Prothrombotic Effects of Fibronectin Isoforms Containing the EDA Domain

Anil K. Chauhan, Janka Kisucka, Maria R. Cozzi, Meghan T. Walsh, Federico A. Moretti, Monica Battiston, Mario Mazzucato, Luigi De Marco, Francisco E. Baralle, Denisa D. Wagner, André F. Muro

Objective—Fibronectin (FN) plays an important role in the formation of stable arterial thrombi at the site of vascular injury. FN containing Extra Domain A (EDA-FN) is absent from normal plasma, but elevated plasma levels of EDA-FN are found in several pathological conditions. We hypothesized that EDA-FN plays a special role in thrombosis.

Methods and Results—We used mouse strains constitutively including (EDA+/+) or excluding (EDA−/−) the EDA domain in all tissues and plasma. Using a flow chamber and the ferric-chloride injury model we found that EDA-FN accelerates thrombosis both in vitro and in vivo at arterial shear rates. In EDA+/+ mice thrombi (>30 μm) grew faster when compared with EDAWT/WT (6.6 ± 0.2 minutes versus 8.3 ± 0.6 minutes, P < 0.05) and the mean vessel occlusion time was shorter (9.9 ± 0.4 minutes versus 14.6 ± 1.7 minutes, P < 0.05). However, the presence of EDA-FN affected neither single platelet adhesion to subendothelium nor thrombosis in veins. In addition, the mortality rate of EDA+/+ mice after collagen/epinephrine infusion was twice that of EDAWT/WT or EDA−/− mice.

Conclusions—Our findings reveal that EDA-FN has prothrombotic activity, and its presence in plasma may worsen pathological conditions in which this form is elevated. (Arterioscler Thromb Vasc Biol. 2008;28:296-301)

Key Words: plasma fibronectin ■ fibronectin splice variants isoforms ■ arterial thrombosis ■ intravital microscopy ■ thromboembolism ■ arterial and venous injury

The formation of a thrombus in an injured vessel wall is a complex process that involves multiple adhesion molecules and their respective receptors on the platelet surface. von Willebrand factor (vWF) and fibrinogen are considered the major ligands mediating platelet adhesion and aggregation. However, in an experimental model 73% of the injured vessels of mice lacking both fibrinogen and vWF still formed occlusive thrombi either at the site of injury or downstream, suggesting that other major adhesive proteins, such as fibronectin (FN), might contribute to the process. Incorporation of FN into a growing thrombus was shown both in vitro and in vivo. Additionally, it was documented in vivo that the depletion or lower levels (50%) of plasma FN result in serious defects in arterial thrombosis. FN is a dimeric multidomain glycoprotein playing an important role in adhesion, migration, growth and differentiation of cells. FN generates protein diversity as a consequence of alternative processing of a single primary transcript at 3 sites: the Extra Domain B (EDB, EDII, or EIII-B), Extra Domain A (EDA, EDI, or EIIIA), and the Type III Homologies Connecting Segment (IICS) (Figure 1A). Two major forms of FN exist: soluble plasma FN (pFN), which lacks both the EDA and EDB domains (Figure 1A); and cellular FN (cFN), which is deposited as insoluble fibrils in the extracellular matrix (ECM) and contains these domains at variable proportions. FN is a ligand for many members of the integrin receptor family and binds to thrombosis-related proteins including heparin, collagen, and fibrin. Platelets contain both EDA-FN and EDA-FN, suggesting that EDA-FN may have some special role in thrombosis. Disease states such as atherosclerosis, pulmonary and acute vascular injury, diabetes, thrombocytopenic purpura, and ischemic stroke are accompanied by elevated plasma levels of EDA-FN. Prothrombotic activity of Fn affects neither single platelet adhesion to subendothelium nor thrombosis in veins. In addition, the mortality rate of EDA+/+ mice after collagen/epinephrine infusion was twice that of EDAWT/WT or EDA−/− mice. Our findings reveal that EDA-FN has prothrombotic activity, and its presence in plasma may worsen pathological conditions in which this form is elevated. (Arterioscler Thromb Vasc Biol. 2008;28:296-301)
pulmonary thromboembolism. We found that mice exclusively having the EDA FN isoform in plasma and platelets had increased thrombosis, revealing that this FN isoform has prothrombotic activity.

Materials and Methods

Animals

The generation of the mice devoid of regulated splicing at the EDA exon has been previously described and is briefly detailed in the Supplemental Data I Section (available online at http://atvb.ahajournals.org). The mice used for intravital microscopy were male and female young mice (approx 3 to 4 weeks old), weighing 14 to 18 g. Infused platelets were isolated from 4- to 6-month-old mice of the same genotype. Animals were bred and housed at the ICGEB and the CBR Institute for Biomedical Research. Experimental procedures were approved by the Animal Care and Use Committees of each institution.

Blood Sampling and Platelet Preparation

Blood sampling and platelet preparation were done as previously described and is briefly detailed in the Supplemental Data I Section.

Platelet Aggregation Test

Platelet count was adjusted to the same concentration (3×10⁶ platelets/mL) with modified Tyrode’s buffer containing 1 mmol/L CaCl₂ and maintained at 37°C. Aggregation was initiated by adding the following agonists: ADP, thrombin, collagen (Nycomed), and protease-activated receptor-4 (PAR4) to platelet rich plasma (PRP) or to washed platelets and was monitored by light transmission that was recorded over 20 minutes on a Chrono-Log 4-channel optical aggregation system.

Pulmonary Thromboembolism

Pulmonary thromboembolism was induced as described previously with slight modifications. Briefly, thromboembolism was induced by intravenous injection of a mixture of soluble-collagen and epinephrine (40 μg [Nycomed] and 4.8 μg [Sigma-Aldrich], respectively, per 30 g body weight) into the tail veins of mice. Mice were randomized and genotypes kept unknown until the end of the experiment. The animals were observed for 1 hour from the time of injection. The time to death was normally less than 3 minutes.

Flow Chamber

Platelet interaction with immobilized collagen under flow conditions was studied as previously described and is briefly detailed in the Supplemental Data I Section.

Thrombus in Arterioles and Venules

A previously described model for arterial and venous thrombosis was used. Mice were anesthetized with 2.5% tribromoethanol (0.15 mL/10 g) and fluorescent platelets (2.5×10⁹ platelets/kg) were infused through the retro-orbital plexus of the eye. An incision was made through the abdominal wall and mesentery arterioles of approx 100 μm in diameter, and mesentery veins of 200 to 300 μm were studied. Whatman paper saturated with ferric chloride (10%) solution was applied topically for 5 minutes, which induced denudation of the endothelium, and the vessel was monitored and video-recorded for 40 minutes after injury or until occlusion.

Statistical Analysis

Results are reported as the mean±SEM, unless otherwise noted. The statistical significance of the difference between means was assessed by the ANOVA followed by the Bonferroni test. A value of P<0.05 was considered statistically significant. Thromboembolism data were analyzed by the Fisher test.

Results

Constitutive Inclusion of EDA Domain in FN

Results in Decreased Levels of FN in Platelets

Previously we have documented that constitutive inclusion of the EDA exon results in ~70% to 80% decrease of FN in plasma of EDA−/− mice compared with EDAWT/WT (Figure 1B). Because most of the FN present in platelets is endocytosed from plasma, we hypothesized that the amount of total FN in platelets was also decreased. As expected, platelet-FN in the EDA−/− mice was ~30% to 35% of the EDAWT/WT levels (Figure 1C). The FN levels in the platelets of the EDA−/− mice were similar to those of EDAWT/WT (Figure 1C). Western blot analysis using a monoclonal antibody specific for the EDA domain showed that EDAWT/WT platelets had roughly 20% of the amount found in the EDA−/− platelets (not shown). No significant differences among the genotypes were observed for vitronectin and fibrinogen in washed platelets (not shown).

Normal Platelet Aggregation, Whole Blood Clotting Time, and Bleeding Time

Because various pathological conditions are accompanied by elevated levels of EDA FN in plasma, we evaluated whether EDA FN will affect platelet aggregation. In vitro aggregation was initiated by adding agonists (ADP, thrombin, collagen, and PAR4) to platelet-rich plasma (PRP) or to washed platelets and was monitored by light transmission. There were no significant differences in platelet aggregation of the EDA−/− or EDA+/− PRP compared with EDAWT/WT PRP, when triggered by different agonists at 2 different concentrations of the agonist (Supplemental Data II Section). Similarly to PRP, washed platelets from either genotype did not show any difference in platelet aggregation (data not shown). These results show that the presence of the EDA FN isoform in plasma or in platelet α-granules does not contribute to platelet aggregation at low shear in vitro.

pFN is an important component of fibrin clot. However, reduction of FN (50% of WT levels) or deficiency of plasma...
FN does not affect clotting time and bleeding time in the mice.3,25 We evaluated whether the presence of EDA FN in plasma may affect hemostatic parameters such as whole blood clotting time, clot rate, and bleeding time. The onset and clotting rate of whole blood was similar in the EDA+/− mice compared with EDAWT/WT mice (Supplemental Data III Section). Additionally, EDA FN neither affected tail bleeding time (Supplemental Data III Section) nor other hemostatic values such as PTT, Antithrombin III, dimer and fibrinogen concentration in plasma (not shown).

Pulmonary Thromboembolism
To explore the in vivo role of EDA FN in platelet aggregation, we used a well-established pulmonary thromboembolism model.20,21 A preliminary dose-response experiment was done using EDAWT/WT mice to select the minimal dose which gave reproducible 40% to 50% mortality (not shown). We found that the mortality rate in EDA+/− mice after collagen/epinephrine infusion was almost twice that of EDAWT/WT (66% as compared with 38% in EDAWT/WT, Fisher Test P<0.007, Figure 2). The mortality rate in EDA−/− mice was similar to EDAWT/WT (43% in EDA−/− mice) and significantly lower than in EDA+/− mice (EDA+/− versus EDA−/−, P<0.025). This observation indicated to us that the presence of EDA FN in the plasma might have a prothrombotic activity.

EDAFN Augments Thrombus Growth at Arterial Shear Rate in a Flow Chamber
Because the EDA−/− mice were more sensitive in the pulmonary thromboembolism model, we hypothesized that EDA FN might be prothrombotic, playing a special role in promoting thrombus growth. We performed in vitro flow chamber studies with whole blood from EDAWT/WT, EDA+/−, and EDA−/− mice at venous and arterial shear rates in a parallel plate chamber at 37°C over glass coverslips coated with fibrillar collagen type I (Figure 3). To determine the size of thrombi, the surface area covered by the mepacrine-labeled fluorescent platelets was quantified. The mean ±95% confidence intervals of 6 different optical fields in 3 separate experiments is plotted in Figure 3B. We found that EDA+/− blood formed significantly bigger thrombi covering a larger area than EDA−/− or EDAWT/WT samples when perfused over collagen for 1 minute at shear rate of 1500 s−1 (mean±SEM, 15.5±2.4%, 30.5±5.2%, and 18.7±2.7% for EDAWT/WT, EDA+/−, and EDA−/−, respectively; ANOVA Test, P≤0.0001, Bonferroni correction EDAWT/WT versus EDA+/−, P≤0.001, EDA+/− versus EDA−/−, P≤0.01) suggesting a role of EDA FN in augmenting thrombus growth. However, at venous shear rate of 250 s−1 the percentage surface area covered were similar among EDAWT/WT, EDA+/−, and EDA−/− mice (mean±SEM, 7.0±0.9%, 9.2±2.6%, and 10.1±2.2% for EDAWT/WT, EDA+/−, and EDA−/−, respectively; Figure 3). These studies suggest that the EDA FN plays a prominent role only at arterial shear rates.

Inclusion of EDA Domain in FN Accelerates Thrombus Formation
After finding that the EDA FN promotes thrombus growth in vitro we asked whether the presence of EDA FN in platelets and plasma would also accelerate thrombosis in vivo. We first evaluated whether EDA containing FN could enhance platelet adhesion at an early time point after ferric chloride-induced endothelial injury. The number of single platelets adhering within 2 to 3 minutes after injury was not significantly different in EDA+/− mice when compared with EDAWT/WT or EDA−/− mice (EDAWT/WT=328±54, EDA+/−=299±33, EDA−/−=317±31) suggesting that the EDA FN does not promote early platelet interaction with the subendothelium.

Figure 2. Increased susceptibility of EDA+/− mice to collagen-epinephrine induced pulmonary thromboembolism. Data represent percentage (% of mortality after the injection of a mixture of collagen and epinephrine through the tail vein. The mortality was twice as high in EDA+/− compared with EDAWT/WT or EDA−/− mice. n=39 to 42 mice per genotype.

Figure 3. EDA+/− mice form larger thrombi at arterial shear rate in a flow chamber. Whole blood was perfused in a parallel plate chamber coated with type I collagen. A, Representative single-frame images at venous and arterial shear rates. B, Quantification of the surface area covered by platelets after perfusion.
Using the ferric chloride injury model, it was previously documented that deficiency or lower levels (50%) of pFN result in serious arterial thrombosis defect. Strikingly, in spite of the decrease in pFN, using the same injury model we found that thrombi grew faster in the EDA mice (Figure 4A). The thrombi ≥30 μm in diameter were seen in the EDA/ mice at 6.6±0.2 minutes compared with 8.3±0.6 minutes in the EDA mice (P<0.05, Figure 4B). The mean vessel occlusion time was also shorter in EDA/ mice compared with EDA mice (9.9±0.4 minutes and 14.6±1.7 minutes for EDA/ and EDA mice, respectively; P<0.05, Figure 4C). In the EDA/ mice the mean time to form the first thrombus and occlusion of the artery was similar to EDA mice (Figure 4A through 4C). Mice from both sexes were used and there were no significant differences in the occlusion time between male and female mice (not shown).

These results show that EDA FN promotes platelet aggregation/cohesion in the growing thrombus under arterial shear rate.

**Thrombogenesis in Veins**

Previously it was documented that venous thrombosis is not affected by the deficiency or decrease in FN levels. Because the EDA/ mice had such a striking increase in arterial thrombogenesis, we were interested to determine whether EDA FN could also affect venous thrombosis. Similar to the artery injury model used above, endothelial damage was induced by ferric chloride. The mean time to form the first thrombus ≥30 μm in diameter (EDA+/ =9.5±0.5 minutes, EDA−/ =9.2±0.6 minutes, EDA WT/WT=9.7±0.6 minutes, Figure 5A) and the mean occlusion time (EDA+/ =17.6±1.6 minutes, EDA−/ =17.3±1.3 minutes, EDA WT/WT=18.2±1.4 minutes, Figure 5B) were similar in the EDA/ when compared with EDA WT/WT or EDA−/ mice. Thus it appears that the presence of EDA FN in plasma does not stimulate venous thrombosis.

**Discussion**

Plasma fibronectin plays an important role in thrombus growth and stability under arterial shear conditions, as was demonstrated using pFN-deficient mice and heterozygous mice having one null FN allele (FN+/). The absence of the EDA domain was previously shown not to affect thrombus formation in the ferric chloride injury model, but nothing was known about the role of the EDA FN isoform in thrombosis and hemostasis.
To study the role of EDA, we used mouse strains unable to undergo regulated splicing of the EDA exon. In this study we show physiological features of EDA–FN in thrombosis, revealing a prothrombotic role. Despite decreased plasma and platelet FN levels in EDA−/− mice, the presence of EDA–FN in whole blood produced larger thrombi in vitro using a flow chamber, accelerated thrombosis and stabilized thrombi in injured arterioles. Additionally, the EDA−/− mice were more sensitive to collagen–epinephrine induced pulmonary thromboembolism. The plasma contains particularly low levels of the EDA domain (cFN, 1.3 to 1.4 μg/mL) but several pathological conditions are accompanied by 3- to 6-fold increase in the plasma levels of EDA–FN, including diabetes (4.3 μg/mL) and acute stroke (7.3 μg/mL). Consequently, elevated EDA–FN levels in plasma of patients should be considered a risk factor for thrombosis. EDA−/− mice used in the present work have higher plasma EDA–FN concentration than those found in human pathological conditions (roughly 10 to 15 times more elevated). It will be interesting to determine the correlation between EDA–FN levels in plasma and arterial thrombosis in patients affected by the disease states mentioned above.

During blood coagulation, Factor XIIIa mediates the cross-linking of pFN to fibrin, enhancing the stability of the clot. Additionally, differential incorporation of pFN and cFN into clots has been reported. Previous studies done in different mouse models documented that polymerization and gelation of fibrinogen was not affected by either a partial reduction in FN levels or depletion of pFN. However, nothing is known about the role played by EDA–FN. In this study, we document that EDA–FN in plasma and platelets does not affect hemostatic parameters such as clotting rate and clot retraction. Thus, the effects of EDA are likely linked to its interaction with platelet receptors.

At arterial shear, vWF and GPIb mediate the initial interaction of platelets with the subendothelium. FN available for interaction with platelets in vivo is pooled from different sources: FN from plasma, tissue FN released at the site of injury, and FN released from α-granules of platelets. FN present in EDA−/− mice is similar to cFN, as the EDB domain is almost absent in adult cFN. We observed no differences in platelet adhesion to the subendothelium in EDA−/− animals when compared with EDAWT/WT or EDA−/− mice suggesting that the EDA–FN present in the subendothelium has a minor role in initial platelet adhesion. Similar results have been also obtained with FN−/− mice and pFN null mice, which have decreased pFN levels, using the same ferric chloride injury model.

There may be several possible explanations for the enhanced thrombus formation observed in the presence of EDA–FN. The main integrin involved in thrombus formation is αIIbβ3, a receptor for adhesive proteins such as vWF, fibrinogen and pFN. Other integrins such as αvβ3 and α5β1 are also capable of platelet adhesion. FN can bind to αIIbβ3, αvβ3, and α5β1 through the Arg-Gly-Asp (RGD) sequence present in the FN type III-10 domain. The EDA domain is present between type III domains 11 and 12 and is a ligand for integrins α9β1 and α4β1, but neither of those integrins is present on platelets. Mechanical stretching of FN or inclusion of the EDA domain are known to augment FN–FN adhesion and binding to integrins, its cell binding and spreading activity. We hypothesize that the enhanced function of the EDA–FN isoform could be the consequence of a conformational change in the FN molecule caused by the inclusion of the EDA domain, leading to a more extended form of fibronectin displaying increased exposure or local unfolding of the type III-10 module, as suggested previously.

Cryptic sites present in FN-type III domains 10 to 12 could be more exposed in the extended conformation when the EDA exon is included in the FN molecule, thus allowing a more efficient interaction with the platelet integrins. Another possibility could be the increased exposure of the 70 kDa N-terminal FN domain and subsequent binding to fibrin or to activated platelets facilitating the interaction with the αIIbβ3 integrin on the platelet surface as suggested previously.

We showed that the presence of EDA–FN in whole blood did not increase thrombus growth at venous shear rate using an in vitro flow chamber. This result is consistent with those obtained in experimental venous thrombosis, where we showed the presence of EDA–FN in plasma and platelets influenced neither first thrombus formation nor mean occlusion time in veins, strongly suggesting that the presence of EDA–FN in plasma plays a prominent role only at arterial shear. This result is similar to that observed in the FN heterozygous (FN+/−) mice where a concentration dependent role of FN was documented only at arterial shear.

We conclude that the presence of the EDA domain gives FN an important prothrombotic potential that is revealed at arterial shear rates. Thus the presence of the EDA–FN may aggravate peripheral arterial disease, coronary artery disease, stroke, and other conditions in which thrombi may develop at arterial shear rates.

Acknowledgments
We thank Lesley Cowan for help in preparing the manuscript.

Sources of Funding
This work was supported by National Heart, Lung, and Blood Institute of the National Institute of Health grant R37 HL41002 to D.D.W. and by Telethon Grant GGP06147 to F.E.B. L.D.M. was supported by a grant of the ASI-DCMC.

Disclosures
None.

References
Chauhan et al  Prothrombotic Role of the Fibronectin EDA Domain 301


Prothrombotic Effects of Fibronectin Isoforms Containing the EDA Domain
Anil K. Chauhan, Janka Kisucka, Maria R. Cozzi, Meghan T. Walsh, Federico A. Moretti, Monica Battiston, Mario Mazzucato, Luigi De Marco, Francisco E. Baralle, Denisa D. Wagner and Andrés F. Muro

Arterioscler Thromb Vasc Biol. 2008;28:296-301; originally published online November 8, 2007;
doi: 10.1161/ATVBAHA.107.149146
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 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/28/2/296

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/12/19/ATVBAHA.107.149146.DC1

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/
Supplemental Data - Section I

Supplemental Materials and Methods

Animals

The generation of the mice devoid of regulated splicing at the EDA exon has been previously described. Briefly, to obtain constitutive inclusion of the EDA exon into the FN mRNA (EDA⁺ allele), both splice sites of the EDA exon were optimized: the 5’ splice site was mutated to match that of the consensus sequence and the 3’ splice site was replaced by that of the constitutively spliced-in second exon of the apolipoprotein A1 gene, which matches exactly the 3’ splice site consensus. In the EDA⁻ allele, the EDA exon was deleted from the genome by CRE-mediated recombination, using loxP sites inserted in the EDA flanking introns. Mice used in the model of pulmonary thromboembolism were 2-3 month-old and had been backcrossed with C57BL/6 mice for two generations (they had a mixed genetic background of 25% and 75% of c129J and C57Bl/6J, respectively). Mice used for all other studies were backcrossed 10 times on C57BL/6J background. Mice homozygous for the EDA⁺ and EDA⁻ alleles (EDA⁺⁺ and EDA⁻⁻ mice) and their WT controls (EDAWT/WT) were obtained by the mating of heterozygous progenitors.

Detection of FN and Fibrinogen

The platelets were isolated from pooled blood of 3 mice per genotype. The same number
of platelets was lysed for each genotype. Plasma samples were obtained from single animals (3 mice per genotype), pooled and the same amounts of total proteins were analyzed as previously described by Western Blot using a polyclonal antibody to FN.\textsuperscript{1} For fibrinogen determination, washed platelet were lysed by the addition of 10% of Triton X 100 and concentration was determined by using the Mouse fibrinogen Elisa kit supplied by ICL (Newberg, OR, USA).

**Whole blood clotting time and clot rate**

The whole blood clotting time was measured with a Sonoclot Coagulation and Platelet Function Analyzer (Sienco) as described.\textsuperscript{2} Analysis was performed with 280 µl citrated (3.8%) blood recalcified with 20 µl of calcium chloride (150 mM) in 37°C pre-warmed cuvettes. The following variables were analyzed: (1) the initial clot formation and (2) the clot rate. Clot rate is defined as the gradient of the primary slope measured as clot signal units per minute, which is an index of fibrinogen conversion into fibrin gel.

**Bleeding time**

Mice (8 to 10 weeks old) were anesthetized with ketamine/xylazine (150/15 mg/kg, IP) and a 3 mm segment of tail was amputated. The tail was immersed in 0.9% isotonic saline at 37°C, and the time required for the stream of blood to stop for 30 s was defined as the bleeding time.
Quantitative analysis of thrombus

Analysis of the recorded tape was performed blinded to the genotype. The following parameters were evaluated: (1) single platelet-vessel wall interaction, determined as the number of fluorescent platelets that deposited on the 250 µm vessel wall segment during 1 min (2-3 min after injury) and the count was normalized with the number of platelets infused, which was checked by flow cytometer after infusion; (2) the time required for formation of a thrombus larger than 30 µm in diameter; and (3) occlusion time of the vessel measured as the time required for blood to stop flowing for 30 sec.

Blood sampling and platelet preparation

Blood sampling and platelet preparation were done as previously described. Briefly, blood was drawn from the retro-orbital venous plexus by puncture and collected in polypropylene tubes containing heparin. Platelet-rich plasma (PRP) was obtained by centrifugation at 1200 rpm for 5 min. The PRP was transferred to fresh tubes containing 2 µl of PGI$_2$ (2 µg/ml) and incubated at 37°C for 5 min. After centrifugation at 2800 rpm, pellets were resuspended in 1 ml modified Tyrode’s-HEPES buffer (137 mM NaCl, 0.3 mM Na$_2$HPO$_4$, 2 mM KCl, 12 mM NaHCO$_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA) containing 2 µl of PGI$_2$ and incubated at 37°C for 5 min. The suspended pellet was centrifuged at 2800 rpm for 5 min. In order to remove PGI$_2$, the washing step was repeated twice and platelets were fluorescently labeled with calcein AM 2.5 µg/ml (Molecular Probes) for 10 min at room temperature. Analysis of classical platelet receptors, including P-selectin, GPIb and activated αIIbβ3, by fluorescent-activated cell sorting (FACS) showed that platelets were not activated.
Flow Chamber

Platelet interaction with immobilized collagen under flow conditions was studied as previously described. We used a modified Richardson’s parallel flow chamber mounted on an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD, Nikon Instech, Japan) and an intensifying CCD videocamera (C-2400-87, Hamamatsu Photonics, Japan). Briefly, blood was collected from EDA\textsuperscript{WT/WT}, EDA\textsuperscript{−/−}, EDA\textsuperscript{+/+} mice by cardiac puncture and mepacrine-labeled platelets in whole blood (10 µM, final mepacrine concentration) were perfused into the flow chamber onto coverslips (flow path height of 125 µm) coated with type I collagen from Bovine Achilles tendon. Flow rates of 0.08 and 0.47 ml/min produced wall shear rate of 250 s\textsuperscript{-1} and 1500 s\textsuperscript{-1}, respectively. Experiments were videotaped and selected images corresponding to representative areas of the substrate were digitized using a Matrox-Degisuite board (Matrox Graphics, Canada). To calculate the size of surface area covered, images were elaborated using Microimage software (image-processing software, CASTI Imaging, Venice, Italy).

Supplemental References


Supplemental Data - Section II

Supplementary Figure

Legend to the Supplemental Figure. Normal in vitro platelet aggregation was observed in EDA<sup>+/+</sup> platelet rich plasma (PRP). Aggregation of PRP prepared from mice of each genotype was initiated by adding the following agonists (at two different concentrations): ADP, PAR4 and Collagen. The exclusive presence of EDA<sup>+</sup>FN in plasma and platelets in the EDA<sup>+/+</sup> mice did not show any difference in platelet aggregation at low shear stress when compared to EDA<sup>-/-</sup> or EDA<sup>WT/WT</sup> mice. Representative images from 3 experiments are shown.
Supplemental Data - Section III

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Clotting time (s)</th>
<th>Clot rate</th>
<th>Tail bleeding time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDA&lt;sup&gt;WT/WT&lt;/sup&gt;</td>
<td>208 ± 27.7 (n=8)</td>
<td>16 ± 4.7 (n=8)</td>
<td>89.5 ± 33.7 (n=8)</td>
</tr>
<tr>
<td>EDA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>216 ± 14.6 (n=3)</td>
<td>15 ± 4.0 (n=3)</td>
<td>59.6 ± 13.9 (n=9)</td>
</tr>
<tr>
<td>EDA&lt;sup&gt;++&lt;/sup&gt;</td>
<td>207 ± 51.6 (n=9)</td>
<td>17 ± 11.7 (n=9)</td>
<td>79.4 ± 26.8 (n=10)</td>
</tr>
</tbody>
</table>

**Legend to the Supplemental Table.** Whole blood clotting time, clot rate and bleeding time in EDA<sup>++</sup>, EDA<sup>+</sup> and EDA<sup>WT/WT</sup> mice. Data are shown as Mean ± SD. The mean among groups was not statistically significant as analyzed by ANOVA followed by Bonferroni test.