Plasma Protein Growth Arrest-Specific 6 Levels are Associated with Altered Glucose Tolerance, Inflammation and Endothelial Dysfunction

Running title: Gas6 in diabetes and endothelial dysfunction

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Objectives - Plasma protein growth arrest-specific 6 (Gas6) is important to the inflammatory process and is involved in the development of diabetic renal and vascular complications. We set out to determine whether plasma Gas6 levels are associated with altered glucose tolerance, insulin sensitivity, inflammation, and endothelial dysfunction.

Research design and methods - A total of 278 adults, including 96 with normal glucose tolerance (NGT), 82 with impaired glucose tolerance (IGT) and 100 with type 2 diabetes were recruited. Plasma Gas6 concentration, biochemical, proinflammatory, and endothelial variables were determined. Insulin sensitivity was examined by homeostasis model assessment.

Results - Plasma Gas6 concentration was significantly lower among patients with type 2 diabetes compared with subjects with NGT (P<0.001). The plasma Gas6 value was inversely correlated with fasting glucose, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and vascular cell adhesion molecule-1 (VCAM-1). In multivariate logistic regression analysis, after adjustment for established diabetes risk factors, higher plasma Gas6 concentrations were significantly associated with a decreased risk of type 2 diabetes. Moreover, the association became slightly stronger after further adjustment for TNF-α, IL-6, high sensitive C-reactive protein (hsCRP), E-selectin, intercellular adhesion molecule 1 (ICAM-1), and VCAM-1.

Conclusions – Plasma Gas6 is associated with altered glucose tolerance, inflammation, and endothelial dysfunction. It also may represent a novel independent risk factor of type 2 diabetes and a potential surrogate marker of inflammation and endothelial dysfunction.
The epidemic of type 2 diabetes and impaired glucose tolerance (IGT) is one of the main causes of morbidity and mortality worldwide (1). In both disorders, tissues such as muscle, fat, liver, and endothelial cells become less responsive or, in some cases, resistant to insulin (2). Although it is well established that insulin resistance and impaired insulin secretion are central to the pathogenesis of type 2 diabetes, it has been unclear how these abnormalities arise and how they are related to many different clinical and biochemical features common in type 2 diabetes, including central obesity, hypertension, accelerated atherosclerosis, chronic inflammation, dyslipidemia, and disordered hemostasis.

Protein growth arrest-specific 6 (Gas6) was the last addition to the family of plasma vitamin K-dependent proteins. Gas6 was cloned and characterized in 1993 and found to be similar to plasma anticoagulant protein S (3). Soon after, it was recognized as a growth factor-like molecule, as it interacted with receptor tyrosine kinases of the TAM (Tyro-3, Axl, Mer) family (4). The Gas6/TAM system regulates an intriguing mix of processes, including cell survival and proliferation, cell adhesion and migration, blood clot stabilization, and inflammatory cytokine release (5-8). Therefore, the role of the Gas6/TAM system has been found to be important in inflammation, hemostasis, autoimmune disease, nervous, reproductive and vascular systems, and cancer (9).

Recently, several reports revealed that the Gas6/TAM system was involved in the pathogenesis of diabetic renal and vascular disease (10-12). Expression of Gas6/TAM was increased in the glomerulus of diabetic rats, which led to mesangial and glomerular hypertrophy (10). In vascular smooth muscle cells (VSMC), Gas6/TAM signaling increased cell survival in the presence of low glucose and increased cell migration in the presence of high glucose (11). VSMC migration was increased in patients with diabetes, and diabetes accelerated the accumulation of VSMCs in atherosclerotic lesions (12). These pre-clinical studies indicate that Gas6/TAM likely represents an important pathogenic mechanism for renal and cardiovascular complications associated with diabetes. However, little is known about the clinical significance of the Gas6/TAM system in patients with diabetes and its association with various biochemical variables that are common in diabetic patients. We have addressed this issue by conducting a cross-sectional study to determine whether plasma Gas6 levels are associated with altered glucose tolerance, insulin sensitivity, inflammatory, and endothelial dysfunction markers in humans.

**RESEARCH DESIGN AND METHODS**

A total of 278 adults were recruited from the outpatient clinics of Tri-Service General Hospital, Taipei, Taiwan. Criteria for inclusion into this study were as follows: 20 to 75 years of age, body mass index (BMI) <35 kg/m², absence of infection within the previous weeks, absence of taking oral anticoagulants and anti-diabetic therapy, including oral hypoglycemic agents, insulin and glucagons-like peptide 1, and absence of malignant tumor history. Exclusion criteria included women who were pregnant or breast feeding; patients with impaired renal function (serum creatinine ≥132.6 μmol/l); patients with abnormal serum aspartate aminotransferase or alanine aminotransferase (2.5 times above the upper normal ranges); patients with acute or chronic pancreatitis; patients with a history of cerebrovascular accident, myocardial infarction or heart failure; patients with autoimmune disorders or psychiatric diseases, including mood disorders and alcoholism; and patients taking concomitant drugs such as beta-blockers,
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diuretics, cholestyramine or systemic steroids. A 75 g oral glucose tolerance test (OGTT) was performed in all subjects after they had fasted for at least 10 hours. According to the American Diabetes Association criteria, participants were divided into normal glucose tolerance (NGT, n=96, fasting glucose <5.6 mmol/l with a 2-hour post-load plasma glucose of <7.8 mmol/l, IGT (n=82, fasting glucose <7.0 mmol/l and 2-hour post-load glucose between 7.8 and 11.1 mmol/l) and previously unknown type 2 diabetes (n=100, fasting glucose ≥ 7.0 mmol/l or 2-hour post-load glucose >11.1 mmol/l).

The institutional review board of the Tri-Service General Hospital approved the protocol and all subjects gave written informed consent.

Analytic methods: After 10 hours of fasting, blood samples were obtained to determine plasma glucose, insulin, creatinine, and lipid profiles. Plasma circulating high-sensitive C-reactive protein (hsCRP), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were subsequently measured. Serum total cholesterol, triglyceride, and low-density lipoprotein cholesterol (LDL-C) were measured using the dry, multilayer analytical slide method in the Fuji Dri-Chem 3000 analyzer (Fuji Photo Film Corporation, Tokyo, Japan). The intra-assay and inter-assay coefficients of variance (CV) for LDL-C were 0.8% and 2.5%, respectively. Serum levels of high-density lipoprotein cholesterol (HDL-C) were determined by an enzymatic cholesterol assay method after dextran sulfate precipitation. The intra-assay and inter-assay CV for HDL-C were 1.1% and 1.7%, respectively. The levels of HbA1c were 1.3% and 2.2%, respectively. Plasma glucose concentrations were determined by the glucose oxidase method on a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA). The intra-assay and inter-assay CV for glucose were 0.6% and 1.5%, respectively. Plasma insulin was measured with a commercial immunoradiometric kit (BioSource Europe S.A., Nivelles, Belgium). The intra-assay and inter-assay CV for insulin were 2.2% and 6.5%, respectively. Plasma hsCRP levels were measured using the Tina-quant (Latex) high sensitivity assay (Roche, Mannheim, Germany). The intra-assay and inter-assay CV for hsCRP were 3.7% and 4.9%, respectively. Serum IL-6 concentrations were determined by a human high-sensitivity enzyme-linked-immunosorbent assay (ELISA) (Besancon Cedex, France). The intra-assay and inter-assay CV for IL-6 were 1.5% and 5.3%, respectively. Serum TNF-α was measured with the Biotrak™ high-sensitivity human ELISA kit from Amersham Biosciences (Buckinghamshire, UK). The intra-assay and inter-assay CV for TNF-α were 3.5% and 5.3%, respectively. Levels of E-selectin, ICAM-1, and VCAM-1 were measured by commercial ELISA (R&D Systems, Minneapolis, USA). The intra-assay and inter-assay CV for E-selectin were 4.5% and 6.2%; ICAM-1 were 3.5% and 7.1%; and VCAM-1 were 5.0% and 8.7%, respectively. All concentrations of the above biochemical variables were determined in duplicate and the values of the two samples were averaged.

Insulin sensitivity was assessed using the homeostasis model assessment (HOMA), in which the homeostasis model of insulin resistance (HOMA-IR) = [(fasting insulin (uU/ml) × fasting glucose (mmol/l))/ 22.5]. (13). Measurement of Gas6: The Gas6 protein was measured with a sandwich enzyme-linked immunoassay (ELISA). The method has been validated according to the Food and Drug
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Administration (FDA) guidelines in a previous study (intra-assay and inter-assay CV were 6.5% and 8.5%, respectively, mean recovery on 10 patients of 97%, lower limit of quantification – LLOQ – 0.26ng/ml) (14). Briefly, a 96-well microtitre plate was coated overnight at room temperature with 4 μg/ml of polyclonal mouse anti-human Gas6 antibody (R&D Systems, Lille, France). After three washes with 0.05% Tween 20 in phosphate-buffered saline, wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for one hour at room temperature. Three additional washes were then performed and 100 μl plasma or standards (recombinant human Gas6; R&D Systems, Lille, France) were added for two hours at room temperature. Washes were repeated and 100 ng/ml biotinylated monoclonal goat anti-human Gas6 antibody (R&D Systems, Lille, France) was added for two hours at room temperature. Detection was performed with peroxysdase-conjugated streptavidin. Measurements were repeated three times.

**Statistical methods:** Descriptive results of continuous variables were expressed as means ± standard error of the mean (S.E.M.). Before statistical analysis, normal distribution and homogeneity of the variables was evaluated using Levene’s test for quality of variance, and variables were then given a base logarithmic transformation if necessary. The parameters HOMA-IR, fasting insulin, triglyceride, TNF-α, IL-6, hsCRP, E-selectin, ICAM-1, and lower HDL cholesterol than subjects with NGT. Plasma Gas6 concentrations were significantly lower among patients with type 2 diabetes (11.5 ± 0.42 ng/ml) compared with subjects with NGT (14.3 ± 0.66 ng/ml) (P<0.001) as illustrated in Figure 1.

**RESULTS**

Characteristics of the subjects according to glucose tolerance status are shown in Table 1. Patients with type 2 diabetes had higher BMI, waist-to-hip ratio, blood pressure, HOMA-IR, triglyceride, hsCRP, E-selectin, ICAM-1, and lower HDL cholesterol than subjects with NGT. Plasma Gas6 concentrations were significantly lower among patients with type 2 diabetes (11.5 ± 0.42 ng/ml) compared with subjects with NGT (14.3 ± 0.66 ng/ml) (P<0.001) as illustrated in Figure 1.

In all subjects as a whole, the plasma Gas6 value was significantly inversely correlated with fasting, TNF-α, IL-6, and VCAM-1 after adjustment for age (Table 2).

A multivariate logistic regression analysis to investigate whether plasma Gas6 values were related to type 2 diabetes independent from other established diabetes risk factors is shown in Table 3. After adjustment for age, sex, BMI, waist-to-hip ratio, blood pressure, smoking, and alcohol consumption, higher plasma Gas6 concentrations were significantly associated with a decreased risk of type 2 diabetes (type 2 diabetes vs. NGT, 0.93 [0.87-0.99]; type 2 diabetes vs. IGT+NGT, 0.94 [0.89-0.99]). This association remained significant after further adjustment for other covariates (including TNF-α, IL-6, and hsCRP). Moreover, the association became slightly stronger after further adjustment for TNF-α, IL-6, hsCRP, E-selectin, ICAM-1, and VCAM-1 (type 2 diabetes vs. NGT, 0.90 [0.83-0.97]; type 2 diabetes vs. IGT+NGT, 0.92 [0.86-0.98]).
CONCLUSIONS
Numerous studies have shown that the Gas6/TAM system regulates cell survival, proliferation, migration, adhesion, and phagocytosis. Consequently, altered activity/expression of Gas6/TAM components has been detected in a variety of pathologies such as inflammation, coagulopathy, cancer, autoimmune disease, and diabetic vascular and renal diseases (9). However, direct clinical evidence of the Gas6/TAM system is lacking. Our results, described for the first time herein, revealed that plasma Gas6 concentrations were significantly lower among patients with new onset of type 2 diabetes and were associated with glucose levels, inflammation, and endothelial dysfunction markers. These findings demonstrate that Gas6/TAM signaling is associated with type 2 diabetes, inflammation, and endothelial dysfunction. Therefore, we hypothesized that inflammatory effects of high glucose may be at least in part mediated through low Gas6 levels as well as reduced TAM signaling and, consequently, activated innate immunity.

Several studies have suggested that the Gas6/TAM system may play a role in vascular diseases such as atherosclerosis, which are characterized by accumulation of VSMCs. Cavet et al. (11) investigated the effects of varying glucose concentration on Axl signaling in VSMCs and demonstrated a role for glucose in altering Axl signaling through coupling to binding partners. Recently, Jiang et al. (18) demonstrated that the Gas6 plasma concentrations correlated with cardiovascular disease, especially in patients with acute coronary syndrome. In addition, Gas6 c.834+7G>A polymorphism was associated with a lower risk for cardiovascular disease. With the exception of VSMCs, prospective evidence linked endothelial dysfunction with atherosclerosis, demonstrating that endothelial dysfunction was the first step in atherosclerosis (19). Endothelial dysfunction contributes to cardiovascular diseases, including hypertension, atherosclerosis, and coronary heart disease, which are also characterized by insulin resistance (20). Two recent studies in humans provide evidence that plasma Gas6 originates from endothelial cells and leukocytes (21, 22). Our results demonstrated that plasma Gas6 values are significantly, but negatively correlated with the endothelial dysfunction marker - VCAM-1. Meanwhile, using in vitro studies (unpublished data), we provided evidence that hyperglycemia can cause endothelial dysfunction with down-regulation of Gas6/TAM signaling. Hence, we hypothesize that hyperglycemia will lead to diminished Gas6/TAM receptors signaling, which may result in cross-talk between Gas6/TAM signaling and insulin signaling, thereby inducing an imbalance in the production of nitric oxide and endothelin-1 in...
endothelial cells.
It can be concluded from this study that plasma Gas6 levels are associated with altered glucose tolerance, inflammation, and endothelial dysfunction. Plasma Gas6 concentration may represent an independent risk factor of type 2 diabetes, and a potential surrogate marker of inflammation and endothelial dysfunction. These results support the hypothesis that modulation of Gas6 activity may provide an important point for intervention. Gas6/TAM signaling represents a new class of therapeutic targets. Understanding the nature of the Gas6/TAM interaction would ultimately help in the development of novel small molecules or neutralizing monoclonal antibodies for therapeutic applications for diseases in which the interaction between Gas6 and TAM receptors contributes to their progression or pathology (23).

**Authors’ Contributions:** We declare that all authors listed have actively participated in the study and met the requirements of the authorship. YJH wrote the manuscript, researched data. CHL researched data, reviewed/edited manuscript. NFC contributed to discussion and statistical analyses, reviewed/edited manuscript. YSS supervised the project, reviewed/edited manuscript. All authors have read and approved the final version of the manuscript.

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**FIGURE LEGEND**
Figure 1 - Plasma Gas6 concentrations in normal glucose tolerance subjects (NGT), impaired glucose tolerance subjects (IGT), and patients with type 2 diabetes. The lines represent the median values in each group. The type 2 diabetes group had significantly lower plasma Gas6 levels than NGT ($P<0.001$) group

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Table 1. Anthropometric and biochemical variables among different glucose tolerance subjects

|                          | NGT (n=96) | IGT (n=82) | Type 2 Diabetes (n=100) | *P value |
|--------------------------|------------|------------|-------------------------|----------|
| Age (years)              | 50.2 ± 1.54| 56.2 ± 1.45| 52.4 ± 1.53             | 0.026    |
| Sex (M/F)                | 43/53      | 26/56      | 57/43                   | 0.003    |
| BMI (kg/m²)              | 23.9 ± 0.37| 25.4 ± 0.50| 26.0 ± 0.39             | 0.002‡   |
| Waist-to-hip ratio       | 0.85 ± 0.01| 0.86 ± 0.01| 0.90 ± 0.01             | <0.001‡  |
| Blood pressure (mmHg)    |            |            |                         |          |
| systolic                 | 118.8 ± 1.67| 125.0 ± 1.77| 126.2 ± 1.80             | 0.006‡   |
| diastolic                | 76.9 ± 0.91 | 78.1 ± 1.16 | 81.1 ± 0.91             | 0.008‡   |
| OGTT glucose (mmol/L)    |            |            |                         |          |
| Fasting glucose          | 5.05 ± 0.06 | 5.42 ± 0.07 | 8.30 ± 0.44             | <0.001‡  |
| 2 h glucose              | 6.32 ± 0.09 | 9.29 ± 0.10 | 16.9 ± 0.54             | <0.001‡  |
| OGTT insulin (pmol/L)    |            |            |                         |          |
| Fasting insulin§         | 54.0 ± 3.85 | 75.5 ± 11.82| 84.1 ± 6.19             | 0.013‡   |
| 2 h insulin§             | 388.2 ± 32.5| 659.1 ± 61.49| 528.2 ± 52.05           | 0.001    |
| HbA1c (%)                | 5.6 ± 0.03  | 6.0 ± 0.05  | 8.2 ± 0.33              | <0.001‡  |
| HOMA-IR§                 | 2.04 ± 0.15 | 3.19 ± 0.61 | 4.85 ± 0.38             | <0.001‡  |
| Triglyceride (mmol/L)§   | 2.94 ± 0.17 | 3.64 ± 0.27 | 4.97 ± 0.30             | <0.001‡  |
| HDL cholesterol (mmol/L)| 1.49 ± 0.05 | 1.45 ± 0.06 | 1.18 ± 0.03             | <0.001‡  |
| Inflammatory markers     |            |            |                         |          |
| TNF-α (ng/ml)§           | 3.19 ± 0.20 | 3.20 ± 0.21 | 3.31 ± 0.23             | 0.914    |
| IL-6 (pg/ml)§            | 2.6 ± 0.45  | 4.5 ± 1.31  | 5.9 ± 1.44              | 0.113    |
| hsCRP (mg/L)§            | 0.70 ± 0.08 | 0.92 ± 0.09 | 1.15 ± 0.10             | 0.002‡   |
| Endothelial dysfunction markers | | | | |
| E-selectin (ng/ml)       | 45.1 ± 1.92 | 47.4 ± 2.54 | 60.9 ± 3.00             | <0.001‡  |
| VCAM-1 (ng/ml)           | 527.6 ± 31.08| 506.7 ± 38.72| 660.2 ± 40.38           | 0.006‡   |
| ICAM-1 (ng/ml)           | 248.8 ± 9.08| 244.9 ± 8.36| 293.6 ± 10.39           | <0.001‡  |
| Gas6 (ng/ml)§            | 14.3 ± 0.66 | 13.3 ± 0.63 | 11.5 ± 0.42             | 0.002‡   |

*Assessed by one-way ANOVA, data shown as mean ± standard error mean
§The logarithms of these variables were used for the analysis
‡P<0.0025, NGT vs. type 2 diabetes. All assessed by post-hoc LSD test.
NGT, normal glucose tolerance; IGT, impaired glucose tolerance; BMI, body mass index; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; hsCRP, high sensitive C-reactive protein; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1
Table 2. Age-adjusted Spearman partial correlation coefficients between plasma Gas6 concentration and biochemical variables

|                          | Spearman partial correlation coefficient* | All (n=278) |
|--------------------------|------------------------------------------|------------|
|                          | r            | P            |
| BMI                      | -0.074       | 0.222       |
| Waist-to-hip ratio       | -0.131       | 0.031       |
| OGGT glucose (mmol/L)    |                                          |            |
| Fasting glucose          | -0.195       | **0.001**    |
| 2 h glucose              | -0.157       | 0.009       |
| OGGT insulin (pmol/L)    |                                          |            |
| Fasting insulin§         | -0.031       | 0.612       |
| 2 hr insulin§            | 0.002        | 0.975       |
| HOMA-IR§                 | -0.107       | 0.076       |
| HbA1c (%)§               | -0.152       | 0.027       |
| TNF-α (ng/ml)§           | -0.221       | **<0.001**  |
| IL-6 (pg/ml)§            | -0.230       | **<0.001**  |
| hsCRP (mg/L)§            | -0.071       | 0.242       |
| E-selectin (ng/ml)       | -0.157       | 0.009       |
| VCAM-1 (ng/ml)           | -0.269       | **<0.001**  |
| ICAM-1 (ng/ml)           | -0.026       | 0.675       |

§ The logarithms of these variables were used for the analysis.

*Corrected for age.

BMI, body mass index; OGGT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; hsCRP, high sensitive C-reactive protein; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1

Table 3. Multivariate logistic regression analyses of plasma Gas 6 concentration among different glucose tolerance subjects

|                          | Type 2 diabetes vs. NGT | Type 2 diabetes vs. IGT | Type 2 diabetes vs. (IGT+NGT) |
|--------------------------|-------------------------|-------------------------|--------------------------------|
| Model 1                  | 0.93 (0.87-0.99)        | 0.94 (0.88-1.01)        | 0.94 (0.89-0.99)               |
| Model 2                  | 0.92 (0.86-0.99)        | 0.93 (0.87-0.99)        | 0.94 (0.89-0.99)               |
| Model 3                  | 0.90 (0.83-0.97)        | 0.92 (0.85-0.99)        | 0.92 (0.86-0.98)               |

Model 1: Adjusted for age, sex, BMI, waist-to-hip ratio, blood pressure, smoking, and alcohol consumption
Model 2: Further adjustment for TNF-α, IL-6 and hsCRP
Model 3: Further adjustment for TNF-α, IL-6, hsCRP, E-selectin, ICAM-1 and VCAM-1
NGT, normal glucose tolerance; IGT, impaired glucose tolerance

Data shown as odds ratio (95% CI)
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![Graph showing Gas6 levels in NGT, IGT, and Type 2 Diabetes groups](image)

- NGT (n=96)
- IGT (n=82)
- Type 2 Diabetes (n=100)

Significance levels:
- p<0.001
- p=0.027