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Plasma levels of growth arrest specific protein 6 are increased in idiopathic recurrent pregnancy loss

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Abstract. – AIM: The aim of this study was to determine plasma Growth Arrest Specific Protein 6 (GAS6) protein levels in patients with unexplained recurrent pregnancy loss (RPL) and compare them to those of pregnant and to healthy non-pregnant women with a history of at least one live delivery.

PATIENTS AND METHODS: A total of 205 women were included in the study. Of these, 68 were diagnosed with unexplained RPL and were not pregnant at the time of the study. The second group consisted of 67 pregnant women in the third trimester of pregnancy. The third group constituted the control group of 70 healthy non-pregnant women who had at least one live birth. Plasma levels of GAS6 protein were measured by ELISA.

RESULTS: Mean plasma GAS6 levels were found to be different between RPL group and healthy non-pregnant women (12.17 ± 4.39 μg/L and 9.18 ± 4.65, respectively, $p = 0.0013$). Although it was not statistically significant, plasma levels of GAS6 in the third trimester pregnant group (10.65 ± 3.74 μg/L) were found to be slightly higher than non-pregnant controls, and slightly lower than RPL group. In comparison of those with 2 or more than 2 pregnancy losses in the RPL group, there was no difference between these two subgroups in terms of GAS6 levels (12.39 ± 4.27 μg/L and 11.83 ± 4.63 μg/L, respectively, $p = 0.62$).

CONCLUSIONS: Our findings revealed that plasma GAS6 levels were increased in patients with RPL. The prothombotic and proinflammatory nature of the GAS 6 protein makes it a likely culprit involved in the pathologic process in patients with RPL. Further studies are warranted to determine the possible role of GAS6 protein in the pathophysiology of idiopathic RPL.

Key Words:
Growth arrest specific protein 6, Recurrent pregnancy loss, Idiopathic, Miscarriage, Thrombosis, Inflammation.

Introduction

Recurrent pregnancy loss (RPL) affects 1-2% of women in the reproductive age group[1]. Although a variety of anatomic, immunological, genetic, endocrine, infectious, thrombophilic and environmental etiologies have been shown to be responsible for RPL, a definite cause cannot be shown in more than 40% of the cases[2]. Impaired trophoblastic invasion and thrombosis in decidual vessels due to hypoxic or inflammatory conditions have been suggested to cause RPL[3]. Consequently, conditions associated with hypercoagulability have been studied extensively as risk factors for RPL[4].

Growth arrest specific protein (GAS6) is a vitamin K dependent protein composed of 678 amino acids in humans. Although GAS6 shows 44% structural homology to Protein S, it has not been shown to have anticoagulant activity[5]. GAS6 exerts its biological functions by binding to the TAM family of receptors, Tyro 3, Axl and Mer[6]. Initially detected in fibroblasts, GAS6 has been shown to be expressed in endothelial, smooth muscle and bone marrow cells[7]. A growing body of evidence suggests that GAS6 may be a potential biomarker of inflammatory and thrombotic states. GAS6 and its receptors have been shown to modify platelet activation and aggregation, thus, mediating thrombus formation[8]. Previous experimental studies on GAS6 null mice have demonstrated GAS6 to have a role in thrombus stabilization[9,10]. Additionally, GAS6 has been shown to promote activation of the endothelium by inducing P-selectin expression, and possibly to reinforce thrombus adhesion onto the vascular wall[11]. Gas6 also has been shown to promote tissue factor (TF) up-regulation by binding to TAM receptors on endothelial cells. TF initiates coagu-

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lation by binding to factor VIIa, which ultimately leads to further thrombin generation and the formation of a fibrin clot[12]. Although previous studies reported the role of GAS6 in reproductive system, the significance of Gas6 plasma levels in recurrent pregnancy loss remains to be established. We hypothesized that because of its pro-inflammatory and prothrombotic actions, GAS6 protein might play a role in the development of RPL.

The aim of this study was to determine plasma GAS6 levels in patients with unexplained RPL and compare them to those of pregnant and to healthy non-pregnant women with a history of at least one live delivery.

Patients and Methods

Patients and Controls

The medical records of 116 women under 42 years of age with a history of RPL attending the Obstetrics and Gynecology outpatient clinic of our tertiary center were retrospectively analyzed. Of the 116 patients, 68 were not pregnant at the time of the study and were diagnosed with unexplained RPL following nonpathological hysterosalpingographic findings and normal test results for hormonal profiles, fasting blood glucose and parental karyotypes. The remaining 48 patients were found to have positive testing for the various etiologies of RPL and were excluded from the study. RPL was defined as 2 or more losses at or before the 10th week of gestation in which clinical pregnancy was documented by ultrasonography or histopathological examination. The second study group consisted of 67 pregnant women in the third trimester of pregnancy known to have delivered healthy babies upon follow up. The third group constituted the control group of 70 healthy non-pregnant women who had at least one live birth, no history of pregnancy loss or concurrent disease, and were not on any medication. Both pregnant and non-pregnant control cases were randomly assigned during the study period. The local Ethics Committee of the Zeynep Kamil Woman's and Children's Disease Training and Research Hospital in Istanbul, Turkey, approved the study (report no: 2013-67).

Measurement of Plasma GAS6 Levels

All blood samples were taken into a citrate tube for plasma GAS6 analysis. We validated ELISA assay for the determination of GAS6 in plasma using kit provided by R&D Systems (Minneapolis, MN, USA) as previously described in detail[13]. Briefly, a 96-well ELISA plate was coated with capture antibody (4 µg/mL) overnight at 4°C and then blocked with wash buffer containing 5% bovine serum albumin (BSA) for 2 h at room temperature. After washing steps, samples or standard solutions were added to the wells and incubated for 1 h at 37°C. The biotinylated secondary antibody (100 ng/mL) was added to wells for 1 h at 37°C. Then, 100 µL of streptavidin peroxidase conjugate were added to each well and incubated for 20 min at room temperature. The wells were washed 5 times with washing buffer, and 100 µL of color reagents A and B (R&D Systems Minneapolis, MN, USA) was added to each well, and incubated for 15 minutes at room temperature. The reaction was then stopped by adding 50 µL of stop solution. The absorbance was measured at 450 nm. All samples were analyzed in duplicate. GAS levels were expressed as µg/L.

Statistical Analysis

Descriptive results of continuous variables were expressed as mean ± standard deviation. The ANOVA test was used to compare data among the study groups. Homogeneity of the variance was established by the Levene's test and Tukey HSD test was used in the post-hoc analysis. Correlations between GAS6 plasma levels and age, and BMI were assessed using the Spearman's correlation test, $p < 0.05$ value was accepted as significant.

Results

The demographic characteristics of the study population and GAS6 levels are presented in Table I. There was no difference among the groups regarding the age. BMI of pregnant women was found to be significantly higher in comparison to RPL and non-pregnant healthy controls. Correlation analysis revealed no significant relationship between GAS6 plasma levels and age, and BMI ($r = 0.109$, $p = 0.38$; $r = 0.035$, $p = 0.78$, respectively) (Figure 1). Mean GAS6 plasma values revealed a statistical difference among the groups ($p = 0.0017$) (Figure 2). In multiple comparison analysis; however, a significant difference was only observed between the non-pregnant healthy controls and the RPL group ($p = 0.0013$). GAS6 levels were

Table I. The demographic characteristics and GAS6 levels of the study population.

	Control group (n=70)	Pregnant group (n=67)	RPL group (n=68)
Age (Years)	31.05 ± 5.78	29 ± 5.95	29.04 ± 6.15
BMI (kg/m^2)	23.29 ± 4.39$^{\alpha}$	28.87 ± 3.11$^{\alpha,\beta}$	24.98 ± 3.84 $^{\beta}$
GAS6 levels (μg/L)	9.18 ± 4.65*	10.65 ± 3.74	12.17 ± 4.39*

Mean ± SD values were given, *Stands for the significant difference between RPL and control groups. $^{\alpha}$Stands for the significant difference between pregnant and healthy controls; $^{\beta}$Stands for the significant difference between RPL group and pregnants.

not significantly different between the RPL and pregnant groups ($p = 0.095$), and between the pregnant and non-pregnant healthy women ($p = 0.189$). In comparison of those with 2 or more than 2 pregnancy losses in the RPL group, there was no difference between these two subgroups in terms of GAS6 levels (12.39 ± 4.27 μg/L and 11.83 ± 4.63 μg/L, $p = 0.62$).

Discussion

To our knowledge, this is the first study examining the relationship between plasma GAS 6 levels and idiopathic RPL. Previously, the role of GAS6 in reproductive system was defined in spermatogenesis and pregnancy related conditions such as preeclampsia and intrauterine growth retardation[14-16]. The alterations in the levels of GAS6 did not correlate with age and BMI of cases in whole study population.

In this study, plasma GAS6 levels were found to be significantly higher in patients with RPL than non-pregnant women. This finding might be related to the possible role of GAS6 in reproduction and also in pathophysiology of RPL. In the process of reproduction, various components of the coagulation, fibrinolytic and inflammatory pathways are involved in normal embryonic implantation, trophoblast invasion, angiogenesis and placentation[3]. The success of a pregnancy depends on the proper functioning of the closely related hemostatic and inflammatory pathways and the development of a healthy placenta[17]. Despite the heterogenous nature of RPL, vascular dysfunction at the maternal-fetal interface has been implicated as the pathophysiological mechanism behind most cases of RPL, including unexplained pregnancy losses[18]. Common inflammatory and thrombotic changes involving perivillous fibrin deposition, fibrinoid necrosis of the decidual bed and thrombi in intervillous spaces have been demonstrated in placentas of patients with RPL[4]. Ekman et al[19] found Gas6 levels to be increased in patients with a wide range of inflammatory conditions, suggesting GAS6 as a potential biomarker of inflammation. Furthermore, Balogh et al[20] suggested that overexpression of

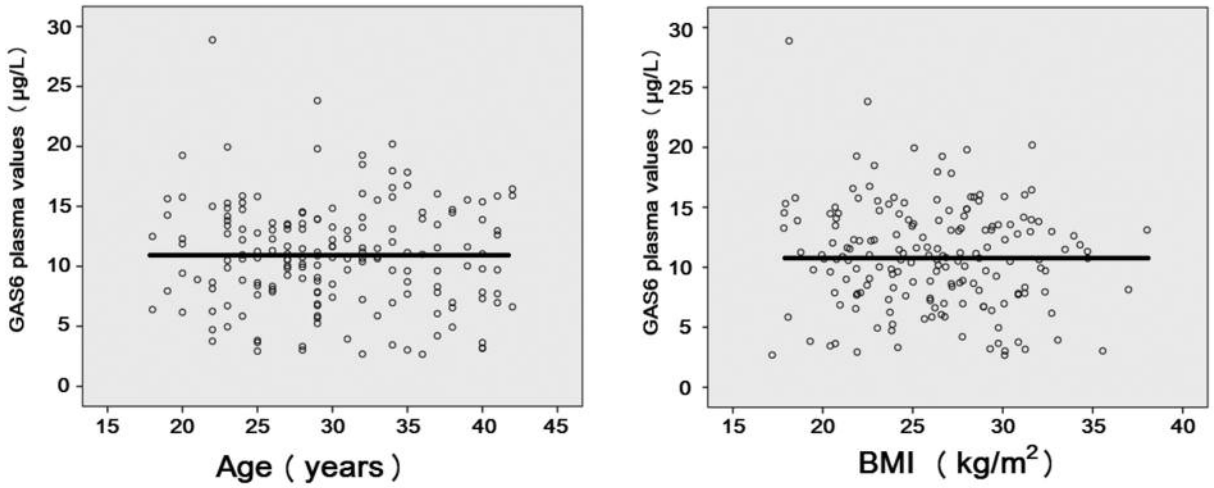

Figure 1. Correlation between GAS6 plasma levels and age and BMI.

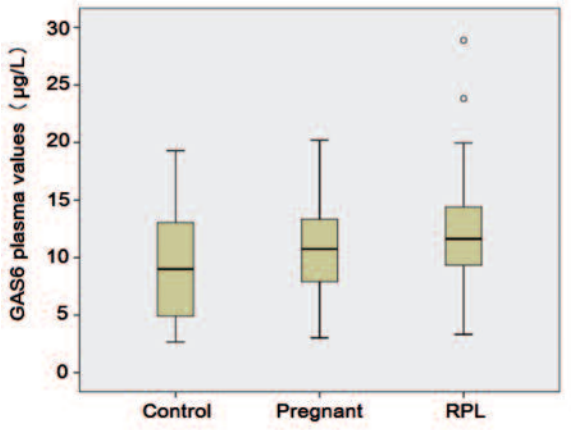

Figure 2. GAS6 plasma levels in the study groups.

GAS6 rather than a relative or absolute deficiency might be a suitable marker for pathophysiological situations. In an experimental study on hamsters, it was reported[21] that GAS6 had a role in endometrial transformation during decidualization and trophoblastic invasion, in such a way that translation of the protein from GAS6 mRNA takes place when normal development of pregnancy was threatened. Based on all these findings, it may be postulated that overexpression of GAS6 may play a role in the pathophysiology of pregnancy loss, and threaten the course of normal pregnancy in patients with RPL.

In this study, we could not demonstrate a difference in GAS6 levels between two subgroups regarding the number of pregnancy losses either 2 or more than 2 consecutive abortions. This finding may suggest that increased plasma levels of GAS6 in patients with RPL seem to be independent from number of miscarriages.

Although it was not statistically significant, plasma levels of GAS6 in the third trimester pregnant group were found to be slightly higher than controls, and slightly lower than RPL group. During the pregnancy, substantial changes take place in the hemostasis mechanism. Significant rise in the majority of the coagulation factors, and decrease in the level of natural anticoagulants and fibrinolytic activity are the most important physiological changes notable through pregnancy. These alterations lead to a state of hypercoagulability[22]. In the current study, slight increase in the level of GAS6 in pregnants may be related to additive role of this protein to the hypercoagulable state of pregnancy. A recent study[15] reported GAS6 levels increased significantly in preclamptic pregnants compared to uncomplicated pregnancies. Similarly, pregnancies complicated with intrauterine fetal growth restriction were found to have higher levels of GAS6 in umblical venous samples at the time of delivery[16]. In light of these findings, it may be postulated that GAS6 levels might further rise in case of a pregnancy in those with RPL and shift the equilibrium from physiologic hypercoagulation of pregnancy toward inflammatory state causing pregnancy loss. In order to validate this, future studies comparing the levels of GAS6 in pregnant and non-pregnant state in patients with RPL are needed. Although GAS6 levels were found to be higher in RPL group, it is not clear whether it is a cause or result of pathophysiological process behind RPL.

Gas 6 has been suggested as one of the key components in the initiation of the coagulation cascade, leading to TF expression on endothelial cells upon possible stimulation by thrombin[23]. Increased plasma levels of TF antigen have been identified in patients with RPL[24,25]. TF is the main initiator of the coagulation cascade and is known to be expressed by placental tissue[26]. Blockade of TF with monoclonal antibodies in antiphospholipid-treated mice prevented pregnancy complications, suggesting causative and crucial role of TF in the pathogenesis of miscarriages[27]. Based on these findings, high levels of GAS6 in RPL might trigger more TF expression, leading to placental thrombosis, thus, might, play a role in the pathogenesis of RPL.

Conclusions

Even though the definite pathology behind recurrent pregnancy loss is not yet evident, inflammatory and thrombotic events at the fetomaternal interface are implicated in its pathogenesis. The prothombotic and proinflammatory nature of the Gas 6 protein makes it a likely culprit involved in the pathologic process in patients with RPL. Our findings revealed that plasma GAS6 levels were increased in patients with RPL. Further studies are warranted to determine the possible role of GAS6 protein in the pathophysiology of idiopathic RPL.

Conflict of interest

The Authors declare that they have no conflict of interests.

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