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Serum osteoprotegerin and tumor necrosis factor related apoptosis inducing-ligand (TRAIL) are elevated in type 2 diabetic patients with albuminuria and serum osteoprotegerin is independently associated with the severity of diabetic nephropathy

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ABSTRACT

Osteoprotegerin (OPG) and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) have recently been reported to be associated with diabetic nephropathy in an in vitro study. However, the literature regarding serum OPG and TRAIL in type 2 diabetes mellitus patients is scarce. To investigate the role of OPG/TRAIL in diabetic nephropathy, we measured the serum concentrations of OPG and TRAIL in type 2 diabetes mellitus patients with different stages of nephropathy by enzyme-linked immunosorbent assay. One hundred seventy-nine subjects with type 2 diabetes mellitus were studied and stratified according to urinary microalbumin and serum creatinine measurements. The serum concentrations of OPG and TRAIL were significantly elevated in patients with microalbuminuria (OPG, 2154.2 ± 922.1 pg/mL; TRAIL, 80.2 ± 24.1 pg/mL) and macroalbuminuria (OPG, 2251.5 ± 925.7 pg/mL; TRAIL, 88.1 ± 23.8 pg/mL) as compared with patients with normoalbuminuria (OPG, 1690.1 ± 627.2 pg/mL; TRAIL, 70.7 ± 23.3 pg/mL). Serum OPG and TRAIL levels were increased in parallel and were significantly associated with each other. Using multivariate stepwise regression analysis, serum OPG was found to be an independent factor associated with the severity of diabetic nephropathy. Our results suggested that serum OPG may be a marker for the severity of diabetic nephropathy. Further studies are necessary to investigate the role of elevated serum OPG in the pathogenesis of diabetic nephropathy.

1. Introduction

Diabetic nephropathy is a major microvascular complication and the leading cause of end-stage renal disease worldwide. Under hyperglycemic conditions, it has been demonstrated that the renal tubular epithelium highly expresses apoptosis regulatory genes [1]. Recently, Kumar et al [2] demonstrated that the apoptosis process was

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ongoing in tubular epithelial cells, endothelial cells, or interstitial cells from renal biopsy samples obtained from diabetic patients. Based on these reports, it is reasonable to speculate that apoptosis is one of the major pathogenic pathways inducing diabetic nephropathy.

Very recently, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was reported to be associated with diabetic nephropathy. Lorz et al [3] found that TRAIL expression was higher in diabetic kidneys than healthy controls with respect to glomerular and proximal tubular cells; they also demonstrated that osteoprotegerin (OPG), a decoy receptor for TRAIL, might interfere with the apoptosis process induced by TRAIL. Therefore, both OPG and TRAIL might play an important role in the pathogenesis of diabetic nephropathy.

To date, increased serum OPG has been reported in diabetic patients with or without micro- and macrovascular diseases [4-9]. However, investigation of TRAIL in type 2 diabetes mellitus patients is lacking, especially in cases of diabetic nephropathy. The aim of this study was to examine the expression of TRAIL and OPG in type 2 diabetes mellitus patients in different stages of albuminuria.

2. Research design and methods

2.1. Subjects and ethics

From August 2008 to December 2009, a total of 179 type 2 diabetes mellitus patients attending the Endocrinology and Metabolism outpatient department at Kaohsiung Medical University Hospital were selected to participate in this study. These patients were diagnosed with type 2 diabetes mellitus according to the American Diabetes Association criteria. All diabetic patients had been treated with oral antidiabetic medications but not with thiazolidinediones or insulin. The participants were randomly selected; and diabetic patients showing evidence of active infection, high-sensitivity C-reactive protein (hs-CRP) greater than 10 mg/L, hyperglycemic crisis, congestive heart failure, coronary artery disease, liver cirrhosis, or genitourinary tract disorders (other than diabetic nephropathy) were excluded from the study. The subjects were classified into 3 groups based on their urine albumin-to-creatinine ratio (ACR) taken from 3 measurements of spot urine samples over 6 months before enrollment in this study: the normoalbuminuria group (ACR <30 mg/g, n = 68), the microalbuminuria group (ACR = 30-299 mg/g, n = 67), or the macroalbuminuria group (ACR >300 mg/g, n = 44). Height and body weight measurements of all subjects were taken. *Hypertension* was defined as a systolic blood pressure greater than 140 mm Hg, a diastolic pressure greater than 90 mm Hg, or current use of antihypertensive drugs. Informed consent was obtained from all participants. This study was approved by the international review board of Kaohsiung Medical University (KMUH-IRB-960329).

2.2. Laboratory measurements

Blood samples were collected after at least 12 hours of overnight fasting; the samples were kept on ice and then centrifuged at 1500*g* at 4°C for 15 minutes. Serum was then aliquoted, stored at –80°C, and thawed before analysis. A biochemical automatic analyzer (Beckman-Coulter, Fullerton, CA) was used to analyze blood samples and measure plasma glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG). Hemoglobin A_{1c} (HbA_{1c}) was measured in whole blood using ion exchange high-performance liquid chromatography (VARIANT II Turbo; Bio-Rad, Hercules, CA). Serum insulin and urine albumin were measured by radioimmunoassay (DPC, Los Angeles, CA). C-reactive protein was measured using a highly sensitive assay (DPC). Albumin-to-creatinine ratio was calculated as the urine albumin concentration divided by the urine creatinine concentration. The serum concentrations of OPG and TRAIL were measured using a commercially available kit (R&D Systems, Minneapolis, MN). All of the blood samples from the different albuminuria groups were evenly distributed in each assay. In each experiment, the samples were in duplicate; and the blinded quality control sample was repeated 6 times. The intraassay and interassay coefficients of variation for OPG were 6.2% and 11.5%, respectively. The intraassay and interassay coefficients of variation for TRAIL were 3.5% and 6.8%, respectively. Body mass index (BMI) was calculated as body weight divided by the square of height (kilograms per square meter). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the insulin reading (micro–international units per milliliter) multiplied by the plasma glucose level (millimoles per liter) and divided by 22.5. The estimated glomerular filtration rate (eGFR) was calculated using the Cockcroft-Gault equation.

2.3. Statistical analyses

All continuous variables are presented as means ± standard deviation. Skewed variables were log-transformed before statistical analysis. *P* values for group comparison were computed by analysis of variance, and Scheffe tests were used for post hoc analysis. χ^2 tests were performed to compare categorical variables. Linear regressions were used to examine the relationship between the investigated factors, including serum TRAIL and OPG, and various parameters. Model 1 consisted of simple linear regression analysis of each parameter in relation to TRAIL or OPG. Model 2 used multiple stepwise linear regression analysis to determine the contribution of various factors to TRAIL or OPG. Logistic regressions were used to determine the independent factors associated with diabetic nephropathy. Model 1 consisted of simple logistic regression analysis of each parameter to nephropathy. Model 2 used a multiple stepwise logistic regression analysis, which included OPG, TRAIL, OPG/TRAIL ratio, and related factors (eg, age, sex, smoking, hypertension, HbA_{1c}, total cholesterol, HDL-C, LDL-C, TG, statin, and angiotensin-converting enzyme inhibitor [ACEI]/angiotensin receptor blocker [ARB] treatment) as relevant variants. All analyses were performed using SPSS (Chicago, IL) version 14. A *P* value (2-sided) < .05 was considered statistically significant.

3. Results

Table 1 presents the clinical characteristics of the patient groups. No significant differences regarding age, sex, or BMI were found among the 3 diabetic groups; and laboratory examinations indicated that there were no significant differences in fasting plasma glucose, total cholesterol, HDL-C, LDL-C, or hs-CRP between the 3 groups. More participants were diagnosed with hypertension in the macroalbuminuria group as compared with the other 2 groups. Significant differences were found in TG and HOMA-IR between the diabetic groups. Hemoglobin A_{1c} was higher in the macroalbuminuric group than in the normoalbuminuric group, whereas eGFR was lower in the microalbuminuric and macroalbuminuric groups than in the normoalbuminuric group. Serum OPG was significantly elevated in type 2 diabetes mellitus patients with microalbuminuria (2154.2 ± 922.1 pg/mL, $P = .045$) and macroalbuminuria (2251.5 ± 925.7 pg/mL, $P = .007$) as compared with those with normoalbuminuria (1690.1 ± 627.2 pg/mL). Serum TRAIL was also significantly elevated in type 2 diabetes mellitus patients with microalbuminuria (80.2 ± 24.1 pg/mL, $P = .037$) and macroalbuminuria (88.1 ± 23.8 pg/mL, $P = .012$) as compared with the normoalbuminuric group (70.7 ± 23.3 pg/mL). In addition, the OPG/TRAIL ratio was significantly higher in patients with microalbuminuria (30.1 ± 13.2, $P = .048$) and macroalbuminuria (30.1 ± 12.3, $P = .045$) than in those with normoalbuminuria (24.4 ± 12.1). With regard to medication, there was no significant difference in statin treatment between the diabetic groups; but ACEI/ARB use was significantly higher in the macroalbuminuric group than in the other 2 groups.

From Table 2, it can be seen that serum OPG in the diabetic subjects was positively correlated with age, ACR, hypertension, and TRAIL and was negatively correlated with the male sex and eGFR (model 1). Multiple stepwise linear regression analysis showed that age, the male sex, HbA_{1c}, ACR, and TRAIL were significantly correlated with serum OPG (model 2). Serum TRAIL in the diabetic subjects was positively correlated with total cholesterol, ACR, and OPG (model 1); and after multiple stepwise linear regression analysis, only ACR and OPG remained significantly correlated with serum TRAIL (model 2). Simple logistic regression showed that HOMA-IR, HbA_{1c}, TG, eGFR, TRAIL, OPG, and OPG/TRAIL were significantly associated with albuminuria (model 1). Multiple stepwise logistic regression analysis showed that only OPG, HbA_{1c}, and TG were independent predictors of albuminuria.

4. Discussion

This study demonstrated that OPG and TRAIL are significantly increased in type 2 diabetes mellitus patients with albuminuria as compared with type 2 diabetes mellitus patients with normoalbuminuria. In addition, the study proved a close connection between OPG and TRAIL and identified OPG as a significant marker of diabetic nephropathy. In the past, OPG has been regarded as an important inhibitor of bone resorption. However, in vivo studies have shown that OPG-deficient mice are prone to calcification of the aorta and renal arteries; and OPG is able to act as a survival factor for smooth muscle cells by inducing matrix metalloproteinase–9 activity [10,11]. In postmenopausal white women, Siepi et al [12] found that OPG is closely related to features of vascular damage such as the intima-media thickness and brachial flow-mediated vasodilatation. There is also increasing clinical evidence showing that elevated OPG concentration is associated with diabetic neuropathy, diabetic maculopathy and silent

Table 1 – Basic characteristics of the type 2 diabetes mellitus patients by albuminuric group

	Normoalbuminuric	Microalbuminuric	Macroalbuminuric
Age (y)	60.7 ± 11.2	64.1 ± 10.7	62.1 ± 10.8
Sex (M/F)	32/36	25/42	23/21
BMI (kg/m^2)	25.0 ± 2.6	25.6 ± 3.8	25.6 ± 3.2
Hypertension (%)	64.7	64.2	86.4[†]
Smoking (%)	7.4	13.4	11.4
Glucose (mg/dL)	170.9 ± 51.2	184.3 ± 59.5	187.9 ± 59.1
HOMA-IR[a]	3.20 ± 3.69	5.20 ± 4.19[*]	6.71 ± 7.23[†]
HbA_{1c} (%)	8.27 ± 1.02	8.87 ± 1.70	9.23 ± 1.75[†]
Cholesterol (mg/dL)	184.9 ± 34.2	187.7 ± 37.3	207.7 ± 49.4
HDL-C (mg/dL)	46.2 ± 12.1	45.1 ± 11.3	43.2 ± 10.6
LDL-C (mg/dL)	118.1 ± 30.9	117.3 ± 33.3	126.2 ± 40.9
TG (mg/dL)[a]	116.6 ± 60.5	152.9 ± 66.2[*]	192.1 ± 123.0[†,‡]
hs-CRP (mg/L)[a]	1.66 ± 1.37	2.06 ± 1.76	2.78 ± 1.67
eGFR (mL/min)	85.2 ± 23.5	74.6 ± 28.7[*]	68.1 ± 29.3[†]
Statin (%)	49.9	54.4	48.4
ACEI/ARB (%)	61.8	59.7	86.4[†]
OPG (pg/mL)[a]	1690.1 ± 627.2	2154.2 ± 922.1[*]	2251.5 ± 925.7[†]
TRAIL (pg/mL)	70.7 ± 23.3	80.2 ± 24.1[*]	88.1 ± 23.8[†]
OPG/TRAIL ratio	24.4 ± 12.1	30.1 ± 13.2[*]	30.1 ± 12.3[†]

All continuous data are presented as mean ± standard deviation.
[a] Log-transformed before analysis.
[*] Microalbuminuric group vs normoalbuminuric group; $P < .05$.
[†] Macroalbuminuric group vs normoalbuminuric group; $P < .05$.
[‡] Macroalbuminuric group vs microalbuminuric group; $P < .05$.

Table 2 – Linear regression analysis for OPG and TRAIL with different variables

	OPG [a]				TRAIL				Albuminuria			
	Model 1		Model 2		Model 1		Model 2		Model 1		Model 2 [b]	
	β	P value	β	P value	β	P value	β	P value	Odds ratio	P value	Odds ratio	P value
Age (y)	0.022	<.001	0.021	<.001	0.076	.686			1.023	.120		
Male sex	−0.218	.002	−0.138	.017	−0.479	.906			0.857	.618		
BMI (kg/m^2)	−0.013	.225			1.001	.110			1.053	.289		
Hypertension	0.210	.005			8.021	.068			1.473	.243		
Smoking	0.128	.257			6.061	.356			0.550	.273		
Glucose (mg/dL)	0.000	.840			0.023	.518			1.005	.091		
HOMA-IR [a]	0.049	.213			1.481	.521			2.027	.001		
HbA_{1c} (%)	0.031	.147	0.037	.038	1.886	.124			1.348	.005	1.329	.013
Cholesterol (mg/dL)	0.001	.315			0.102	.041			1.007	.087		
HDL-C (mg/dL)	0.003	.354			0.078	.659			0.986	.280		
LDL-C (mg/dL)	0.000	.897			0.091	.119			1.002	.607		
TG (mg/dL) [a]	0.104	.106			1.314	.725			3.045	<.001	2.930	.002
hs-CRP (mg/L) [a]	0.017	.664			1.785	.417			1.187	.307		
eGFR (mL/min)	−0.008	<.001			−0.127	.081			0.982	.003		
ACR (mg/g) [a]	0.064	<.001	0.041	.009	2.995	.005	2.434	.025				
Statin treatment	0.076	.278			4.971	.224			1.811	.057		
ACEI/ARB	0.103	.104			7.656	.074			1.463	.241		
TRAIL (pg/mL)	0.003	.009	0.002	.039					1.013	.036		
OPG (pg/mL) [a]					11.381	.009	8.819	.045	3.072	<.001	2.698	.009
OPG/TRAIL ratio									1.022	.048		
R^2 for model 2				.365				.066			0.203	

Model 1: simple regression for OPG, TRAIL, and albuminuria with each variable. Model 2: multiple forward stepwise regression analysis for OPG and TRAIL adjusted with all variables except OPG/TRAIL ratio.

[a] Log-transformed before analysis.

[b] Model 2: multiple logistic regression analysis for albuminuria included sex, hypertension, smoking, HbA_{1c}, cholesterol, HDL-C, LDL-C, TG, statin, ACEI/ARB, TRAIL, OPG, and OPG/TRAIL as relevant factors.

myocardial ischemia in type 2 diabetes mellitus patients [4-6]. Very recently, Xiang et al [8] demonstrated that OPG was increased in microalbuminuric and macroalbuminuric type 2 diabetes mellitus patients as compared with normoalbuminuric type 2 diabetes mellitus patients. Taken together with the results of this study, these findings suggest that OPG might play an important role in diabetic nephropathy, especially when albuminuria becomes severe.

Tumor necrosis factor–related apoptosis-inducing ligand, a member of the tumor necrosis factor superfamily, has been found to be closely related to the apoptosis process and has therefore been targeted as a potential regulating factor in anticancer therapy [13]. Recently, Lorz et al [3] identified TRAIL and OPG genes as being most up-regulated among cell death–related genes in tissue samples of human diabetic nephropathy as compared with control tissue. In addition, they further demonstrated that TRAIL is able to induce tubular cell loss and that addition of recombinant OPG increases tubular cell survival by interfering with the TRAIL-induced apoptosis pathway [3]. We demonstrated that serum TRAIL concentrations were significantly increased in microalbuminuric and macroalbuminuric type 2 diabetes mellitus patients, which, in association with the results of Lorz et al, supports the notion that TRAIL may be involved in the process of diabetic nephropathy.

Although our results support the implication that TRAIL induces diabetic nephropathy, other reports have indicated that TRAIL may also play a protective role in vascular diseases. Secchiero et al [14] showed that TRAIL is able to promote the survival of endothelial cells by activating the Akt and ERK pathways; and Zauli et al [15] demonstrated that TRAIL could induce endothelial nitric oxide and prostanoid production synthesis, both of which are known to protect endothelial cell function. In clinical studies, Michowitz et al [16] showed that patients with stable angina and individuals with normal coronary arteries had higher serum TRAIL than patients with acute coronary syndrome. Secchiero et al [17] demonstrated in a cohort study that serum TRAIL was significantly decreased in patients with acute myocardial infarction and then gradually elevated after discharge from hospital. These results suggested that the lowered serum TRAIL concentration in subjects with acute coronary syndrome is an expression of increased consumption of serum TRIAL in the cellular environment to protect from vascular injury. Very recently, based on a streptozotocin-induced diabetes model, treatment with recombinant TRAIL showed its potential in ameliorating hyperglycemia [18]. According to the above studies, these results hinted that TRAIL may also act as a protective cytokine.

It is important to point out that TRAIL is a transmembrane protein with an extracellular protein and that the serum soluble TRAIL we detected is mainly generated through enzymatic cleavage of this extracellular domain, which then acts as a multifunctional cytokine by binding to several cellular receptors [19]. Therefore, serum OPG may be important in the regulation of the TRAIL-activated response, as it acts as a soluble serum decoy receptor to soluble TRAIL [19]. To prove the connection between OPG and TRAIL and investigate significant factors related to TRAIL and OPG separately, we performed both simple

and multiple variant analyses. Our findings regarding the association of age and HbA_{1c} with OPG were consistent with those of other groups [8,20]. That the female sex is a positive factor related to OPG is also reasonable, as estrogen was shown to up-regulate OPG expression in an in vitro study [21]. Our results also indicated that diabetic participants with a higher severity of albuminuria tended to have a higher OPG and TRAIL expression. Interestingly, we found a significant relationship between serum OPG and serum TRAIL in our regression analyses, which supported the hypothesis that OPG is capable of regulating the TRAIL pathway.

The interaction of OPG and TRAIL has attracted much attention recently. Shaker et al [22] clearly demonstrated a trend of increase in OPG and decrease in TRAIL accompanied by an increase in affected coronary arteries. Secchiero et al [23] showed that a higher OPG/TRAIL ratio is a risk factor for the development of heart failure in patients who suffer from acute myocardial infarction. In our results, we demonstrated that patients with significant albuminuria had a higher OPG/TRAIL ratio. This evidence suggested that a connection between OPG and TRAIL is noteworthy with regard to vascular complications.

To further clarify the contribution of OPG and TRAIL to diabetic nephropathy, logistic regression analyses were performed. Although serum TRAIL, as well as the OPG/TRAIL ratio, was initially significant with regard to albuminuria (model 1), it failed to achieve independent significance after multiple variants regression analysis (model 2). In contrast, serum OPG remained significant in relation to albuminuria in both regression models. Associated with our previous results, this might hint that serum OPG could be more important to the pathogenesis of diabetic nephropathy.

Although Lorz et al [3] demonstrated that OPG could interfere with the process of TRAIL-induced apoptosis in renal-tubular cells and suggested a protective role of OPG, they also identified that the OPG gene exhibited the highest degree of expression in the sample obtained from diabetic kidneys. Their results may also show a different perspective in that OPG could act directly on the diabetic kidney. In an in vivo study, Toffoli et al [24] found that OPG-treated mice showed a reduction in islet function resulting in increased islet inflammatory cell infiltration, fibrosis, and apoptosis. They found that this OPG-induced remodeling of the islet structure was associated with increases in the expression of the renin-angiotensin system, which is known to be a major pathophysiologic response when diabetic nephropathy develops [24]. Recently, O'Sullivan et al [9] showed that serum OPG, not TRAIL, was elevated in type 2 diabetes mellitus patients without vascular complications as compared with normal healthy subjects. This result indicated that a higher serum OPG precedes the development of diabetic vascular complications. In light of those and our results, OPG appears to be a more sensitive marker in the diabetes state and may play a vital role in diabetic nephropathy.

The interpretations of our study were limited by its cross-sectional design. Although we demonstrated a close association between serum OPG, TRAIL, and diabetic nephropathy, the elevated serum TRAIL and OPG levels observed in the present study could also be an incidence of impaired degradation or excretion by deteriorated renal function in diabetic participants with significant albuminuria. Therefore, we can neither conclude the physiologic role of TRAIL and OPG nor build their causality to diabetic nephropathy by simply investigating their serum concentrations. However, based on the above-described literature and our results, it would be worthwhile to investigate the complex interaction between soluble TRAIL and OPG, or OPG itself, which activates different intracellular signal pathways in the experimental microenvironment.

In conclusion, we first demonstrated that serum OPG and TRAIL concentrations are closely related to the severity of diabetic nephropathy, which supports the hypothesis of close interplay between OPG and TRAIL. The study also suggested that OPG may be a marker of the severity of diabetic nephropathy. Further studies are necessary to determine the pathophysiologic role of increased OPG in diabetic nephropathy.

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