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ORIGINAL ARTICLE

Plasma Growth Arrest-Specific Protein 6 Expression in Uremic Patients with Type 2 Diabetes

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Background: Diabetic kidney disease is a major cause of uremia worldwide; recently, controversial roles for growth arrest-specific protein 6 (GAS 6) have been revealed in the pathogenesis of diabetic nephropathy. A better understanding of the association between GAS6 and diabetic nephropathy may lead to the development of novel therapeutic approaches for the prevention and treatment of diabetic uremia. **Objectives:** The aim of this study was to investigate the levels of GAS6 and its role in uremic patients with Type 2 diabetes. **Materials and Methods:** A total of 109 adults were recruited, of whom 23 had Type 2 diabetes and uremia and 56 had newly diagnosed Type 2 diabetes without remarkable nephropathy; thirty individuals with normal glucose tolerance without significant clinical comorbidities served as controls. Plasma GAS6 concentration and common anthropometric and biochemical variables were analyzed. **Results:** Plasma GAS6 levels were significantly lower in patients with Type 2 diabetes than in controls regardless of nephropathy ($P < 0.001$). A trend in declined level of GAS6 among the three groups was also observed. In addition, GAS6 levels were significantly inversely correlated with plasma creatinine, blood urea nitrogen, and uric acid levels in all patients ($P < 0.01$ for all comparisons). In multivariate logistic regression analysis, higher plasma GAS6 concentration was significantly associated with decreased risk of diabetic uremia, even after adjusting for age, sex, and fasting glucose (C-statistic, 0.72; 95% confidence interval 0.57–0.92; $P < 0.01$). **Conclusions:** Our results suggest that plasma GAS6 levels are associated with Type 2 diabetes and may play a role in development of diabetic uremia.

Key words: Growth arrest-specific protein 6, type 2 diabetes, uremia

INTRODUCTION

Type 2 diabetes mellitus (DM) is a chronic disorder and is associated with various macrovascular and microvascular complications such as diabetic nephropathy.[1] Diabetic nephropathy is characterized by structural and functional changes.[2] In the early stage of diabetic nephropathy, tubular hypertrophy occurs, followed by interstitial fibrosis with tubular atrophy in the advanced stage, as well as loss of podocytes with decreased endothelial cell fenestration.[3] Functionally, overt proteinuria with reduced glomerular filtration rate usually occurs after glomerular hyperfiltration and increased albumin excretion.[4] However, the effects of interactions between metabolic derangements and hemodynamic alterations and the potential mechanisms for the development and progression of diabetic uremia have yet to be elucidated.[5]

The growth arrest-specific gene 6 (GAS 6) encodes for a Vitamin K-dependent protein secreted primarily by immune cells, adipocytes, vascular smooth muscle cells, and endothelial cells.[6] GAS6 has growth factor-like properties through binding to receptor tyrosine kinases of the

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TYRO3-AXL-MERTK (TAM) family, via carboxy-terminal LamG domains.[7] A large number of signaling pathways for the GAS6/TAM system has been reported, including activation of antiapoptotic kinase AKT, mitogen-activated protein kinase, PLC-γ, and Janus kinase pathways.[8] Consequently, the GAS6/TAM system is considered to be involved in inflammation, insulin resistance, hemostasis, autoimmune disorders, vascular biology, and cancer.[9]

Recent research has indicated that GAS6 may play multiple roles in the pathogenesis of chronic kidney disease and diabetic nephropathy. One study indicated that GAS6 levels were elevated in patients with early-stage diabetic nephropathy, but gradually declined during progression of the disease; [10] however, progressively increased levels of GAS6 were observed in patients with chronic renal failure, while their renal function appeared deteriorated in the other research group.[11] The controversy about the role of GAS6 may be due to the different characteristics of the participants involved in these studies, including the diabetes incidence, cerebrovascular accident, myocardial infarction, heart failure, and history of long-term dialysis (>10 years). Furthermore, both hypertrophy and proliferative responses during the progression of diabetic nephropathy have been reported to be associated with fluctuation in GAS6 levels, hence the change of GAS6 levels in diabetic nephropathy appeared to be complicated.[12,13] Nevertheless, no definite results have been reported with regard to diabetic uremia, particularly in humans. Therefore, the aims of this study were to investigate the possible association between GAS6 and metabolic derangement and the role of GAS6 in patients with Type 2 diabetic uremia.

MATERIALS AND METHODS

Study design and ethics committee statement

This study was approved by the Internal Review Board of the Ethics Committee of Tri-Service General Hospital, Taipei, Taiwan, and all enrolled individuals provided written informed consent. Ambulatory adults were recruited from the outpatient clinics of Tri-Service General Hospital. The age of patients ranged from 50 to 70 years, and they all had a body mass index (BMI) <35 kg/m^2. The inclusion criteria were no recorded infections within the previous 12 weeks; no administration of oral anticoagulants or any types of statins; no history of stroke, myocardial infarction, or angina; and no history of malignant tumors before participating in this study. The following exclusion criteria were applied: lactation or pregnancy, acute or chronic disorders, malignant tumor history, psychiatric diseases, and recent use of medication in general. The patients without diagnosis of diabetes underwent a 75-g oral glucose tolerance test after they had fasted for at least 10 h. According to the American Diabetes Association criteria, participants were then classified as having normal glucose tolerance (NGT; n = 30, fasting glucose <5.6 mmol/l with a 2-h postload plasma glucose of < 7.8 mmol/l) or newly diagnosed Type 2 diabetes (n = 56, fasting glucose ≥7.0 mmol/l or 2-h postload glucose >11.1 mmol/l). Patients with preexisting diabetes were eligible for the study as the diabetic uremia group; all patients had a history of >10 years of diabetes and dialysis dependency (n = 23). A total of 109 adults (male:female ratio 2:1) were therefore enrolled in this study for cross-sectional research. The height, weight, BMI, and systolic and diastolic blood pressure of all patients were recorded when blood samples were collected. Blood samples were collected from patients in the diabetic uremia group before starting dialysis.

Laboratory measurements

Following a 10-h fast, blood samples were obtained for general biochemistry and total cholesterol using an enzymatic colorimetric method and a Roche Cobas C501 chemistry analyzer (Diamond Diagnostics, Holliston, MA, USA). Plasma glucose, uric acid, creatinine, and GAS6 levels were also measured. Serum triglycerides were assayed using a colorimetric enzymatic test on a Hitachi 717 system (Biomedilines, San Diego, CA), while plasma glucose concentrations were determined with the hexokinase method (the ultraviolet test) and a Roche Cobas C501 chemistry analyzer. All concentrations were determined in duplicate, and the values from the two samples were averaged.

Plasma GAS6 protein was measured using a DuoSet® ELISA Development kit (R and D Systems, Minneapolis, MN, USA), which contained the basic components required to develop a sandwich ELISA to measure endogenous and recombinant human GAS6 levels. For each plasma sample, 100 μl was directly transferred to the microtest strip wells of the ELISA plate coated with the capture mouse antihuman GAS6 antibody and incubated for 2 h at room temperature. After three washes, the detection antibody was added, and the reaction mixture was incubated for another 2 h at room temperature. Antibody binding was detected using streptavidin-conjugated horseradish peroxidase and developed with a substrate solution. The reaction was stopped after adding stop solution, and optical density was determined using a microplate reader at 450 nm. The concentration of GAS6 was quantitated with a human GAS6 standard curve. Each plasma sample was assayed in duplicate according to the instructions of the manufacturer, and values were within the linear portion of the standard curve. The intra-assay and interassay CVs of

GAS6 were 6.5% and 8.5% respectively, with a mean recovery in 10 patients of 97%, and a lower limit of quantification of 0.26 ng/ml. High- or low-plasma GAS6 levels were defined as beyond the upper reference limit (97.5 percentile) of 18.8 (18.0–22.3) μg/L or below the lower reference limit (2.5 percentile) of 2.5 (1.9–3.1) μg/L.[14]

Statistical methodology

Statistical differences in the demographic characteristics and GAS6 concentrations between the NGT, Type 2 diabetes, and diabetic uremia groups were determined according to the Kruskal–Wallis one-way analysis of variance. Associations between age, uric acid, total cholesterol, triglycerides creatinine, blood urea nitrogen (BUN), glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, fasting glucose, and GAS6 in all enrolled patients were estimated using Spearman rank correlation analysis. All statistical analyses were performed with SPSS software version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Furthermore, we performed multivariate logistic regression and step-wise regression analyses to estimate the regression coefficients among GAS6 and other independent variables such as age, sex, and fasting glucose in three comparable subgroups of the enrolled individuals. All values were expressed as mean ± standard error. $P < 0.05$ were considered to be statistically significant.

RESULTS

Table 1 presents the anthropometric profiles and clinical information of the three groups. The mean age of the participants in the NGT, Type 2 DM, and diabetic uremia groups was 60.1 years, 58.8 years, and 69.7 years, respectively (range 50–70 years), and the male-to-female ratio was approximately 2:1. Significant differences were noted between the three groups in most anthropometric values and baseline biochemical profiles except for triglyceride levels. Individuals in the diabetic uremia group were older, had higher levels of uric acid, and worse renal function than those of the other two groups. The diabetic uremia group showed a lower concentration of GAS6 than the NGT group [Figure 1]; however, there was no significant difference in GAS6 levels between the Type 2 DM and diabetic uremia groups.

Table 2 shows the correlation coefficients between plasma GAS6 concentration and biochemical variables in the enrolled individuals. The results revealed significant negative correlations between the concentration of GAS6 and uric acid or renal function after adjusting for age. There were no significant correlations between age or fasting plasma glucose and the concentration of GAS6.

Table 3 shows the results of multivariate logistic regression analysis performed to determine whether plasma GAS6 levels were related to Type 2 diabetic uremia, regardless of other well-known factors. After adjusting for age and sex, higher plasma GAS6 levels were significantly associated with decreased risk of both Type 2 diabetes and diabetic uremia (T2DM versus NGT, 0.89 [0.81–0.98]; DM uremia versus NGT, 0.76 [0.61–0.96]). However, this association became nonsignificant after further adjusting for fasting plasma

Table 1: Anthropometric and biochemical variables among the three groups

	NGT (*n*=30)	T2DM (*n*=56)	DM uremia (*n*=23)	*P**
Age (years)	60.1±1.1	58.8±1.1	69.7±3.0	<0.001‡,¶
Sex (male/female)	20/10	36/20	15/8	1.000
TC (mg/dL)	202.17±6.36	202.74±5.44	157.22±6.53	<0.001‡,¶
Triglyceride (mg/dL)	122.67±12.59	186.63±15.71	165.52±27.51	0.061
Uric acid (mg/dL)	5.78±0.35	5.73±0.22	8.50±0.32	<0.001‡,¶
Creatinine (mg/dL)	0.86±0.05	0.84±0.03	10.70±0.49	<0.001‡,¶
BUN (mg/dL)	17.04±1.07	15.20±0.72	85.30±3.85	<0.001‡,¶
GOT (U/L)	21.69±1.22	29.83±2.16	19.09±1.59	<0.001†,‡
GPT (U/L)	21.78±2.62	33.85±3.27	14.74±1.47	<0.001†,‡
Fasting glucose (mg/dL)	93.53±1.77	126.91±5.33	165.2±16.62	<0.001†,‡,¶
GAS6 (ng/mL)	14.35±1.23	11.44±0.47	9.29±0.25	<0.001†,¶

*Assessed by Kruskal–Wallis one-way analysis of variance. Data shown are mean±SEs; †NGT versus T2DM; ‡T2DM versus DM uremia; ¶NGT versus DM uremia. All assessed by *post hoc* LSD test; **P* value: variables compared among the three study groups. NGT=Normal glucose tolerance; T2DM=Type 2 diabetes mellitus; DM uremia=Type 2 diabetic uremia; TC=Total cholesterol; BUN=Blood urine nitrogen; GOT=Glutamate oxaloacetate transaminase; GPT=Glutamic pyruvic transaminase; GAS6=Growth arrest-specific protein 6; SEs=Standard errors; LSD=Least significant difference

Table 2: Age-adjusted Spearman partial correlation coefficients between plasma growth arrest-specific protein 6 concentration and biochemical variables

*Spearman partial correlation coefficient (*n*=109)		
	R	*P*
TC (mg/dL)	0.061	0.563
Triglyceride (mg/dL)	-0.110	0.282
Uric acid (mg/dL)	-0.328	**0.002**
Creatinine (mg/dL)	-0.295	**0.003**
BUN (mg/dL)	-0.470	**<0.001**
GOT (U/L)	0.053	0.690
GPT (U/L)	0.064	0.559
Fasting glucose (mg/dL)	-0.148	0.126

* Corrected for age. TC, total cholesterol; BUN, blood urea nitrogen; GOT, glutamate oxaloacetate transaminase; GPT, glutamic pyruvic transaminase

Table 3: Multivariate logistic regression analyses of plasma growth arrest-specific protein 6 concentration among different patients

	T2DM versus NGT		DM uremia versus NGT		DM uremia versus T2DM	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Model 1	0.89 (0.81−0.98)	0.015	0.75 (0.61−0.94)	0.011	0.76 (0.61−0.94)	0.010
Model 2	0.89 (0.81−0.98)	0.017	0.77 (0.62−0.96)	0.019	0.75 (0.59−0.94)	0.014
Model 3	0.89 (0.81−0.98)	0.014	0.76 (0.61−0.96)	0.018	0.75 (0.59−0.94)	0.014
Model 4	0.95 (0.84−1.10)	0.348	0.87 (0.67−1.15)	0.337	0.72 (0.57−0.92)	0.009

Model 1=Crude odds ratio; Model 2=Adjusted for age; Model 3=Adjusted for age, sex; Model 4=Adjusted for age, sex, and fasting glucose. T2DM=Type 2 diabetes mellitus; NGT=Normal glucose tolerance; OR=Odds ratio; CI=Confidence interval; DM uremia=Type 2 diabetic uremia

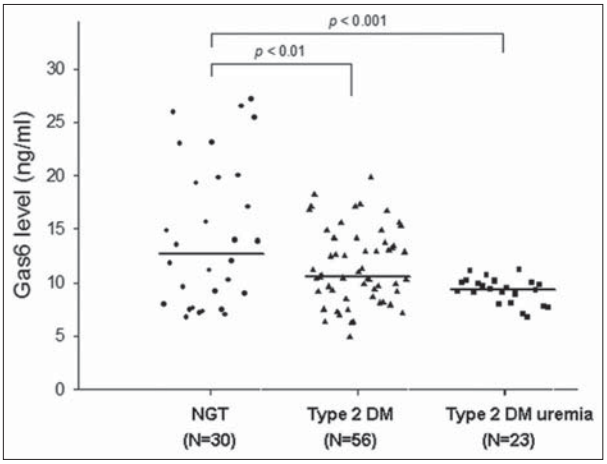

Figure 1: Plasma growth arrest-specific protein 6 concentrations in patients with normal glucose tolerance, patients with Type 2 diabetes, and patients with Type 2 diabetes with uremia. The lines stand for the mean values in each group. The participants with normal glucose tolerance had the highest plasma growth arrest-specific protein 6 levels among the three groups

glucose concentration. In logistic regression analysis between the Type 2 DM and diabetic uremia groups, higher plasma GAS6 concentrations were associated with lower risk of diabetic uremia (DM uremia versus T2DM, 0.75 [0.59–0.94]), and this association became slightly stronger after further adjusting for fasting plasma glucose (DM uremia versus T2DM, 0.72 [0.57–0.92]).

DISCUSSION

GAS6, a gamma-carboxyglutamic acid domain-containing protein, was first identified in 1988[15] and has been strongly associated with altered glucose tolerance, endothelial dysfunction, and renal diseases. It has even been reported to be an independent risk factor for Type 2 DM.[16] The mean amplitude of glycemic excursion and chronic low-grade inflammation in patients with diabetes can lead to downregulation of GAS6/TAM signaling.[17] Diabetic nephropathy can be caused or deteriorated by complicated interactions between metabolic derangements and hemodynamic alterations,[18] and GAS6 and GAS6 receptors have been shown to play important roles in protecting from progression of diabetic glomerular hypertrophy, which is a hallmark of the early phase of nephropathy.[19] On the other hand, high glucose stimulation of mesangial cells, followed by activation of the GAS6/AXL and AXL/mTOR pathways, has been reported to result in glomerular hypertrophy and subsequent mesangial expansion, followed by glomerulosclerosis for pathophysiological adaptation.[20] In addition, plasma GAS6 levels have been associated with albuminuria in patients with Type 2 diabetes and higher plasma GAS6 concentrations in patients with micro- or macro-albuminuria compared to those with normoalbuminuria.[21] However, our results showed a trend of a decrease in GAS6 levels among the groups of NGT, Type 2 diabetes, and Type 2 diabetes with uremia, with the lowest level being in the diabetic uremia group, in contrast to the previous studies.[22,23] The actual mechanism underlying this phenomenon is unclear; however, we assume that it is relevant to the gradual depletion of GAS6 either due to longer duration of diabetes with its related micro- and macro-vascular complications, or poor control of hyperglycemia with remarkable fluctuation. Therefore, lower GAS6 concentration would be expected in the late stage of diabetic nephropathy, although elevated GAS6 levels have been reported in patients with early stage chronic renal failure.

To our knowledge, this is the first study to investigate the relationship between GAS6 and diabetic uremic state in clinical practice, although various functions of the GAS6/TAM system have been identified in humans. Our findings also showed an inverse relationship between GAS6 and other factors including uric acid, creatinine, and BUN in all patients, which is consistent with some previous studies.[10,17] In contrast, other studies indicated that increased GAS6 levels were related with deterioration of renal function in patients with Type 2 diabetes.[11,22] Given the discrepancies observed between studies, confounding factors leading to these controversial results should be investigated. In this respect, GAS6 can be secreted from various types of cells and tissues, primarily

generated by endothelial cells.[24] Therefore, elevation of GAS6 levels may be anticipated in response to endothelial dysfunction in early stage chronic renal failure or diabetes.[25] Thereafter, dysregulated crosstalk between endothelial cells, the immune system and systemic inflammatory cytokines can develop as diabetic uremia progresses, followed by declining GAS6 concentration. In addition, DM nephropathy has been reported to cause various vascular complications through fluctuations in blood sugar levels and ultimately contributed to impaired GAS6/TAM interactions[26] that strongly affected GAS6 levels in the circulation. Apart from interacting with the corresponding TAM family of receptor tyrosine kinases, GAS6 polymorphisms may result in different outcomes.[27] Moreover, the persistent decline in renal function with elevated levels of creatinine and plasma uric acid may trigger production of interleukin (IL)-1β and IL-18, thereby enhancing the dysfunction of endothelial cells[28] to reduce circulatory GAS6. Some studies have also reported that hypoxia can occur in this situation, and that the AXL pathway can lose its ability to downregulate higher expression of the soluble extracellular domain of AXL to attenuate plasma GAS6 levels under conditions of persistent low-grade systemic inflammation.[29] Although the reason behind the controversial results regarding the levels of GAS6 is still not clear, our results indicated that high plasma GAS6 concentrations ameliorated the risk of deteriorating Type 2 diabetic nephropathy. Moreover, GAS6 levels showed remarkably significant lower odds ratio in Type 2 DM with uremia, even after adjusting for fasting glucose. GAS6 may, therefore, be relevant to diabetic nephropathy, and lower levels of GAS6 may represent deterioration of Type 2 diabetic nephropathy.

There are several limitations to this study. First, our data may have been biased due to the cross-sectional design and lower sample size, which may account for the fact that although plasma GAS6 levels were not significantly lower in uremic patients with diabetes compared with patients with diabetes, higher plasma GAS6 levels were significantly associated with decreased risk of diabetic uremia compared with that of diabetes. Furthermore, the selection of patients included in our study could also be considered as biased. Nevertheless, the results showed statistically significant findings using Kruskal–Wallis one-way analysis of variance. Second, we collected only plasma GAS6 levels for evaluation without examination of other serum inflammation markers or serum vessel calcification markers. However, our research was a pilot study with the aim to investigate the role of GAS6 in diabetic nephropathy, and the results indicated that GAS6 is actually involved in the pathogenesis of diabetic kidney disease. Further important markers would be investigated in our following studies. Third, sex differences in plasma GAS6 have been demonstrated in several studies, and plasma GAS6 is associated with sex hormones in females and age in males.[30] However, no significant difference in GAS6 levels was observed between older male and female participants in all groups in our study (data not shown).

CONCLUSIONS

Our results support that fasting plasma glucose induces variations in GAS6 level in patients with Type 2 DM compared to those with NGT, but also suggest that GAS6 may be important for the pathogenesis of diabetic uremia as shown by its unique expression in uremic patients with Type 2 diabetes.

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Conflicts of interest

There are no conflicts of interest.

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