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Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis

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The growth arrest-specific gene 6 product (Gas6) is a secreted protein related to the anticoagulant protein S but its role in hemostasis is unknown. Here we show that inactivation of the *Gas6* gene prevented venous and arterial thrombosis in mice, and protected against fatal collagen/epinephrine-induced thrombo embolism. *Gas6*^{-/-} mice did not, however, suffer spontaneous bleeding and had normal bleeding after tail clipping. In addition, we found that Gas6 antibodies inhibited platelet aggregation *in vitro* and protected mice against fatal thrombo embolism without causing bleeding *in vivo*. Gas6 amplified platelet aggregation and secretion in response to known agonists. Platelet dysfunction in *Gas6*^{-/-} mice resembled that of patients with platelet signaling transduction defects. Thus, Gas6 is a platelet-response amplifier that plays a significant role in thrombosis. These findings warrant further evaluation of the possible therapeutic use of Gas6 inhibition for prevention of thrombosis.

Gas6, the product of the growth arrest-specific gene 6 (*Gas6*), is a new member of the vitamin K-dependent protein family^{1,2}. Proteins belonging to this family are characterized by post-translational γ -carboxylation of certain glutamic acid residues by a carboxylase, using vitamin K as cofactor. The γ -carboxyglutamic acid (Gla)-containing module in prothrombin, coagulation factors VII, IX and X, protein C, protein Z, protein S and Gas6 allows these vitamin K-dependent plasma proteins to bind to negatively charged phospholipid membranes³. Gas6 is structurally similar to protein S, but lacks a loop, crucial for the anticoagulant activity of protein S (ref. 2). The latter is a cofactor for activated protein C, which inactivates the coagulation factors Va and VIIIa (ref. 4). Genetic deficiency of protein S in humans is one of the most severe inherited risk factors for thrombosis⁵. To date, Gas6 is the only protein among Gla-module-containing proteins which has not been reported to play a role in hemostasis or thrombosis.

Apart from a Gla-domain-dependent interaction with phospholipid membranes⁶, Gas6 also binds as a ligand to the receptor tyrosine kinases Axl (Ark, Ufo, Tyro7), Sky (Rse, Tyro3, Dtk, Etk, Brt, Tif) and Mer (c-Mer, Eyk, Nyk)⁷⁻¹¹ by its carboxy-terminal globular G domains⁹. It has been implicated in reversible cell growth arrest², survival¹², proliferation¹²⁻¹⁴ and cell adhesion^{6,15,16}. Mice with a triple deficiency of Axl, Sky and Mer are viable, but have not been reported to suffer spontaneous bleeding or thrombosis¹⁷.

Here, we generated *Gas6*^{-/-} mice to investigate the role of Gas6 in hemostasis and thrombosis. We found that deficiency of Gas6 protected mice against fatal thrombosis, but did not induce

bleeding. Gas6 was found to amplify the aggregation and secretion response of platelets to known agonists, and the platelet dysfunction in *Gas6*-deficient mice resembled the platelet defects of patients with primary platelet signal transduction defects¹⁸. Gas6 antibodies protected mice against fatal thrombo embolism without inducing a bleeding tendency, indicating that inhibition of Gas6 might provide a novel means to safely block thrombosis.

Results

Normal hemostasis in *Gas6*^{-/-} mice

We inactivated *Gas6* by deleting the transcription start site, the translation initiation codon, the signal peptide and the Gla module that is required for binding of Gas6 to phospholipid membranes. We confirmed correct targeting at the DNA, RNA and protein level (Fig. 1a-c; Fig. 5b). Homozygous *Gas6*^{-/-} mice were born at the expected mendelian frequency: of 317 offspring from heterozygous *Gas6*^{+/-} breeding pairs, 72 were wild-type (*Gas6*^{+/+}), 168 were *Gas6*^{+/-} and 77 were *Gas6*^{-/-}. *Gas6*^{+/-} and *Gas6*^{-/-} mice were viable, fertile, appeared normal and showed no obvious differences in size, weight or behavior. No genotypic differences in litter size were observed (9.7 ± 2 for wild-type mice; $n = 23$ litters versus 9.3 ± 3 for *Gas6*^{-/-} mice; $n = 44$ litters; $P = \text{n.s.}$).

Gas6^{-/-} mice did not suffer spontaneous bleeding or thrombosis. Bleeding (estimated as the amount of blood loss) after tail clipping was comparable for both genotypes ($166 \pm 48 \mu\text{l}$ in wild-type mice versus $172 \pm 68 \mu\text{l}$ in *Gas6*^{-/-} mice; $n = 10$; $P = \text{n.s.}$). There were also no genotypic differences in the plasma levels of

Fig. 1 Targeting of the *Gas6* gene. **a**, Southern blot of HindIII-digested genomic DNA hybridized with an internal probe (within the 5'-flank), generating a 4.2 kb wild-type and a 5.8 kb *Gas6*-null fragment. **b**, Northern-blot analysis of total RNA from cultured mouse embryonic fibroblasts, hybridized with a *Gas6* or *protein S* cDNA probe; ethidium bromide stained rRNA bands indicate equal loading. **c**, Immunoblot analysis of kidney extracts using a rabbit polyclonal antibody against the murine C-terminal *Gas6* peptide.

coagulation factors (percent of wild-type: 88 ± 13 , 110 ± 8 , 94 ± 9 , 111 ± 21 , 93 ± 8 and 90 ± 5 for fibrinogen, factor II, V, VIII, IX and X; $n = 7-12$ mice; $P = \text{n.s.}$), the prothrombin and activated partial thromboplastin (aPTT) times (percent of wild-type: 100 ± 5 and 85 ± 25 , respectively; $P = \text{n.s.}$), and the counts of peripheral red cells, leukocytes and platelets (percent of wild-type: 102 ± 5 , 89 ± 14 and 98 ± 5 , respectively; $n = 7-12$; $P = \text{n.s.}$).

Protection of *Gas6*^{-/-} mice against thrombosis

We used three thrombosis models to determine the effects of *Gas6* deficiency. In the first model, thrombosis was induced by ligation of the abdominal caval vein. Stasis-induced venous thrombosis is known to result from both coagulation and platelet activation¹⁹. Compared with wild-type mice, thrombi in *Gas6*^{-/-} mice were on average 85% smaller ($P < 0.001$; Fig. 2a). In the second model, we induced a platelet- and fibrin-rich thrombus by photochemical denudation of the carotid artery²⁰. Compared with wild-type mice, the arterial thrombus in *Gas6*^{-/-} mice was on average 60% smaller ($P < 0.05$; Fig. 2b). In the third model, where we induced platelet-dependent thromboembolism by intravenous injection of collagen and epinephrine in anesthetized mice, 80% of wild-type mice died within 1 to 3 minutes as compared with 20% of *Gas6*^{-/-} mice that died within 10 to 15 minutes ($n = 10$; $P < 0.03$; Fig. 2c). These data indicate that thrombus formation in *Gas6*^{-/-} mice did not develop as rapidly and extensively as in wild-type mice. Macroscopic and histological analysis revealed extensive pulmonary thromboembolism in wild-type mice (Fig. 2e). In contrast, surviving *Gas6*^{-/-} mice lacked signs of pulmonary embolization (Fig. 2f). Thus, loss of *Gas6* significantly protected mice against venous and arterial thrombosis.

The resistance to thrombosis of *Gas6*^{-/-} mice was not due to increased thrombolysis. Lysis of [¹²⁵I]fibrin-labeled pulmonary plasma clots was comparable in both genotypes, regardless of whether platelet-poor (*P*) or platelet-rich plasma (PRP) was used to make these clots. Lysis of PRP-clots after 16 hours was $33 \pm 10\%$ in wild-type mice versus $30 \pm 7\%$ in *Gas6*^{-/-} mice ($n = 6$; $P = \text{n.s.}$), while lysis of *P*-clots after 16 hours was $67 \pm 8\%$ in wild-type mice versus $52 \pm 5\%$ in *Gas6*^{-/-} mice ($n = 6$; $P = \text{n.s.}$).

Impaired platelet aggregation in *Gas6*^{-/-} mice

The prothrombotic mechanisms of *Gas6* were further studied by analyzing its effects on blood coagulation and fibrin formation. Recombinant murine *Gas6* protein (r*Gas6*; 0.2–1 $\mu\text{g/ml}$) did not affect the aPTT of *Gas6*^{-/-} plasma (in seconds: 33 ± 2 , 34 ± 1 , 35 ± 1 and 34 ± 3 at 0, 0.2, 0.4 and 1 $\mu\text{g/ml}$ r*Gas6*; $n = 6$; $P = \text{n.s.}$), consistent with previous findings in human plasma that r*Gas6* only minimally altered the aPTT or the degradation of factor V induced by activated protein C (ref. 21).

As platelets are known to play an essential part in the venous and arterial thrombosis models used to demonstrate the anti-thrombotic *Gas6*^{-/-} phenotype, we examined the role of *Gas6* in platelet function. *Gas6*^{-/-} mice had a normal platelet count and number and morphology of megakaryocytes (data not shown). In addition, *Gas6*^{-/-} platelets appeared ultrastructurally normal (data not shown). However, platelet aggregation studies revealed significant functional defects in *Gas6*^{-/-} mice. Platelets from wild-type mice dose-dependently aggregated in response to ADP (Fig. 3a–d), collagen (Fig. 3e–h) or the TXA₂ analogue U46619 (Fig. 3i and j). We achieved maximal aggregation of platelets from wild-type mice at similar concentrations of these agonists as used previously²². In contrast, platelets from *Gas6*^{-/-} mice failed to irreversibly aggregate in response to low concentrations of ADP ($< 10 \mu\text{M}$), collagen (2 $\mu\text{g/ml}$) or U46619 (10 μM). At low agonist concentration, *Gas6*^{-/-} platelets only displayed shape change as revealed by an immediate decrease in light transmission after stimulation. However, higher concentrations of ADP (50 μM ; Fig. 3d), collagen (5–15 $\mu\text{g/ml}$; Fig. 3f–h) or U46619 (100 μM ; Fig. 3j) induced irreversible aggregation of *Gas6*^{-/-} platelets. Both wild-type and *Gas6*^{-/-} platelets aggregated normally in response to the phorbol ester phorbol-12-myristyl-13-acetate (PMA) or the Ca²⁺

Fig. 2 Inactivation or inhibition of *Gas6* protects mice against thrombosis. **a**, Stasis-induced thrombosis in the inferior vena cava ($n = 10$; mean $\pm$ s.e.m.; *, $P < 0.001$); recombinant murine *Gas6* restored thrombosis in *Gas6*^{-/-} mice (□). **b**, Thrombosis in the carotid artery induced by endothelial denudation ($n = 5$; *, $P < 0.05$). **c** and **d**, Thromboembolism induced by collagen/epinephrine injection in both genotypes (**c**; $n = 10$) and in wild-type mice injected with anti-*Gas6* antibodies or control antibodies (**d**; ▨, $n = 12$; ▩, $n = 16$; *, $P < 0.03$). **e** and **f**, Light microscopy (H&E staining) of the lungs after collagen/epinephrine injection, revealing extensive platelet thromboembolism (arrows) in wild-type mice (**e**) but not in surviving *Gas6*^{-/-} mice (**f**). Scale bars, 400 μm .

Fig. 3 Effect of Gas6 deficiency or of anti-Gas6 antibodies on platelet aggregation. **a–j**, Aggregation of wild-type (+/+) and Gas6 deficient (–/–) platelet-rich plasma. Platelets from Gas6^{–/–} mice were unable to fully aggregate in response to concentrations of ADP at 2.5 μM (**a**), 5 μM (**b**) and 10 μM (**c**), of collagen at 2 μg/ml (**e**) or of the thromboxane A₂ analogue U46619 at 10 μM (**i**), while higher concentrations of ADP (**d**; 50 μM), collagen (**f–h**; 5–15 μg/ml) or U46619 (**j**; 100 μM) induced irreversible platelet aggregation. Representative example of 4 independent experiments using PRP pooled from 4 to 6 wild-type or Gas6^{–/–} mice. Squares represent 2 min (X-axis) and 10% change in light transmission (Y-axis). **k**, Restoration of the impaired aggregation of Gas6^{–/–} platelets in response to ADP (5 μM) by recombinant Gas6 (1 μg/ml) to comparable levels as in wild-type platelets. Representative example of 3 independent experiments. **l** and **m**, Aggregation response to ADP (5 μM) of washed human platelets after preincubation with anti-Gas6 antibodies (**l**) or isotype-matched control antibodies (**m**), revealing that anti-Gas6 antibodies prevent platelet aggregation. Representative example of 3 independent experiments. Arrows in all panels indicate application of the platelet agonists.

immunogold-labeling of resting wild-type platelets revealed that Gas6 colocalized with fibrinogen in α-granules (data not shown). Upon activation of wild-type platelets with thrombin (1 U/ml), Gas6 became detectable on the surface of platelets by immunogold-labeling (data not shown). Flow cytometry confirmed that the levels of surface-bound Gas6 were minimal in resting human platelets but significantly increased upon activation by ADP (5 μM; Fig. 5c and d). These results extend previous observations of Gas6 in human megakaryocytes²⁴ and rat platelets²⁵.

Platelets also expressed Gas6 receptors. By RT-PCR analysis, transcripts of Axl, Sky and Mer were detected in human platelets (Fig. 5a). Immunogold-labeling revealed that Axl was localized on the surface of resting platelets (data not shown). Comparable amounts of Axl and Sky (Fig. 5b) were detectable in wild-type and Gas6^{–/–} platelets, indicating that the reduced response of Gas6^{–/–} platelets was not due to differences in Gas6 receptor expression. Thus, platelets produce and release Gas6, and express Gas6 receptors.

Impaired secretion in Gas6^{–/–} platelets

Ultrastructural analysis of the thrombin-induced platelet aggregates indicated a reduced ability of Gas6^{–/–} platelets to degranulate. Secretion of ADP from dense granules is essential for the formation of stable macro-aggregates after initial formation of small, unstable platelet aggregates²⁶. Secretion of dense granule

ionophore A23187 (data not shown).

Thrombin stimulated platelet aggregation comparably in both genotypes at all doses tested (data not shown). However, ultrastructural analysis revealed that thrombin-induced aggregates of Gas6^{–/–} platelets were abnormal. In wild-type aggregates, platelets were densely packed, made tight contacts with each other and were completely degranulated (Fig. 4a). In contrast, platelets in Gas6^{–/–} aggregates were loosely packed, displayed fewer and smaller contact sites and were incompletely degranulated (Fig. 4b). Fibrinogen is released from α-granules upon platelet activation and forms bridges, linking adjacent activated platelets²³. Flow cytometry of washed platelets revealed that fibrinogen was detectable on the surface of wild-type platelets after stimulation with ADP (20 μM; Fig. 4c). In contrast, levels of surface-bound fibrinogen in Gas6^{–/–} platelets did not increase upon stimulation (Fig. 4d), which may contribute to the loose assembly of Gas6^{–/–} platelet aggregates. The reduced amount of fibrinogen on Gas6^{–/–} platelet surfaces was not due to a defect of Gas6^{–/–} platelets to sequester fibrinogen, as revealed by the comparable amounts of immunoreactive fibrinogen in platelet lysates in both genotypes (Fig. 4e). Thus, impaired platelet aggregation in response to known agonists in Gas6^{–/–} mice contributed to their resistance to thrombosis.

Expression of Gas6 and its receptors in platelets

The defect of platelet aggregation in Gas6^{–/–} mice indicated that platelets produce and respond to Gas6. Therefore, the expression of Gas6 and its receptors Axl, Sky and Mer was studied in resting and stimulated platelets. By reverse transcriptase (RT)-PCR analysis, GAS6 mRNA transcripts were detected in human platelets (Fig. 5a). In addition, immunoblotting revealed the presence of Gas6 in platelet extracts and in the releasate of thrombin-activated platelets from wild-type but not from Gas6^{–/–} mice (Fig. 5b). Ultrastructural analysis combined with double

Table 1 ATP release in response to various agonists.

Agonist	Concentration	Wild-type mice	Gas6 ^{–/–} mice
ADP	20 μM	0.8 ± 0.08	0.2 ± 0.09*
	20 μM + rGas6	n.d.	0.75 ± 0.1
	50 μM	2.6 ± 0.8	1.3 ± 0.3*
Collagen	1 μg/ml	1.8 ± 0.3	< 0.1
	10 μg/ml	10 ± 1.0	11 ± 0.8
U46619	10 μM	6.5 ± 1.5	< 0.1
	100 μM	8.2 ± 2.0	7.6 ± 2.3
Thrombin	1 U/ml	17 ± 1.6	11 ± 1.2*
PMA	100 μM	2.3 ± 0.4	1.9 ± 0.6
A23187	8 μM	9.2 ± 3.1	7.3 ± 3.0

ATP release expressed in μM. The data represent the mean ± s.e.m. of 3 experiments using platelet-rich plasma (for ADP, collagen, U46619, PMA or A23187 stimulation) or washed platelets (for thrombin stimulation). Note that rGas6 (200 ng/ml) rescued the impaired ATP secretion of Gas6^{–/–} platelets. Each experiment was performed with a pool of 4 to 6 wild-type or Gas6^{–/–} mice. n.d. not done. *, *P* < 0.05 versus wild-type.

Fig. 4 Role of Gas6 in the formation of platelet macro-aggregates. **a** and **b**, Electron microscopy revealed that platelets at the borders of platelet aggregates in wild-type mice (+/+) were densely compacted and completely degranulated (**a**). In contrast, Gas6 deficient (-/-) platelet aggregates of a comparable size were loosely packed, had fewer contact sites and were incompletely degranulated (**b**). Arrows indicate α -granules and arrowheads indicate dense granules in **a** and **b**. Scale bars, 4 μ m. **c** and **d**, Flow-cytometry analysis of fibrinogen on washed wild-type (**c**) and Gas6^{-/-} (**d**) platelets (black line denotes resting platelets, green line denotes ADP-activated platelets), revealing that surface-bound fibrinogen levels only increased in stimulated wild-type but not in Gas6^{-/-} platelets. **e**, Western-blot analysis of washed platelet lysates reveals comparable amounts of fibrinogen in wild-type and Gas6^{-/-} platelets.

stores (evaluated by measuring release of ATP) was significantly impaired in Gas6^{-/-} platelets. Compared with wild-type platelets, release of ATP from Gas6^{-/-} platelets was significantly decreased in response to ADP, collagen or U46619, when these agonists were used at low concentrations, which only caused platelet shape changes or reversible platelet aggregation (Table 1). ATP release from Gas6^{-/-} platelets was also reduced in response to high concentrations of ADP (50 μ M) or thrombin (1 U/ml), consistent with the incomplete degranulation of thrombin-stimulated Gas6^{-/-} platelets (ultrastructural analysis). However, release of ATP was normal or only slightly reduced when Gas6^{-/-} platelets were stimulated with high concentrations of collagen (10 μ g/ml) or U46619 (100 μ M; Table 1), which cause irreversible platelet aggregation. PMA and the Ca²⁺ ionophore A23187 induced a normal secretory response in both genotypes (Table 1). Secretion of α -granules, as assessed by measurement of surface expression of P-selectin during platelet activation, was also impaired in Gas6^{-/-} platelets (Fig. 5e and f). Thus, we found a close correlation between the defects in the aggregation and secretion response of Gas6^{-/-} platelets to various agonists.

Production of TXA₂, which contributes to the formation of stable macro-aggregates²⁶, was normal in both genotypes. Production of TXA₂ in serum and upon activation of platelets by thrombin (5 U/ml) was estimated by measurement of TXB₂. Levels of TXB₂ were 110 $\pm$ 63 ng/ml in serum and 53 $\pm$ 23 ng/ml after thrombin activation of wild-type mice versus 120 $\pm$ 41

ng/ml in serum and 55 $\pm$ 16 ng/ml after thrombin activation of Gas6^{-/-} mice (n = 6; P = n.s.).

Restoration of the Gas6^{-/-} phenotype by recombinant Gas6

To confirm that the platelet dysfunction in Gas6^{-/-} mice was due to deficiency of Gas6, we evaluated the effect of recombinant murine Gas6 (rGas6) on the impaired aggregation and secretion of Gas6^{-/-} platelets *in vitro*. Whereas rGas6 itself—at a concentration of up to 10 μ g/ml—was unable to induce a shape change or aggregation of wild-type or Gas6^{-/-} platelets, a concentration of 200 ng/ml restored the defective aggregation (Fig. 3k) and ATP secretion (Fig. 5g) of Gas6^{-/-} platelets in response to ADP. Moreover, the thrombotic defect in Gas6^{-/-} mice *in vivo* was restored by administering rGas6 at a dose of 100 μ g/kg (n = 8; P > 0.05 by comparison to wild-type mice; Fig. 2a). These findings indicate that Gas6 stimulates platelet function by amplifying the response to other platelet activators.

Antibodies against Gas6 inhibit platelet function

In order to examine whether inhibitors of Gas6 might be useful to prevent thrombosis, we studied the effect of antibodies specific for Gas6 on platelet aggregation *in vitro* and on thrombo

Fig. 5 Expression and role of Gas6 in platelets. **a**, RT-PCR analysis of Gas6 and its receptors Axl, Sky and Mer in human platelets. **b**, Western-blot analysis revealing Gas6 in extracts of resting platelets and in releasates of thrombin-activated platelets from wild-type but not from Gas6^{-/-} mice and comparable expression of the Gas6 receptors Axl and Sky. **c** and **d**, Flow cytometry using Gas6-antibodies, revealing minimal Gas6 on the surface of resting human platelets (**c**) and increased levels of Gas6 on human platelets stimulated by ADP 5 μ M (**d**). **e** and **f**, Flow cytometry of P-selectin on resting platelets (black line) and on ADP-activated platelets (green line), revealing that surface expression of P-selectin increased in stimulated wild-type (**e**) but not in Gas6^{-/-} (**f**) platelets. **g**, Rescue by rGas6 (200 ng/ml) of the defective Gas6^{-/-} platelet ATP secretion in response to ADP 20 μ M. A representative tracing of ATP secretion by Gas6^{-/-} platelets with and without rGas6, and by wild-type platelets is displayed. The average ATP levels (μ M) after rGas6 rescue are indicated in Table 1. The arrow indicates the time of application of ADP.

embolism after a collagen/epinephrine challenge *in vivo*. We used antibodies directed against the C-terminal part of Gas6—responsible for binding of Gas6 to its receptors²⁷. In contrast to isotype-matched control antibodies, Gas6-neutralizing antibodies dose-dependently blocked aggregation of washed human platelets in response to ADP (5 μ M; Fig. 3*l* and *m*), but had no effect in the absence of ADP. Since no rGas6 was exogenously added, the antibodies blocked Gas6 released from platelet stores and acting extracellularly to stimulate platelet aggregation.

Importantly, Gas6-neutralizing antibodies protected wild-type mice against the fatal collagen/epinephrine-induced thrombo embolism to the same degree (75% survival, $n = 12$; Fig. 2*d*) as genetic loss of Gas6 (80% survival in *Gas6*^{-/-} mice; Fig. 2*c*). Control antibodies were ineffective in preventing fatal thrombo embolism in wild-type mice (25% survival, $n = 16$; Fig. 2*d*). Gas6 antibody-treated mice did not show any signs of bleeding. These results indicate that inhibition of Gas6 effectively blocks thrombosis.

Discussion

Here we provide genetic evidence for a novel role of Gas6 in thrombosis. *Gas6*^{-/-} mice are protected against arterial and venous thrombosis, but do not suffer spontaneous or trauma-induced bleeding. The antithrombotic mechanism of Gas6 deficiency is at least partly due to defective platelet aggregation and secretion. Gas6, though ineffective itself, amplifies the response to known platelet agonists. Neutralizing Gas6 antibodies protect wild-type mice against fatal thrombo embolism without causing spontaneous bleeding.

Gas6^{-/-} mice were resistant to thrombosis as assessed using models known to depend on coagulation and platelets^{19,20,28}. Their resistance to thrombosis was not due to differences in coagulation, fibrinolysis or megakaryopoiesis, but to platelet dysfunction. Though ineffective by itself, Gas6 significantly enhanced the formation of stable platelet macro-aggregates in response to several platelet agonists. In the absence of Gas6, low concentrations of these agonists could only induce reorganization of actin filaments, responsible for the shape change preceding initial platelet micro-aggregation. Signaling by the ADP, collagen, TXA₂ or thrombin receptors was not completely blocked in *Gas6*^{-/-} platelets, as a shape change did occur in response to low concentrations and irreversible platelet aggregation proceeded in response to high concentrations. Only thrombin induced aggregation of *Gas6*^{-/-} platelets at low concentrations, but these aggregates were smaller, loosely packed and incompletely degranulated. Thus, secretion and aggregation of platelets could occur, but both were less efficient in *Gas6*^{-/-} platelets. As secretion of ADP is essential to secure formation of stable platelet macro-aggregates, only unstable *Gas6*^{-/-} platelet microaggregates formed at low agonist concentration. Higher concentrations of platelet agonists or a potent agonist like thrombin were required to induce formation of stable *Gas6*^{-/-} platelet macro-aggregates. An additional mechanism that could contribute to the defective platelet aggregation in *Gas6*^{-/-} mice may relate to the reduced formation of fibrinogen bridges linking adjacent activated platelets.

An autocrine role for Gas6 in platelets is indicated by the finding that Gas6 is present in α -granules and, following platelet activation, becomes secreted and bound to Gas6 receptors. As *Gas6*^{-/-} platelets have normal expression of the Gas6 receptors Axl or Sky, the platelet defects were not related to downregulation of these receptors. Collectively, our data are consistent with

a model where Gas6 is released from the α -granules upon initial stimulation of platelets by several agonists. Subsequently, Gas6 amplifies—by signaling through one or more of its receptors—the intracellular signals generated from the ADP, collagen, TXA₂ and thrombin receptors. Gas6 might exert this amplification signal at the level or downstream of the platelet agonist receptors, but most likely upstream of protein kinase C activation or Ca²⁺ mobilization. Indeed, the downstream pathways mediating granule secretion and platelet aggregation¹⁸ were functional in *Gas6*^{-/-} platelets, since PMA or the Ca²⁺ ionophore A23186 induced normal secretion and aggregation.

Consistent with Evenas *et al.*²¹, Gas6 does not seem to proteolytically activate existing coagulation factors, but we cannot exclude the possibility that Gas6 triggers expression of some essential coagulation factors. Indeed, Gas6 could also activate leukocytes and endothelial cells, as these cells express Gas6 receptors^{29,30}. Gas6 might also assist platelet aggregation by physically linking platelets together, for example its C-terminal G-domain could bind a Gas6 receptor on one platelet and form a bridge to another platelet via binding of its N-terminal Gla-domain to phospholipids on an adjacent platelet. This cell-adhesion activity of Gas6 may assist, but does not appear to mediate, platelet aggregation, as Gas6 by itself was unable to induce platelet aggregation. In addition, *Gas6*^{-/-} platelets formed aggregates when stimulated by high concentrations of agonists.

Congenital abnormalities in platelet aggregation and secretion have been identified in a number of patients suffering from mild bleeding syndromes¹⁸. Some of these patients may have a 'signal transduction defect'¹⁸. Like *Gas6*^{-/-} mice, patients with primary signaling transduction defects have impaired secretion of dense granules in response to weak agonists or to low concentrations of potent agonists. Fibrinogen levels on platelets are also reduced in these patients, but their number of platelet granules, TXA₂ production and initial aggregation are normal³¹. The present investigation indicates that Gas6 defects might constitute a possible mechanism of some of these primary signal transduction defects.

A function for Gas6 or its receptors in thrombosis has not been demonstrated previously. Deficiency of the purinergic P2Y₁ receptor^{32,33} or of GTP-binding G α_q (ref. 22) displays a comparable protection against fatal collagen-induced thrombo embolism. However, in contrast to these other mouse models, Gas6 deficiency did not increase bleeding time after tail clipping. Thus, Gas6 appears to be redundant for baseline hemostasis, but constitutes an important 'amplification' system in pathological conditions. Precisely because Gas6 only amplifies the response of other platelet agonists—while not evoking a response itself—inhibition of Gas6 might constitute an attractive treatment to prevent thrombosis without causing bleeding side effects.

Methods

Generation of *Gas6*^{-/-} mice. We screened a SVJ mouse genomic library in lambda FIX II (Stratagene) with cDNA probes of mouse *Gas6* (from C. Schneider). A total homology of 6.2 kb was used to construct the targeting vector *pPNT.gas6*. R1 embryonic stem cells were electroporated with NotI-linearized *pPNT.gas6*. The correctly targeted ES cell clones were used for aggregation with Swiss morula embryos to generate chimeric animals, which were test bred for germline transmission, and the resulting heterozygous mice intercrossed to obtain homozygous offspring. Genotyping was performed by Southern-blot analysis or PCR amplification of mouse tail DNA using allele-specific probes. Housing and procedures involving experimental animals were approved by the Institutional Animal Care and Research Advisory Committee of the University in Leuven³⁴.

Hemostasis, thrombosis models and thrombolysis. We anesthetized mice by intraperitoneal injection of sodium pentobarbital (60 mg/kg). Thrombus formation due to stasis was induced by tightening two sutures, separated 0.7 cm apart in the inferior *vena cava*, for 20 min³⁵. Thrombosis was quantified by weighing the thrombus after rinsing, blotting on filter paper and drying overnight at 60 °C (ref. 35). For rescue experiments, *Gas6*^{-/-} mice received 100 µg/kg of r*Gas6*. Thrombus formation in the carotid artery by photochemical denudation of the endothelium was established by irradiation of the exposed artery with green light (wavelength: 540 nm) from a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) after intravenous administration of Rose Bengal as described³⁷. To induce thrombo embolism, a mixture of collagen (0.5 mg/kg, equine collagen; Hormon Chemie, München, Germany) and epinephrine (60 µg/kg) was injected into the jugular vein²⁸. When indicated, mice received 100 µg goat-anti-human *Gas6* (directed against the C-terminal part of *Gas6*) or control isotype-matched antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Thrombolysis of PRP or *P* plasma clots embolized into the lungs was measured as described³⁸. Bleeding was measured by tail tip transsection as described³⁹.

Platelet aggregation and secretion. Whole blood, drawn from anesthetized mice from the inferior *vena cava* into 4% citrate (1 volume anticoagulant/9 volumes blood), was centrifuged at 100g (10 min) to obtain PRP and additionally at 2,000g (10 min) to obtain *P*. PRP and *P* were pooled from four *Gas6*^{-/-} or wild-type mice. Washed platelets were prepared with blood drawn from the inferior *vena cava* into acid-citrate-dextrose solution (ACD) (1 volume ACD /6 volumes blood). Apyrase was added to PRP (final concentration, 1 U/ml), and platelets were washed by adding 2 vol ACD and centrifuged at 2,000g (10 min). The platelet pellet was resuspended in Tyrode's buffer containing 1% BSA. For experiments using washed human platelets, blood from volunteers (9 volumes) was anticoagulated with 3.13% citrate (1 volume). Washed platelets were prepared as mentioned above.

We measured platelet aggregation turbidimetrically using an optical Chronolog aggregometer (model 490, Coulter, Hialeah, Florida). When indicated, human washed platelets were preincubated with anti-*Gas6* or irrelevant antibodies (Santa Cruz) before stimulation with ADP (4 min). Platelet ATP release was monitored by adding firefly luciferase and luciferin and comparing the luminescence generated by platelet ATP release or an ATP standard (Chrono-Lume, Kordia, The Netherlands). When indicated, platelet aggregation or ATP release were performed in the presence of recombinant murine *Gas6*. TXA₂ production was measured by TXB₂ assay using an EIA Biotrak kit (Amersham International, UK).

Production of r*Gas6*, western-blot analysis and PCR analysis. r*Gas6* was produced in 293 cells stably transfected with the pcDNA3 expression vector encoding the mouse *Gas6* cDNA. r*Gas6* was purified using a modification of a calcium affinity method used for Gla-containing proteins^{40,41}. Washed platelet extracts were immunoblotted after separation by SDS-PAGE using the following antibodies: anti-human *Gas6* and anti-human Axl (both from Santa Cruz), anti-murine Sky or anti-murine fibrinogen (Nordic Immunology, Tilburg, The Netherlands). PCR was performed on a human platelet cDNA library as described⁴² using the following primers: for *Gas6*, 5'-AGCTGCTCGAGGCGCTGTTGCCGCGC-3' and 5'-AGCTGCTCGAGGACCACTGCACGCCCAACC-3' (ref. 27); for *axl*, 5'-GGTGGCTGTGAAGACG ATGA-3' and 5'-CTCAGATACTCCATGCCATCT-3' (ref. 29); for *mer*, 5'-CACCTCTGCCTTACCACAT CT-3' and 5'-ATCCACAAAGCAGCCCAA AGA-3' (ref. 43); for *sky*, 5'-CAATCTGAGCAGCTACCAA-3' and 5'-GGACAGAAGAGGCTGTCCAG-3'.

Electron microscopy and flow cytometry. Washed platelets, stimulated by thrombin 1 U/ml (5 min) under stirring conditions, were fixed with 2% glutaraldehyde in sodium cacodylate-HCl buffer (pH 7.2-7.4) and post-fixed in 1% osmium tetroxide in the same buffer before embedding in Epon resin. For immuno EM, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 was used as described⁴⁴. The sections were examined using a Philips 201 electron microscope. To analyze surface expression of *Gas6*, washed resting and stimulated platelets were incubated with goat anti-human *Gas6* antibody (Santa Cruz) and, subsequently, with fluorescein isothiocyanate (FITC)-rabbit anti-goat antibody (Dako, Glostrup,

Denmark). For surface expression of fibrinogen or P-selectin, a FITC-fibrinogen rabbit polyclonal antibody (Dako, Glostrup, Denmark) or a FITC-P-selectin rat anti-mouse antibody (BD Pharmingen, San Diego, California) were used. After incubation, samples were diluted and immediately analyzed on a FACS calibur flow cytometer (Becton Dickinson, Rungis, France).

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