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ORIGINAL ARTICLE

Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization

J. M. E. M. COSEMANS,* R. VAN KRUCHTEN,* S. OLIESLAGERS,† L. J. SCHURGERS,* F. K. VERHEYEN,‡ I. C. A. MUNNIX,* J. WALTENBERGER,† A. ANGELILLO-SCHERRER,§ M. F. HOYLAERTS,¶ P. CARMELIET** and J. W. M. HEEMSKERK*

**Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht; †Department of Cardiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht; ‡Department of Molecular Cell Biology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands; §Faculty of Medicine and University Hospital of Geneva, Geneva, Switzerland; ¶Centre for Molecular and Vascular Biology, KU Leuven, Leuven; and **Vesalius Research Centre, KU Leuven, Leuven, Belgium*

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Summary. *Background:* Interaction of murine Gas6 with the platelet Gas6 receptors Tyro3, Axl and Mer (TAM) plays an important role in arterial thrombus formation. However, a role for Gas6 in human platelet activation has been questioned. *Objective:* To determine the role of Gas6 in human and murine platelet activation and thrombus formation. *Methods and Results:* Gas6 levels appeared to be 20-fold higher in human plasma than in platelets, suggesting a predominant role of plasma-derived Gas6. Human Gas6 synergizes with ADP–$P2Y_{12}$ by enhancing and prolonging the phosphorylation of Akt. Removal of Gas6 from plasma impaired ADP-induced platelet aggregation. Under flow conditions, absence of human Gas6 provoked gradual platelet disaggregation and integrin $\alpha_{IIb}\beta_3$ inactivation. Recombinant human Gas6 reversed the effects of Gas6 removal. In mouse blood, deficiency in Gas6 or in one of the TAM receptors led to reduced thrombus formation and increased disaggregation, which was completely antagonized by external ADP. In contrast, collagen-induced platelet responses were unchanged by the absence of Gas6 in both human and mouse systems. *Conclusions:* The ADP–$P2Y_{12}$ and Gas6–TAM activation pathways synergize to achieve persistent $\alpha_{IIb}\beta_3$ activation and platelet aggregation. We postulate a model of thrombus stabilization in which plasma Gas6, by signaling via the TAM receptors, extends and enhances the platelet-stabilizing effect of autocrine ADP, particularly when secretion becomes limited.

Keywords: Akt, Gas6, platelet, TAM, thrombus stabilization.

Correspondence: Judith M. E. M. Cosemans, Department of Biochemistry, CARIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, the Netherlands.
Tel.: +31 43 3881534; fax: +31 43 3884159.
E-mail: judith.cosemans@bioch.unimaas.nl

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Introduction

Gas6, encoded by growth arrest-specific gene 6, is a vitamin K-dependent protein that is expressed in and released by many cell types, including endothelial cells, vascular smooth muscle cells, macrophages, and bone marrow cells [1–3]. Consistent with its wide distribution, Gas6 has been implicated in a variety of cellular functions, such as reversible growth arrest, survival, proliferation, and inflammation [1,4,5]. The current understanding is that Gas6 exerts these functions by acting as an extracellular ligand for receptor tyrosine kinases of the TAM family, namely Tyro3, Axl, and Mer [6–8]. Activation of phosphoinositide 3-kinase (PI3K) is an important downstream effector of Gas6–Gas6 receptor signaling, as demonstrated by studies in several cell types [5].

In mice, there is substantial evidence that the interaction of Gas6 with TAM receptors on platelets has a stimulatory role in thrombus formation *in vivo*. Interestingly, mice deficient in Gas6 or any one of the TAM receptors were found to be protected against venous and arterial thrombosis, without having a bleeding phenotype [9,10]. As Gas6 also contributes to plaque instability in $Apoe^{-/-}$ mice [3], it has become an attractive target for the prevention or treatment of atherothrombosis. In mice, the binding of autocrine-secreted Gas6 to the TAM receptors on activated platelets leads to integrin $\alpha_{IIb}\beta_3$ activation via PI3K [10]. However, ADP–$P2Y_{12}$-

mediated signaling also causes activation of $\alpha_{IIb}\beta_3$ by a similar route [11–13]. How the roles of Gas6 and ADP in platelet activation and thrombus formation interact or overlap is still unknown.

Whether Gas6 has a function in human platelet activation is controversial. Whereas initial studies showed that Gas6 mRNA was detectable in human platelets [9,14], others have found that human platelets neither store nor secrete Gas6 protein [15]. In human plasma, a variable level of Gas6 has been measured, ranging from 15 to 65 μg/L [15–17]. However, this variation in plasma Gas6 levels did not correlate with the extent of initial platelet aggregation [18]. It has therefore been questioned whether Gas6 contributes to the activation process of human platelets. In the present study, we investigated the contribution of plasma-derived Gas6 to human platelet activation under static and flow conditions. We also compared the roles of human and murine Gas6 in thrombus formation and stabilization. The results lead to a model in which Gas6 enhances and extends the thrombus-stabilizing role of ADP.

Materials and methods

Materials

Fibrillar Horm type I collagen was from Nycomed (Munich, Germany). ADP and bovine serum albumin (BSA) were from Sigma (St Louis, MO, USA). H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA, USA). Fluorescein isothiocyanate (FITC)-labeled annexin A5 was from PharmaTarget (Maastricht, The Netherlands), and FITC-labeled PAC-1 monoclonal antibody (mAb) was from Becton-Dickinson (San Jose, CA, USA). Recombinant human Gas6 (rhGas6) was from Abnova (Taipei, Taiwan). Anti-human Gas6 antibody (Ab) (sc-1936), anti-human Gas6 Ab (sc-22759) and anti-human Axl Ab (sc-20741) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-phosphoserine-473 Akt Ab and polyclonal anti-Akt Ab were from Cell Signaling Technology (Danvers, MA, USA).

Human blood and platelets

Human blood was drawn from healthy volunteers after full informed consent had been obtained. Blood was collected into 17 vol.% acid–citrate–dextrose, 10 vol.% 129 mM trisodium citrate, or 40 μM PPACK, as required. Platelet-rich plasma (PRP), platelet-free plasma and washed platelets were obtained as described [19]. For flow experiments, citrate-anticoagulated plasma was recalcified with 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ in the presence of 40 μM PPACK [19].

Mouse blood and platelets

Experiments were approved by the local animal experimental committees. Mice deficient in Gas6, Tyro3, Axl or Mer were generated as previously described [9,20], and compared with matched control wild-type mice of the same genetic background. All mice had platelet counts in the normal range. Mouse blood was collected into 10 vol.% 129 mM trisodium citrate and 1 U mL^{-1} heparin. Prior to flow experiments, citrate-anticoagulated blood was recalcified, as described above.

Immunoelectron microscopy

Resting and thrombin-stimulated platelets were fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The samples were further processed for cryoimmunoelectron microscopy, basically as described previously [21]. In brief, fixed platelets were infiltrated into 2.3 M sucrose and frozen in liquid nitrogen. Immunogold labeling was performed with rabbit anti-human Gas6 or Axl Abs on thin, frozen sections, using protein A/gold with 20-nm gold particles. The sections were embedded in a mixture of 1.8% methylcellulose and 0.4% uranyl acetate. No labeling reaction was visible in preparations incubated with a control antibody.

Detection of Gas6 in human plasma and platelets

Human platelets (100×10^8 mL^{-1}) were washed three times in HEPES buffer (pH 6.6) (136 mM NaCl, 10 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% BSA), and then lysed in RIPA buffer containing a cocktail of protease inhibitors [10]. The lysates were centrifuged at 15 000 ×*g* for 5 min to remove membrane fragments. Supernatants were incubated overnight at 4 °C with an anti-Gas6 polyclonal Ab, coupled to protein A Sepharose beads. Likewise, citrate-anticoagulated pooled platelet-free plasma was incubated with beads containing anti-Gas6 Ab. Beads were collected by centrifugation, washed twice in phosphate-buffered saline, and eluted in sodium dodecylsulfate (SDS) sample buffer. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide), transferred onto a poly(vinylidine fluoride) membrane, and stained with goat anti-human Gas6 Ab. Detection was with horseradish peroxidase-conjugated rabbit anti-goat IgG, followed by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Preparation of human Gas6-depleted plasma

Anti-human Gas6 Ab was coupled to aldehyde-activated bead agarose resin by using the Seize primary immunoprecipitation kit from Pierce. Pooled human citrate-anticoagulated plasma was incubated overnight with a Gas6 Ab-coupled resin at 4 °C. Supernatants of Gas6 Ab-treated and sham-treated plasmas were frozen at − 80 °C until use. Full depletion of Gas6 was verified by barium citrate precipitation of plasma samples, followed by SDS-PAGE and western blotting, as described previously [22].

Platelet aggregation

Human platelet aggregation was measured by light-transmission aggregometry (Chronolog, Havertown, PA, USA) under constant stirring at 1000 r.p.m. at 37 °C [13]. Control and Gas6-depleted plasmas (anticoagulated with citrate) were reconstituted with washed human platelets at a final concentration of 2.5×10^8 mL^{-1}. Prior to addition of agonist, plasmas were recalcified with 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ in the presence of 40 μM PPACK. Alternatively, platelet aggregation was determined by measuring single cell platelet counts with a Coulter counter (Coulter Electronics, Hialeah, FL, USA).

Measurement of thrombus formation and phosphatidylserine (PS) exposure

Degreased rectangular coverslips (24 × 60 mm) were coated with fibrillar type I Horm collagen (25 μL of 200 μg collagen mL^{-1}), as described previously [13]. Coated coverslips were inserted into a transparent parallel-plate perfusion chamber, through which blood was perfused at a shear rate of 1000 s^{-1}. Secondary perfusion was with HEPES buffer (pH 7.45) or recalcified citrate-anticoagulated plasma at the same shear rate. Post-staining was with FITC-labeled annexin A5 (0.5 μg mL^{-1}) in $CaCl_2$-containing HEPES buffer, as indicated. Phase-contrast and fluorescence images were taken in real time with a sensitive camera system [13]. Video recordings were made simultaneously. Images were analyzed for surface area coverage and for distribution of areas of individual segmented features, as described previously [23]. For analysis of disaggregation, the number of detaching (single or clustered) platelets per individual aggregate were counted offline from video recordings, as described previously [13].

Coverslips with thrombi were also examined with a Bio-Rad 2100 multiphoton microscopic system (Zeiss, Jena, Germany) [24]. Two-photon excitation was with a Spectra-Physics Tsunami Ti:Sapphire laser (Mountain View, CA, USA), tuned and mode-locked at 800 nm, producing pulses of 100 fs in width (repetition rate of 82 MHz). The fluorescence from FITC-labeled PAC-1 (1 : 20) was detected at an emission wavelength of 508–523 nm. Data for PAC-1 binding to platelets in a thrombus were normalized for the extent of platelet deposition. Analysis of confocal images was performed ImagePro/LaserPix software (Media Cybernetics, Silver Spring, MD, USA).

Measurement of Akt activation

Akt activation was measured by western blot analysis of platelet lysates [11]. A polyclonal anti-phosphoserine-473 Akt Ab was used to detect active Akt, and a polyclonal anti-Akt Ab to determine total Akt.

Statistics

Significance of differences was determined with the Mann–Whitney *U*-test by using the statistical package for social sciences (SPSS 11.0; SPSS, Chicago, IL, USA). Size distribution of platelet aggregates was evaluated by chi-square analysis.

Results

Quantitative determination and localization of Gas6 and Axl in human platelets

The literature is unclear on whether [9,14] or not [15] human platelets express physiologically relevant amounts of Gas6. To investigate this, we measured Gas6 levels in human platelets and a corresponding volume of human plasma. Samples of 150 μL of plasma and 5×10^7 lysed platelets were subjected to immunoprecipitation with beads coupled to anti-Gas6 Ab. A normal platelet count of 3×10^8 mL^{-1} was assumed. Western blots showed a single weak band at the size of Gas6 (75 kDa) in the platelet immunoprecipitate. In contrast, precipitation of plasma Gas6 gave a much denser band of the same size (Fig. 1A). Staining of the blots with a mAb against γ-carboxyglutamate residues indicated that the Gas6 present in both plasma and platelets was γ-carboxylated (not shown). By comparison with a reference sample of full-length rhGas6, the Gas6 content in human plasma was estimated to be 20 μg L^{-1}. In contrast, the platelet Gas6 content was only 1 μg L^{-1} (equivalent to 5 ng per 10^9 platelets). These results thus indicate that plasma is the major source of Gas6 in human blood.

Immunoelectron microscopy was performed to determine the intracellular location of Gas6 and its main receptor, Axl. Quantitative analysis of sections from resting and thrombin-stimulated platelets indicated that the fraction of Gas6 gold label located near the plasma membrane increased from 14% to 27%, with a corresponding decrease in the α-granules, the remainder being predominantly in the open canicular system (Fig. 1B,C). Similarly, stimulation of the platelets increased the fraction of Axl gold label near the plasma membrane from 13% to 44%, again at the expense of granule-associated label (Fig. 1D,E).

Plasma-derived Gas6 stabilizes aggregation of human platelets

To study its role in platelet activation, Gas6 was depleted from human pooled plasma. Depleted and control plasmas were reconstituted with freshly isolated washed platelets from the same donor. Just prior to addition of platelet agonists, the PRP was recalcified with $CaCl_2/MgCl_2$ solution to obtain millimolar levels of both divalent cations. Millimolar levels of Ca^{2+} and Mg^{2+} are required for physiologic regulation of integrin activation [13]. PPACK was added to stop thrombin activity. Treatment of the reconstituted Gas6-depleted PRP with rhGas6 alone, up to 2 μg mL^{-1}, did not evoke platelet aggregation or shape change (Fig. 2A). Stimulation with a suboptimal dose of ADP (10 μM) caused near-irreversible aggregation in reconstituted control PRP, but transient aggregation in reconstituted Gas6-depleted PRP. To confirm that

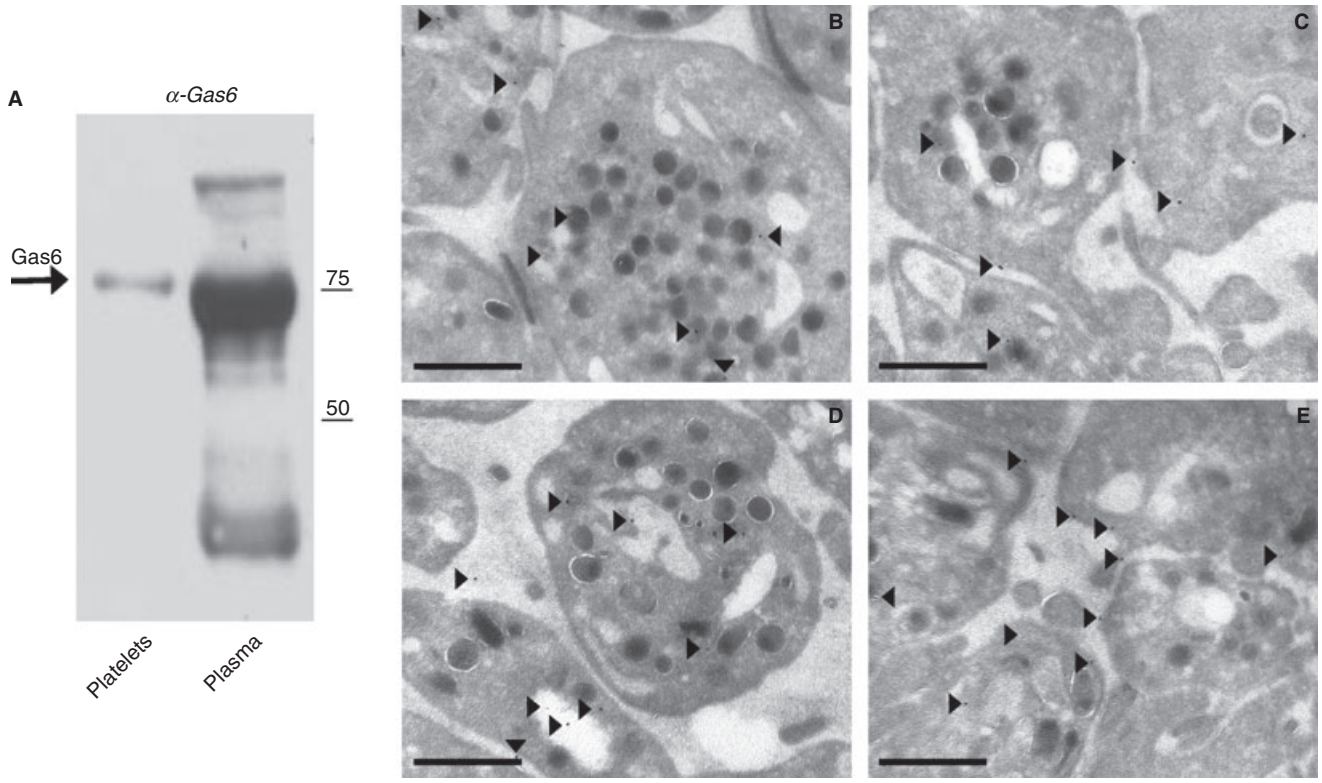


Fig. 1. Quantitative determination and localization of Gas6 and Axl in human platelets. (A) Levels of Gas6 in human plasma and platelets were compared by immunoprecipitating Gas6 from washed platelets (5×10^7) and from an equivalent amount of plasma (150 μL). A normal platelet count of 3×10^8 mL^{-1} was assumed. A representative western blot of the immunoprecipitates, after staining with anti-Gas6 antibody, is shown. Only plasma immunoprecipitate gave a dense 75-kDa Gas6 band. (B–E) Immunoelectron microscopy localization of Gas6 and Axl in human platelets. Resting (B, D) and thrombin-activated (C, E) platelets were fixed and incubated with anti-Gas6 antibody (B, C) or anti-Axl antibody (D, E), and this was followed by immunogold labeling. Arrowheads denote gold label (scale bar: 1 μM). Note the presence of Axl staining near the plasma membrane in activated platelets.

the difference was attributable to Gas6 depletion, rhGas6 was pre-added to the PRP. Addition of rhGas6 (500 ng mL^{-1}) restored the impaired ADP-induced aggregation in Gas6-depleted PRP, but did not influence the aggregation in control PRP (Fig. 2B). Platelet aggregation induced by collagen (5 μg mL^{-1}) was only slightly impaired in Gas6-depleted PRP in comparison with control PRP (Fig. 2C). A similar level of impairment was observed at higher collagen concentrations (not shown). The rhGas6 was used here at a concentration similar to that used in earlier studies [9]. Dose–response curves of ADP-induced aggregation indicated half-maximal stimulation at 70 ng rhGas6 mL^{-1} (range: 25–500 ng mL^{-1}) (Fig. 2D). This concentration is in the order of the estimated plasma concentration of about 25 ng Gas6 mL^{-1}. Together, these data suggest that, in the human system, plasma-derived Gas6 contributes to the stabilization of platelet aggregate formation, particularly in response to ADP.

Synergy of Gas6 and ADP in Akt phosphorylation in human platelets

In mouse platelets and other cell types, it has been demonstrated that ligand occupation of the Gas6 receptors leads to phosphorylation and activation of Akt by PI3K [5,10]. As the PI3Kβ/γ isoforms and Akt play crucial roles in the ADP–$P2Y_{12}$-dependent sustained activation of integrin $\alpha_{IIb}\beta_3$ [12,13], we investigated how, in human platelets, Gas6 and ADP receptor-mediated signaling contribute to Ser473 phosphorylation of Akt. Platelet stimulation with 10 μM ADP caused a two-fold transient increase in Akt phosphorylation, with an optimum between 10 and 20 min (Fig. 3A,B). In comparison, stimulation with rhGas6 (500 ng mL^{-1}) led to a gradual increase in Akt phosphorylation, which crossed the level of ADP-stimulated platelets after 30 min. The rhGas6-induced Akt phosphorylation, however, completely relied on autocrine $P2Y_{12}$-mediated effects, as it was fully blocked by the $P2Y_{12}$ antagonist AR-C69931MX (Fig. 3B). Strikingly, platelet activation with the combination of rhGas6 and ADP caused greatly enhanced Akt phosphorylation in comparison with ADP alone. This enhancement persisted for up to 45 min (Fig. 3A,B). In sum, these results demonstrate synergy between Gas6-evoked and ADP–$P2Y_{12}$-evoked Akt phosphorylation.

Human plasma Gas6 contributes to stabilization of human thrombi under flow by maintaining $\alpha_{IIb}\beta_3$ activation

To investigate the functional implications of the synergy between Gas6 and ADP in persistent platelet activation, studies

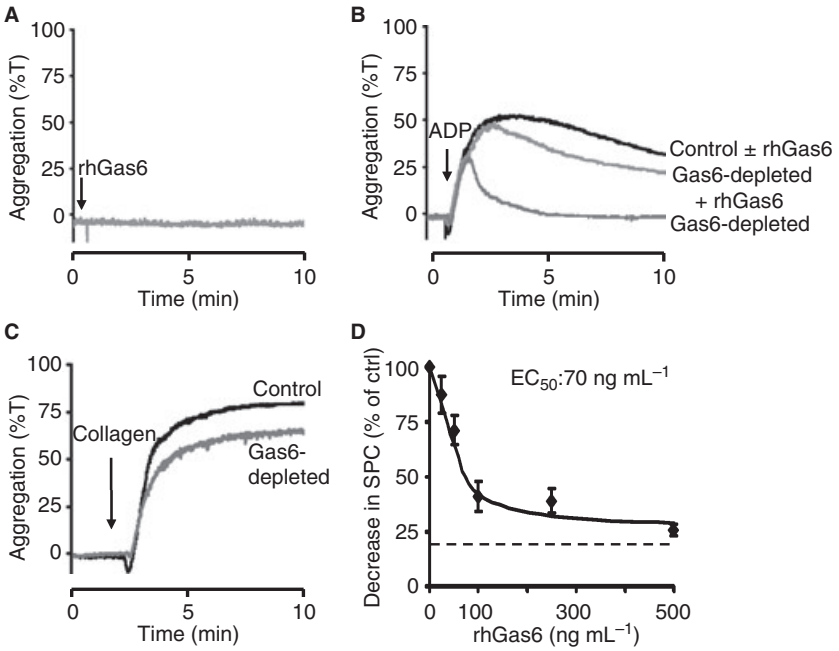


Fig. 2. Gas6 in human plasma enhances platelet aggregation. (A–C) Human Gas6-depleted and control plasmas (citrate-anticoagulated) were reconstituted with washed platelets from one donor (final concentration: 2.5×10^8 platelets mL^{-1} plasma). Prior to addition of agonist, plasmas were recalcified with 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ in the presence of 40 μM PPACK. Platelet aggregation was measured in response to (A) 500 ng mL^{-1} recombinant human Gas6 (rhGas6), (B) 10 μM ADP ± 500 ng mL^{-1} rhGas6, or (C) 5 μg mL^{-1} collagen. Aggregation traces are representative of four independent experiments. (D) Dose–response curve of the effect of rhGas6 on single cell platelet count (SPC) in Gas6-depleted platelet-rich plasma (PRP) after stimulation with 10 μM ADP for 2 min. The dotted line is the SPC of control (ctrl) PRP after stimulation with 10 μM ADP for 2 min. %T, percentage transmission.

were carried out with whole blood under flow conditions. An earlier established procedure was used, in which platelet aggregates were preformed on immobilized collagen, after which their tendency to disaggregate was measured upon secondary perfusion with plasma [13]. Secondary perfusion for 10 min with control plasma resulted in the disappearance of only a few platelets from the top of aggregates, the core remaining stable (Fig. 4A). This contrasted with the results of secondary perfusion experiments in the presence of the $P2Y_{12}$ antagonist AR-C69931MX (ARC), where aggregates immediately started to disintegrate [13]. Secondary perfusion with Gas6-depleted plasma also caused nearly complete thrombus disintegration (Fig. 4A). However, interestingly, the majority of platelets left the aggregates only after a period of 3 min (Fig. 4B). Addition of rhGas6 (500 ng mL^{-1}) to Gas6-depleted plasma reduced the number of disaggregation events almost to the level seen in control plasma. After secondary perfusion, the size distribution of remaining platelet features on the coverslip was measured. Large platelet structures were present for both control plasma and Gas6-depleted plasma with rhGas6, but only smaller structures (< 40 platelets) were left after perfusion with Gas6-depleted plasma (Fig. 4C). Further experiments showed that addition of 20 μM ADP to Gas6-depleted plasma completely prevented the disaggregation events. It was also examined whether rhGas6 can contribute to thrombus stability in the absence of $P2Y_{12}$ activity. Therefore, secondary perfusion was performed with plasma supplemented with ARC (10 μM) with or without rhGas6 (1 μg mL^{-1}). In the first 3 min, rhGas6 addition insignificantly changed the disaggregation events from 5.3 ± 0.3 to 5.5 ± 0.25 per aggregate [mean ± standard error of the mean (SEM), $N = 3$, $P = 0.67$]. However, after 3 min of perfusion with rhGas6, the disaggregation events tended to be reduced from 5.1 ± 0.4 to 4.4 ± 0.3 ($P = 0.08$). These results suggest that Gas6 can compensate for a gradual decrease in ADP activity, but that it cannot prevent platelet disaggregation upon full blockage of the $P2Y_{12}$ receptors.

The activation state of $\alpha_{IIb}\beta_3$ in aggregates formed on collagen-coated coverslips was investigated by post-staining with fluorescence-labeled PAC-1 mAb and quantitative confocal image analysis. After 10 min of secondary perfusion with control plasma, platelets on the surface showed high PAC-1 staining, indicating a high level of active $\alpha_{IIb}\beta_3$ (Fig. 4D,E). Whereas secondary perfusion with Gas6-deficient plasma led to a significant reduction in PAC-1 staining, the addition of rhGas6 to Gas6-depleted plasma completely restored this staining. To verify that the reduced PAC-1 staining was not a consequence of a reduction in platelet number, platelets on the surface were post-stained with carboxyfluorescein succinimidyl ester (CFSE). The ratio of PAC-1 to CFSE fluorescent intensities was determined, and set at 1 for secondary perfusion with control plasma. Secondary perfusion with Gas6-depleted plasma reduced the PAC-1/CFSE ratio to 0.28 ± 0.06, and this ratio increased to 0.75 ± 0.17 in the presence of rhGas6 (means ± SEM, $n = 3$, $P = 0.05$). Together, these data indicate that the removal of

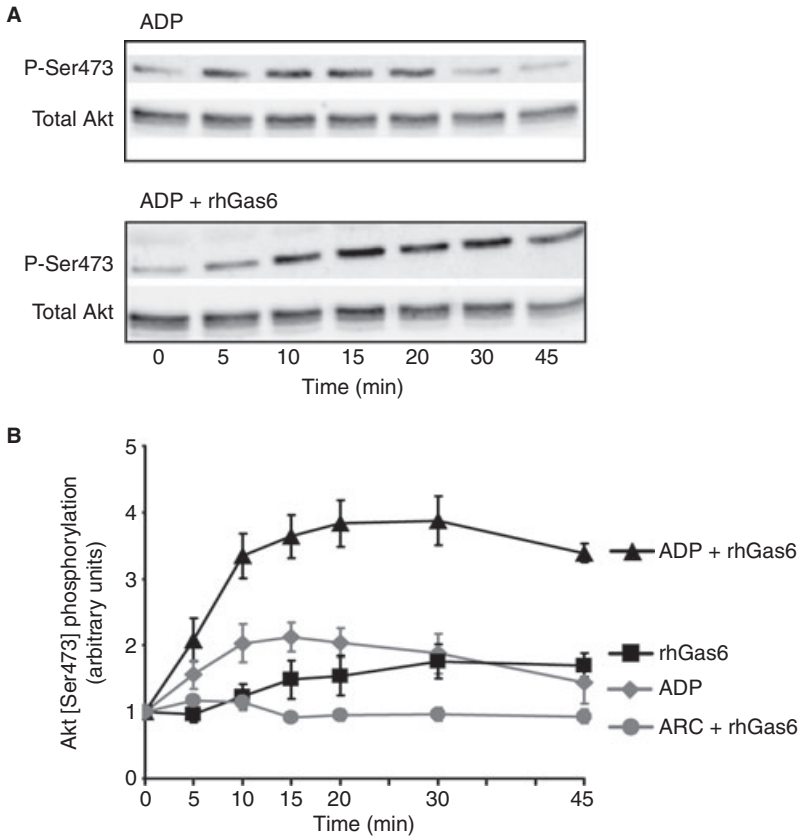


Fig. 3. Gas6 potentiates phosphorylation of Akt in ADP-stimulated human platelets. Washed human platelets (5×10^8 mL^{-1}) were stimulated with ADP (10 μM) and/or recombinant human Gas6 (rhGas6) (500 ng mL^{-1}) for different time periods. Preincubation was with the P2Y12 receptor blocker AR-C69931MX (10 μM) for 10 min, as indicated. (A) Platelet samples taken at indicated time points were analyzed for Ser473 phosphorylation of Akt (P-Ser473) and for total Akt by western blotting. Representative blots are shown. (B) The graph represents quantification of the phosphorylation of Akt on Ser473 (phosphorylated/total ratio). Data are means ± standard errors of the mean (n = 4–5).

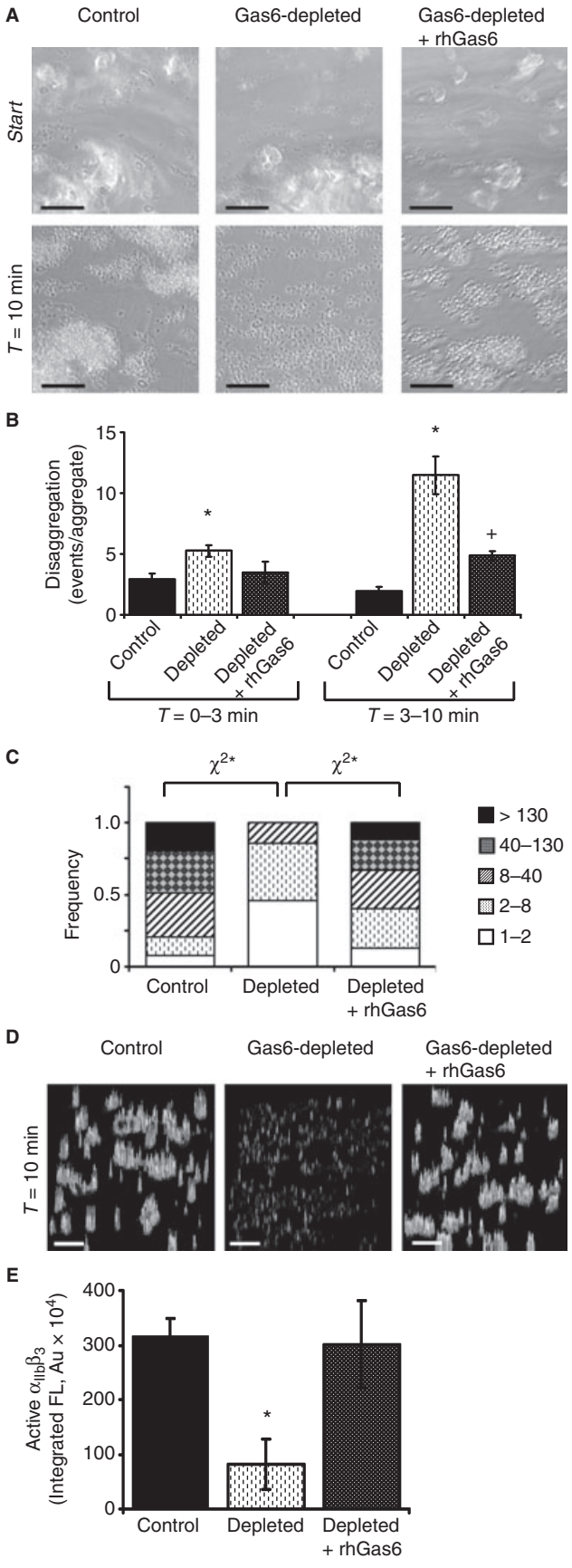
Gas6 from plasma leads to $\alpha_{IIb}\beta_3$ inactivation and subsequent disaggregation. In conclusion, these findings point to an important role for human plasma-derived Gas6 in late phases of aggregate stabilization, probably by maintaining $\alpha_{IIb}\beta_3$ in the active state.

Murine Gas6 and TAM receptors are required for stable thrombus formation under flow

Mice lacking Gas6 or any of the three TAM receptors display reduced arterial thrombus formation in various experimental models [9,10,14]. In the present study, we used blood from these mice to investigate the role of Gas6–Gas6 receptor interaction in the stabilization of thrombi formed on collagen under moderately high shear flow conditions. With blood from Gas6$^{-/-}$ mice, the platelet aggregates formed on collagen were smaller in size and covered less of the surface than wild-type aggregates (Fig. 5A,B). The reduced aggregate formation was accompanied by a strong increase in disaggregation events (platelets detaching from the aggregates) (Fig. 5C). The ability of collagen-adhered platelets to elicit glycoprotein (GP) VI-induced exposure of PS was investigated by staining with FITC-labeled annexin [24]. The numbers of PS-exposing platelets were similar with Gas6$^{-/-}$ and Gas6$^{+/+}$ blood (Fig. 5D), implying that Gas6 deficiency did not affect GPVI-induced procoagulant function. This was confirmed by experiments indicating that collagen-induced thrombin generation was unaltered in PRP from Gas6$^{-/-}$ mice in comparison with wild-type mice (not shown).

Similar flow studies were performed with blood from mice lacking one of the three TAM receptors (Fig. 5E–H). Again smaller aggregates formed on collagen in comparison with wild-type blood. Video analysis showed that platelets from Tyro3$^{-/-}$, Axl$^{-/-}$ or Mer$^{-/-}$ mice detached more frequently from the aggregates than corresponding wild-type platelets, whereas the staining with FITC–annexin A5 remained unchanged (Fig. 5G,H).

Given the synergy between Gas6 and ADP signaling, we then investigated whether ADP could overcome the reduced thrombus formation that was found in the absence of Gas6 or Gas6 receptors. When ADP was coinfused with Gas6$^{-/-}$ blood, normal platelet aggregates formed on collagen with Gas6$^{-/-}$ blood, and the number of disaggregation events was greatly reduced (Fig. 6A–C). Similarly, coperfusion of ADP normalized the formation of stable aggregates of Tyro3$^{-/-}$ or Axl$^{-/-}$



platelets (Fig. 6D,E). Thus, the Gas6–Gas6 receptor interaction seems to become redundant for stable thrombus formation once ADP is not a limiting factor.

Discussion

This study provides first evidence that Gas6 contributes to platelet activation and thrombus stabilization in the human circulatory system. The data show that, in human platelets, Gas6 has a potent, enhancing effect on the $P2Y_{12}$-dependent activation of PI3K to phosphorylation of Akt. Interestingly, Gas6 was unable to promote Akt phosphorylation if the $P2Y_{12}$ receptors were blocked, but it potently enhanced and prolonged the phosphorylation resulting from ADP–$P2Y_{12}$ receptor stimulation. As the $P2Y_{12}$-induced PI3K pathway is a major route to $\alpha_{IIb}\beta_3$ activation [12,25], this Gas6 effect leads to more prolonged activation of the integrin.

In contrast to a report suggesting that Gas6 is absent in human platelets [15], we were able to detect low levels of this protein in human platelet preparations (5 ng per 10^9 platelets). The difference is probably a result of the use of a more sensitive detection method for protein on western blot by enhanced chemiluminescence. For human blood, we report a 20-fold higher concentration in pooled plasma ($\sim$ 20 μg L^{-1}) than in platelets. The Gas6 concentration measured in plasma pools is in agreement with the levels of 15–65 μg L^{-1} measured by others [15–17]. For the murine system, almost six-fold higher platelet Gas6 levels have been reported, whereas the plasma Gas6 concentration is comparable [26]. The present data suggest that plasma-derived Gas6 is responsible for the majority of the effects of this protein in the human system. However, this does not exclude the possibility that locally, for example near a damaged vessel wall, levels of Gas6 can be higher because of rupture of endothelial cells and smooth muscle cells.

Fig. 4. Gas6 in human plasma preserves late thrombus stabilization and $\alpha_{IIb}\beta_3$ activation under flow. Human PPACK-anticoagulated whole blood was perfused over a collagen-coated coverslip at a shear rate of 1000 s^{-1} for 4 min. Thrombi formed on the coverslip were secondarily perfused for 10 min with either control plasma or Gas6-depleted plasma at the same flow rate. Supplementation with recombinant human Gas6 (rhGas6) (500 ng mL^{-1}) was as indicated. (A) Representative phase contrast images (120 × 120 μm) of thrombi at the start and after 10 min of secondary perfusion. (B) Number of disaggregation events per platelet aggregate during initial (T = 0–3 min) and later (T = 3–10 min) periods of secondary perfusion. (C) Histogram of features left on the coverslip at the end of secondary perfusion. Estimated numbers of platelets per feature are 1–2, 2–8, 8–40, 40–130, and > 130, as indicated. (D, E) Thrombi were stained with fluorescein isothiocyanate (FITC)–PAC-1 monoclonal antibody (1 : 20) to detect activated $\alpha_{IIb}\beta_3$. Shown are representative fluorescent images (180 × 180 μm) after secondary perfusion (D), and normalized quantification of integrated FITC–PAC-1 fluorescence (FL) intensity (E). Scale bar: 30 μm. Data are means ± standard errors of the mean (n = 4). *P < 0.05 as compared with control. (B, E), Mann–Whitney U-test. (C) χ^2 analysis. AU, arbitrary units.

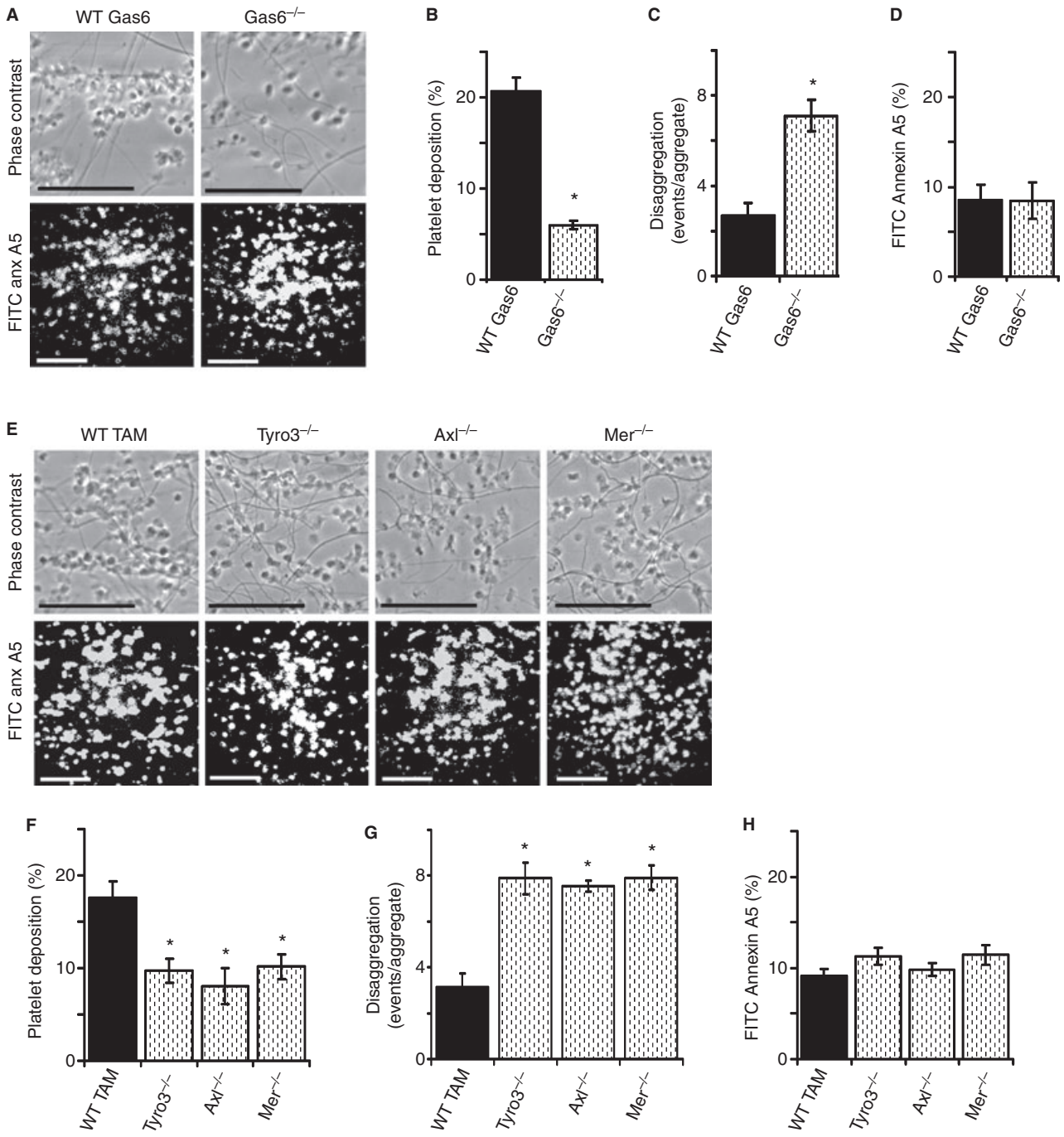


Fig. 5. Deficiency in murine Gas6 or one of the Gas6 receptors, Tyro3, Axl, or Mer (TAM), reduces thrombus formation and increases platelet disaggregation under flow. Blood from wild-type (WT) mice or mice deficient in Gas6, Tyro3, Axl or Mer was passed over collagen-coated coverslips at a shear rate of 1000 s^{-1} for 4 min. Phosphatidylserine (PS)-exposing platelets were stained by post-perfusion with fluorescein isothiocyanate (FITC)–annexin A5. (A, E) Representative phase contrast (50 × 50 μm) and fluorescent (100 × 100 μm) images after perfusion. (B, F) Surface area coverage of deposited platelets. (C, G) Quantification of detachment of platelets from thrombi (disaggregation) monitored during thrombus formation by real-time video recording. Numbers indicate events per aggregate. (D, H) Surface area coverage of PS-exposing deposited platelets. Scale bar: 30 μm. Means ± standard errors of the mean ($n = 4$); *$P < 0.05$.

Experiments to assess the role of plasma-derived Gas6 in platelet aggregation and stabilization indicate that plasma-derived Gas6 potentiates aggregation in stimulated human platelets under static conditions. Under conditions of moderate high shear, the use of Gas6-depleted plasma, unlike $P2Y_{12}$ blockage, did not lead to immediate embolization of platelets, but to delayed disaggregation, starting after several minutes. Apparently, at earlier time points, other autocrine factors – in particular, ADP – mask the involvement of Gas6. Given the limited ADP content of platelets, the continuous washout of

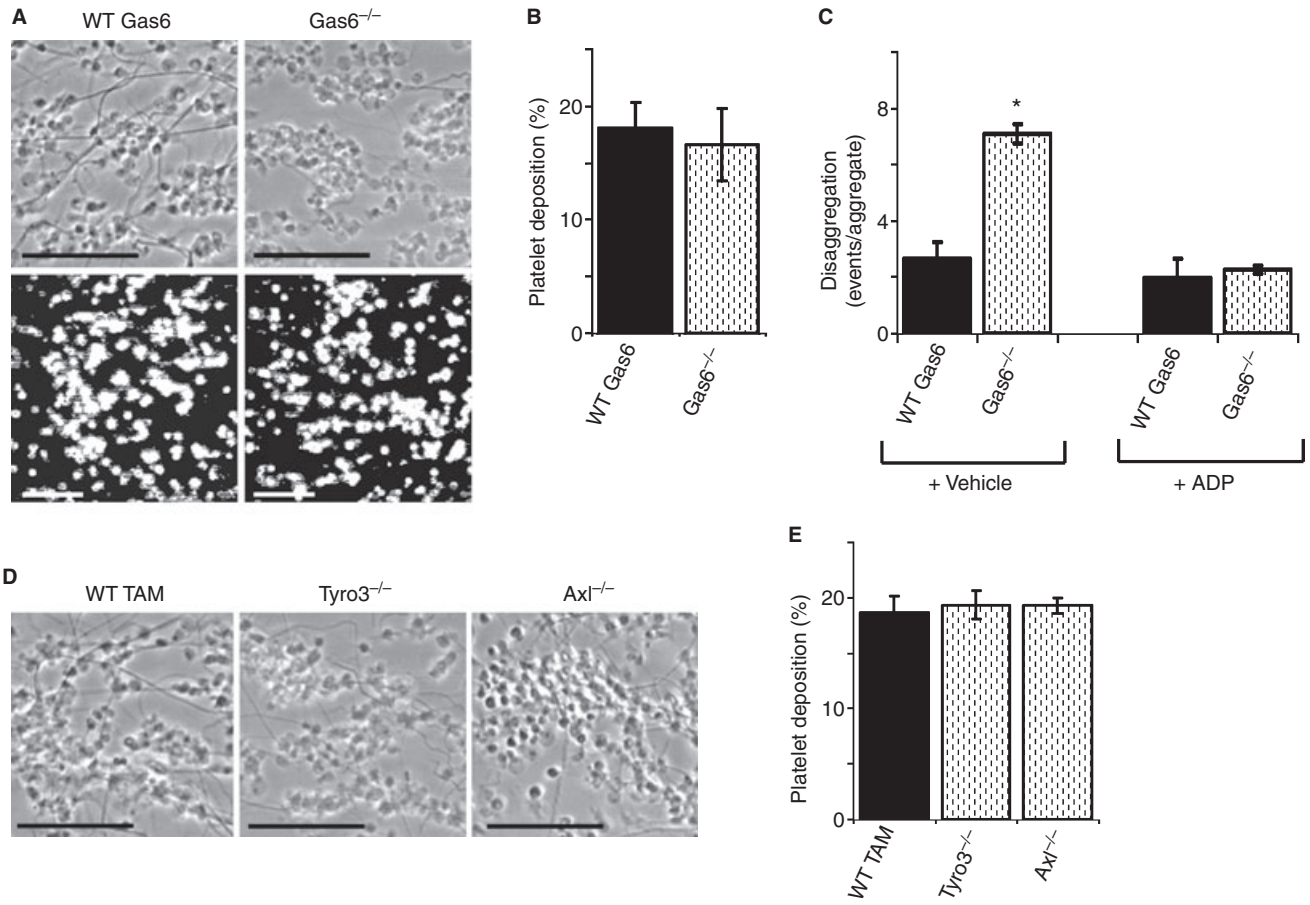


Fig. 6. Coperfusion with ADP normalizes thrombus formation in blood from mice deficient in Gas6 or Tyro3, Axl or Mer (TAM). Blood from mice deficient in Gas6, Tyro3 or Axl or corresponding wild-type (WT) mice was coperfused with ADP (40 μM, final concentration) during flow over collagen at 1000 s^{-1} for 4 min. Phosphatidylserine-exposing platelets were stained by post-perfusion with fluorescein isothiocyanate–annexin A5. (A, D) Representative phase contrast (50 × 50 μm) and fluorescent (100 × 100 μm) images after perfusion. (B, E) Surface area coverage of deposited platelets. (C) Effect of coinfusion with ADP on disaggregation during flow with $Gas6^{+/+}$ and $Gas6^{-/-}$ blood. Scale bar: 30 μm. Data are means ± standard errors of the mean ($n = 4$); $^*P < 0.05$.

ADP by flow [13], and the rapid degradation of ADP by plasma ectonucleotidases [27], it is conceivable that the contribution of Gas6 signaling to $\alpha_{IIb}\beta_3$ activation becomes critical under these conditions. In human platelets, it is still unclear which of the TAM receptors contributes to platelet function. One study has shown that anti-TAM receptor Abs impair agonist-induced platelet aggregation [28]. However, we have been unable to reproduce this finding with preparations of anti-human TAM receptor Abs, which were dialyzed to remove azide (data not shown). Recently, it has been suggested that the majority of Gas6 in plasma is bound to soluble Axl and therefore incapable of stimulating platelet TAM receptors [29]. However, our functional studies do not support this suggestion, as we found a consistent increase in Akt phosphorylation, $\alpha_{IIb}\beta_3$ activation and thrombus formation with native Gas6-containing plasma or blood.

For mice, the data point to a remarkable similarity in the phenotype of deficiency in Gas6 or in one of the TAM receptors, in that thrombus formation on collagen under flow is diminished, whereas the embolization of platelets from the thrombi is increased. In both Gas6-deficient and TAM-deficient mice, the defective thrombus formation could be fully restored by coinfusion of ADP. This is compatible with a mechanism whereby Gas6–TAM receptor and ADP–$P2Y_{12}$ receptor signaling synergize to induce the same platelet responses, leading to integrin activation and thrombus stabilization. In contrast, the absence of Gas6 or TAM receptors does not appear to impair platelet activation by collagen receptors, as the GPVI-induced exposure of PS of platelets on collagen was unchanged in all types of knockout blood. Combined, these findings provide a mechanistic explanation for earlier *in vivo* data showing reduced experimental thrombosis in mice lacking Gas6 or any of the TAM receptors [9,10], and also for data showing that deficiencies in two or three of the TAM receptors lead to a somewhat more severe platelet dysfunction [30].

With the present findings, Gas6 is added to the growing list of platelet agonists that are unable to stimulate platelets by

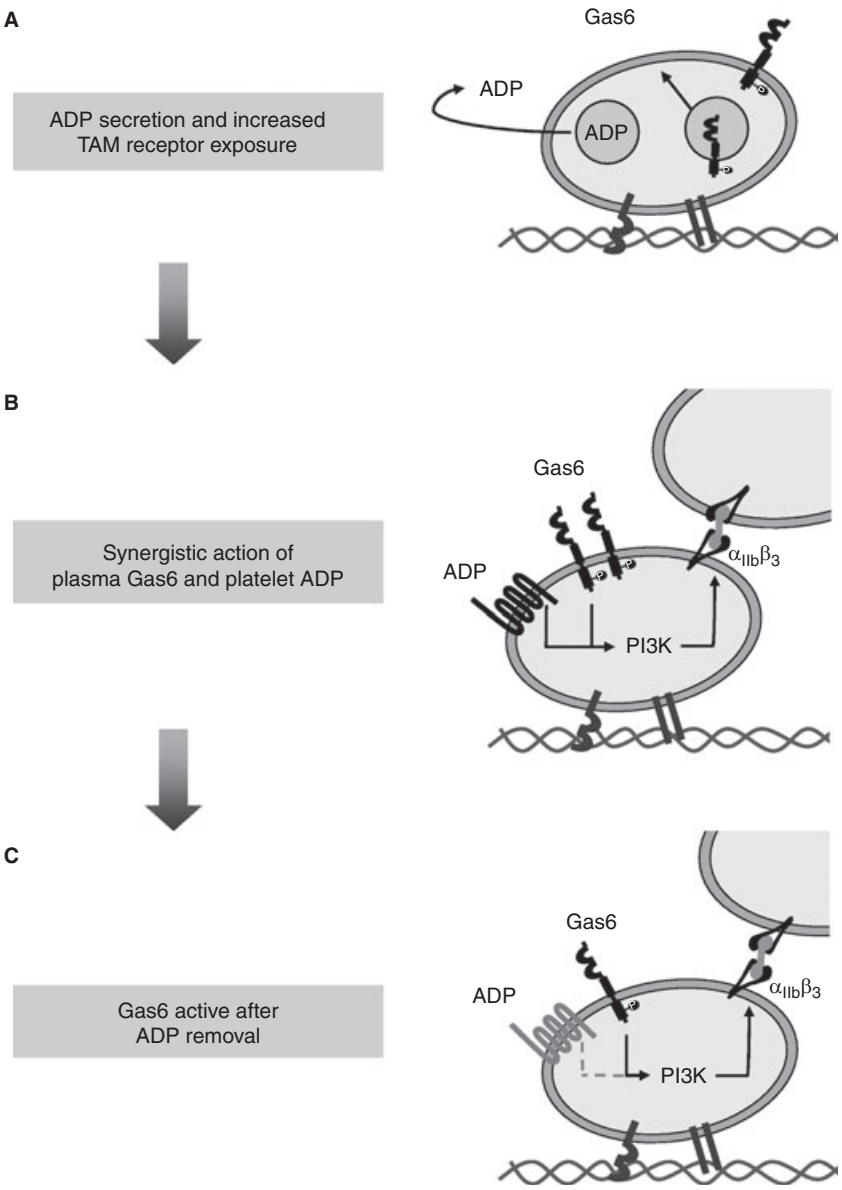


Fig. 7. Synergistic roles of ADP and Gas6 in thrombus formation and stabilization. (A) Activation of platelets results in the secretion of autocrine agents such as ADP and in increased exposure of Tyro3, Axl and Mer (TAM) receptors. (B) ADP (acting via $P2Y_{12}$) and Gas6 (acting via TAM) jointly elicit strong signaling via phosphoinositide 3-kinase (PI3K)–Akt to keep $\alpha_{IIb}\beta_3$ in the activated state. (C) The availability of autocrine ADP is time-restricted, owing to the limited amount of it in platelets and its rapid degradation by ectonucleotidases. At that point, Gas6-induced signaling of exposed TAM receptors via PI3K becomes critical for persistent $\alpha_{IIb}\beta_3$ activation and thrombus integrity.

themselves, but potentiate activation processes evoked by other agonists. Interestingly, two such potentiating compounds, insulin-like growth factor [31,32] and thrombopoietin [33], also enhance G_i-induced or GPVI-induced signaling pathways via PI3K and Akt phosphorylation. Also, the amplifying effects of matrix metalloproteinase-2 and ephrinB1 are suggested to be mediated, at least in part, by PI3K [34,35].

Together, the present results lead to an altered concept of the roles of Gas6 and TAM receptors in thrombus formation (Fig. 7). Receptors for ADP ($P2Y_{12}$) and Gas6 (Tyro3, Axl, and Mer) cooperate in the activation of PI3K to achieve persistent activation of $\alpha_{IIb}\beta_3$ and thrombus stabilization. Initially, secreted ADP is sufficient to prevent embolization by continuous $P2Y_{12}$-mediated signaling [13]. However, the role of autocrine ADP via $P2Y_{12}$ receptor stimulation is time-restricted, owing to the limited amount of ADP in platelets and its rapid degradation by ectonucleotidases. At this point, Gas6-induced signaling of TAM receptors via PI3K becomes critical for persistent $\alpha_{IIb}\beta_3$ activation and thrombus integrity. Thus, whereas others have shown that, under shear, platelets can also aggregate in the absence of integrin activation [36], our data point also to a time-dependent and place-dependent role of platelet-derived and plasma-derived platelet activators or amplifiers in regulating the highly dynamic process of throm-

bus formation. Targeting one of these amplifiers, such as Gas6, might provide a safe strategy for the development of new antithrombotic agents.

Addendum

J. M. E. M. Cosemans performed experiments, analyzed data, designed research, participated in discussions, and wrote the manuscript. R. van Kruchten performed experiments and analyzed data. S. Olieslagers performed experiments and analyzed data. L. J. Schurgers performed experiments, analyzed data, and contributed analytical tools. F. K. Verheyen performed experiments and analyzed data. I. C. A. Munnix performed experiments and analyzed data. J. Waltenberger contributed analytical tools. A. Angelillo-Scherrer contributed analytical tools. M. F. Hoylaerts contributed analytical tools and participated in discussions. P. Carmeliet contributed analytical tools. J. W. M. Heemskerk designed research, participated in discussions, and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 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**: 4976–85.

2 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**: 1219–26.

3 Lutgens E, Tjwa M, Garcia de Frutos P, Wijnands E, Beckers L, Dahlback B, Daemen MJ, Carmeliet P, Moons L. Genetic loss of Gas6 induces plaque stability in experimental atherosclerosis. *J Pathol* 2008; **216**: 55–63.

4 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**: 471–80.

5 Fernandez-Fernandez L, Bellido-Martin L, Garcia de Frutos P. Growth arrest-specific gene 6 (Gas6) An outline of its role in haemostasis and inflammation. *Thromb Haemost* 2008; **100**: 604–10.

6 Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, Masiakowski P, Ryan TE, Tobkes NJ, Chen DH, DiStefano PS, Long GL, Basilico C, Goldfarb MP, Lemke G, Glass DJ, *et al*. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* 1995; **80**: 661–70.

7 Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, Hunt RW, Trail G, Clogston C, Toso RJ, Yanagihara D, Bennett L, Sylber M, Merewether LA, Tseng A, Escobar E, Liu ET, Yamane HK. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature* 1995; **373**: 623–6.

8 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**: 30022–7.

9 Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlbäck B, Carmeliet P. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med* 2001; **7**: 215–21.

10 Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, Herbert JM, Lemke G, Goff SP, Matsushima GK, Earp HS, Vesin C, Hoylaerts MF, Plaisance S, Collen D, Conway EM, Wehrle-Haller B, Carmeliet P. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. *J Clin Invest* 2005; **115**: 237–46.

11 Hirsch E, Bosco O, Tropel P, Laffargue M, Calvez R, Altruda F, Wymann M, Montrucchio G. Resistance to thromboembolism in PI3Kγ-deficient mice. *FASEB J* 2001; **15**: 2019–21.

12 Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE, Kenche V, Anderson KE, Dopheide SM, Yuan Y, Sturgeon SA, Prabaharan H, Thompson PE, Smith GD, Shepherd PR, Daniele N, Kulkarni S, Abbott B, Saylik D, Jones C, *et al*. PI 3-kinase p110β: a new target for antithrombotic therapy. *Nat Med* 2005; **11**: 507–14.

13 Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Continuous signaling via PI3K isoforms β and γ is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood* 2006; **108**: 3045–52.

14 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**: 1118–23.

15 Balogh I, Hafizi S, Stenhoff J, Hansson K, Dahlbäck B. Analysis of Gas6 in human platelets and plasma. *Arterioscler Thromb Vasc Biol* 2005; **24**: 1280–6.

16 Clauser S, Peyrard S, Gaussem P, Crespin M, Emmerich J, Aiach M, Borgel D. Development of a novel immunoassay for the assessment of plasma Gas6 concentrations and their variation with hormonal status. *Clin Chem* 2007; **53**: 1808–13.

17 Alciato F, Sainaghi PP, Castello L, Bergamasco L, Carnieletto S, Avanzi GC. Development and validation of an ELISA method for detection of growth arrest specific 6 (Gas6) protein in human plasma. *J Immunoassay Immunochem* 2008; **29**: 167–80.

18 Clauser S, Bachelot-Lozat C, Fontana P, Gaussem P, Remones V, Aiach M, Borgel D. Physiological plasma Gas6 levels do not influence platelet aggregation. *Arterioscler Thromb Vasc Biol* 2006; **26**: e22.

19 van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak LP, Heemskerk JW. Platelet $P2Y_{12}$ receptors enhance signalling towards procoagulant activity and thrombin generation. A study with healthy subjects and patients at thrombotic risk. *Thromb Haemost* 2005; **93**: 1128–36.

20 Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, Lai C, Skinner MK, Klein R, Matsushima GK, Earp HS, Goff SP, Lemke G. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature* 1999; **398**: 723–8.

21 Heijnen HF, van Lier M, Waaijenborg S, Ohno-Iwashita Y, Waheed AA, Inomata M, Gorter G, Mobius W, Akkerman JW, Slot JW. Concentration of rafts in platelet filopodia correlates with recruitment of c-Src and CD63 to these domains. *J Thromb Haemost* 2003; **1**: 1161–73.

22 Dahlbäck B. Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin. *Biochem J* 1983; **209**: 837–46.
23 Siljander PR, Munnix IC, Smethurst PA, Deckmyn H, Lindhout T, Ouwehand WH, Farndale RW, Heemskerk JW. Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood. *Blood* 2004; **103**: 1333–41.
24 Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2484–90.
25 Cosemans JM, Iserbyt BF, Deckmyn H, Heemskerk JW. Multiple ways to switch platelet integrins on and off. *J Thromb Haemost* 2008; **6**: 1253–61.
26 Tjwa M, Bellido-Martin L, Lin Y, Lutgens E, Plaisance S, Bono F, Delesque-Touchard N, Herve C, Moura R, Billiau AD, Aparicio C, Levi M, Daemen M, Dewerchin M, Lupu F, Arnout J, Herbert JM, Waer M, Garcia de Frutos P, Dahlbäck B, *et al*. Gas6 promotes inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes. *Blood* 2008; **111**: 4096–105.
27 Cauwenberghs S, Feijge MA, Hageman G, Hoylaerts M, Akkerman JW, Curvers J, Heemskerk JW. Plasma ectonucleotidases prevent desensitization of purinergic receptors in stored platelets: importance for platelet activity during thrombus formation. *Transfusion* 2006; **46**: 1018–28.
28 Gould WR, Baxi SM, Schroeder R, Peng YW, Leadley RJ, Peterson JT, Perrin LA. Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic responses. *J Thromb Haemost* 2005; **3**: 733–41.
29 Ekman C, Stenhoff J, Dahlback B. Gas6 is complexed to the soluble tyrosine kinase receptor Axl in human blood. *J Thromb Haemost* 2010; **8**: 838–44.
30 Wang H, Chen S, Chen Y, Wang H, Wu H, Tang H, Xiong W, Ma J, Ge Y, Lu Q, Han D. The role of Tyro 3 subfamily receptors in the regulation of hemostasis and megakaryocytopoiesis. *Haematologica* 2007; **92**: 643–50.
31 Hers I. Insulin-like growth factor-1 potentiates platelet activation via the IRS/PI3Kα pathway. *Blood* 2007; **110**: 4243–52.
32 Kim S, Garcia A, Jackson SP, Kunapuli SP. Insulin-like growth factor-1 regulates platelet activation through PI3-Kα isoform. *Blood* 2007; **110**: 4206–13.
33 Pasquet JM, Gross BS, Gratacap MP, Quek L, Pasquet S, Payrastre B, van Willigen G, Mountford JC, Watson SP. Thrombopoietin potentiates collagen receptor signaling in platelets through a phosphatidylinositol 3-kinase-dependent pathway. *Blood* 2000; **95**: 3429–34.
34 Prevost N, Woulfe DS, Tognolini M, Tanaka T, Jian W, Fortna RR, Jiang H, Brass LF. Signaling by ephrinB1 and Eph kinases in platelets promotes Rap1 activation, platelet adhesion, and aggregation via effector pathways that do not require phosphorylation of ephrinB1. *Blood* 2004; **103**: 1348–55.
35 Falcinelli E, Guglielmini G, Torti M, Gresele P. Intraplatelet signaling mechanisms of the priming effect of matrix metalloproteinase-2 on platelet aggregation. *J Thromb Haemost* 2005; **3**: 2526–35.
36 Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* 2009; **15**: 665–73.