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Blood serum of patients with diabetic kidney disease stimulates thrombospondin 1 gene expression in peripheral blood mononuclear cells

Surowica krwi od pacjentów z cukrzycową chorobą nerek wzmacnia ekspresję genu dla trombospondyny 1 w komórkach jednojądrzastych krwi obwodowej

ABSTRACT

Background. The thrombospondin 1 and cyclooxygenase 1 genes were over-expressed and the matrix metalloproteinase 9 and cyclooxygenase 2 genes have lower expression in peripheral blood mononuclear cells (PBMC) of type 1 diabetic patients with diabetic kidney disease (DKD). The aim of the study was to examine if the expression of those genes could be stimulate by incubating PBMC of healthy volunteers in blood serum of patients with DKD.

Material and methods. Blood serum samples from five type 2 diabetic patients with DKD and five type 2 diabetic patients without DKD were collected. PBMC of four healthy volunteers were incubated in blood serum of 5 type 2 diabetic patients with DKD and 5 without DKD. The expression of the thrombospondin 1, cyclooxygenase 1, matrix metalloproteinase 9 and cyclooxygenase 2 genes was examined using a method based on the real-time PCR. The expression was compared between cells incubated in blood serum of type 2 diabetic patients with and without DKD.

Results. We observed a higher expression of the thrombospondin 1 gene in cells incubated in blood serum of patients with DKD. The expression of other genes did not differ between cells incubated in blood serum of patients with and without DKD.

Conclusions. Blood serum of type 2 diabetic patients with DKD induces expression of thrombospondin 1 gene in PBMC. Blood serum of type 2 diabetes patients with DKD may contain an intrinsic factor which may be involved in vascular complications accelerated by DKD. (Diabet. Klin. 2012; 1, 2: 55–60)

Key words: diabetic kidney disease, gene expression, peripheral blood mononuclear cells, blood serum, thrombospondin 1, type 2 diabetes

STRESZCZENIE

Wstęp. Wykazano, że komórki jednojądrzaste krwi obwodowej u chorych na cukrzycę typu 1 z cukrzycową chorobą nerek charakteryzują się zwiększoną ekspresją genów dla trombospondyny 1 i cyklooksygenazy 1 oraz zmniejszoną ekspresją genów dla metaloproteinazy 9 i cyklooksygenazy 2. Celem badania było sprawdzenie, czy ekspresja tych genów może zostać zmieniona przez inkubację komórek jednojądrzastych krwi obwodowej od zdrowych ochotników w surowicy krwi chorych z cukrzycową chorobą nerek.

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Materiał i metody. Pobrano próbki surowicy krwi od 5 chorych na cukrzycę typu 2 z cukrzycową chorobą nerek oraz 5 chorych na cukrzycę typu 2 bez cukrzycowej choroby nerek. Komórki jednojądrzaste krwi obwodowej od 4 zdrowych ochotników były inkubowane w surowicy krwi od 5 chorych na cukrzycę typu 2 z cukrzycową chorobą nerek i 5 chorych bez cukrzycowej choroby nerek. Zbadano ekspresję genów dla trombospondyny 1, cyklooksygenazy 1, metaloproteinazy 9 i cyklooksygenazy 2, używając metody opartej na reakcji łańcuchowej polimerazy DNA z analizą ilości produktu w czasie rzeczywistym. Porównano ekspresję genów pomiędzy komórkami inkubowanymi w surowicy krwi od chorych na cukrzycę typu 2 z i bez cukrzycowej choroby nerek.

Wyniki. Stwierdzono większą ekspresję genu dla trombospondyny 1 w komórkach inkubowanych w surowicy chorych z cukrzycową chorobą nerek. Ekspresja pozostałych genów nie różniła się między komórkami inkubowanymi w surowicy od chorych z cukrzycową chorobą nerek i bez tej choroby.

Wnioski. Surowica krwi od chorych na cukrzycę typu 2 z cukrzycową chorobą nerek powoduje wzrost ekspresji genu dla trombospondyny 1 w komórkach jednojądrzastych krwi obwodowej. Surowica krwi od chorych na cukrzycę typu 2 z cukrzycową chorobą nerek może zawierać czynnik biorący udział w powikłaniach naczyniowych, których postęp jest szybszy przy obecności cukrzycowej choroby nerek.

(Diabet. Klin. 2012; 1, 2: 55–60)

Słowa kluczowe: cukrzycowa choroba nerek, ekspresja genu, komórki jednojądrzaste krwi

Background

Diabetic kidney disease (DKD) is a frequent cause of chronic kidney disease. The pathomechanism of DKD is not well explained. Approximately one third of patients with diabetes is predisposed to DKD. Numerous studies have searched genetic marker which predicts predisposition to DKD. No marker useful for clinical practice has been found yet. An alternative way to find a marker of genetic predisposition to DKD may be an analysis of gene expression. Different models of gene expression were used to analyze gene expression in diabetic kidney disease. The most popular model was based on cultured skin fibroblasts of diabetes patients [1]. We started a new model using peripheral blood mononuclear cells (PBMC) of patients with DKD [2]. Using this model we analyzed expression of over 20 thousand genes in peripheral blood mononuclear cells (PBMC) of patients with DKD [2]. Using the method based on microarray we found that the thrombospondin 1 (*THBS1*) and cyclooxygenase 1 (*PTGS1*) genes were over-expressed and matrix metalloproteinase 9 (*MMP9*) and cyclooxygenase 2 (*PTGS2*) genes had lower expression in PBMC of type 1 diabetic men with DKD [2]. The different expression of those four genes might be primary marker of genetic predisposition to DKD or secondary consequence of development of DKD.

To examine the hypothesis that different gene expression might be secondary to development of DKD we examined the effect of blood serum from patients with DKD on gene expression in PBMC from healthy volunteers. PBMC of healthy volunteers were incubated in blood serum samples of type 2 diabetes patients with DKD. Gene expression of the thrombospondin 1, cyclooxygenase 1, metalloproteinase 9 and cyclooxygenase 2 genes were examined and compared to gene expression in PBMC incubated in blood serum of type 2 diabetes patients without DKD.

Material and methods

Blood serum sample collection

Venous blood (10 ml) was drawn from 10 type 2 diabetic patients into serum tube. 5 patients had urine albumin excretion in the range of macroalbuminuria and 5 patients had normal urine albumin excretion. Macroalbuminuria was defined as albumin to creatinine ratio (ACR) $\geq$ 300 mg/g and normoalbuminuria as ACR $<$ 30 mg/g. Blood samples were centrifuged at 2000 g for 10 min at room temperature (20°C) and blood serum was transfered to separate tubes and frozen in –20°C. All enrolled patients gave written informed consent for participation in the study. The Ethics Committee of the Medical University of Lodz approved the study protocol.

Incubation of PBMC and RNA isolation

Venous blood was drawn from four healthy volunteers (4 men in the age from 27 to 43 years) into EDTA-coated tubes. The aliquots of each sample (2.7 ml) blood were carefully layered on the top of 2.7 ml Gradisol L solution (Polfa, Kutno, Poland). Samples were then centrifuged at 400 g for 30 min at room temperature (20°C). The interphase (500 μl) containing PBMC was then transferred to 1500 μl of 1 $\times$ phosphate-buffered saline (PBS) and mixed by inverting the tubes. The sample centrifuged at 250 g for 10 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 500 μl of blood serum from type 2

diabetic patients and transferred to 2-ml tubes, and the lids were tightly closed.

Peripheral blood mononuclear cells from each healthy volunteers were incubated in 10 samples from type 2 diabetic patients, 5 with and 5 without DKD. Altogether 40 incubations experiments were performed (4 healthy volunteers × 10 blood serum samples of type 2 diabetic patients). The samples were incubated for 2 h at 37°C and then centrifuged at 10000 g for 30 s at room temperature. The supernatant was discarded and the cell pellet was re-suspended in 500 μl of the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Total *RNA was isolated* using the TRIZOL reagent *according* to the *manufacturer's* instructions (Invitrogen, Carlsbad, CA, USA).

Reverse transcription and real-time PCR

Total RNA (1,0 μg) was reverse transcribed using random decamers RETROscript™ First Strand Synthesis Kit for RT-PCR (Ambion, Austin, USA). Reagents were incubated for 60 min at 42°C followed by 10 min at 92°C. KAPA SYBR® FAST qPCR Master Mix (2×) Bio-Rad iCyclerTM Kit (Kapa Biosystems Inc., Boston, MA, USA) was used on the Bio-Rad iQ^{TM}5 Real Time PCR System (*Bio-Rad* Laboratories, Hercules, CA, USA) to amplify human cDNAs as well as the cDNA for *GAPDH* gene as the internal control. PCR reactions were carried out in a total volume of 25 μl containing 7.5 μl of cDNA with the following thermocycling steps: 95°C for 20 s, then 40 cycles of 95°C for 3 s, and 60°C for 30 s, followed by melting curve data collection.

In the real-time PCR experiment, the data analysis and calculations were performed using the $2^{-\Delta\Delta CT}$ method described previously [3]. Expression was assessed by analyzing threshold cycle (C_T) values. The relative amount of expressed mRNA was calculated by comparison with the expression of the housekeeping *GAPDH* gene amplified in a separate tube.

Using the $2^{-\Delta\Delta CT}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (*GAPDH*) and relative to the expression in cells incubated in blood serum of patients without DKD.

All primers used for real-time semiquantitative PCR were described previously [4–7]. Primers used for semiquantitative real-time PCR were purchased from MolBiol (Poznan, Poland).

The following primers were used for *GAPDH*, thrombospondin 1 (*THBS1)*, cyclooxygenase 1 (*PTGS1*), cyclooxygenase 2 (*PTGS2*) and matrix metalloproteinase 9 (*MMP9*), respectively, in real-time PCR:

- GAPDH antisense, 5'-CGCCCAATACGACCAAAT-3';
- GAPDH sense, 5'-AGTCAGCCGCATCTTCTT-3' [4];
- THBS1 antisense, 5'-CTGATCTGGGTTGTGGTTGTA-3';
- THBS1 sense, 5'-CCTGTGATGATGACGATGA-3' [4];
- PTGS1 sense 5'-GCTATTCCGGCCCCAACT-3';
- PTGS1 antisense 5'-GATGAAGGTGGCATTGACAAACT-3' [5];
- PTGS2 sense 5'-CTTTGCCCAGCACTTCA-3';
- PTGS2 antisense 5'-CTAGCCAGAGTTTCACCGTAA-3' [6];
- MMP9 sense 5'-CGGCTTGCCCTGGTGCAGT-3';
- MMP9 antisense 5'-CGTCCTGGGTGTAGAGTCTCTCG-3' [7].

The results for the *THBS1* gene expression were confirmed in the repeated real-time PCR assay. For final analysis of the *THBS1* gene expression mean values of both assays were used.

Statistical analysis

Statistical analysis was performed using software R version 2.11.1 (The R Foundation for Statistical Computing). Analysis of variance was used to compare the gene expression in PBMC after incubation in blood serum of type 2 diabetes patients. Gene expression was a dependent variable, and the origin of PBMC from healthy volunteers and the presence of DKD in blood serum donor were the independent variables in the model.

Results

Clinical characteristics of type 2 diabetes patients was presented in table 1. Five patients had DKD with urine albumin excretion in the range of macroalbuminuria. Five patients with normal urine albumin excretion.

Peripheral blood mononuclear cells of four healthy volunteers were incubated 2 hours in blood serum samples from 5 type 2 diabetic patients with DKD and from 5 type 2 diabetic patients with normal urine albumin excretion. The expression of the *THBS1*, *PTGS1*, *PTGS2* and *MMP9* genes in the PBMC incubated in blood serum of patients with DKD was compared to gene expression of PBMC incubated in blood serum of patients without DKD (table 2). The expression of the *THBS1* gene was higher in PBMC incubated in blood serum of patients with DKD. Figure 1 presents the boxplot of the *THBS1* gene expression depending on the origin of blood serum. There was no difference in the expression of the *PTGS1*, *PTGS2* and *MMP9* genes between PBMC incubated in blood serum of type 2 diabetic patients with and without DKD (tab. 2).

Table 1. Clinical characteristics of type 2 diabetic donors of blood sera. Data are presented as median (minimum––maximum)

	Patients with DKD	Patients without DKD
n	5	5
Age (years)	66 (54–75)	64 (45–71)
Duration of diabetes after diagnosis (years)	10 (5–19)	16 (10–17)*
HbA_{1c} (%)	7.2 (6.1–7.6)	10.6 (8.0–12.5)*
BMI [kg/m^2]	31.6 (30.4–32.6)	29.3 (22.5–32.4)
Serum creatinine concentration [μmol/L]	207 (134–220)	82 (72–110)*
GFR MDRD [ml/min/1,73 m^2]	23 (20–36)	71 (61–110)*
C-reactive protein [mg/l]	4.4 (2.1–13)	4.9 (2.4–13.1)
Retinopathy (yes/no)	5/0	2/3
History of myocardial infarction, CABG, PTCA or stroke (yes/no)	1/4	0/5

*$p < 0.05$

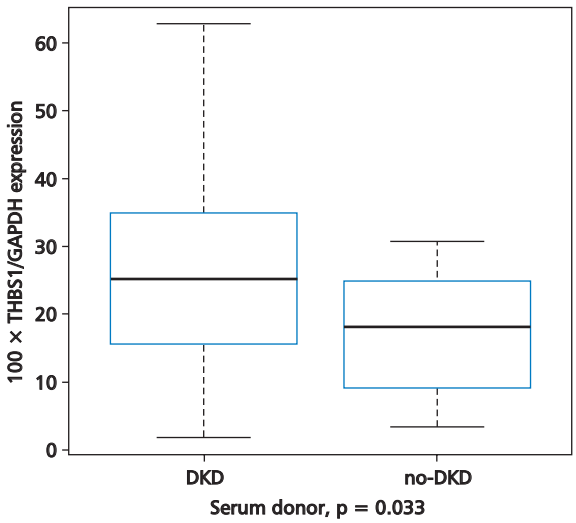

Figure 1. Boxplot of the expression of the *THBS1* gene depending on the presence of DKD in blood serum donor. The data are presented as 100 × THBS1/GAPDH expression. DKD — incubation in blood serum of type 2 diabetic patient with DKD; no-DKD — incubation in blood serum of type 2 diabetic patient without DKD

Discussion

We found that blood serum of patients with DKD induced the *THBS1* gene expression in PBMC of healthy volunteers. We had found previously that the *THBS1* gene expression was higher in type 1 diabetic men with DKD than in type 1 diabetic men without DKD. Those observations indicate that an increased expression of the *THBS1* gene is secondary to development of DKD. It may suggest presence of an intrinsic factor in blood serum of patients with DKD, which stimulates expression of the *THBS1* gene in PBMC.

Table 2. Gene expression in PBMC of healthy volunteers incubated with blood serum of five type 2 diabetic patients with DKD compared to cells incubated in blood serum of five type 2 diabetic patients without DKD

Gene name	Change in gene expression	P
Thrombospondin 1 (*THBS1*)	2.06	**0.033**
Cyclooxygenase 1 (*PTGS1*)	0.76	0.311
Cyclooxygenase 2 (*PTGS2*)	0.63	0.120
Matrix metalloproteinase 9 (*MMP9*)	0.93	0.808

The increased expression of the *THBS1* gene may be induced by renal damage in the course of DKD. The effect of kidney diseases on other organs was described in epidemiological studies [8-10]. Increased urine albumin excretion and impaired renal function accelerated atherosclerosis [8, 9] and induced progression of diabetic retinopathy [10]. In the presented study all patients with diabetic kidney disease had retinopathy, however the presence of retinopathy was one of the inclusion criteria in the DKD group to avoid non-diabetic kidney disease. An increased expression of the *THBS1* gene might be associated with arterial diseases accelerated by DKD. A linear correlation between peripheral arterial disease and thrombospondin 1 plasma level was observed [11]. Thrombospondin 1 was implicated in profibrotic, proinflamatory, antiangiogenic and proapoptotic effects [12]. Thrombospondin 1 expression was increased in glumeruli from the earliest stage of DKD [13]. Thrombospondin 1 was an endogenous activator of TGF-β in experimental DKD

[14]. Its expression was also increased in kidney during hypoxia [15].

Thrombospondin 1 is expressed by a variety of cell types such as platelets, vascular smooth muscle cells as well as diverse renal cells including mesangial cells, podocytes, endothelial cells, tubular and interstitial cells [12]. Thrombospondin 1 is regulated by various cytokines such as platelet derived growth factor, fibroblast growth factor 2 or transforming growth factor beta, and is frequently expressed at sites of inflammation, wound healing and tissue remodeling [12, 16]. Thrombospondin 1 might be a target in prevention and treatment of DKD what has been already shown in animal model [17, 18]. Proteinuria was significantly improved in mice treated with LSKL peptide [18]. LSKL is a peptide which inhibits thrombospondin 1 activation of TGF-ß. LSKL treatment reduced urinary TGF-β activity and improved markers of tubulointerstitial injury [18].

A similar model was used by other research group, which investigated an exposure of normal human podocytes to the sera of diabetic patients with albuminuria [19]. They observed cytoskeleton remodeling, cellular insulin resistance and susceptibility to apoptosis in cells exposed to sera of diabetic patients with DKD. They found significant modulation of several genes involved in cytokines action, actin remodeling and insulin signaling [19]. Presence of a circulating factor responsible for podocyte malfunction was suggested in patients with albuminuria [19]. The factor that might stimulates thrombospondin 1 gene expression in PBMC exposed to blood serum of patients with DKD is not known. In the recent study C-reactive protein (CRP) induced thrombospondin 1 protein release and mRNA expression from human renal tubular epithelial cells incubated with purified CRP via activation of the p38MAPK and NF-κB signaling pathways [20]. CRP might be one of the circulating factors inducing thrombospondin-1 gene expression. However, in our study no correlation was found between thrombospondin 1 gene expression and CRP concentration in blood serum donors.

Cells isolation and material preparation might have an influence on gene expression *per se*. To avoid that potential effect we coded and mixed the samples of patients with and without DKD during all steps of material preparation.

Patients without DKD had longer duration of diabetes after diagnosis than patients with DKD. They may be protected against DKD by some genetic background, what might have an impact on the observed results.

In summary, blood serum of type 2 diabetic patients with DKD increased the expression of thrombospondin 1 gene in peripheral blood mononuclear cells of healthy volunteers. Blood serum of type 2 diabetes patients with DKD may contain an intrinsic factor which may be involved in vascular complications accelerated by DKD.

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Conflict of interest statement — none declared.

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