Development of a Novel Immunoassay for the Assessment of Plasma Gas6 Concentrations and Their Variation with Hormonal Status

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Background: Gas6 is a vitamin K–dependent antiapoptotic protein that has been implicated in cardiovascular pathophysiology. We report the development and validation of an ELISA for Gas6, and the variation of plasma Gas6 with hormonal status in a study designed to evaluate the effect of oral contraception on plasma markers.

Methods: After validation of the main stages of the ELISA assay, we measured plasma Gas6 concentrations in 94 male and 88 female healthy volunteers ages 18 to 38 years. Forty-five of the women then received an oral contraceptive, which contained ethinylestradiol and levonorgestrel, for 3 months before a new measurement was performed at the same time point in their menstrual cycles.

Results: Interassay imprecision was 5.8%–11.8%, and the detection limit was 5.9 μg/L. Mean Gas6 plasma concentrations were significantly lower in men (52.0 μg/L) than in women not receiving oral contraceptives (63.8 μg/L, P <0.001). In the women who received oral contraceptives, Gas6 concentrations decreased after 3 months of therapy from 63.6 μg/L to 51.9 μg/L (P <0.001).

Conclusions: We have developed a simple and reproducible ELISA assay for measuring plasma Gas6 concentrations, which vary with sex and are decreased by oral contraceptive use. These results suggest regulation of plasma Gas6 concentrations by sex hormones. Future clinical studies may require participants to be stratified by sex.

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Gas6 belongs to the family of plasma vitamin K–dependent proteins, sharing 44% homology with protein S but lacking its anticoagulant activity (1). Gas6 is the ligand for tyrosine kinase receptors Axl, Mer, and Sky (2) and was shown to have antiapoptotic properties (3).

A role of Gas6 in hemostasis has been shown by the study of mouse models, in which mice with Gas6 deficiency were protected against thrombosis (4). However, Gas6 participation in human platelet aggregation is not clearly established, because Gas6 was found to circulate in plasma but was not present in human platelets (5), as opposed to murine platelets. Moreover, we have shown that human plasma Gas6 concentrations did not influence platelet aggregation with low doses of agonists (6), but correlated with a higher risk of aspirin pseudoresistance (7).

Gas6 may play a role in cardiovascular pathophysiology. Indeed, a polymorphism in the growth arrest-specific 6 (GAS6)5 gene was found to be associated with stroke (8), for which male sex is a risk factor (9). A recent study reported the presence of a functional estrogen-responsive element in the GAS6 promoter (10). Thus, assessing if plasma Gas6 varies with sex and hormonal status seems important for future clinical studies to better understand its role as a potential marker in cardiovascular disease.

We have developed and validated a rapid and specific ELISA assay for Gas6. This assay allowed us to demon-

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Received March 19, 2007; accepted August 2, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.089102

strate that plasma Gas6 concentrations were influenced by sex hormones, in a study designed to evaluate the effects of oral contraceptive use on plasma markers.

Materials and Methods

ELISA Assay for Plasma Gas6
To quantify total plasma Gas6, microtiter plates (Nunc U96 Maxisorp-Immuno Plate) were coated with 60 μL 10 mg/L goat antihuman Gas6 polyclonal antibody (AB 885; R&D Systems) in 50 mmol/L carbonate buffer, pH 9.0, overnight at 4 °C. We washed the wells 5 times with Tris-buffered saline (TBS)6 (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) containing 2 mmol/L CaCl2 and 0.2% Tween, and then treated them with 60 μL TBS containing 2 mmol/L CaCl2 and 50 g/L BSA for 1 h at room temperature. After washing, we added 50 μL plasma diluted 1:50 in TBS containing 2 mmol/L CaCl2, 2 g/L BSA, and 30 mmol/L hirudin (Refudian, Berlex) to the wells and incubated them for 1 h at room temperature. We constructed calibration curves by diluting a reference plasma pool of known Gas6 concentration, 1:12.5, 1:25, 1:50, 1:100, and 1:200. After washing, we added 50 μL 2 mg/L biotinylated goat antihuman Gas6 polyclonal antibody (BAF 885; R&D Systems) in TBS containing 2 mmol/L CaCl2 and 2 g/L BSA. After a 1-h incubation at room temperature and washing, we added 50 μL 2.2 mg/L horseradish peroxidase-labeled streptavidin (Sigma-Aldrich) in TBS containing 2 mmol/L CaCl2 and 2 g/L BSA and incubated the wells at room temperature for 1 h. After washing, we added 50 μL 2-phenylene diamine substrate and incubated the wells at room temperature for 5 min. The reaction was then stopped by adding 50 μL 3 mol/L H2SO4, and we measured end-point absorbance at 490 and 630 nm on a microplate reader (Fluostar Optima; BMG Labtech).

Expression and Purification of Recombinant Human Gas6
We amplified full-length human Gas6 cDNA, which was obtained by reverse transcription of human umbilical vein endothelial cell mRNA, by PCR using 2 specific primers. This 1st PCR product was used as template for a nested PCR step, with 2 internal primers designed to add an EcoRI restriction site at the 5′ extremity of the cDNA and an XbaI restriction site at the 3′ extremity. Nested PCR product was then digested with EcoRI and XbaI and ligated into pCI-neo mammalian expression vector (Promega), from which the corresponding fragment was removed. We sequenced Gas6 cDNA in the final expression vector (ABI Prism 377 DNA Sequencer; Perkin-Elmer) by use of the ABI Prism dRhodamine Terminator Cycle Sequencer Ready Reaction Kit (Applied) to rule out inadvertent mutations introduced by PCR.

We performed transfection and clone selection as previously described (11) and selected recombinant-producing clones using our Gas6 ELISA assay. For Gas6 production, cells were cultured in OptiMEM serum-free medium (Invitrogen) with 5 mg/L vitamin K1 (Roche). Purification was performed by an anion-exchange chromatography step and elution with a Ca2+ ion gradient (0–50 mmol/L). We measured protein concentrations by monitoring absorbance at 280 nm, using an ε1%1 cm value of 9.5 and a molecular mass of 75 000 Da.

We ran 2 μg of recombinant Gas6 on 10% SDS-PAGE in unreduced condition and after reduction by β-mercaptoethanol. The gel was then stained with Coomassie blue.

Preparation of Gas6-Depleted Plasma
A specific anti-Gas6 polyclonal antibody was prepared by Agro-Bio by immunization of chicken with our recombinant Gas6. One-half (0.5) mg of this antibody was coupled to a HiTrap N-hydroxysuccinimide-activated HP 1 mL column (Amersham Biosciences). This immunoaffinity column was loaded with 1 mL of a commercial lypophilized plasma (Coag Control N®, Diagnostica Stago) diluted 1:2 in buffer (50 mmol/L Tris, 500 mmol/L NaCl, pH 7.4). After 1 h at room temperature, 1 mL Gas6-depleted plasma was washed out of the column using 1 mL buffer.

Study Population
Women. Ninety healthy female volunteers ages 18 to 38 (mean 27.7) years were included in a study to evaluate the effects of oral contraceptive use on plasma markers. They did not take any oral contraceptives, or had a 2-month washout period. The volunteers had unremarkable personal and familial medical histories, and women with thrombophilia (antithrombin, protein C or protein S deficiency, factor V Leiden, or prothrombin G20210A mutation) were excluded. Routine laboratory tests and physical examinations were normal. For all women, a blood sample was obtained on the inclusion visit (visit 1), which took place within the 1st 3 days of menstrual bleeding. Then, half of these women (n = 45) received a monophasic oral contraceptive containing ethinylestradiol (EE) 20 μg/levonorgestrel (LNG) 100 μg capsules for 21 days, and placebo capsules for the remaining 7 days, during 3 consecutive cycles of 28 days. In those women, a 2nd blood sample was collected at day 17 to 21 of the 3rd cycle of oral contraceptive treatment (visit 2).

Men. We recruited 100 unrelated, healthy, nonsmoking white male volunteers ages 18 to 35 (mean 24.3) years at the Clinical Investigation Centre of Hôpital Européen Georges Pompidou, Paris, for a study designed to evaluate the effects of genetic polymorphisms on hemostasis variables (12). The volunteers had unremarkable personal and familial medical histories and denied taking any medication for at least 10 days before blood collection. Before inclusion, all volunteers underwent a physical
examination and routine laboratory tests that confirmed the absence of clinical anomalies.

Both studies were conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki and were approved by a local ethics committee. All participants gave informed consent.

SAMPLE PREPARATION
We collected venous blood in tubes containing 0.11 mol/L trisodium citrate (1:10) and centrifuged them within 2 h. Plasma was obtained by centrifugation at 12 °C and 2300 g for 15 min, then divided into aliquots and stored at −80 °C until analysis.

STATISTICAL ANALYSIS
Statistical analyses were performed using ANOVA including 3 variables: group (men, women), time (2 visits separated by 3 months), and group × time interaction. We tested pairwise comparisons by Student t-test and adjusted P-values with the Bonferroni-Holm method. Results in text are expressed in observed mean (SD). After removal of 2 outliers, we performed a sensitivity analysis to confirm the robustness of our results. All analyses were carried out using SAS Statistical Software (version 8.2). A P value <0.05 was considered to be significant.

Results
GAS6 ELISA VALIDATION
During the development of our ELISA assay, we found that the highest signal was obtained in a solution containing 2 mmol/L calcium, with a polyclonal goat antihuman Gas6 antibody for the capture antibody and a biotinylated polyclonal goat antihuman Gas6 antibody for the detection antibody, visualized via an avidin-coupled enzymatic reaction. The optimal reagent concentrations and sample dilutions were as described in Materials and Methods.

For routine testing, our assay was calibrated using a reference plasma pool, and results were expressed as a percentage of this reference, with the 1:50 dilution of the pool amounting to 100%. As described in Materials and Methods, we traced a 5-point linear calibration curve ranging from 25–400%; values outside those limits were rejected.

Using recombinant Gas6 as a calibrator, we measured Gas6 concentration of the reference plasma pool at 58.8 μg/L, and this value was used to convert results from percentage to micrograms per liter. We assessed the purity of recombinant Gas6 by migration of the protein on SDS-PAGE under reduced and unreduced conditions, as shown in Fig. 1.

We tested interassay imprecision by analyzing 2 different commercial lyophilized plasma samples (Coag Control N + P®, Diagnostica Stago) 2 times per assay in a total of 11 runs, over a period of 8 months. Gas6 concentrations in these samples were 68.2 and 25.9 μg/L. The total imprecision values (CVs) were 5.8% and 11.8%, respectively. Intraassay imprecision was tested by analyzing the same commercial plasma samples 8 times in 1 assay. Intraassay imprecision values were 7.5% and 4.1%, respectively.

The analytical sensitivity of the assay (slope of the calibration curve) was 8.2 mAbsorbance per μg/L (mean from 11 assays). The detection limit (3 SD above the mean for a calibrator that is free of analyte) was 5.9 μg/L.

When the reference plasma pool was enriched with an equal volume of solution containing 100, 50, 25, 12.5, and 6.25 μg/L recombinant Gas6, the observed recovery, (final concentration – initial concentration)/added concentration, was 95–106% of expected recovery.

The specificity of our ELISA assay was further assessed by analyzing Gas6-depleted plasma. Gas6 depletion was performed using a different antibody than those used in the ELISA assay, as described in Materials and Methods. Before depletion, Gas6 concentration was measured at 58.2 μg/L. In Gas6-depleted plasma, Gas6 concentration was undetectable.

Given the strong homology between Gas6 and another vitamin K–dependent protein, protein S, we tested cross-reactivity with protein S. Supplementation of a protein S–depleted plasma with up to 600 nmol/L recombinant protein S did not change the Gas6 concentration detected by our assay (physiological plasma protein S concentration is approximately 350 nmol/L). The same protein S concentrations in assay buffer were below the detection limit of the assay.

Finally, we examined the influence of preanalytical conditions on our assay. To assess whether plasma Gas6
originates from platelet activation during blood collection, we collected blood samples from 5 donors in standard citrate tubes and in citrate, theophylline, adenosine, and dipyridamole tubes, which contain strong inhibitors of platelet activation. No significant difference in Gas6 concentrations was observed. Regarding the effect of the delay between blood sampling and centrifugation, no significant differences in Gas6 concentrations were observed when centrifugation was performed 2 h, 1 h, or immediately after blood collection. Moreover, detected Gas6 concentrations were not modified by up to 10 freeze-thaw cycles of plasma samples.

**PLASMA GAS6 IN THE 2 STUDY POPULATIONS**

We measured Gas6 concentration of 94 available plasma samples from the 100 healthy male volunteers; plasma Gas6 concentration was 52.0 (22.2) µg/L.

Among the plasma samples from 90 healthy women volunteers, 88 from the 1st visit were available for analysis. Plasma Gas6 for the 88 female donors not receiving oral contraceptives was 63.8 (11.7) µg/L. Gas6 values were significantly higher in women than in men (P <0.001), suggesting a hormonal influence on plasma Gas6 concentrations.

In the 45 female volunteers who then received EE and LNG for 3 months, Gas6 concentrations decreased significantly from 63.6 (10.5) µg/L at visit 1 to 51.9 (10.1) µg/L at visit 2 (P <0.001), reaching values comparable to those observed in men (not significant).

Two male volunteers had very high plasma Gas6 concentrations (235.0 and 107.9 µg/L). After removal of these 2 outliers, a sensitivity analysis confirmed the previous results, with differences in Gas6 concentrations between men and the different groups of women becoming more pronounced.

**Discussion**

Using commercially available antibodies, we developed a specific and reproducible ELISA for quantification of Gas6 in human plasma samples, allowing us to detect Gas6 in human plasma. This result is consistent with the findings of Balogh et al. (5), using an assay that included 1 in-house antibody.

With our assay, Gas6 plasma concentration was 52.0 (22.2) µg/L in men and 63.8 (11.7) µg/L in women not receiving oral contraceptives. Balogh et al. (5) measured lower Gas6 concentrations in plasma from men and women (17.2 and 18.5 µg/L, respectively). This difference could be explained by the different designs of the ELISA assays, each using a different detection antibody. Little is yet known about plasma Gas6, which may circulate in different forms; therefore, detection antibodies may differ in their ability to detect total plasma Gas6. The use of 2 mmol/L calcium in buffers for our assay could also make a difference, because calcium modifies the conformation of the Gla domain of vitamin K-dependent proteins, potentially influencing their binding to antibodies. More importantly, the use of different recombinant proteins to calibrate the assays could explain the discrepancy. Protein S, which is structurally similar to Gas6, is known to polymerize during purification (13), and this effect could modify reactivity to antibodies. The same phenomenon may occur with Gas6, making preparation of a calibrator and accurate quantification difficult. Differences in glycosylation of the calibrator protein can also account for discrepancies between ELISA assays (14). The lack of a common reference standard has been shown to be responsible for large differences in quantification between ELISA tests for proteins such as thrombomodulin (15), plasminogen activator inhibitor-1 (16), and D-dimers (17).

Very recently, a study by Gibot et al. (18) in patients presenting with septic shock reported a median Gas6 plasma concentration of 51 ng/L. This result is surprising, because it shows a striking difference from the concentrations reported by us and Balogh et al. (5). More information about the antibodies and the calibrator used by Gibot et al. (18) is required to understand this difference. Nevertheless, important differences in reported plasma Gas6 concentrations raise the question of the expression of Gas6 plasma concentration results. Expressing results as a percentage of the Gas6 concentration in a reference pool of normal plasma could allow an easier comparison between different studies.

In the present study, we showed that plasma Gas6 concentration varies with sex and may be decreased after oral contraceptive use. Gas6 plasma concentrations in premenopausal women without oral contraception were significantly higher than in men. This difference was not observed by Balogh et al. (5), probably because of the smaller number of people studied and lack of data regarding use of oral contraception and hormonal status of patients. Moreover, the design of our study allowed us to measure Gas6 in women at the same time point in their menstrual cycle.

Our results suggest regulation of plasma Gas6 concentrations by sex hormone. The differences observed between men and women without oral contraceptives may be related to up-regulation of Gas6 concentrations by estrogens or down-regulation by androgens. Three cycles after EE + LNG was initiated, Gas6 plasma concentrations in women decreased significantly, reaching values measured in men. This decrease rules out up-regulation of Gas6 by estrogens. On the other hand, LNG is known to have an androgenic effect on some biological variables (19). Indeed, 100 µg of LNG induces changes in lipid and lipoprotein concentrations and carbohydrate metabolism (20). Therefore, our results suggest that plasma Gas6 concentrations are down-regulated by androgens, a theory that explains both the higher Gas6 values in women than men and the decrease of Gas6 concentrations in women after oral contraceptive treatment by EE + LNG.

Although Mo et al. (10) reported that the GAS6 gene was upregulated by estrogens in mammalian epithelial cells, evidence exists for other mechanisms of Gas6 hor-
monal regulation. Indeed, Gas6 and its receptors were found to be involved in spermatogenesis and male gonad development (21), and these mechanisms strongly depend on upstream androgenic stimulation. These contradictory findings, along with ours, suggest that the hormonal regulation of Gas6 in vivo is likely to be more complex than that observed in 1 cell line or tissue.

Although the effect of Gas6 on human platelet aggregation remains unclear, Gas6 seems to play a key role in the vascular system. Indeed, the binding of Gas6 to Axl favors survival of human endothelial cells during acidification (22) and protects rat vascular smooth muscle cells from apoptosis (23). Moreover, mice deficient for Gas6 receptors present vessel wall abnormalities, including altered histology, increased apoptosis, and cellular degeneration (21). Because vessel wall response to injury is a major component of human atherothrombotic pathology, Gas6 could be a key player in the pathophysiology of cardiovascular diseases. Consistent with this theory, Munoz et al. (8) have shown that a polymorphism of the GAS6 gene was associated with stroke. Therefore, lower plasma Gas6 concentrations could result in higher sensitivity to vascular injury, with plaque fibrous cap thinning, vascular smooth muscle cell and endothelium apoptosis; and, thus, a higher susceptibility to plaque rupture or erosion. Interestingly, Boddaert et al. (24) found a higher apoptotic index in the arterial wall in men than in women, a finding that could be related to the difference in Gas6 concentrations between the sexes. It would be of interest to evaluate plasma Gas6 variations in stroke and, more generally, in cardiovascular diseases; our results suggest that future clinical studies on this parameter will require participants to be stratified by sex. The variations of Gas6 with hormonal status that we have seen here may also contribute to the long-reported difference in cardiovascular morbidity between the 2 sexes (25).

Grant/funding support: S.C. received a grant from Association Claude Bernard. This study was supported in part by a fund from Leducq Transatlantic Network of Excellence on Atherothrombosis Research.

Financial disclosures: None declared.

Acknowledgments: A specific chicken polyclonal antibody against Gas6 was kindly provided by Agro-Bio (La-Ferté-Saint-Aubin, France).

References


