Growth Arrest-specific 6 Protein and Matrix Gla Protein in Hemodialysis Patients

Jamal Hallajzadeh,[1] Amir Ghorbanihaghjo,[1] Hassan Argani,[2] Siavoush Dastmalchi,[1] Nadereh Rashtchizadeh[3]

[1]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Division of Nephrology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Drug Applied Research Center Tabriz University of Medical Sciences, Tabriz, Iran

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Introduction. Plasma protein growth arrest-specific 6 (GAS6) and matrix Gla protein (MGP) are crucial mediators of vascular calcification and are involved in the development of vascular complications in chronic kidney diseases. This study was set out to investigate the relationship between plasma GAS6 levels and MGP in patients with end-stage renal disease on maintenance hemodialysis.

Materials and Methods. Forty-six hemodialysis and 46 healthy individuals with normal kidneys were recruited. Plasma GAS6 and MGP concentrations and related biochemical factors were quantified as well as collection of data on clinical characteristics.

Results. Plasma GAS6 levels were significantly higher in the hemodialysis patients as compared with the control group (763.52 ± 187.91 pg/mL versus 421.63 ± 189.91 pg/mL, $P < .001$). Plasma MGP concentration was significantly lower in the hemodialysis patients than the control group (52.35 ± 12.35 ng/mL versus 6.60 ± 19.54 ng/mL, $P < .001$). The levels of GAS6 were inversely associated with MGP (r = -0.341, $P = .02$) in the hemodialysis patients.

Conclusions. Increased GAS6 and decreased MGP levels in hemodialysis patients, as mediators of induction or prevention of vascular calcification, and their inverse correlation may suggest that there might be a role in increased calcification process in hemodialysis patients or only as a secondary phenomenon of advanced kidney failure. Their direct role on vascular calcification needs further studies in the future.

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INTRODUCTION

In patients with chronic kidney disease (CKD), especially those on dialysis therapy, the risk of cardiovascular death is particularly 10 to 20 times greater than in the general population.[1] Although severe vascular calcifications are thought to be associated with raised cardiovascular mortality in this population,[2] pathological mechanisms causing it remain incompletely understood. Many studies have shown that patients with CKD are at risk for vascular calcification because of multiple risk factors that induce vascular smooth muscle cells to change into a chondrocyte or osteoblast-like cell; high total body burden of calcium and phosphorus due to abnormal bone metabolism; low levels of circulating and locally produced inhibitors such as Fetuin-A; and impaired renal excretion.[3]

Looking beyond traditional markers and identifying novel mediators of vascular calcification in CKD may be helpful in developing therapeutic interventions to reduce vascular complications.[3,4] Beyond coagulant factors, there are other vitamin

K-dependent proteins with widespread physiologic activities. Recent studies have focused on 2 particularly important vitamin K-dependent proteins, growth arrest-specific 6 (GAS6) protein and matrix γ-carboxyglutamate (Gla) protein (MGP).[5-7] Growth arrest-specific 6 protein was the last addition to the family of plasma vitamin K-dependent proteins. Various cell types express GAS6, including endothelial cells, vascular smooth muscle, leukocytes, and platelets. It was cloned and characterized in 1993 and found to be similar to plasma anticoagulant protein S.[2] Soon after, it was recognized as a growth factor-like molecule, as it interacted with receptor tyrosine kinases of the TAM family; Tyro3, Axl, and Mer receptor tyrosine kinase.[8] The GAS6-axl system imparts signals via the PI3K/Akt pathway, resulting in cell survival, proliferation, adhesion, and protection from cellular death.[9] Altered activity or expression of GAS6/TAM components has been detected in a variety of pathologies such as inflammation, coagulopathy, cancer, autoimmune disease, diabetic vascular disease, kidney disease, and chronic kidney failure.[10-13] The TAM ligands and receptors modulate inflammation, regulating toll-like receptor signaling and pro-inflammatory cytokine signaling in macrophages and dendritic cells.[9,14] Without the TAM receptors, animals develop unregulated immunity, autoimmunity, and inflammation.[15-17] The inflammatory reaction could be considered a vascular response to harmful stimuli, where the regulation of cell traffic through the vessel wall is a crucial regulatory step. Growth arrest-specific 6 has been shown to play an important role in this part of the inflammatory response. Role of GAS6 in vascular system is complex. It promotes inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes.[18] Numerous studies demonstrate a role of GAS6-Axl interaction in cell migration, cell adhesion, atherosclerosis, endothelial cell activation, platelet aggregation, thrombosis, and vascular calcification. The process of vascular calcification has also been related to GAS6. Growth arrest-specific 6 signaling through axl inhibits mineral deposition by cultured vascular smooth muscle cells.[19-21] However, little is known about the clinical significance of the GAS6/TAM system in patients with CKD, especially end-stage renal disease, and its association with various inflammation and calcification variables that are common in hemodialysis patients.

Matrix Gla protein is produced in bone and vascular smooth muscle cells and is a potent inhibitor of vascular calcification.[22] Matrix Gla protein exerts its effects on vascular calcification, directly through inhibition of calcium crystal formation in conjunction with other calcification inhibitors such as fetuin-A, and indirectly, by influencing transcription factors that inhibit vascular cell differentiation to an osteoblast-like phenotype. [21,23,24] Growth arrest-specific 6 and MGP are mediated to induce or prevent vascular calcification, respectively. We investigated the hypothesis that these factors would also play roles in groups of hemodialysis patients, who are prone to vascular calcification.

MATERIALS AND METHODS

Patients

The study was performed in the Department of Biochemistry of Tabriz University of Medical Sciences. After obtaining informed written consent, blood samples were obtained from 46 hemodialysis patients, and 46 healthy volunteers with no known medical history. The hemodialysis group was recruited from dialysis units affiliated to the Department of Nephrology, Tabriz University of Medical Sciences. All of the hemodialysis patients were stable and were under regular hemodialysis for at least 6 months (range, 6 to 84 months), three times per week, each for 4 hours. Hemodialysis patients with a history of cardiovascular disease and patients on vitamin D, warfarin therapy, and hormone therapy with parathyroid hormone were excluded from the study.

Sample Collection

All of the blood samples were obtained from the peripheral vein puncture after 12 hours of overnight fasting in the control group, and at the beginning of hemodialysis in hemodialysis patients. Samples were centrifuged and plasma was separated within 30 minutes. Plasma samples were stored frozen at -70°C for estimation of high-sensitivity C-reactive protein (HSCRP), interleukin-6 (IL-6), intact parathyroid hormone, MGP, and GAS6 till assessment.

Enzyme-Linked Immunosorbent Assay

Plasma growth arrest-specific 6 protein levels. To quantify total plasma GAS6, we used the Human

GAS6 enzyme-linked immunosorbent assay (ELISA) Kit (CAT No SK00098-01, Avisceera Bioscience Inc, Santa Clara, CA, USA). In brief, the microtiter plates were coated with 100 µL of standards, specimens, and positive control and incubated 2 hours on the plate shaker at room temperature. The wells were washed 4 times with washing buffer; 100 µL of detection antibody working solution was added to each well, gently mixed for 15 seconds, and incubated for 2 hour at 36°C. The wells were washed 4 times with washing buffer, and 100 µL of streptavidin-horseradish peroxidase conjugate working solution was added to each well, mixed for 15 seconds, and incubated for 60 minutes at 36°C and protected from light. Onehundred microliter of substrate solution was added to each well and incubated 3 to 6 minutes on plate shaker. The reaction was then stopped by adding 100 µL of stop solution, followed by gentle mixing for 30 seconds until all the blue color changed into yellow. The absorbance was measured at 450 nm in a microplate reader within 15 minutes after the addition of stop solution. Intra-assay and inter-assay coefficients of variation of the test were 4% to 6% and 8% to 10 %, respectively.

Plasma interleukin-6 levels. Immunoenzymetric assay for the in vitro quantitative measurement of human IL-6 in serum (DIAsource IL-6-ELISA Kit, Cat No KAP1261, DIA Source ImmunoAssays SA, Belgium) uses monoclonal antibodies directed against distinct epitopes of IL-6.100µl Calibrators and samples react with the capture monoclonal antibody 1 coated on microtiter well and with a monoclonal antibody monoclonal antibody 2 labelled with horseradish peroxidase. After an incubation period, allowing the formation of a sandwich, coated MAb 1–human IL-6–monoclonal antibody 2–horseradish peroxidase, the microtiter plate was washed to remove unbound enzyme labeled antibody. Bound enzyme-labelled antibody was measured through a chromogenic reaction. Chromogenic solution (tetramethylbenzidine) was added and incubated. The reaction was stopped with the addition of stop solution, and the microtiter plate was then read at 450 nm wavelength. A calibration curve was plotted and IL-6 concentration in samples was determined by interpolation from the calibration curve. Detection limit was 2 pg/mL with inter-assay coefficient of variation of 5.4% and intra-assay coefficient of variation of 4.3%.

Plasma matrix Gla protein levels. This assay was a solid phase-phase sandwich ELISA (TSZ ELISA, Cat No HU8370, Framingham, USA). Samples, including standards of known target protein concentrations and unknowns, were pipetted into these wells. During the first incubation, the MGP protein antigen and a biotinylated monoclonal antibody specific for MGP protein were simultaneously incubated. After washing, the enzyme (streptavidin peroxidase) was added. After incubation and washing to remove the entire unbound enzyme, a substrate solution, which was acting on the bound enzyme, was added to induce a colored reaction product. The reaction was terminated by addition of an acidic stop solution and the microtiter plate was then read at the 450 nm wavelength. The concentration of MGP calculated according to standard curve assay range was between 15 ng/mL and 500 ng/mL.

High-sensitivity C-Reactive Protein

Plasma analysis for HSCRP was performed by the nephelometry method (Pars Azmoon Co, Tehran, Iran).

Statistical Analyses

Statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences, version 18.0, SPSS Inc, Chicago, Ill, USA). All data were tested for normality of distribution, using the Kolmogorov-Smirnov test and the assumption of satisfactory normal distribution was met for all of the examined variables. Quantitative data are presented as mean with ± standard deviation for parametric data and as median (range) for nonparametric data. The two study groups were compared using the Mann-Whitney U test for the nonparametric data and independent sample *t* test for parametric data. The Spearman coefficient or Pearson correlation was calculated to determine the correlation between biochemical parameters, where appropriate. A *P* value less than .05 was considered significant.

RESULTS

Demographic and clinical characteristics data for the study groups are shown in Table 1. When phosphate-chelating agent was necessary calcium carbonate, 500 mg, was used accompanying with each meal. None of the patients were

Table 1. Demographic Features and Biochemical Parameters in Hemodialysis Patients and Healthy Controls*

Characteristic	Hemodialysis Patients (n = 46)	Healthy Controls (n = 46)	*P*
Age, y	61.08 ± 13.92	61.84 ± 11.52	.78
Sex			
Male	28	23	
Female	18	23	.14
Glomerular filtration rate, mL/min/1.73 m^2	5 (3 to 9)	68 (34 to 136)	< .001
Underlying diagnoses of kidney failure			
Diabetic nephropathy	18 (40.0)	…	…
Chronic glomerulonephritis	3 (6.7)	…	…
Polycystic kidney disease	5 (11.1)	…	…
Hypertensive ischemic nephropathy	10 (22.2)	…	…
Obstructive nephropathy	7 (15.6)	…	…
Unknown etiology	2 (4.4)	…	…
Duration of dialysis, mo	44.0 ± 34.4	…	…
Alkaline Phosphatase, U/L	411.15 ± 310.05	185.76 ± 59.93	< .001
Calcium, mg/dL	8.81 ± 0.90	9.50 ± 0.56	< .001
Calcium-phosphorus product, mg2/dL^2	53.43 ± 9.74	38.73 ± 7.82	< .001
Albumin, g/dL	3.48 ± 0.77	3.98 ± 0.49	< .001
Total protein, g/dL	8.25 ± 1.01	7.88 ± 1.30	.13
Phosphorus, mg/dL	6.05 ± 0.91	4.08 ± 0.87	< .001
Intact parathyroid hormone, pg/mL	367.29 ± 133.38	26.04 ± 15.34	< .001
Creatinine, mg/dL	9.20 ± 2.44	1.10 ± 0.28	< .001
Urea, mg/dL	104.00 ± 17.47	39.56 ± 16.05	< .001
High-sensitivity C-reactive protein, mg/L	4.40 ± 1.26	1.38 ± 1.61	< .001
Interleukin-6, pg/mL	5.77± 2.55	1.59 ± 1.61	< .001
Serum glucose, mg/dL	126.00 ± 7.21	92.43 ± 28.83	.75
Growth arrest-specific 6, pg/mL	763.52 ± 187.91	421.63 ± 189.91	< .001
Matrix Gla protein, ng/mL	52.35 ± 12.35	66.07 ± 19.54	< .001

*Values are frequencies (percentage) for qualitative parameters and mean ± standard deviation, for quantitative parameters, except for glomerular filtration rate, which is median (range).

on glucocorticosteriods. The mean estimated glomerular filtration rate (GFR), by the Modification of Diet in Renal Disease study equation, was 5.5 mL/min/1.73 m^2. The control group consist of 46 healthy volunteers with comparable age and sex distributions with the hemodialysis group, normal kidney function (mean estimated GFR, 71.2 ± 21.7 mL/min/1.73 m^2), and no significant albuminuria.

As shown in Table 1, plasma GAS6 levels were significantly higher in the hemodialysis patients as compared with the control group (763.52 ± 187.91 pg/mL versus 421.63 ± 189.91 pg/mL, $P < .001$). Plasma MGP concentration was significantly lower in the hemodialysis patients than the control group (52.35 ± 12.35 ng/mL versus 6.60 ± 19.54 ng/mL, $P < .001$). Comparisons of the serum levels of GAS6 and MGP between the men and women in the two hemodialysis and control groups are shown in Table 2. Serum levels of GAS6 did not differ between males and females in the hemodialysis patients and not either in the control group, while women in the hemodialysis group had a significantly lower level of MGP in the hemodialysis samples as compared to the men. Both indicators were significantly different

Table 2. Mean Plasma Levels of Growth Arrest-specific 6 and Matrix Gla Protein by Sex in Hemodialysis Patients and Healthy Controls

Parameters	Men	Women	*P*
Growth arrest-specific 6			
Hemodialysis group	748.93 ± 175.04	787.50 ± 209.25	.49
Control group	473.30 ± 199.62	369.90 ± 168.32	.06
Matrix Gla protein			
Hemodialysis group	55.46 ± .11.24	47.52 ± 12.76	.03
Control group	63.90 ± 16.26	68.24 ± 22.50	.46

in the subgroups of men and women between the two groups ($P < .001$ for all comparisons; Table 2).

An inverse linear relationship was confirmed between GAS6 levels and estimated GFR in the hemodialysis patients (r = -0.424; $P = .003$). As shown in Table 1, serum HSCRP level was lower in the control group than the hemodialysis patients, and serum IL-6 level was higher in hemodialysis group than the controls. However, no significant correlation was found between HSCRP and GAS6 levels in the hemodialysis group (r = 0.05, $P = .74$), it significantly correlated with IL-6 (r = 0.56, $P = .001$). Conversely, the level of serum albumin was inversely associated with GAS6 concentrations (r = -0.301, $P = .04$). A significant negative correlation was found between GAS6 and MGP levels in the hemodialysis patients (r = -0.341, $P = .02$; Table 3).The correlations of GAS6 and MGP in the hemodialysis and the control groups are shown by sex in Table 4. A significant correlation was seen between GAS6 and MGP levels among the male hemodialysis patients.

DISCUSSION

In this study, we compared the main proteins involved in vascular calcification including serum levels of GAS6 and MGP between hemodialysis patients and healthy individuals. Lee and coworkers showed that GAS6 level was markedly elevated in the nonhemodialysis CKD and hemodialysis patients.[6] In our study, the GAS6 level was significantly higher in hemodialysis patients than the normal individuals. Increased GAS6 levels did not seem to be a direct product of the dialysis procedure itself.[6] Given the role of GAS6 as mediating potential pro-inflammatory signals, particularly those related with mortality such as IL-6, it is of interest that GAS6 levels were significantly elevated in hemodialysis patients compared with controls.[9,25] Several studies have demonstrated that various inflammatory biomarkers, such as HSCRP, IL-6, serum albumin, are robust independent predictors of both all-cause and cardiovascular mortality in end-stage renal disease patients. Our results revealed that plasma GAS6 values in hemodialysis patients negatively correlated with inflammation markers including albumin and IL-6.

Monocytes-macrophages and endothelial cells play important roles in inflammatory processes in CKD patients. Studies on endothelial cells, monocytes-macrophages and smooth muscle cells support a direct role for HSCRP in atherogenesis. Although the study of Ekman and colleagues demonstrated an association of GAS6 levels with HSCRP in patients with critical limb ischemia,[26] recently, a study of Lee and colleagues did not show such an association between HSCRP and GAS6 in the uremic milieu nondialysis and dialysis. Our study did not find an association between GAS6 and HSCRP in hemodialysis patients. In part, this may be due to the small sample of our study population.

Under inflammatory conditions, addition of GAS6 has been shown to inhibit adhesion of granulocytes to endothelial cells in culture.[27] While this observation seems to imply an anti-inflammatory role of GAS6 by decreasing cellular extravition, results from axl- and GAS6-deficient animals indicate an opposite effect. During intimal thickening, axl-/- deficient mice have less leukocyte recruitment.[18] Growth arrest-specific 6-deficient animals had diminished endothelial cell-leukocyte interactions in vivo after endothelial cell stimulation.[28] It is well accepted that the endothelium in CKD is subject to particular stresses that are thought to play a role in accelerated vascular disease and cardiovascular mortality.[29-31] Oxidative stress, endothelial damage, and structural disintegration of the endothelium

Table 3. Correlation Between Selected Clinical Variables and Growth Arrest-specific 6 Levels in Hemodialysis Patients*

Parameter	Coefficient	*P*
Serum albumin	-0.301	.04
Interleukin-6	0.560	< .001
Estimated glomerular filtration rate	-0.424	.003
Matrix Gla protein	-0.341	.02

*Parathyroid hormone, serum phosphorous, hemoglobin, age, serum calcium, calcium-phosphorus product, and high-sensitivity C-reactive protein were not significantly associated with growth arrest-specific 6.

Table 4. Correlation Between Growth Arrest-specific 6 and Matrix Gla Protein in Hemodialysis Patients and Healthy Controls

Parameter	Coefficient	*P*
Hemodialysis group		
All	-0.341	.02
Women	-0.067	.79
Men	-0.548	.003
Control group		
All	-0.192	.20
Women	-0.272	.21
Men	-0.058	.79

leading to vascular micro-inflammation can destruct both in the large vessels as well as in the glomerular capillaries, impacting both peripheral vascular disease and CKD progression.

It is known that several pro-inflammatory cytokines such as IL-6, IL-1β, and tumor necrosis factor-α are elevated in the blood of both nonhemodialysis CKD and hemodialysis patients. Expression analyses in the mouse indicate that endothelial cells, platelets, and leukocytes produce and release Gas6. Endothelial cells release GAS6 constitutively. On activation by tumor necrosis factor-α, endothelial cells do not produce increased amounts of GAS6 but respond to GAS6 (a process requiring the presence of Axl), possibly suggesting that GAS6 is only able to bind to axl on activated endothelial cells. Tjwa and coworkers showed that GAS6 determined the response of human activated endothelial cells to stimulation of tumor necrosis factor-α in vitro for increasing of expression of vascular cell adhesion molecule 1 and intercellular cell adhesion molecule 1.[18] These results support the hypothesis that modulation of GAS6 activity may provide an important point for intervention. The 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.

Matrix Gla protein is one of the major calcification inhibitors released from bone and vascular smooth muscle cells and is a potent inhibitor of vascular calcification. We found that serum MGP levels were significantly decreased in dialysis patients than in healthy controls which was inconsistent with the results of other studies.[32] Gluba-Brzózka and colleagues showed no significant difference in the level of MPG between the control group and patients with CKD (all stages). Matrix Gla protein originating from human is one of the most insoluble proteins known. MGP may be bound to a soluble carrier protein and circulate in the blood. Matrix Gla protein forms a complex with another calcification inhibiting protein known as fetuin-A and mineral (calcium and phosphorus); this high molecular weight complex is detectable in serum.[33] We previously reported that fetuin-A levels in serum are lower in dialysis patients than in healthy controls.[34] On the other hand, the development of calcium crystals in the vasculature may further decrease circulating MGP levels because the gla residues in MGP have a high affinity for calcium phosphate and hydroxyapatite crystals.[35]

Despite these contributions, this study has certain limitations. First, because this was a cross-sectional study, interpretation of the results is limited. Therefore, it is difficult to prove causality or the direction of influence based on the findings. Further longitudinal studies are needed to confirm our results. Second, we did not assess the uncarboxylated MGP fraction, but total MGP, that limits conclusions of their role for vascular calcification development.

CONCLUSIONS

Increased GAS6 and decreased MGP levels in hemodialysis patients, as mediators of induction or prevention of vascular calcification, and their inverse correlation may suggest that there might be a role in increased calcification process in hemodialysis patients or only as a secondary phenomenon of advanced kidney failure. Their direct role on vascular calcification needs further studies in the future.

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CONFLICT OF INTEREST

None declared.

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Correspondence to:
Amir Ghorbanihaghjo
Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz 51664, Iran
Tel: +98-411 336 3234
Fax: +98 411 336 3231
E-mail: ghorbaniamir@hotmail.com

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