ORIGINAL RESEARCH

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ADPase CD39 Fused to Glycoprotein VI-Fc Boosts Local Antithrombotic Effects at Vascular Lesions

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Background—GPVI (Glycoprotein VI) is the essential platelet collagen receptor in atherothrombosis. Dimeric GPVI-Fc (Revacept) binds to GPVI binding sites on plaque collagen. As expected, it did not increase bleeding in clinical studies. GPVI-Fc is a potent inhibitor of atherosclerotic plaque-induced platelet aggregation at high shear flow, but its inhibition at low shear flow is limited. We sought to increase the platelet inhibitory potential by fusing GPVI-Fc to the ectonucleotidase CD39 (fusion protein GPVI-CD39), which inhibits local ADP accumulation at vascular plaques, and thus to create a lesion-directed dual antiplatelet therapy that is expected to lack systemic bleeding risks.

Methods and Results—GPVI-CD39 effectively stimulated local ADP degradation and, compared with GPVI-Fc alone, led to significantly increased inhibition of ADP-, collagen-, and human plaque–induced platelet aggregation in Multiplate aggregometry and plaque-induced platelet thrombus formation under arterial flow conditions. GPVI-CD39 did not increase bleeding time in an in vitro assay simulating primary hemostasis. In a mouse model of ferric chloride–induced arterial thrombosis, GPVI-CD39 effectively delayed vascular thrombosis but did not increase tail bleeding time in vivo.

Conclusions—GPVI-CD39 is a novel approach to increase local antithrombotic activity at sites of atherosclerotic plaque rupture or injury. It enhances GPVI-Fc–mediated platelet inhibition and presents a potentially effective and safe molecule for the treatment of acute atherothrombotic events, with a favorable risk–benefit ratio. **(*J Am Heart Assoc*. 2017;6:e005991. DOI: 10.1161/JAHA.117.005991.)**

Key Words: glycoprotein • platelet • thrombosis

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Ischemic stroke is the most frequent disabling disease. Stroke and myocardial infarction are leading causes of death.[1] Frequently, the underlying alteration is the rupture or erosion of atherosclerotic plaques, leading to platelet adhesion and thrombus formation and to embolization, as observed in cerebral arteries.[2] Platelet adhesion and activation mediated by GPVI (glycoprotein VI) and dependent on collagen-bound von Willebrand factor play important roles in human plaque-triggered thrombus formation and subsequent development of cardiovascular syndromes such as stroke and could be a target for pharmacological inhibition of pathological thrombus formation.[3–6] The alternative collagen receptor, $\alpha 2\beta 1$-integrin, is not involved in plaque-induced platelet aggregation.[4,6] Targeting collagen-induced activation of GPVI should allow preferential inhibition of atherosclerotic plaque-induced thrombosis without affecting systemic hemostasis. GPVI expression is specifically observed in platelets and megakaryocytes.[7,8]

The interaction of GPVI with collagen can be inhibited competitively by a dimeric GPVI-Fc fusion protein (Revacept)[9,10] or by antibodies that have been developed to block GPVI.[11–13] Whereas anti-GPVI antibodies are systemic and potent inhibitors of plaque- and collagen-induced platelet aggregation in static and dynamic models, GPVI-Fc acts locally at the site of plaque rupture and is most effective under high shear flow.[12] Anti-GPVI antibodies

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Received March 3, 2017; accepted June 8, 2017.

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Clinical Perspective

What Is New?

- Dimeric glycoprotein VI (GPVI-Fc; Revacept) binds to collagen in atherothrombosis, GPVI. GPVI-Fc is a potent inhibitor of atherosclerotic plaque-induced platelet aggregation at high shear flow, but its inhibition at low shear flow is limited.
- Fusing GPVI-Fc to the ectonucleotidase CD39, which inhibits local ADP accumulation at vascular plaques, creates a lesion-directed dual antiplatelet therapy that is expected to lack systemic bleeding risks.

What Are the Clinical Implications?

- The fusion of GPVI-Fc and CD39 potently reduces intravascular thrombus formation, adding to the therapeutic potency of GPVI-Fc.
- Further development should investigate optimal dosing to prepare clinical trials.

might increase bleeding propensity, as observed in some patients with anti-GPVI autoantibodies,[13] whereas GPVI-Fc does not interact directly with platelets, did not increase bleeding times in clinical studies, and thus may be safer.[10] Consequently, this drug circumvents important shortcomings of existing platelet inhibitors and antithrombotics, which all incur a moderately to greatly increased bleeding risk.[14,15]

Sites of platelet adhesion and aggregation are also characterized by local release of ADP, several cytokines, and other biologically active substances from these platelets.[16] Released ADP activates additional platelets and leads to further platelet aggregation and secretion and thrombus propagation.[16] The endothelial ecto-ADPase CD39 (ENTPDase1) degrades ADP to AMP and inorganic phosphate (Pi) and thus locally inactivates an important platelet stimulus that may cause occlusive thrombi.[17–19] Gayle et al developed a soluble form of CD39 that can inhibit platelet function in vitro[20] and in vivo.[17,21] Hence, the potential of each, soluble CD39 and soluble GPVI (GPVI-Fc) alone, to inhibit platelet function has been characterized appropriately. General, nonspecific CD39 activation, however, results in bleeding propensity in CD39-transgenic mice[22] and after systemic application of soluble CD39 in vivo.[23]

In this study, we combined soluble CD39 and GPVI-Fc to form a recombinant, bifunctional fusion protein (GPVI-CD39) and showed that this molecule potently inhibits collagen- and plaque-induced platelet thrombus formation in vitro and arterial thrombus formation after vascular injury in vivo. Tail bleeding time in mice was not prolonged. GPVI-CD39 can bind to vascular lesions locally and concentrate in plaques, which should allow for markedly lower effective doses than soluble CD39, thus minimizing its bleeding propensity.

Methods

Reagents and Antibodies

Standard laboratory chemicals were purchased from Carl Roth. Ham's F-12 growth medium, fetal bovine serum, PBS, and glutamine were from Biochrom. Blasticidin S was obtained from InvivoGen. Hygromycin B came from Carl Roth. ProCHO4 growth medium was from Lonza. All enzymes for cloning were bought from New England Biolabs. Doxycycline hydrochloride, ADP sodium salt, and ATP disodium salt hydrate were purchased from Sigma-Aldrich. Part of the recombinant hirudin was a kind gift from Prof. Christian P. Sommerhoff (University of Munich), and another part was purchased from Celgene. Collagen-related peptide was synthesized at AnaSpec and chemically cross-linked. DiOC6 (3,3′-dihexyloxacarbocyanine iodide) was from Life Technologies. midazolam (Dormicum; Roche) was purchased from Roche, and medetomidine (Dormitor; Pfizer) and fentanyl were both from Janssen-Cilag. Recombinant soluble human CD39 (solCD39) was obtained from R&D Systems. Goat–antihuman Fcγ and goat–antihuman IgG (H+L)-POD (peroxidase) were purchased from Jackson ImmunoResearch.

Cloning and Protein Production

GPVI-Fc was taken from existing stocks. The cDNA coding for the fusion protein (GPVI-CD39), consisting of the extracellular domain of platelet GPVI, Fc (partial hinge region, CH2 and CH3 domain) of human IgG2, and the extracellular domain of human CD39, which was connected by a 15-amino acid linker, was established by gene synthesis. For steric reasons, the sequence coding for the Fc part of human IgG2 was inserted into the GPVI-CD39 fusion protein, whereas the original GPVI-Fc (also termed Revacept, which is currently in clinical investigation) is composed of the Fc derived from human IgG1. The cDNA was cloned into the mammalian expression vector pcDNA5/FRT/TO using HindIII and BamHI sites. Flp-In-CHO cells (Life Technologies) that had been genetically modified to harbor the cDNA for a Tet repressor protein were stably transfected with the expression construct and pOG44 helper plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) transfection reagent according to the instructions of the supplier (Life Technologies). Stable adherent cells were adapted to growth in suspension in the chemically defined growth medium ProCHO4 supplemented with 4 mmol/L glutamine, 600 μg/mL hygromycin B, and 20 μg/mL blasticidin S. Recombinant protein expression was induced in dense cultures by addition of 30 ng/mL doxycycline followed

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by incubation at 31°C and 5% CO_2 for 6 to 7 days in a humidified atmosphere. The construct for the control protein was produced by gene synthesis, accordingly. Expression of Fc(IgG2) control proteins was performed in stably transfected Flp-In-CHO cells grown at 37°C, 5% CO_2 for 3 to 4 days in Ham's F-12 medium with 2% fetal bovine serum that had been depleted for bovine IgG in advance. Recombinant proteins were purified from cell culture supernatants using HiTrap Protein G HP affinity chromatography columns (VWR), according to the manufacturer's manual. All proteins were dialyzed against PBS. Purified GPVI-CD39 protein was separated in nonreducing and reducing sample buffer in a Tris-HEPES NH 4% to 20% gradient gel that was stained with Coomassie Brilliant Blue G250.

ADPase Activity

Various concentrations of GPVI-CD39 or solCD39 protein or plasma samples from the pharmacokinetic study diluted 1:500 to 1:2000 in assay buffer were incubated in 25 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.5, with 200 μmol/L ADP in a total volume of 100 μL for 30 minutes or 5 minutes at 37°C. Enzymatically released Pi was detected using the Malachite Green phosphate detection kit, according to the supplier's manual (R&D Systems). A serial dilution of Pi standard was analyzed in parallel, which facilitates quantification of released Pi. Absorbance was measured at a wave length of 630 nm using a Tecan Infinite 200 ELISA reader. Enzymatic activity of purified protein was calculated taking incubation time and protein amount into account and expressed as units per milligram of protein.

Blood Collection

Blood was withdrawn from healthy volunteers who did not take any anticoagulative medication within the past 14 days with either recombinant hirudin (200 U/mL; 13 μg/mL) or citrate as anticoagulant. Informed consent was obtained, as approved by the local ethics committee. In total, 58 healthy volunteers were included into the study: Blood samples from 39 participants were taken for in vitro experiments using vascular agonists, and blood samples from 19 participants were used for experiments involving human plaque material.

Human Carotid Atherosclerotic Plaque Material

Atherosclerotic plaques were donated from patients undergoing endarterectomy for high-grade carotid artery stenosis.[24] Patients' informed consent was obtained, as approved by the ethics committee of the Faculty of Medicine of the University of Munich, in accordance with the Declaration of Helsinki. Plaque material from 10 patients was included. The carotid plaque tissue was processed and preserved, as described.[12] Plaque homogenates from 5 patients were mixed to obtain plaque pools that were kept in aliquots at −80°C. Plaque homogenates were used to stimulate platelets in blood under static conditions or coated onto glass coverslips for flow studies.[12]

Platelet Aggregation

The effect of GPVI-CD39 and control proteins on platelet aggregation was analyzed using the Multiplate (Roche) device.[25] 1:1 diluted hirudin-anticoagulated blood was preincubated with antagonist for 3 minutes in the test cell without stirring to avoid platelet preactivation.[26] Agonist was added, and samples were incubated for 6 minutes at 37°C with stirring. The following agonists were used: 6.5 μmol/L ADP, 12 μg/mL collagen isolated from rabbit aorta, 103 μg/mL collagen type I secreted by human fibroblasts (VitroCol; Advanced BioMatrix), or 333 μg/mL pooled human plaque homogenate. Platelet aggregation was measured in arbitrary units over the time period (arbitrary units×minutes; cumulative aggregation values).

Platelet Aggregation Under Flow Conditions

Glass coverslips were coated with pooled human plaque homogenates, as described,[12] and mounted into parallel plate flow chambers using sticky slides (0.1 Luer sticky slides; ibidi) previously blocked with 4% human serum albumin in PBS. The flow chamber was then mounted on the stage of a fluorescence microscope (TE2000-E; Nikon) within an incubation chamber (37°C). The flow chamber was rinsed with PBS and blocked with 4% human serum albumin in PBS and subsequently perfused with hirudin-anticoagulated human blood from healthy donors that had been preincubated for 10 minutes at 37°C with DiOC6 (1 μmol/L) to stain platelets and with an antagonist or control protein. Blood was perfused with continuous flow at shear rates of 600/s and 1500/s or with pulsatile flow (60 pulses/min, 0.5 seconds: shear rate 0/s; 0.5 seconds: shear rate 1000/s; resulting in a mean shear rate of about 600/s) using a withdrawal syringe pump. Fluorescence of adhering platelets and platelet aggregates was continuously recorded and quantified, as described in detail.[12]

In Vivo Thrombus Formation After Ferric Chloride Injury in Carotid Arteries

For examination of arterial thrombus formation in vivo, C57BL/6J wild-type mice aged 6 to 8 weeks were anesthetized by injection of midazolam (5 mg/kg body weight), medetomidine (0.5 mg/kg body weight), and fentanyl

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(0.05 mg/kg body weight). In the ferric chloride model, 21 mice were studied.

The common carotid artery was dissected free, and the mice were injected intravenously 30 minutes before carotid injury with GPVI-CD39 (3 mg/kg body weight) or its control and with GP1bα-488 for platelet visualization. The carotid was exteriorized and injured by topical application of a filter paper saturated with 15% ferric chloride for 1 minute. Thrombus formation in arteries was monitored for 20 minutes or until complete occlusion (stop of blood flow for >1 minute). Digital films and images were recorded with a Nikon Eclipse intravital microscope and analyzed off-line.

Pharmacokinetic Analysis

Male and female wild-type C57BL/6J mice aged 12 to 22 weeks were used in this small-scale study. Nine mice were investigated. GPVI-CD39 or solCD39 was applied at a volume of 5 mL/kg into the right tail vein. At time points indicated in the figure7C, blood sampling was performed by incision of the left tail vein using 25 µL heparinized capillaries. Blood samples were recovered by centrifugation at 2400*g* for 10 minutes. The upper plasma phase was transferred to fresh tubes and stored frozen at −20°C.

Determination of Protein Concentration in Plasma Samples of Mice

The concentration of GPVI-CD39 or Fc control protein in plasma samples was determined by Fc-specific sandwich ELISA. Wells of a MaxiSorp 96-well plate (Thermo Fisher Scientific) were coated with 0.1 µg per well of goat–antihuman Fcγ antibody. Wells were washed 3 times with PBST (PBS and Tween-20) between incubations. After blocking with 3% skimmed milk in PBST, wells were incubated for 1 hour with 50 µL plasma from mice treated with GPVI-CD39 (1:200) or Fc (1:500) diluted in PBST. Wells were incubated for 1 hour with 100 µL of 80 ng/mL goat–antihuman IgG

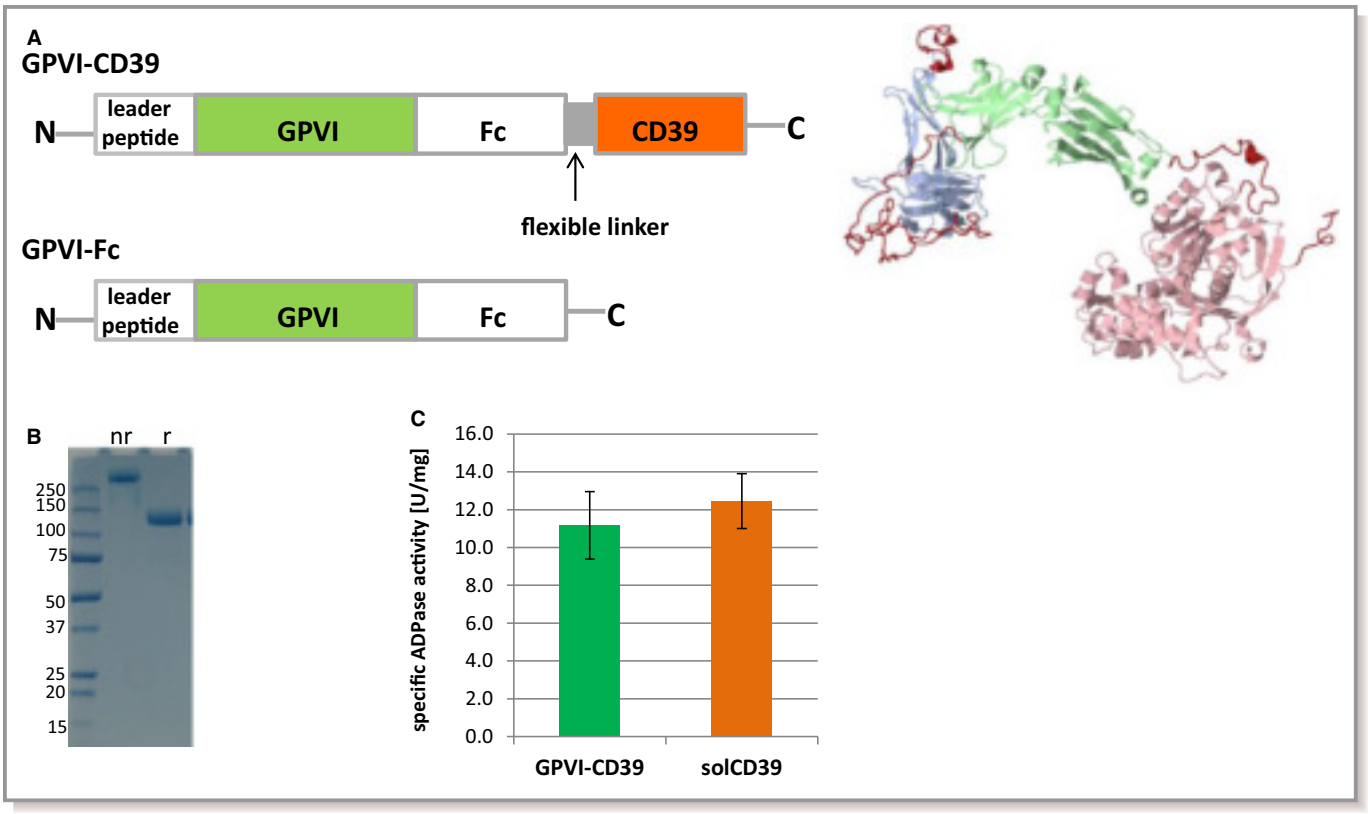

Figure 1. Structure scheme and biochemical characterization of bifunctional, recombinant GPVI-CD39 compared to GPVI-Fc. A, Structure (left panel) and putative 3D-modeling (right panel) of bifunctional, recombinant GPVI-CD39 compared with GPVI-Fc. The 3D model shows the extracellular GPVI domains in blue, the Fc part in green, and the CD39 domain in light red. The N-terminal leader peptides of each protein are cleaved before secretion of each protein. B, Coomassie staining of GPVI-CD39 as purified from supernatants of GPVI-CD39–expressing cells. C, Specific ADPase activity of recombinant GPVI-CD39 (333 nmol/L) in comparison to that of commercially available soluble CD39 (666 nmol/L; n=3, mean±SEM). Both specific activities were determined at other enzyme molarities and did not differ, as expected, because substrate concentrations were not limiting. GPVI indicates glycoprotein VI; GPVI-CD39, dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI; nr, nonreducing conditions; r, reducing conditions.

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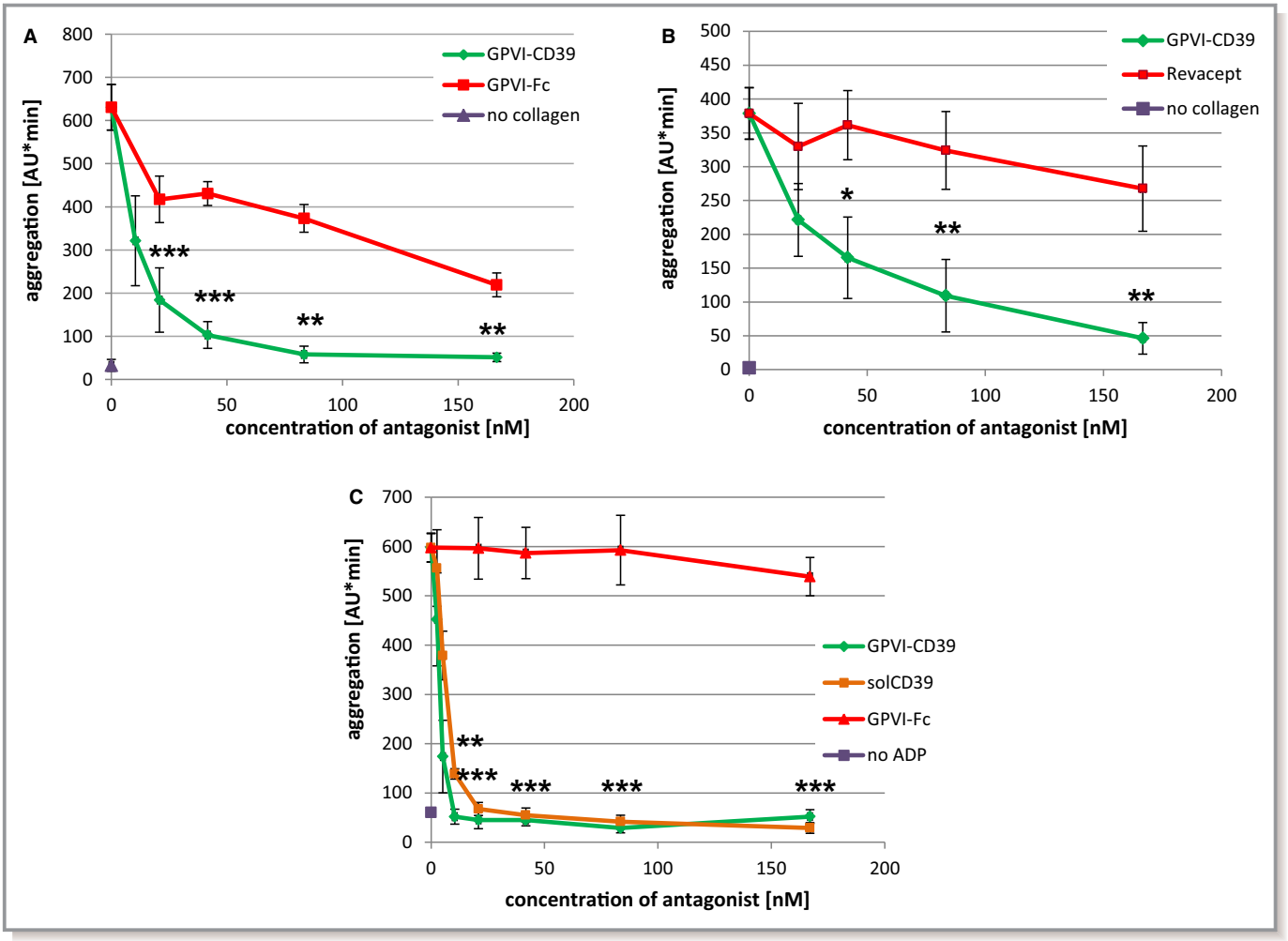

Figure 2. Effects of GPVI-CD39 and GPVI-Fc fusion proteins and of control proteins on static platelet aggregation in blood after stimulation with collagen or ADP. Platelet aggregation was determined by impedance aggregometry. Values are mean±SEM. A, Platelet aggregation after stimulation with 12 µg/mL collagen extracted from rabbit aorta. Preincubation with increasing concentrations of GPVI-CD39 reduces platelet aggregation more strongly than GPVI-Fc alone (n=5; **P<0.01 and ***P<0.001, compared with GPVI-Fc). B, Platelet aggregation after stimulation with 103 µg/mL collagen from cultured human fibroblasts (VitroCol; Advanced BioMatrix), as determined by impedance aggregometry. Preincubation with increasing concentrations of GPVI-CD39 reduces platelet aggregation more strongly than GPVI-Fc alone (n=5; *P<0.05 and **P<0.01, compared with GPVI-Fc). C, Platelet aggregation after stimulation with 6.5 µmol/L ADP. Preincubation with increasing concentrations of either GPVI-CD39 (n=8) or of soluble CD39 markedly reduces ADP-induced platelet aggregation, whereas GPVI-Fc alone has no effect (**P<0.01 and ***P<0.001). AU indicates arbitrary unit; GPVI-CD39, dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI; solCD39, soluble CD39.

(H+L)-POD detection antibody. POD activity was visualized using 100 µl of Ultra TMB-ELISA substrate (Thermo Fisher Scientific), and signal intensities were read with a Tecan Infinite F200 ELISA reader.

Analysis of In Vivo Tail Bleeding Time in Mice

Test or control substances were applied into the tail vein of C57BL/6J mice. Animals were anesthetized by intraperitoneal injection of 0.5 mg/kg medetomidine, 5 mg/kg midazolam, and 0.05 mg/kg fentanyl. At 10 minutes after protein delivery, a blood sample of 20 µL was drawn for analysis of recombinant protein content and ADPase activity using a heparinized capillary. At 15 minutes after protein application, the distal 2 mm of the tail were cut off, and the tail was immediately immersed in prewarmed PBS (37°C) and time-monitored until bleeding stopped for at least 30 seconds. The process was standardized to yield comparable results over time, and results were reproducible. Animals were euthanized, and a final blood sample was stored for analysis.

Determination of In Vitro Closure Time

Citrated blood of healthy donors was mixed with antagonist in concentrations indicated in the figures 6 and added to Dade PFA collagen/epinephrine, Dade PFA collagen/ADP, or

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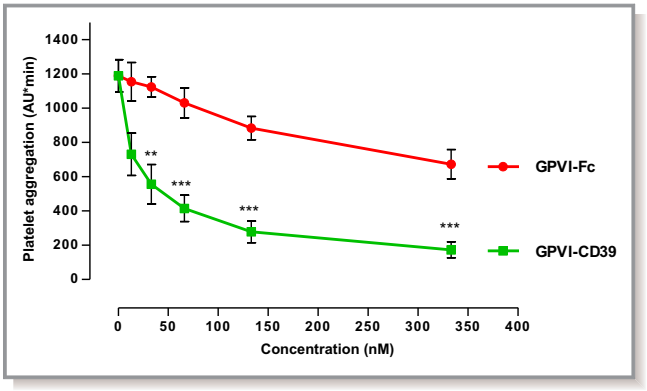

Figure 3. GPVI-CD39 inhibits static platelet aggregation in blood stimulated by human plaque more potently than GPVI-Fc. Blood samples were preincubated for 3 min with increasing concentrations of GPVI-CD39 or GPVI-Fc before stimulation with plaque homogenate (333 μg/mL) for 10 min. Values are mean ±SEM (n=8). ***P<0.001 by 2-way ANOVA for factor concentration and drug and secondary pairwise comparisons of isomolar GPVI-CD39 vs GPVI-Fc by Fisher least significant difference. The asterisks indicate significant differences between the 2 drugs at isomolar concentrations. In addition, direct-pair comparisons between isomolar drug concentrations by Student t testing resulted in the same significance levels. AU indicates augmented unit; GPVI-CD39, dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI.

Innovance PFA P2Y test cartridges (Siemens Healthcare). Blood was aspirated under high shear conditions (>5000/s) through a capillary onto a membrane with a small aperture coated with substances that activate platelets and lead to closure of this aperture. The time until closure of this aperture is monitored and expressed as in vitro closure time with a maximum closure time of 300 seconds.

Statstical Analysis

Normal distribution of all analyzed parameters was verified and confirmed by Kolmogorov–Smirnov testing. Differences between ≥2 experimental groups were analyzed by ANOVA using SPSS software (version 19; IBM Corp), followed by Fisher least significant difference post hoc testing. Specifically, 2-way repeated-measures ANOVA was used as indicated. The Student t test with Bonferroni method was also used when absence of differences in ADPase activity was investigated.

Results

Description of GPVI-CD39 Protein and its Properties

To enhance the antithrombotic potential of GPVI-Fc, we created a fusion protein that combines the extracellular collagen binding domain of GPVI with the extracellular domain of CD39 harboring enzymatic ADPase activity (Figure 1A). The Fc domain in between facilitates dimerization of the molecule, as was confirmed by nonreducing polyacrylamide gel analysis (Figure 1B). A flexible linker of 15 amino acids facilitates proper folding of the CD39 domain. The protein was successfully expressed by doxycycline-inducible, stably transfected CHO cells and was purified from cellular supernatants by protein G affinity chromatography. At various concentrations, the fusion protein exhibited mean ADPase activity of 11.2±4.0 U/mg, which was similar to that of commercially available solCD39 (12.5±3 U/mg).[27] These results are shown in Figure 1C. Statistical comparison of GPVI-CD39 with solCD39 activities (equal amounts) yielded no significant difference by either Student t testing or ANOVA.

Effect of GPVI Fusion Proteins on Collagen-, ADP-, or Plaque-Induced Platelet Aggregation in Human Blood Under Static Conditions

The effect of GPVI-CD39 on collagen-induced aggregation of human platelets was analyzed in blood using collagens from different sources as well as human plaque material and the secondary agonist ADP. GPVI-CD39 exhibited a highly significant, dose-dependent inhibition of platelet aggregation induced by 12 μg/mL collagen isolated from rabbit aorta (Figure 2A). Similarly, using 103 μg/mL collagen secreted by human fibroblasts (VitroCol; mainly type I collagen) as agonist, GPVI-CD39 inhibited platelet aggregation significantly, whereas GPVI-Fc resulted in only minor inhibition at the same concentration (Figure 2B). Effective inhibition of ADP-induced platelet aggregation occurred by GPVI-CD39 as well as by equimolar concentrations of solCD39, using 6.5 μmol/L ADP, whereas GPVI-Fc lacking the CD39 component displayed no inhibitory effect (Figure 2C). Adding GPVI-CD39 to platelet aggregation triggered by human plaque material (333 μg/mL) also resulted in dose-dependent inhibition with an approximate IC_{50} value of 30 nmol/L (Figure 3). GPVI-Fc tested at the same concentrations was markedly and significantly less effective than GPVI-CD39.

Effect of GPVI Fusion Proteins on Plaque-Induced Platelet Aggregate Formation Under Flow Conditions

To mimic the situation found in arteries after human carotid plaque rupture in vivo, pooled human plaque homogenates from samples taken during carotid surgery were coated onto glass coverslips, and human blood was perfused in a parallel flow chamber over the coated surface at various arterial shear rates in the presence or absence of GPVI fusion proteins.

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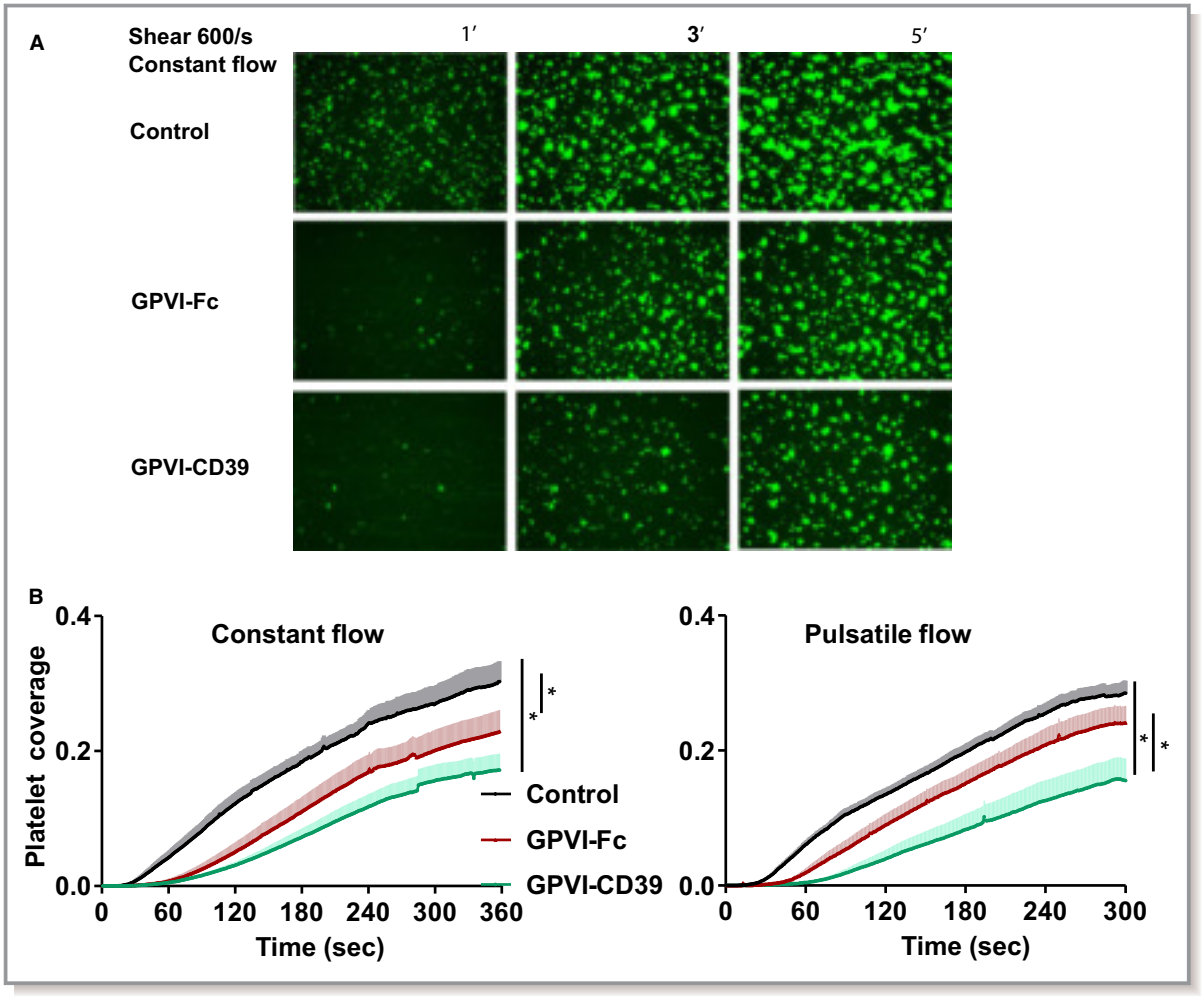

Figure 4. Effects of GPVI-CD39 and GPVI-Fc on plaque-induced platelet deposition from flowing blood at 2 arterial shear rates. A, Representative micrographs display platelet coverage of pooled plaque homogenate at different times after start of blood flow at 600/s. Blood was preincubated for 10 min with DiOC6 for platelet visualization in the absence (control) or presence of GPVI-CD39 (150 nmol/L) or GPVI-Fc (150 nmol/L). B, Effects of GPVI-Fc and GPVI-CD39 on the kinetics of platelet deposition onto human plaques from flowing blood at constant (shear rate 600/s) or pulsatile flow (60 pulses/min, mean shear rate 600/s). The binary fluorescent area fraction (1.0=total area) was quantified, as detailed in Methods. Values are mean±SEM of 8 experiments performed in parallel with the same blood donors. *P<0.05, by repeated-measures ANOVA at 300 s and secondary pairwise comparison by Fisher least significant difference. Repeated measures refer to the comparison of aliquots from samples of each donor under different concurrent experimental conditions at the same time. C, Comparison of the effects of either 75 or 150 nmol/L GPVI-CD39 on plaque- induced platelet deposition from flowing blood at low and high arterial shear rates. Blood was preincubated with DiOC6 for platelet visualization in the absence (control) or presence of GPVI-CD39 (75 or 150 nmol/L) for 10 min at 37°C before start of flow at shear rates of 600/s or 1500/s. Values are mean±SEM (n=6). *P<0.05 and **P<0.01 by repeated-measures ANOVA at 300 s and secondary pairwise comparison by Fisher least significant difference. DiOC6 indicates 3,3′-dihexyloxacarbocyanine iodide; GPVI-CD39, dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI.

A shear rate of 600/s was selected to represent the mean physiological wall shear rates in carotid and coronary arteries, whereas a mean shear rate of ≈1500/s has been described to prevail in mildly stenotic coronary lesions.[28] In addition, pulsatile flow conditions were tested to simulate the stop-and-go blood flow in coronary arteries. GPVI-CD39 exhibited stronger potency than GPVI-Fc to inhibit aggregate formation when tested at concentrations of 150 nmol/L at continuous flow at a shear rate of 600/s (Figure 4A and 4B). The difference was larger and significant when pulsatile flow was applied (Figure 4B, right panel).

At the shear rate of 1500/s, the effects of GPVI-CD39 were more pronounced compared with its effects at the lower shear rate. GPVI-CD39 at 150 nmol/L led to nearly complete inhibition (−97%) of plaque-induced platelet aggregation.

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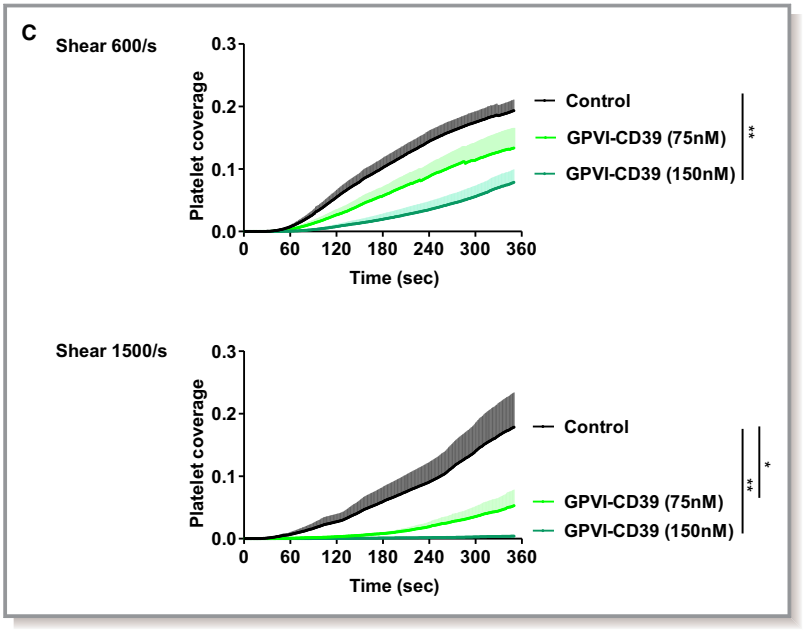

Figure 4. Continued.

GPVI-CD39 at 75 nmol/L still significantly inhibited plaque-induced platelet aggregation (Figure 4C).

Antithrombotic Effects of GPVI-CD39 In Vivo in a Ferric Chloride Model

To investigate the antithrombotic effects of GPVI-CD39 in vivo in mice, the common carotid artery was injured using 15% ferric chloride, and the time to occlusion of the vessel was monitored. In wild-type mice, the mean time to vessel occlusion was 480 seconds (not shown). Intravenous administration of either vehicle (NaCl) or 1.5 mg/kg (10 nmol/kg) GPVI-Fc resulted in similar occlusion times, whereas 3 mg/kg (10 nmol/kg) GPVI-CD39 significantly delayed vessel occlusion to 1083 seconds (Figure 5).

Assessment of Closure Times

The efficacy of GPVI-CD39 was also analyzed by measuring closure times in response to various agonists with a platelet function analyzer (Innovance PFA-200). Four different concentrations of GPVI-CD39 were tested, ranging from 21.7 to 217 nmol/L. Using either COL/ADP or COL/EPI cartridges, no significant increase in closure times of the aperture compared with the buffer control (PBS) was observed (Figure 6A and 6B). Using the COL/EPI cartridges, only acetylsalicylic acid, used as a positive control, inhibited the closure of the aperture completely over the analyzed time period. As expected, P2Y cartridges, which allow for sensitive detection of P2Y antagonism, showed a statistically significant increase in closure time using 47 to 217 nmol/L GPVI-CD39 but not with 21 nmol/L (Figure 6C). The prolongations with higher concentrations of GPVI-CD39 corresponded to those observed with 1.9 μmol/L of the $P2Y_{12}$ inhibitor ticagrelor (Figure 6C). These results confirmed effective inhibition of the secondary agonist ADP by GPVI-CD39.

Pharmacokinetic Analysis and Determination of In Vivo Bleeding Time in Mice

Because the application of effective doses of solCD39 in various animal models of thrombosis caused higher bleeding risks, the hazard potential of GPVI-CD39 was analyzed by measuring in vivo bleeding times in mice. To determine a proper time point after protein application for an in vivo bleeding study, a pharmacokinetic study of GPVI-CD39 plasma concentrations was performed. Mice were injected with 4 mg/kg GPVI-CD39 or 2 mg/kg solCD39; these doses relate to 26.6 nmol of the CD39 moieties of both agents.

Blood sampling was performed at intervals indicated in Figure 7A, and both the content of Fc-containing protein (GPVI-CD39) and ADPase activity in plasma were determined. At 5 minutes after protein application, a mean concentration of GPVI-CD39 of 95 μg/mL was detected. The solCD39 could not be analyzed because of the lack of an Fc portion. Concentration of the protein decreased rapidly in the course of 2 hours. After 48 hours, GPVI-CD39 was still detectable at a low concentration of 6 μg/mL.

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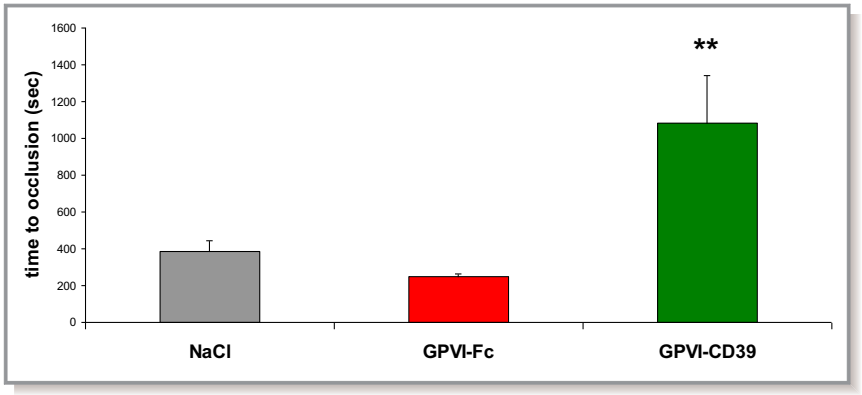

Figure 5. Effect on thrombus formation after ferric chloride injury: mean times to occlusion after administration of vehicle (NaCl), GPVI-Fc, or GPVI-CD39. Administration of 3 mg/kg (10 nmol/kg) GPVI-CD39 strongly delayed ferric chloride–induced thrombus formation in vivo compared with administration of 1.5 mg/kg (10 nmol/kg) GPVI-Fc or vehicle only. Mean values of 7 independent experiments are shown with SEM. $^{**}P<0.01$ by ANOVA. GPVI-CD39 indicates dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI.

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ADPase activity was measured in the same plasma samples for solCD39 and was comparable to that of GPVI-CD39 (Figure 7B). ADPase activity diminished rapidly during the first 60 minutes but was still detectable at very low levels after 2 days.

The analysis of tail bleeding time 15 minutes after intravenous administration showed no differences for 4 mg/kg (13 nmol/kg) GPVI-CD39 compared with 2 mg/kg (13 nmol/kg) GPVI-Fc or vehicle or buffer controls (Figure 7C).

Discussion

In the present study, we generated a recombinant fusion protein consisting of a GPVI domain coupled to the ecto-ADPase CD39 that degrades prothrombotic extracellular ADP.[17,18] We found that this fusion molecule efficiently inhibits ADP-, collagen- and human plaque–induced platelet aggregation under static conditions and plaque-triggered platelet adhesion and thrombus formation under arterial flow at clinically relevant concentrations. In contrast, collagen/epinephrine-triggered closure times, as measured in an Innovance PFA-200 device, were unchanged because epinephrine is a sufficiently strong agonist. Moreover, GPVI-CD39 markedly delayed ferric chloride–induced thrombus formation in mice in vivo but did not prolong tail bleeding times in vivo at any doses.

Our findings imply that the fusion protein GPVI-CD39 is an attractive strategy for a lesion-directed dual antiplatelet therapy (inhibition of collagen- and ADP-induced platelet adhesion/aggregation) at sites of arterial vulnerability (eg plaque ruptures and erosions, stented lesions) but may not incur a relevant systemic bleeding risk. So far, dual antiplatelet therapy, which typically combines acetylsalicylic acid with an ADP receptor antagonist such as clopidogrel, is the standard therapy for patients with acute vascular lesions treated by coronary stenting, and its major limitation is increased bleeding risk.

The endothelial ecto-ADPase CD39/ENTPDase1 degrades ADP to AMP and Pi and thus inactivates an important agent that may cause occlusive thrombi.[17–19] Transgenic mice that overexpressed CD39 showed impaired platelet aggregation and resistance to thrombogenic stimuli but also markedly prolonged tail bleeding time that led to death when unchecked.[22] Similarly, these CD39-transgenic mice were also resistant to ferric chloride–induced thrombus formation,[27] and to myocardial injury.[29] CD39-transgenic pigs were also generated and underwent a model of myocardial ischemia-reperfusion injury by left anterior descending artery balloon inflation.[30] These pigs showed markedly reduced infarct sizes compared with wild-type controls. In contrast, $CD39^{-/-}$ knockout mice were characterized by increased cerebral infarct volumes and reduced postischemic cerebral perfusion.[31] These knockout mice also developed increased atherosclerotic plaque burden when cross-bred with apolipoprotein $E^{-/-}$ knockouts, with especially low CD39 expression in atheroprone regions.[32]

Gayle et al developed a soluble form of CD39 that can inhibit platelet function in vitro[20] and in vivo[18,21,31]: Administration of 4 mg/kg soluble CD39 led to clearly reduced infarct sizes and improved neurological function in experimental mouse stroke (whereas 1 mg/kg had no effects). In this study, bleeding time was prolonged only after administration of ≥8-mg/kg doses of solCD39 in mice. In addition,

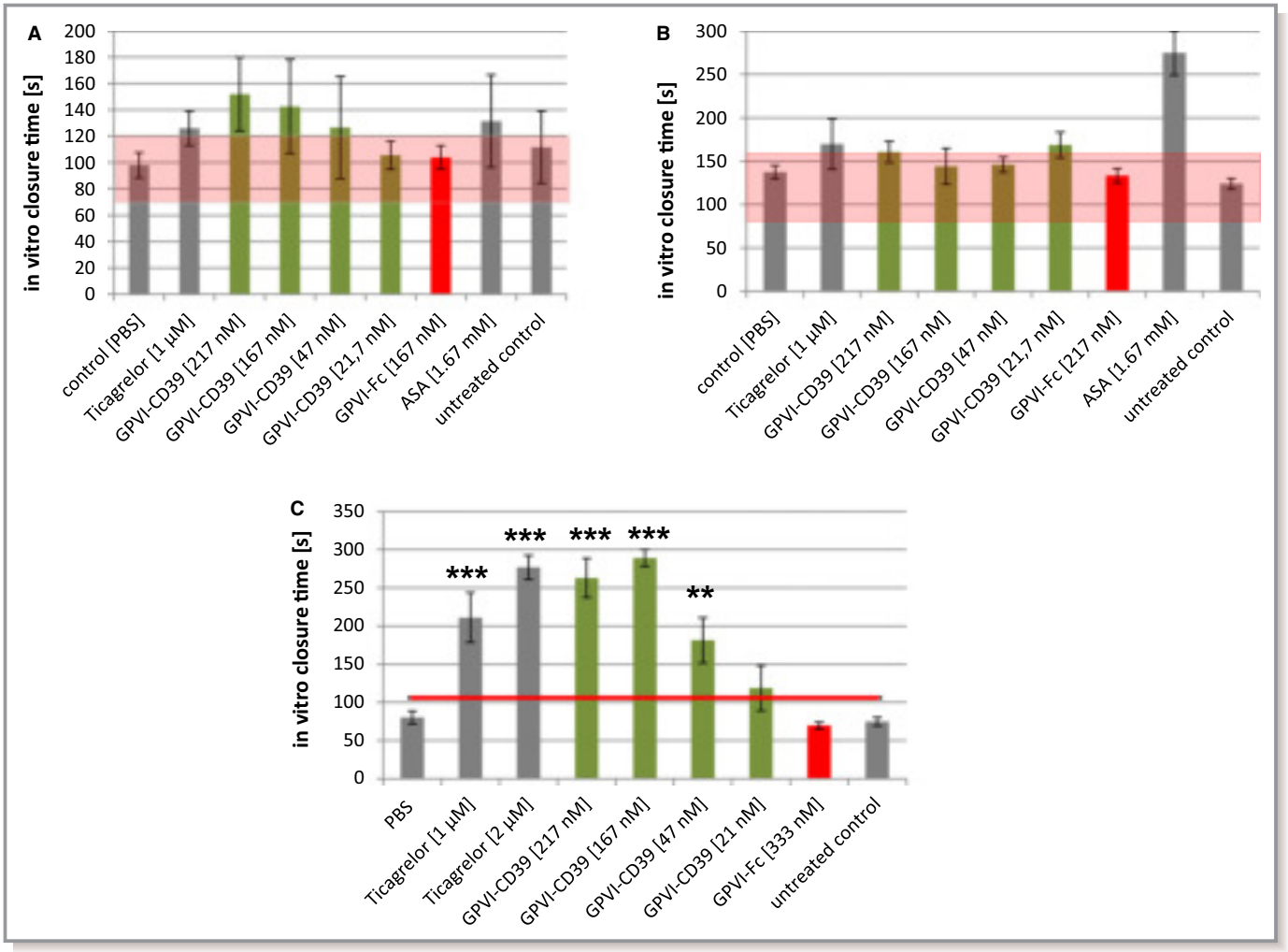

Figure 6. Effects on Innovance PFA-200 closure times of human blood ex vivo. A, Effects of ticagrelor, GPVI-CD39, GPVI-Fc, or ASA at the indicated concentrations on closure times in collagen/ADP cartridges (n=8 samples from independent donors). No significant differences between groups occurred. B, Effects of ticagrelor, GPVI-CD39, GPVI-Fc, or ASA at the indicated concentrations on closure times in collagen/epinephrine cartridges (n=8 samples from independent donors). No significant differences between results for GPVI-CD39 and GPVI-Fc occurred. Closure time was significantly (P=0.04) prolonged after addition of ASA compared with PBS only. C, Effects of ticagrelor, GPVI-CD39, GPVI-Fc at the indicated concentrations on closure times in specific P2Y cartridges (n=8 samples from independent donors; **P<0.01 and ***P<0.001 vs PBS only). ASA indicates acetylsalicylic acid; GPVI-CD39, dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI.

Hohmann et al reported markedly increased bleeding time after 8 mg/kg but not after 0.8 mg/kg[23]; however, this lower dose did not have a beneficial effect on occlusion time in a ferric chloride–induced carotid thrombosis model.[23] In pigs, 700 µg/kg solCD39 increased bleeding times but achieved only a nonsignificant trend to attenuate platelet and fibrin deposition after coronary balloon injury.[21]

In our study, we combined soluble forms of CD39 and GPVI-Fc to form a recombinant, bifunctional protein, GPVI-CD39, to investigate platelet-mediated thrombus formation. We showed previously that GPVI-Fc inhibits platelet-induced thrombus formation at sites of vascular injury.[9] Administration of GPVI-Fc improved myocardial ischemia [33] and cerebral infarction [34] without affecting bleeding time[35] and inhibited progression of atherosclerosis.[36] GPVI-Fc also inhibited collagen-induced aggregation in humans in a phase 1 study.[10]

The combination of GPVI-Fc with CD39 potentiates the antithrombotic effect of GPVI-Fc by blocking not only the primary platelet agonist collagen but also the secondary agonist ADP. Local platelet release of ADP is an important mediator of atherosclerotic plaque-stimulated platelet aggregation at static and flow conditions.[19] GPVI-coupled CD39 should concentrate specifically at collagen fibers within vascular lesions and atherosclerotic plaques and thus act at lower local concentrations in response to lower systemic concentrations than soluble recombinant CD39. Consequently, bleeding risk that results from recombinant CD39 should be minimized.[20,23]

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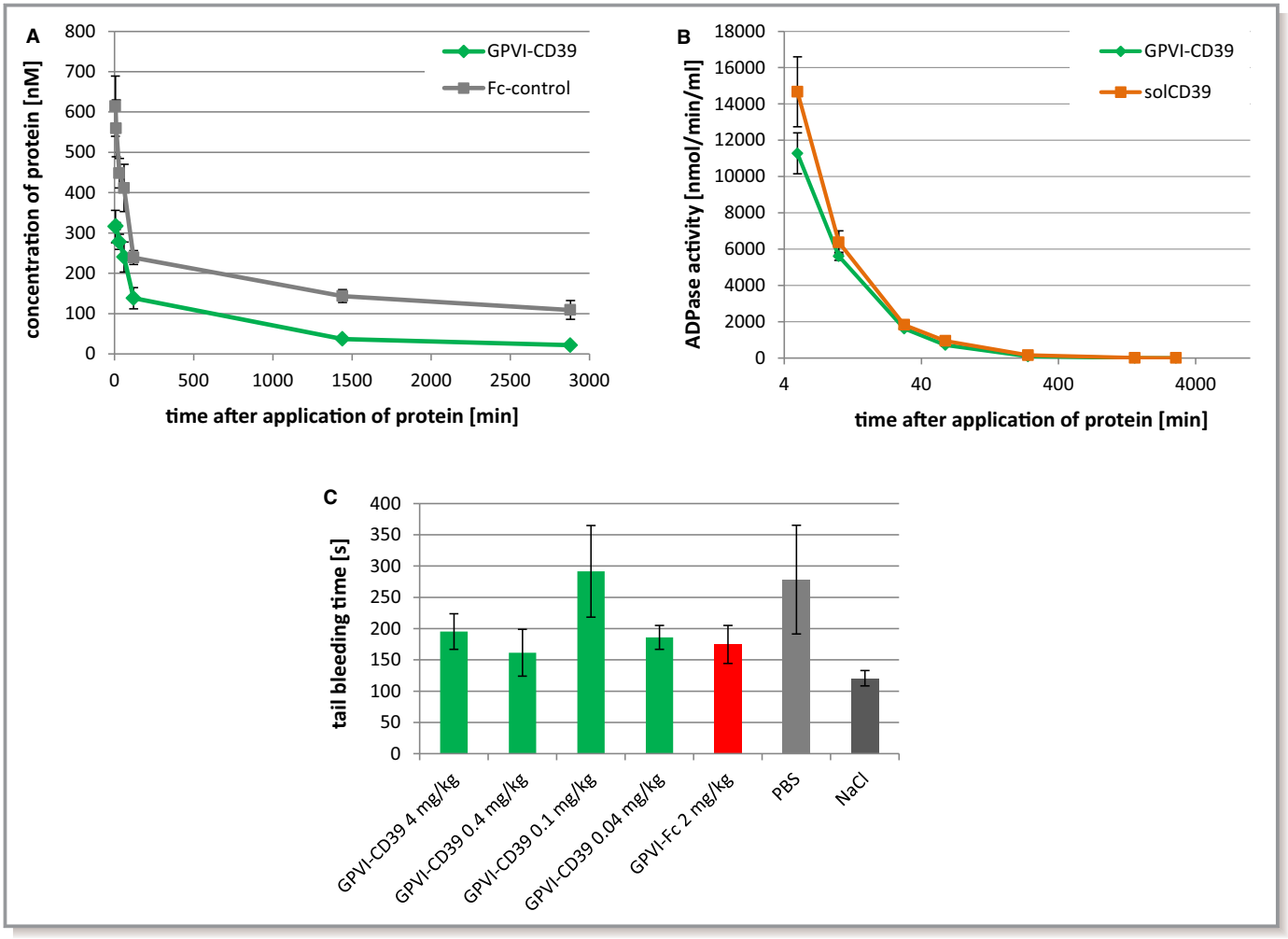

Figure 7. Pharmacokinetic and pharmacodynamic evaluation in mice in vivo; bleeding times in vivo. A, Plasma concentrations in mice up to 48 hours after administration of GPVI-CD39 or Fc control protein. Blood samples were taken at the indicated times after IV administration of 4 mg/kg (13 nmol/kg) GPVI-CD39, and plasma levels were detected by ELISA. Mean±SEM is shown.(n=3 animals). B, ADPase activities in mice up to 48 hours after administration of GPVI-CD39 or Fc controls. Blood samples were taken at the indicated times after IV administration of either 4 mg/kg GPVI-CD39 (13 nmol/kg, corresponding to 26 nmol/kg ADPase moieties) or 26 nmol/kg solCD39, and ADP turnover (mean±SEM) was measured by using a Malachite Green phosphate detection kit. Time is shown at a logarithmic scale to visualize decrease in activity during early time points (n=3 animals). C, Tail bleedings times. Tails were incised 15 minutes after IV administration of the indicated doses of GPVI-CD39, GPVI-Fc, or buffer, and tail bleeding times were determined. Mean values of 8 independent experiments are shown with SEM. No significant differences between groups occurred. GPVI-CD39 indicates dimeric glycoprotein VI and CD39 fusion protein; GPVI-Fc, dimeric glycoprotein VI; IV, intravenous; solCD39, soluble CD39.

Under flow, platelet inhibition by GPVI-CD39 was more pronounced than adding a full ex vivo dose of the ADP receptor inhibitor ticagrelor to GPVI-Fc: Comparing the results of the current study with those of our previous results,[37,38] 150 nmol/L GPVI-CD39 was equally effective in inhibiting plaque-induced platelet aggregation (81% inhibition) as the combination of 150 nmol/L GPVI-Fc with 3.82 µmol/L of the ADP receptor antagonist ticagrelor (79% inhibition). This comparison underscores the relative potency of the GPVI-CD39 fusion protein compared with existing antiplatelet drugs. Similar to previous reports on solCD39,[21,23] the fairly low dose of GPVI-CD39 used in this study had no effect on systemic bleeding times; however, GPVI-CD39 was fully effective in inhibiting arterial thrombosis in response to a ferric chloride challenge. This finding implies that the combination of CD39 with GPVI in a single molecule offers a favorable risk–benefit ratio.

The concept of a fusion protein has been developed in parallel with another interesting fusion concept, namely, the combination of CD39 with an activation-specific anti-GPIIb/IIIa single-chain antibody,[22,39] which also allowed reduction of the systemic doses of applied CD39 due to local enrichment. Both approaches are complementary insofar as this ScFvSCE5-CD39 fusion protein[23] targets growing thrombi, whereas the approach to use GPVI-CD39 focuses on local enrichment at high-risk arterial lesion before a full thrombus has evolved.

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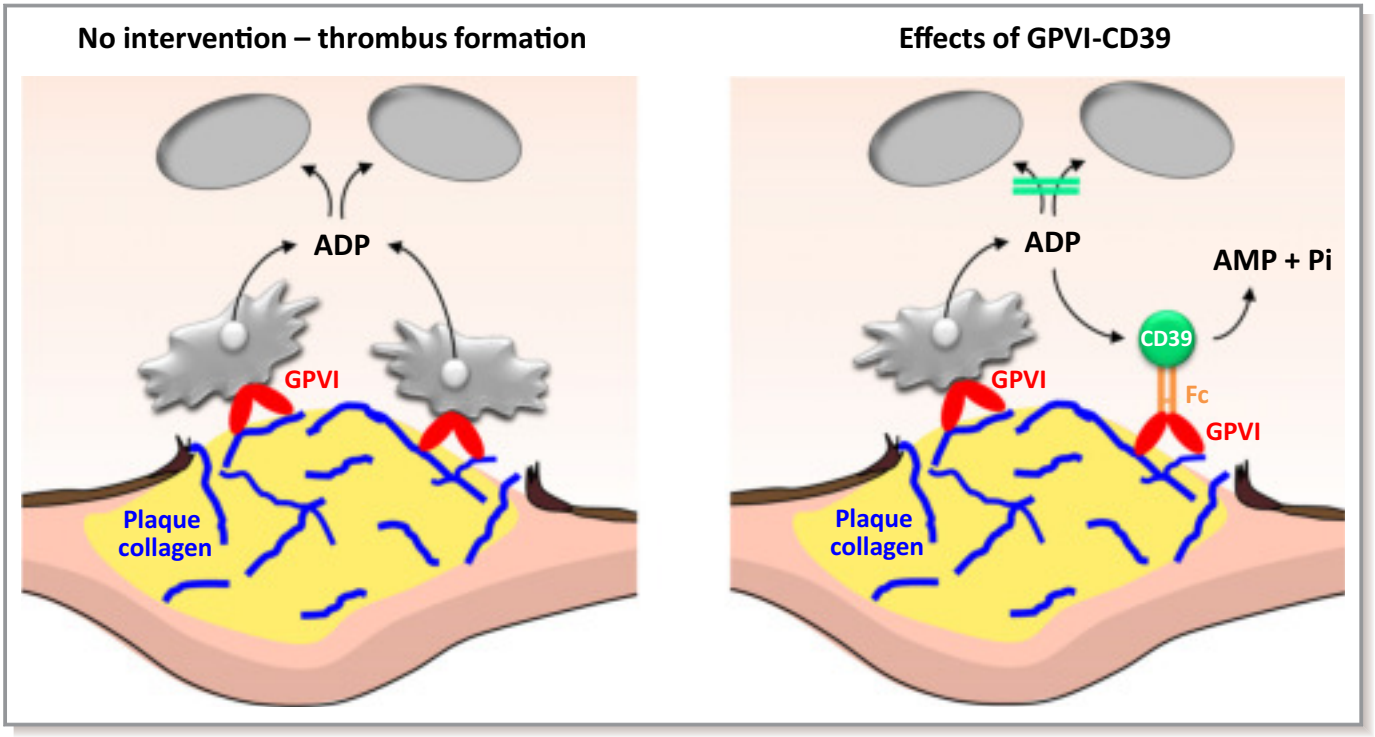

Figure 8. Mode of action of GPVI-CD39 at arterial plaques. GPVI indicates glycoprotein VI; GPVI-CD39 indicates dimeric glycoprotein VI and CD39 fusion protein; Pi, inorganic phosphate.

Such local enrichment of CD39 might provide an attractive alternative therapeutic option in arterial diseases. Reduced CD39 activity was recently shown to be associated with disease progression in patients with peripheral arterial disease.[40] However, the frequently used ADP receptor antagonist drug clopidogrel and ticlopidine further inhibit CD39 activity, especially at the beginning (the first few days) of the respective therapies,[41] so that in comparison, short-term rapid intravenous administration of GPVI-CD39 might be particularly beneficial for acute vascular syndromes and emergency conditions. A schematic overview of the mode of action of GPVI-CD39 is shown in Figure 8.

Generally, CD39 fusion proteins offer perspectives in several regards and indications.[42] CD39 has been proposed as an approach to widen the cardiovascular therapeutic window.[43] We demonstrated in this study that the antiatherosclerotic properties of blocking GPVI binding sites and promoting CD39 activity add up at the site of atherothrombosis when combined in a bifunctional molecule, but this fusion protein does not compromise systemic hemostasis.

Limitations of the Study

The antithrombotic effects of the fusion proteins have been studied in vivo in murine arterial thrombosis models and ex vivo in human atherothrombosis models but not in vivo in cardiovascular patients after plaque ruptures or erosions.

Sources of Funding

This study was supported by a grant of the Bavarian Research Foundation (Bayerische Forschungsstiftung), grant 1145-14, by the Deutsche Forschungsgemeinschaft (SFB 1123, TP 08), and by "Klinische Forschergruppe" KFO240 "Platelets – Molecular mechanisms and translational implications" at the University of Tübingen.

Disclosures

Drs Degen, Münch, Holthoff, Fassbender and Ungerer are employees of the biotech company Advancecor. Meinrad Gawaz is a co-founder of Advancecor, owns shares of Advancecor and is Professor at the Cardiology Department of the University of Tübingen. He further received honoraria payments from Lilly, Bristol-Myers Squibb and Bayer-Schering and is also consultant for Bayer-Schering.

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ADPase CD39 Fused to Glycoprotein VI–Fc Boosts Local Antithrombotic Effects at Vascular Lesions

Heidrun Degen, Oliver Borst, Melanie Ziegler, Ann-Katrin Mojica Munoz, Janina Jamasbi, Britta Walker, Silvia Göbel, Julia Fassbender, Kristin Adler, Richard Brandl, Götz Münch, Reinhard Lorenz, Wolfgang Siess, Meinrad Gawaz and Martin Ungerer

J Am Heart Assoc. 2017;6:e005991; originally published July 27, 2017;
doi: 10.1161/JAHA.117.005991
The *Journal of the American Heart Association* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Online ISSN: 2047-9980

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