30)	(0,7–6.3)	(0,076 +/- 0,07)	(72–128)	(80–120)	(970–1576)	(162–385)	(35–70)
Healthy volunteers	MEDIAN	590.52	< 0.156	276.88	<10	6.16	0.00	136.00	107.00	1200.00	242.00	52.00
	MIN	77.89	< 0.156	224.89	<10	1.54	0.00	75.00	84.00	888.00	131.00	44.00
	MAX	1459.60	< 0.156	538.15	<10	15.61	0.01	248.00	203.00	1410.00	374.00	60.00
Patients with type 2 diabetes	MEDIAN	1353.44	4.42	106.00	35.00	7.00	0.01	73.00	84.00	1280.00	332.00	52.00
	MIN	553.76	0.85	77.00	26.00	1.68	0.00	7.00	6.00	817.00	206.00	46.00
	MAX	3680.00	13.56	371.00	2107.00	16.87	0.50	123.00	148.00	1690.00	496.00	90.00
	M-W test	<0.001	ND	0.007	ND	0.084	0.005	<0.001	0.009	0.116	<0.001	0.049

complement activation has not been convincingly confirmed.

Kaunisto et al. found the median serum MBL concentration to be significantly higher in patients with macro-albuminuria, but in spite of a reasonably high association between *Mbl2* SNPs and MBL protein concentration, neither single SNP, nor any of their haplotype combinations confers a risk of diabetes type 1 or diabetic nephropathy.[26]

Vaidya et al. studied the importance of urinary tubular injury biomarkers KIM-1 (kidney injury molecule-1) and NAG (N-acetyl-β-D-glucosaminidase) and elevated urinary albumin excretion in patients with type 1 diabetes.[27] Although micro-albuminuria was considered to be an important earliest non-invasive biomarker for diagnosis of diabetic nephropathy, it was shown that elevated urinary albumin often regresses to normal albuminuria in patients with diabetes. Elevated NAG values are indicative of tubulointerstitial disease; however, it is not entirely clear whether this injury results in direct tubular toxicity and the cytokines generation enhancing the inflammatory process, or it is a direct consequence of high levels of glucose.[26-29] Since fructosamines are also elevated in hyperglycemia, it is easy to envisage that elevated levels of mannose can be a source of complement activation by the MBL pathway.

It is further known that diabetes is significantly associated with a high risk of morbidity and mortality because of cardiovascular and micro-vascular disease.[30] Chronic low-grade inflammation is believed to play a central role in the development of cardiovascular disease. MBL may aggravate local and systemic inflammation by complement activation. This may contribute to the development of nephropathy and cardiovascular disease.[30,32] A consistent relationship has been found between chronic inflammation and cardiovascular complications of hyperglycemia.[33] Acute hyperglycemia was originally regarded as unproblematic; moreover, it was believed to be potentially beneficial, ensuring an adequate supply of glucose to the immune system. Pavlov et al. found that hyperglycemic mice with wild type MBL presented with severe cardiomyopathy, in

contrast to MBL deficient mice.[34] This finding was the basis for the opinion that an absence or deficiency of MBL is beneficial in some diseases.

Traditionally, activation of the alternative pathway has been recognized as important factor of glomerular injury.[35] The exact mechanism of this activation is still not entirely clear but, after recognition of the lectin pathway of complement activation, new evidence has been accumulating that some molecules able to activate the alternative pathway also activate the lectin pathway. In patients with IgA nephropathy, 25 % had mesangial deposits of MBL, while patients with MBL deficiency tended to show better clinical presentation and lower levels of urinary protein and serum creatinine than MBL-sufficient patients.[19] It was shown recently that N-glycans on secretory IgA may be ligands for MBL and thus for activation of the lectin pathway. Mannose binding lectin plays a critical role in diseases in which vessels are affected, such as in myocardial infarction and reperfusion injury.[36] Patients with diabetes are at risk of cardiomyopathy and acute myocardial infarction.[21,36] Although earlier studies stressed that the classical pathway and natural antibodies are important for the occurrence of pathological changes in the walls of arteries, more recent studies suggest that the lectin complement pathway is the major initiating pathway in myocardial ischemia and reperfusion injury, leading to the conclusion that renal vessels are also affected in the same way.[4]

Mannose-binding lectin deficiency presenting with a low serum concentration of protein or its disfunctionality is envisaged in exon 1 of the *Mbl2* gene.[37,38] MBL in donors homozygous for the normal MBL genotype predominantly contained high molecular weight MBL, sera of individuals heterozygous for the variant alleles contained high and low molecular weight MBL, and sera of individuals homozygous for MBL variant alleles contained mainly low molecular weight MBL. Only high molecular weight MBL is functional. Without genotypisation and molecular weight determination, it is therefore not possible to predict the functional status of MBL in a single individual. This observation is important because people with low complement mannose-binding lectin concentrations have better survival chances in myocardial ischemia/reperfusion injury.[39,40] It appears to be important to understand the mechanisms of vessel injury in patients with type 2 diabetes in the same way, to find ways of protecting them from premature invalidism or death.[41]

In this study, we found that patients with diabetes and nephropathy present with a much higher percentage of *Mbl2* genotype, producing normal or high levels of MBL protein, than healthy controls. From the evidence on the role of mannan pathway activity in the pathogenesis of vascular injury presented above, we suggest that biotransformation of glucose to mannose and fructosamines is important for possible activation of the mannan pathway of complement activation in the course of the pathogenesis of diabetic nephropathy.

5. Conclusions

Patients with type 2 diabetes present with *Mbl2* genotype coding for a high blood concentration of MBL protein in 75 % of cases, in comparison to healthy individuals from the general population, who present these genotypes in 55 %. Patients present with subnormal activity of the classical and alternative pathway of complement characteristic for the consumption of the complement on account of inflammation. Patients have in addition increased levels of urinary C4d, SC5b-9 and MBL, which are present in negligible concentrations or absent from the urine of healthy controls. The data presented support the opinion that all three pathways of the complement system substantially participate in the occurrence of renal injury in patients with type 2 diabetes.

Acknowledgement

We express thanks to Mrs. Jelka Markelj, Mrs. Maja Mrgole, and asist. Rok Kogoj, BSc of microbiology for excellent technical performance of the applied tests, and Mr. Martin Cregeen for providing language help.

Part of the study was supported by Grant No. P3–0083 (2009–2013) from the Slovenian Research Agency.

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