

Analysis of Gas6 in Human Platelets and Plasma

Istvan Balogh, Sassan Hafizi, Jonas Stenhoff, Karin Hansson, Björn Dahlbäck

Objective—Gas6 is a member of the vitamin K-dependent protein family. Gas6-deficient mice were found to be resistant to thrombosis because of defective platelet function. Mouse Gas6 was demonstrated to be present in platelets and found to be involved in platelet aggregation. The aim of this study was to investigate the presence of Gas6 in human platelets and plasma and determine its role in platelet function.

Methods and Results—The presence of Gas6 in human platelets and plasma was analyzed using sensitive immunologic methods. Mass spectrometry and ELISA were used to identify and quantify Gas6 in plasma. Gas6 was demonstrated to be present in human plasma, at a concentration determined to be 13 to 23 ng/mL (0.16 to 0.28 nM). Furthermore, plasma Gas6 levels were found to be lower in patients administered with warfarin. However, Gas6 was undetectable in human platelets.

Conclusions—This is the first report to identify and quantify Gas6 in human plasma. However, Gas6 protein was not detected in human platelets, suggesting that any potential platelet-specific function could be because of Gas6 from the circulation. These findings open up new directions regarding the role of Gas6 in normal and pathophysiological situations such as inflammation, autoimmune disease, thrombosis and arteriosclerosis. (*Arterioscler Thromb Vasc Biol.* 2005;25:1280-1286.)

Key Words: Gas6 ■ protein S ■ vitamin K ■ Gla ■ warfarin ■ platelets

Gas6 is the product of the growth arrest-specific 6 (*gas6*) gene, which was originally identified in fibroblasts as a gene whose expression was upregulated on serum starvation.¹ Gas6 is a multimodular vitamin K-dependent protein consisting of an N-terminal γ -carboxyglutamic acid (Gla) domain, 4 epidermal growth factor-like domains and a C-terminal sex hormone-binding globulin-like (SHBG) domain.

Gas6 and protein S, a negative regulator of the blood coagulation cascade, show a high degree of similarity, both in module organization and at the amino acid level.¹ However, contrary to protein S, the main site of synthesis for Gas6 is not the liver. Gas6 expression is widespread in many tissues, including endothelial cells,¹ vascular smooth muscle cells,² and bone marrow cells.³

Gas6 has been shown to be a ligand for 3 different receptors, Axl, Sky and c-Mer which belong to the Axl receptor tyrosine kinase (RTK) subfamily. These RTKs share a characteristic extra- and intracellular domain organization. The ligand-binding portions are composed of 2 immunoglobulin-like domains followed by 2 fibronectin III-like domains. Ligand binding results in dimerization of the receptor molecules, enabling autophosphorylation of their intracellular tyrosine kinase domains.⁴ Although the SHBG domain is sufficient for receptor activation, a modulatory effect of the Gla domain in receptor binding has been

reported.⁵ Gas6 binds all 3 receptors with markedly different affinities (Axl>Sky>c-Mer).⁶

Gas6 has been implicated in numerous physiological processes, including cell migration,^{7,8} adhesion,^{4,9} cell growth and survival.¹⁰⁻¹⁴ Gas6 might be involved in the etiology of numerous different disorders, like rheumatoid arthritis,¹⁵ nephrotoxic nephritis,¹⁶ and breast cancer.¹⁷

Recently, Gas6 knock-out mice were created and found to be resistant to both venous and arterial thrombosis.¹⁸ This was explained by a deficiency in platelet-associated Gas6, which was suggested to function as an important secondary signal amplifier in normal mouse platelets. In mouse platelets, Gas6 was found to be associated with α -granules and to be released by a number of platelet agonists. The presence of all known Gas6 receptors on the murine platelet surface is still controversial.^{18,19} It was hypothesized that Gas6 provided secondary amplification of weak agonist signals.¹⁸ In the same study, it was suggested that Gas6 was also a normal constituent of human platelets, being exposed on the platelet surface after thrombin activation.¹⁸ In the present study, based on these observations, we developed several highly sensitive antibody-based assays to detect Gas6 in human samples and thus to investigate the potential role of Gas6 in human platelets. We found Gas6 to be present in human plasma at low subnanomolar levels (0.16 to 0.28 nmol/L). However, we were

Original received January 27, 2005; final version accepted March 9, 2005.

From the Department of Laboratory Medicine, Division of Clinical Chemistry, Lund University, Wallenberg Laboratory, University Hospital Malmö, Sweden. Present address for I.B. is the Department of Clinical Biochemistry and Molecular Pathology, Medical and Health Science Center, University of Debrecen, Hungary.

Correspondence to Björn Dahlbäck MD, PhD, Professor of Blood Coagulation Research, University of Lund, Department of Laboratory Medicine, Division of Clinical Chemistry, Wallenberg laboratory, University Hospital, Malmö, S-20502 Malmö, Sweden. E-mail bjorn.dahlback@klkemi.mas.lu.se
© 2005 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000163845.07146.48

unable to detect Gas6 in human platelets. Therefore, we conclude that Gas6 is not a physiologically important constituent of human platelets and thus may not serve an autocrine role in human platelet activation. However, our detection and quantification of plasma-derived Gas6 suggest that Gas6 at these physiological concentrations may influence cellular processes such as angiogenesis, which has recently been reported to occur.²⁰

Methods

Materials

General laboratory reagents were purchased from Sigma. Hi-Trap protein G and NHS columns were obtained from Amersham Biosciences. Cell culture medium and reagents were obtained from Gibco. Streptavidin and biotinylated horseradish peroxidase signal amplification reagent (ABC) for ELISA and for immunoblotting were purchased from DAKO and from Vector Laboratories, respectively.

Antibodies

Polyclonal rabbit antiserum (P05) and monoclonal antibodies M1 and M7 were raised against recombinant human Gas6 (rhGas6) as antigen in standard procedures. Recombinant hGas6 was immunopurified from serum free medium of HEK293 cells that had been stably transfected with human Gas6 cDNA. As affinity ligand, M3B, an immobilized (to Hi-Trap) anti-Gla monoclonal antibody (a generous gift of Prof Johan Stenflo, University of Lund), was used following a procedure previously described.²¹ Antibodies were biotinylated using the biotinamidocaproate NHS ester reagent according to standard procedures. P05, M1 and M7 antibodies were purified using Hi-Trap protein G coupled columns according to the manufacturer's instructions (AP Biotech). Antibodies were tested using ELISA and immunoblotting for their ability to bind rhGas6. Goat polyclonal antibody against rhGas6 (amino acids 118 to 678, AB885) was obtained from R&D Systems (Minneapolis, MN). Goat polyclonal peptide antibodies C-20 and N-20 against Gas6 carboxy- and amino-terminus, respectively, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against human protein S and biotinylated secondary antibodies were obtained from DAKO (Glostrup, Denmark).

Isolation and Purification of Human Plasma Gas6 and Recombinant Gas6

Barium citrate precipitation on 1 L citrate phosphate dextrose-anticoagulated fresh frozen plasma was performed as described previously.²² From the redissolved precipitate, Gas6 was purified using a Hi-Trap NHS immunoaffinity column coupled to either monoclonal M1 or M7 anti-Gas6 antibodies according to the manufacturer's instructions. Eluted fractions were analyzed on SDS-PAGE. For purification of rhGas6,²³ essentially the same protocol was followed. Amino acid composition analysis of the purified rGas6 was used to calculate the molar extinction coefficient using data for Asp (Asp+Asn), Glu (Glu+Gln+Gla), Val, Ile and Tyr residues, essentially as described.²⁴ A molecular weight of 82 kDa was used in the calculations. The calculated molar extinction coefficient was found to be $83676 \text{ L} \times \text{mol}^{-1} \text{cm}^{-1}$. The purified rhGas6 was shown to be functionally active.²³

Mass Spectrometry

Samples were prepared for mass spectrometric analysis using a protocol previously described.²⁵ Mass spectrometry was performed using nano-electrospray ionization (nanoESI) mass spectrometry on an API QSTAR Pulsar-i quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a NanoSpray ion source (MDS Proteomics). Some of the peptide ions (present both in rhGas6 and in the suspected Gas6 gel band) were subjected further to tandem mass spectrometry (MS/MS) analysis to obtain amino acid sequence data.

ELISA Assay for the Quantification of Gas6

An assay volume of 100 μL was used throughout. The washing buffer was phosphate buffered saline (PBS)-0.05% Tween-20 (PBS-T) and all washing steps were carried out 5 times between steps. All incubation steps, unless otherwise stated, were performed at room temperature. Optimal concentrations of the antibodies were determined by serial dilutions. After coating with AB885 (2.9 $\mu\text{g}/\text{mL}$), the microtiter plates were blocked with assay buffer (PBS-1% bovine serum albumin [BSA]) and the samples were added in 10- and 20-fold dilutions. The plates were incubated overnight at 4°C then the secondary antibody was added (biotinylated P05, 2 $\mu\text{g}/\text{mL}$) and incubated for 5 hours. This incubation period was followed by the ABC signal amplification step (DAKO). Subsequently, orthophenylenediamine dihydrochloride (OPD) (DAKO) was used as a substrate for color development, which was stopped after 8 minutes with the addition of 100 μL 1 mol/L H_2SO_4 . The absorbance was measured at 490 nm in a microplate reader.

Platelet Isolation and Activation

For experiments on platelets, venous blood was collected in ACD tubes (BD Vacutainer). The washed platelet suspension was prepared using standard procedures as described previously.²⁶ Platelet count was adjusted to 1000 to $1400 \times 10^9/\text{L}$. The platelet solution was then subsampled and one half was lysed in resting phase using 1% Triton X-100. The other half was activated with 5 U/mL (final concentration) thrombin and incubated at 37°C for 20 minutes, then centrifuged for 5 minutes at 13000 g. The resulting supernatant was separated from the residual pellet, which was solubilized in Tris-buffered saline (TBS) containing 1% Triton X-100. The efficiency of the platelet washing and activation procedures was monitored by immunoblotting using different Gas6 antibodies or as positive control a polyclonal rabbit anti-protein S antibody, as protein S has been shown to be present in platelet α -granules, and is secreted and proteolytically processed by thrombin during platelet activation.²⁷

Immunoprecipitation

For platelet and plasma immunoprecipitation (IP) experiments, the equivalent of 5×10^8 platelets in the resting or activated platelet lysates or supernatants and 120 μL of citrate-anticoagulated plasma were used. Protein A- or streptavidin-coated agarose beads were incubated with the respective antibody (see details in Results) to which the antigen-containing material was added. After overnight incubation at 4°C, the beads were washed in TBS and the bound material was eluted in SDS sample buffer, then separated by SDS-PAGE, blotted and developed using different antibodies.

Immunoblotting

Varying amounts of protein were run on 10% SDS-PAGE. The proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Pall Corporation). After quenching, the membrane was incubated with the respective antibody. Where unlabeled primary antibody was used, the membrane was incubated with a biotinylated secondary antibody. Signal amplification was performed using ABC horseradish peroxidase kit (Vector). Color development was done using diaminobenzidine as substrate (Sigma).

Patients and Reference Population

Plasma samples were obtained from 94 apparently healthy hospital staff members not undergoing anticoagulant therapy (57 females and 37 males). Nine volumes of blood were drawn into a vacuum tube (0.129 mol/L citrate, BD Vacutainer). After centrifugation at 1500 g for 15 minutes at room temperature, plasma was removed and stored at -20°C. For tube comparison experiments, 0.129 mol/L citrate, $\text{K}_3\text{-EDTA}$, serum and lithium-heparin (BD Vacutainer) tubes were used. For the analysis of the effect of warfarin treatment on plasma Gas6 levels, samples from 96 patients on such therapy were measured (46 females and 50 males). The International Normalized Ratio (INR) values of the patients were between 2.0 to 4.5. As this study was done anonymously, no clinical data of the patients were available, except sex, age and INR value.

Statistical Methods

For normality testing in the reference population, Kolmogorov-Smirnov test was used. Reference interval was expressed as mean \pm 2 standard deviations (SD) and was also calculated by a non-parametric method. Differences in Gas6 levels in different groups were tested using the Mann-Whitney test. Gas6 levels measured using different blood collection tubes were compared using paired t-test. $P < 0.05$ was considered significant.

Results

Gas6 is Present in Plasma but Undetectable in Human Platelets

To investigate the content of Gas6 in human platelets, isolated platelets were subjected to Western blotting using 6 different antibodies (2 monoclonal and 4 polyclonal) against Gas6. Protein S, which is known to be present in platelets, was used as control. Gas6 was undetectable in the platelet lysates and in the supernatant after thrombin stimulation (not shown). In contrast, protein S was readily found in lysates of resting human platelets, in the platelet supernatant after thrombin activation but not in the thrombin-activated platelet lysates, suggesting release of protein S during thrombin-mediated activation (Figure 1C). To further elucidate whether Gas6 was present in platelets or in plasma, extracts from 5×10^8 platelets (equivalent to the amount of platelets in 1 to 2 mL blood) and 120 μ L plasma were subjected to IP using 70 μ g of biotinylated monoclonal anti-Gas6 antibody, M1, immobilized on streptavidin-agarose beads. The western blotting membrane was probed using biotinylated polyclonal anti-Gas6 antiserum P05. No signal for Gas6 could be detected in the platelet extracts (Figure 1A). However, a faint band appeared in the IP of plasma (Figure 1A), which migrated at a rate comparable to rhGas6. The IP of Gas6 was repeated with 2 other different antibody combinations. For the IP, either 20 μ g AB885 or 20 μ g of M7 was used while goat polyclonal anti-Gas6 (AB885) was used for detection. Gas6 was not detected in platelets using any of the IP-immunoblotting combinations (not shown).

Isolation of Gas6 from Human Plasma

To further investigate Gas6 in plasma, a purification procedure for plasma Gas6 was devised. Barium citrate adsorption of human plasma was performed as an initial step. Gas6 could be readily identified by immunoblot analysis of the precipitate (Figure 1B), but not of the wash fraction. The monoclonal antibody M7 against Gas6 immobilized on a Hi-Trap column was used to affinity-purify Gas6. The eluted proteins were subjected to SDS-PAGE and a band migrating as recombinant Gas6 was subjected to in-gel trypsin digestion for analysis by mass spectrometry. The primary mass spectrum yielded information about the masses of a number of peptides present in human Gas6. Altogether, the peptides identified constituted 28% of the mature Gas6 sequence. To confirm the primary mass spectrum data, 8 peptides were chosen for further sequence analysis using the MS/MS mode. All derived sequences confirmed that the analyzed material was Gas6. The summary of the mass spectrometry analysis is shown in Figure 2.

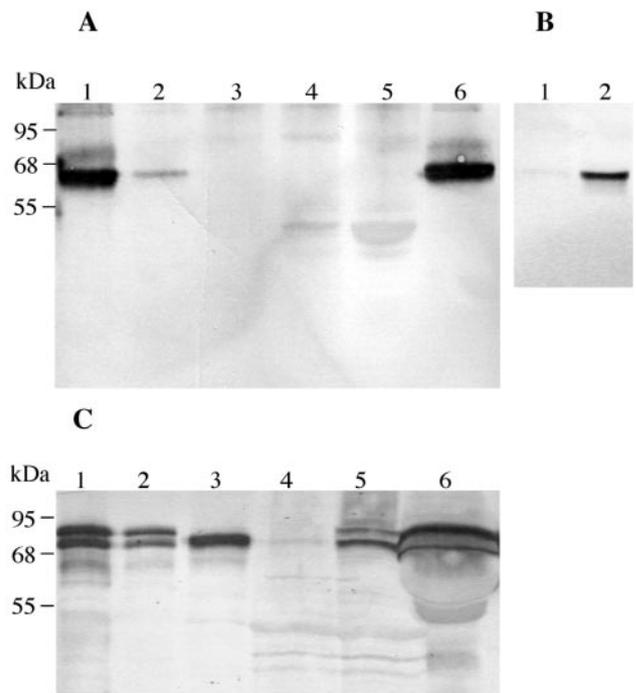


Figure 1. Gas6 is undetectable in platelets but present in human plasma. (A) Immunoprecipitation of 100 ng rhGas6 (lane 1), 120 μ L human plasma (lane 2), supernatant from 5×10^8 isolated platelets activated by thrombin (lane 3), lysate of 5×10^8 isolated resting platelets (lane 5). Samples were analyzed by SDS-PAGE under nonreducing conditions and Western blotting using an anti-Gas6 antibody. RhGas6 (100 ng) was used without IP as a control (lane 6). (B) Gas6 in Ba-citrate precipitate of human plasma. Gas6 is virtually absent in the wash fraction (lane 1) but detectable in the redissolved concentrated precipitate (lane 2). (C) Protein S in platelets. Human platelets were prepared and activated as described in the text. Following SDS-PAGE under reducing conditions, the transferred protein S was visualized using a polyclonal anti-protein S antibody. Human purified protein S (lane 1 and 2; 100 and 10 ng, respectively), proteolytically processed protein S in the supernatant of thrombin-activated platelets (lane 3), absence of protein S in the lysate of activated platelets (lane 4), uncleaved protein S in resting platelets (lane 5), human plasma (0.5 μ L; lane 6).

Development of a Highly Sensitive and Specific ELISA for Gas6

To allow sensitive and specific measurements of Gas6 in different fluids and cell extracts, an ELISA was developed. The combination of antibodies that yielded the highest sensitivity in the ELISA was to use the goat polyclonal anti-Gas6 (AB885) antiserum for capture and biotinylated rabbit polyclonal antiserum (P05) for detection. As the concentration of plasma Gas6 was found to be low, an additional signal amplification step (streptavidin-biotinylated horseradish peroxidase) was introduced. The assay was standardized with purified recombinant Gas6 (Figure 3). The sensitivity limit of the assay was 0.4 ng/mL Gas6 and it was linear up to 6.25 ng/mL. Optimal plasma or serum dilutions were found to be 10- and 20-fold. In a recovery analysis, normal citrated plasma was supplemented with 20 ng/mL rGas6 and then tested in the ELISA assay (Table 1). The recovery was close to the theoretical 100%, suggesting that there were no interfering substances in the assay. Cross-

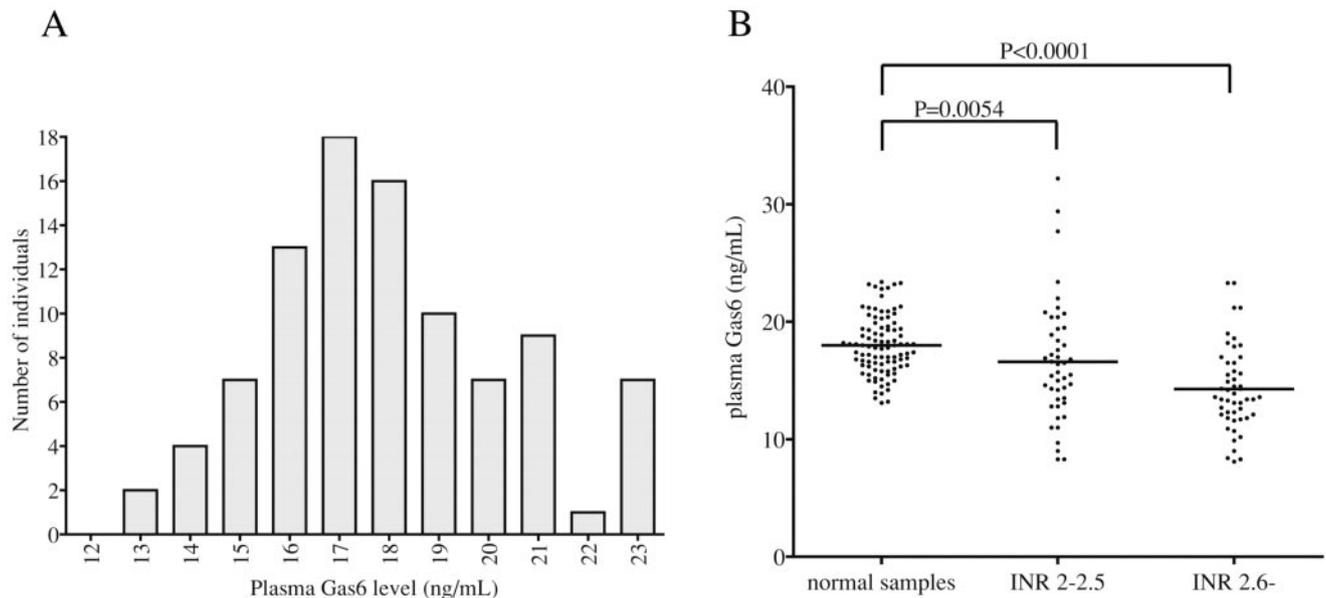


Figure 4. Frequency distribution of plasma Gas6 concentration in healthy individuals and the effect of warfarin on plasma Gas6 levels. (A) Citrate-anticoagulated plasma samples of 94 apparently healthy individuals (57 females and 37 males) were tested. Normal distribution was verified by the Kolmogorov–Smirnov test. (B) Citrate-anticoagulated plasma samples of patients on warfarin anticoagulation ($n=96$, 46 females and 50 males) were analyzed. Gas6 levels were found to be significantly decreased in patients (18.0 ng/mL in controls versus 15.4 ng/mL in patients). In addition, plasma Gas6 level decreased progressively with increases in INR (16.6 ng/mL and 14.3 ng/mL in patients with INR 2 to 2.5, and above 2.6, respectively).

platelet lysate and the activated platelet supernatant, respectively (6 independent experiments).

Reference Interval for Plasma Gas6

The Gas6 concentration was determined using citrated plasmas from 94 apparently healthy individuals (1 outlier was omitted from the analysis). The group consisted of 57 females and 37 males with a mean age of 44 years (range 22 to 65). The normal frequency distribution of plasma Gas6 level was confirmed by Kolmogorov–Smirnov test ($d=0.07459$; P =not significant [NS]; Figure 4A). No age-related differences in Gas6 levels were observed. The established reference interval was 13 to 23 ng/mL for plasma Gas6 concentration (Table 2). Four different blood collection tubes were tested on blood samples drawn from 5 volunteers; citrate, K_3 -EDTA, and Lithium-heparin for plasma and serum from no-additive serum tubes. The following results were obtained (data are expressed as percent \pm SD of the value of the citrate tube): 106.1% \pm 6.9, 107.7% \pm 5.4, and 101.5% \pm 6.1% in the K_3 -EDTA, lithium-heparin and serum tubes, respectively. No significant differences were observed between the citrate

anticoagulated plasma, serum, and the K_3 -EDTA tubes (P =NS), while the difference between citrate and Lithium-heparin was significant ($P=0.04$).

Warfarin Affects Plasma Gas6 Levels

To analyze the possible effect of warfarin on the expression level or secretion efficiency of Gas6, plasma samples from 96 warfarin-treated patients were tested. The mean age of this group was 72 years (range, 31 to 92). No change in Gas6 levels could be observed with increasing age or between the sexes. However, a significant decrease in plasma Gas6 was observed in the treated group. The mean Gas6 plasma level was 18.0 in the controls and 15.4 in the patients ($P<0.0001$). Furthermore, the Gas6 level progressively decreased with increasing INR (Figure 4B).

Discussion

It has previously been reported that Gas6 is present in rat,²⁸ mouse,¹⁸ and human¹⁸ platelets. However, using immunoblotting, combined IP-immunoblotting experiments and a highly sensitive and specific ELISA for Gas6, we could not detect Gas6 in washed human platelets. Three combinations of 4 different antibodies used in IP-immunoblotting experiments yielded no detectable Gas6 in platelet extracts. In contrast, Gas6 present in human plasma (13 to 23 ng/mL) could readily be detected using the same IP-immunoblotting combinations. Assuming that the IP efficiency of Gas6 is similar in both plasma and platelet samples, we could have shown the presence of 2 ng Gas6 from 5×10^8 platelets. This suggests therefore, that 5×10^8 platelets (equivalent to the number of platelets in ≈ 2 mL blood) should contain less than 2 ng Gas6 to escape detection by our methods.

TABLE 2. Reference Interval for Plasma Gas6

	Reference Interval of Plasma Gas6 Concentration		
	No.	Mean \pm SD, ng/mL	Reference Interval, ng/mL
Males	37	17.2 \pm 2.4	12.4–22.0
Females	57	18.5 \pm 2.5	13.5–23.5
Total	94	18.0\pm2.5	13.0–23.0

Citrate-anticoagulated plasma samples of 94 apparently healthy individuals were analyzed. Reference interval was expressed as mean and ± 2 standard deviations.

In an attempt to gain quantitative information and to increase the sensitivity of the detection, an ELISA method was developed. RhGas6 added exogenously to the resting platelet lysate and the activated platelet supernatant could readily be detected in the ELISA. In contrast, no endogenous (platelet) Gas6 could be detected in these samples, even when analyzed in undiluted form. Taking into account the sensitivity limit of the assay (0.04 ng Gas6 in a 100 μ L sample volume) and the number of platelets analyzed (1.4×10^8), it can be concluded that Gas6 is absent in platelets or that the Gas6 level in platelets is less than 0.04 ng per 1.4×10^8 platelets, which is the amount of platelets present in ≈ 1 mL blood. If the latter is true, then these results suggest that less than 1% of the Gas6 in blood is present in the platelets. Thus, even though the sensitivity of the ELISA was almost 2 orders of magnitude higher than that of the IP-immunoblotting, no Gas6 could be detected in human platelets. The estimates from the ELISA measurements can be used to calculate that each platelet contains less than 2 molecules Gas6, which strongly argues against any physiological importance for platelet-derived Gas6. In comparison, 1.4×10^8 platelets contain approximately 230 ng protein S, which corresponds to 12 000 protein S molecules per platelet.^{27,29} Thus, our study suggests a discrepancy between the lack of detectable Gas6 protein in human platelets here and a previously reported demonstration of Gas6 in human platelets.¹⁸ As the concentration of Gas6 in plasma is very low, it is unlikely that plasma contamination of the platelet preparation used could explain the reported presence of Gas6 in platelets in the previous study.¹⁸

Gas6 is capable of binding to different cell surfaces via its N- and C-termini, the N-terminal Gla domain binding to phosphatidylserine-containing phospholipids and the C-terminal SHBG-like domain interacting with the Axl/Sky/c-Mer receptors.^{30,31} Moreover, it appears that, at least in the eye, the binding involving the N-terminal Gla domain of Gas6 to phosphatidylserine is a prerequisite for receptor binding.³² It is also well-documented that Gas6 needs to be carboxylated at its N-terminal Gla domain to function both *in vitro*^{23,33,34} and *in vivo*.³⁵ Thus, it is very likely that the local concentration of Gas6 may increase dramatically on exposure of phosphatidylserine, ie, on the surfaces of apoptotic cells and possibly also on activated platelets, enabling subsequent activation of Axl, Sky, or c-Mer either on the same cells or adjacent cells. Therefore, it is conceivable that activated platelets, although containing no Gas6, are able to sequester Gas6 from the surroundings via their membrane surfaces and the expressed receptors, thus increasing the Gas6 concentration significantly in the local environment. Assuming that the number of Gas6 receptors does not increase during platelet activation (ie, not redirected to the platelet membrane from intracellular stores), an interaction between the activated platelet membrane and the Gla domain of Gas6 could contribute to the platelet-specific function of Gas6. However, the observation of similar Gas6 concentrations in serum and plasma argues against the hypothesis of activated platelet membranes binding and trapping circulating Gas6.

In the present study, we found that human blood plasma contains Gas6 at concentrations that seem very low (13 to 23

ng/mL; 160 to 280 pmol/L). However, Gallicchio et al²⁰ showed very recently that Gas6 might inhibit angiogenesis in endothelial cells via its primary receptor Axl at even lower concentrations (1 ng/mL). In this context, circulating Gas6 might be a physiologically important mediator in endothelial cells even without the need for local accumulation. Yanagita et al¹⁶ have previously demonstrated the presence of 1 to 10 ng/mL Gas6 in the serum of Wistar rats, which agrees well with our data obtained with human plasma. The cell source of plasma Gas6 is not known, but the plasma Gas6 could be derived from synthesis in endothelial cells, vascular smooth muscle cells and fibroblasts. Using immunoblotting and mRNA analysis, Nakano et al reported that the expression level of the Gas6 protein in rat vascular smooth muscle cells was not affected by warfarin treatment, even though the Gas6 produced in the presence of warfarin was functionally defective.³³ To elucidate the influence of warfarin on the plasma level of Gas6 in humans, the ELISA assay was used to measure Gas6 in plasma samples of 96 warfarin-treated patients. We did observe a small but statistically significant decrease in plasma Gas6 levels of patients with high INR. Therefore, our results indicate that warfarin treatment down-regulates Gas6 expression or secretion, as is the case with the vitamin K-dependent clotting factors.^{36,37} In this context, it should be mentioned that ELISAs for vitamin K-dependent proteins show variable results in plasmas from warfarin treated patients depending on the epitopes of the antibodies used. Thus, if one of the antibodies binds the Gla domain then significant quantitative reductions are seen with anticoagulated patients. In the now described ELISA, the antiserum used to coat the ELISA plates was raised using a truncated Gas6 lacking the Gla domain whereas the detecting antiserum was against full-length Gas6. It is therefore likely that the reduction in Gas6 observed in warfarin-treated individuals reflects a decrease in total Gas6 protein rather than a loss of reactivity of antibodies used in the ELISA.

Few studies to date have analyzed the possible association of Gas6 with different diseases. In a mouse model, Gas6 was suggested to be involved in the progression of nephrotoxic nephritis because lower mortality, proteinuria, and fibrin deposition were observed in Gas6^{-/-} mice than in normal mice.¹⁶ Furthermore, Gas6 and Axl have been shown to be upregulated in rheumatoid arthritis.¹⁵ In addition, the expression of the highest affinity Gas6 receptor, Axl, has been shown in many cancer types. These data suggest that over-expression of Gas6 rather than a relative or absolute deficiency might be a suitable marker for different disease states.

In conclusion, using different methods, including immunoprecipitation, BaCl₂ precipitation, immunoaffinity purification and mass spectrometry, we have shown that Gas6 is present at subnanomolar concentrations in the human circulation. However, we could not detect Gas6 in human platelets despite extensive investigation, using several different methods. Therefore, the previously proposed role of Gas6 in platelet aggregation¹⁸ may be because of function of Gas6 originating from the circulation. Furthermore, a highly sensitive ELISA immunoassay was developed, tested and has been shown to be useful in analyzing clinical samples, and

can therefore provide a novel means for analysis of the association of Gas6 levels with different diseases.

Acknowledgments

This work was supported by a Marie Curie Fellowship of the European Community FP5 Programme under Contract No. HPMF-CT-2001-01305, a Magyary Zoltan Postdoctoral Fellowship of the Foundation for the Hungarian Higher Education and Research (I.B.) and by grants from the Swedish Cancer foundation, the Swedish Research Council (#07143), the Albert Pålsson Foundation, the Kock Foundation and by research funds from the University Hospital Malmö.

References

- Manfioletti G, Brancolini C, Avanzi G, Schneider C. The protein encoded by a growth arrest-specific gene (Gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol Cell Biol.* 1993;13:4976-4985.
- Nakano T, Higashino K, Kikuchi N, Kishino J, Nomura K, Fujita H, Ohara O, Arita H. Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. *J Biol Chem.* 1995;270:5702-5705.
- Avanzi GC, Gallicchio M, Cavalloni G, Gammaitoni L, Leone F, Rosina A, Boldorini R, Monga G, Pegoraro L, Varnum B, Aglietta M. GAS6, the ligand of Axl and Rse receptors, is expressed in hematopoietic tissue but lacks mitogenic activity. *Exp Hematol.* 1997;25:1219-1226.
- Bellosta P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. *Mol Cell Biol.* 1995;15:614-625.
- Mark MR, Chen J, Hammonds RG, Sadick M, Godowsk PJ. Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. *J Biol Chem.* 1996;271:9785-9789.
- Nagata K, Ohashi K, Nakano T, Arita H, Zong C, Hanafusa H, Mizuno K. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J Biol Chem.* 1996;271:30022-30027.
- Fridell YW, Villa J Jr, Attar EC, Liu ET. GAS6 induces Axl-mediated chemotaxis of vascular smooth muscle cells. *J Biol Chem.* 1998;273:7123-7126.
- Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, Kandel ER, Heidenreich KA, Wierman ME. Novel mechanism for gonadotropin-releasing hormone neuronal migration involving Gas6/Ark signaling to p38 mitogen-activated protein kinase. *Mol Cell Biol.* 2002;22:599-613.
- McCloskey P, Fridell YW, Attar E, Villa J, Jin Y, Varnum B, Liu ET. GAS6 mediates adhesion of cells expressing the receptor tyrosine kinase Axl. *J Biol Chem.* 1997;272:23285-23291.
- Goruppi S, Ruaro E, Schneider C. Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. *Oncogene.* 1996;12:471-480.
- Fridell YW, Jin Y, Quilliam LA, Burchert A, McCloskey P, Spizz G, Varnum B, Der C, Liu ET. Differential activation of the Ras/extracellular-signal-regulated protein kinase pathway is responsible for the biological consequences induced by the Axl receptor tyrosine kinase. *Mol Cell Biol.* 1996;16:135-145.
- Goruppi S, Ruaro E, Varnum B, Schneider C. Gas6-mediated survival in NIH3T3 cells activates stress signalling cascade and is independent of Ras. *Oncogene.* 1999;18:4224-4236.
- Li R, Chen J, Hammonds G, Phillips H, Armanini M, Wood P, Bunge R, Godowski PJ, Sliwkowski MX, Mather JP. Identification of Gas6 as a growth factor for human Schwann cells. *J Neurosci.* 1996;16:2012-2019.
- Bellosta P, Zhang Q, Goff SP, Basilico C. Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. *Oncogene.* 1997;15:2387-2397.
- O'Donnell K, Harkes IC, Dougherty L, Wicks IP. Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel endothelial cell survival pathway. *Am J Pathol.* 1999;154:1171-1180.
- Yanagita M, Ishimoto Y, Arai H, Nagai K, Ito T, Nakano T, Salant DJ, Fukatsu A, Doi T, Kita T. Essential role of Gas6 for glomerular injury in nephrotoxic nephritis. *J Clin Invest.* 2002;110:239-246.
- Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem.* 2002;277:5209-5218.
- Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlback B, Carmeliet P. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med.* 2001;7:215-221.
- Chen C, Li Q, Darrow AL, Wang Y, Derian CK, Yang J, de Garavilla L, Andrade-Gordon P, Damiano BP. Mer receptor tyrosine kinase signaling participates in platelet function. *Arterioscler Thromb Vasc Biol.* 2004;24:1118-1123.
- Gallicchio M, Mitola S, Valdembrì D, Fantozzi R, Varnum B, Avanzi GC, Bussolino F. Inhibition of vascular endothelial growth factor receptor 2-mediated endothelial cell activation by Axl tyrosine kinase receptor. *Blood.* 2005;105:1970-1976.
- Brown MA, Stenberg LM, Persson U, Stenflo J. Identification and purification of vitamin K-dependent proteins and peptides with monoclonal antibodies specific for gamma-carboxyglutamyl (Gla) residues. *J Biol Chem.* 2000;275:19795-19802.
- Dahlback B. Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin. *Biochem J.* 1983;209:837-846.
- Stenhoff J, Dahlback B, Hafizi S. Vitamin K-dependent Gas6 activates ERK kinase and stimulates growth of cardiac fibroblasts. *Biochem Biophys Res Commun.* 2004;319:871-878.
- Dahlback B, Podack ER. Characterization of human S protein, an inhibitor of the membrane attack complex of complement. Demonstration of a free reactive thiol group. *Biochemistry.* 1985;24:2368-2374.
- Steen H, Pandey A, Andersen JS, Mann M. Analysis of tyrosine phosphorylation sites in signaling molecules by a phosphotyrosine-specific immunon ion scanning method. *Sci STKE.* 2002;2002:PL16.
- Muszbeck L, Polgar J, Boda Z. Platelet factor XIII becomes active without the release of activation peptide during platelet activation. *Thromb Haemost.* 1993;69:282-285.
- Schwarz HP, Heeb MJ, Wencel-Drake JD, Griffin JH. Identification and quantitation of protein S in human platelets. *Blood.* 1985;66:1452-1455.
- Ishimoto Y, Nakano T. Release of a product of growth arrest-specific gene 6 from rat platelets. *FEBS Lett.* 2000;466:197-199.
- Schwarz HP, Heeb MJ, Lammle B, Berrettini M, Griffin JH. Quantitative immunoblotting of plasma and platelet protein S. *Thromb Haemost.* 1986;56:382-386.
- Nakano T, Ishimoto Y, Kishino J, Umeda M, Inoue K, Nagata K, Ohashi K, Mizuno K, Arita H. Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. *J Biol Chem.* 1997;272:29411-29414.
- Ishimoto Y, Ohashi K, Mizuno K, Nakano T. Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. *J Biochem (Tokyo).* 2000;127:411-417.
- Hall MO, Obin MS, Prieto AL, Burgess BL, Abrams TA. Gas6 binding to photoreceptor outer segments requires gamma-carboxyglutamic acid (Gla) and Ca(2+) and is required for OS phagocytosis by RPE cells in vitro. *Exp Eye Res.* 2002;75:391-400.
- Nakano T, Kawamoto K, Kishino J, Nomura K, Higashino K, Arita H. Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. *Biochem J.* 1997;323(Pt 2):387-392.
- Tanabe K, Nagata K, Ohashi K, Nakano T, Arita H, Mizuno K. Roles of gamma-carboxylation and a sex hormone-binding globulin-like domain in receptor-binding and in biological activities of Gas6. *FEBS Lett.* 1997;408:306-310.
- Nagai K, Arai H, Yanagita M, Matsubara T, Kanamori H, Nakano T, Iehara N, Fukatsu A, Kita T, Doi T. Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. *J Biol Chem.* 2003;278:18229-18234.
- Takahashi H, Wada K, Hayashi S, Hanano M, Tatewaki W, Shibata A. Behavior of protein S during long-term oral anticoagulant therapy. *Thromb Res.* 1988;51:241-249.
- Tokunaga F, Wakabayashi S, Koide T. Warfarin causes the degradation of protein C precursor in the endoplasmic reticulum. *Biochemistry.* 1995;34:1163-1170.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Analysis of Gas6 in Human Platelets and Plasma

Istvan Balogh, Sassan Hafizi, Jonas Stenhoff, Karin Hansson and Björn Dahlbäck

Arterioscler Thromb Vasc Biol. 2005;25:1280-1286; originally published online March 24, 2005;

doi: 10.1161/01.ATV.0000163845.07146.48

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2005 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/25/6/1280>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:

<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:

<http://atvb.ahajournals.org/subscriptions/>