The CX3C chemokine fractalkine mediates platelet adhesion via the von Willebrand receptor glycoprotein Ib

Sascha Meyer dos Santos,[1] Ute Klinkhardt,[1] Klaus Scholich,[1] Karen Nelson,[2] Nadejda Monsefi,[3] Hans Deckmyn,[4] Karina Kuczka,[1] Anita Zorn,[1] and Sebastian Harder[1]

Departments of [1]Clinical Pharmacology, [2]Vascular and Endovascular Surgery, and [3]Cardiovascular and Thoracic Surgery, J. W. Goethe University Hospital Frankfurt, Frankfurt, Germany; and [4]Laboratory for Thrombosis Research, Interdisciplinary Research Centre, KU Leuven Campus Kortrijk, Kortrijk, Belgium

The membrane-anchored CX3C chemokine fractalkine (FKN) is expressed on activated endothelium and is associated with the development of atherosclerosis. The potential of FKN in mediating platelet adhesion beyond platelet activation remains unexplored to date. A flow-based adhesion assay was used to study the adhesion of platelets to immobilized FKN under physiologic flow conditions. Platelet adhesion to von Willebrand factor (VWF) was increased in the presence of FKN at 600 inverse seconds. Additional platelet adhesion to FKN coimmobilized with VWF was dependent on the FKN receptor CX3CR1 and activation of glycoprotein (GP) IIb/IIIa. The number of platelets rolling on VWF was likewise enhanced in the presence of FKN. The enhancement of rolling on FKN and VWF was insensitive to anti-CX3CR1 antibody but was fully inhibited by neutralizing GPIbα function. The extracellular domain of GPIbα was covalently coupled to fluorescent microspheres, and microsphere binding was significantly higher in the presence of FKN. Platelet adhesion to activated endothelium in vitro and to intact human arteries was substantially increased in an FKN-dependent manner. These data demonstrate that endothelial expressed FKN activates platelets via its cognate receptor CX3CR1, whereas platelet adhesion is predominantly mediated by GPIbα and independent of CX3CR1. (*Blood*. 2011;117(18):4999-5008)

Introduction

The intact and nonactivated endothelium normally prevents sustained interactions with platelets and the adhesion of platelets or other inflammatory cells to the vessel wall. On injury of the vessel wall, blood is exposed to the extracellular matrix, and circulating von Willebrand factor (VWF) can rapidly bind to several of its components. The attachment of platelets to a thrombogenic surface depends on the velocity of the flowing blood. Above a threshold shear rate of approximately 500 inverse seconds (s^{-1}), the process is considered to be strictly dependent on VWF and its receptor glycoprotein (GP)Ibα.[1] Immobilized VWF mediates only reversible platelet adhesion via GPIb-IX-V because this interaction exhibits a rapid dissociation rate and platelets tethered to VWF move constantly in the direction of flow with a characteristic rolling velocity.[1] This process permits the formation of additional interactions, which have a slower rate of bond establishment but are capable of mediating stable stationary platelet adhesion, spreading, and aggregation. For the most part, these receptors belong to the integrin superfamily, with GPIIb/IIIa (integrin αIIbβ3) being the most prominent, and the function of many of these receptors depends on the platelet activation state.[2]

Fractalkine (FKN) is an unusual chemokine that exists in a soluble and membrane-anchored form[3] and its leukocyte adhesion properties have been well characterized.[4-6] The presence of the FKN receptor CX3CR1 on platelets has recently been demonstrated.[7] In rat platelets, soluble FKN induces a G-protein-dependent activation and expression of P-selectin. In human platelets, stimulation with soluble FKN enhances GPIIb/IIIa-dependent platelet adhesion to collagen and fibrinogen. Furthermore, FKN triggers P-selectin exposure on platelets adhering to the endothelium, which initiates the local accumulation of leukocytes at elevated shear stress.[8] The endothelial expression of FKN has been implicated in the genesis and progression of atherosclerosis.[9-11] To date, the role of surface-bound FKN in platelet adhesion in flowing blood remains unexplored. It may be questioned whether immobilized FKN is capable of mediating platelet capture from flowing blood and whether dynamic properties resemble observations previously reported with leukocytes. Therefore, the adhesive capacity of immobilized FKN toward platelets was tested under physiologic flow conditions.

Methods

More detailed information is available in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Preparation of blood samples

Suspensions of washed platelets were prepared as described elsewhere.[12,13] All studies involving human subjects were approved by the University Hospital Frankfurt ethics committee. Informed consent to donate blood for the experiments was obtained from all subjects in accordance with the Declaration of Helsinki.

Flow-based adhesion assays

The interaction between blood platelets and immobilized FKN under conditions of laminar flow was studied as previously described.[12] μ-slides

Submitted February 7, 2011; accepted March 1, 2011. Prepublished online as *Blood* First Edition paper, March 11, 2011; DOI 10.1182/blood-2011-02-335471.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

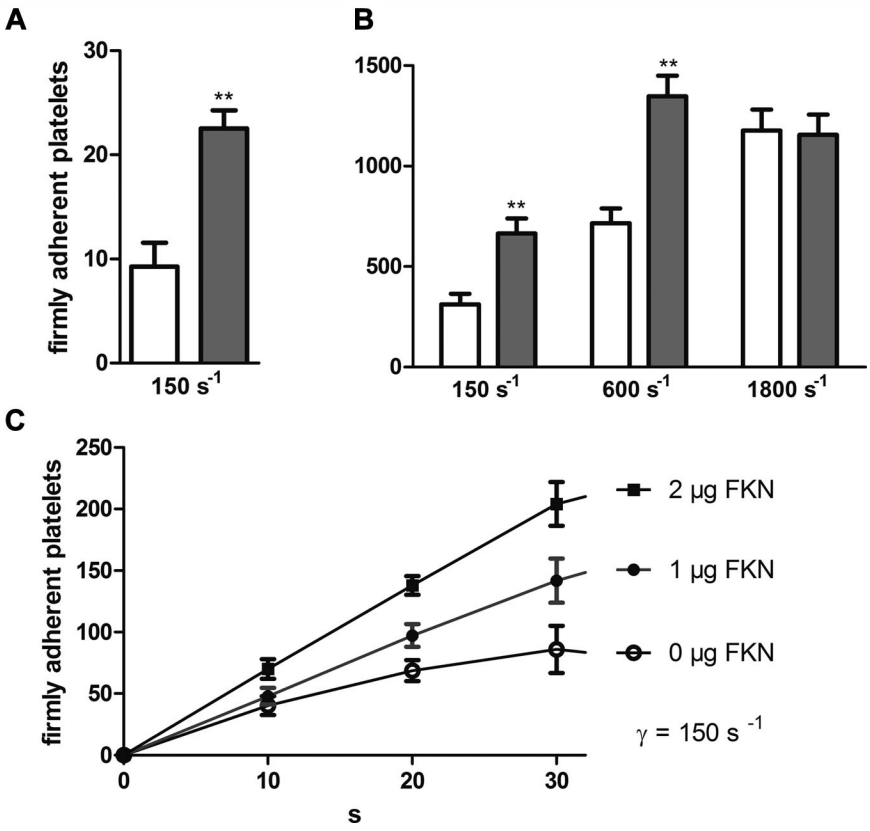

Figure 1. Adhesion of platelets to immobilized FKN under physiologic flow conditions. (A) Adhesion of platelets after 2 minutes at the indicated shear rate to immobilized FKN (closed bars). Control surfaces (open bars) were prepared in the same way with the omission of FKN to determine nonspecific background binding. (B) Adhesion of platelets to FKN coimmobilized with VWF (closed bars) or to VWF solely treated surfaces (open bars). Conditions were identical to panel A, except that VWF was present. The rate of nonspecific background adhesion to the control surface was subtracted from the presented adhesion rates. Results are the mean ± SEM of at least 4 independent experiments with blood from different donors. $^{**}P < .01$ in 1-way analysis of variance with Bonferroni Multiple Comparison Test versus control or VWF adhesion rates. (C) Adhesion of platelets to VWF coimmobilized with FKN at the indicated doses as a function of time. The rate of nonspecific background adhesion to the control surface was subtracted from the adhesion rates. Results are the mean ± SEM of a representative experiment performed 3 times. Adhesion rates for .5 μg and 1.5 μg were omitted for clarity.

(Ibidi) were coated overnight at 4°C with 1 μg antipolyhistidine monoclonal antibody (mAb; R&D Systems) with or without 13.5 μg recombinant human VWF (produced in HEK 293 cells stably transfected with full-length VWF-cDNA as previously described[14]). The slides were blocked with 2% bovine serum albumin (BSA), and 0–2 μg recombinant his-tagged FKN (R&D Systems) was then captured for 2 hours. Nonspecific background binding was evaluated on slides coated only with antipolyhistidine antibody. In some control experiments to inactivate FKN, FKN was inactivated by heating to 95°C for 30 minutes before immobilization. The μ-slides were placed under an epifluorescent microscope (Eclipse, Nikon) for real-time videomicroscopic observation of the platelet interactions with protein-coated surfaces or endothelial cells. Image processing was performed as described previously.[12]

Inhibitors of platelet adhesive interactions

Washed platelets were incubated with a selectively antagonizing anti-CX3CR1 mAb (TP502AF, Torrey-Pines; 20 μg/mL), polyclonal control IgG (Southern Biotechnology), or vehicle alone. Pretreated platelets were combined with washed autologous red blood cells (RBCs) to obtain reconstituted blood as described in "Preparation of blood samples." To define the adhesion molecules engaged in platelet adhesion experiments, the samples were incubated with the anti-GPIIb/IIIa mAb abciximab (C7E3, Eli-Lilly, Centocor; 20 μg/mL) or a function blocking anti-GPIbα mAb (BioLegend; clone HIP1; 40 μg/mL).

Preparation of FKN-coated microspheres

A total of 500 μL of the microsphere suspension (10-μm diameter, Polysciences) was washed 3 times with 1 mL phosphate-buffered saline (PBS) and incubated with 100 μg FKN overnight at 4°C under continuous shaking. After washing, the microspheres were resuspended in 1% BSA in PBS and used within 1 week. Coupling efficiency was controlled by flow cytometric analysis (FACSCalibur; BD Biosciences).

Bead adhesion assay with FKN-coated microspheres

Leukocyte-free platelet concentrates were obtained from the Blutspendedienst Hesse. The absence of leukocytes was confirmed by flow cytometry. Platelets were harvested by centrifugation at 3000*g* for 5 minutes and washed 3 times with modified Tyrode buffer 2. The platelet pellet was adjusted to 1×10^9 platelets/mL, and the resulting suspension was aspirated through μ-slides (Ibidi) precoated with 13.5 μg VWF at a wall shear rate of 1800 s^{-1} to obtain a confluent platelet monolayer. Confluency of the platelet monolayer was confirmed by phase-contrast microscopy. The immobilized platelets were incubated with the function blocking anti-CX3CR1 mAb (20 μg/mL), an isotype control (20 μg/mL), or vehicle alone for 30 minutes at 37°C in a CO_2 incubator. After washing 3 times, the slides were perfused with protein-coated microspheres (1×10^6/mL) suspended in PBS for 15 minutes at a wall shear rate of 150 s^{-1}. After extensive washing, attached microspheres were visualized by fluorescence microscopy, and the number of adherent microspheres was averaged in 5 randomly selected high power fields. Unspecific background binding was determined with BSA-coated microspheres.

Covalent coupling of glycocalicin to carboxyl-modified microspheres

Glycocalicin was covalently coupled to fluorescent polystyrene beads (1.75 μm diameter, Bangs Laboratories) as described elsewhere,[15] with minor modifications. A total of 200 mg of the microsphere suspension was washed with acetate buffer (0.1M acetic acid, pH 4.4) and activated with 100 mg, (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, (EDAC). The activated microspheres were allowed to react with 3 mg glycocalicin for 3 hours. The unmodified functional groups were quenched with 1% BSA in 40mM ethanolamine. Microspheres were washed and stored in 1% BSA in PBS at 4°C until use. Control microspheres were prepared in the same manner, but 1% BSA was substituted for glycocalicin. The presence of glycocalicin on microsphere surface was confirmed by flow cytometry with a phycoerythrin-labeled anti-GPIbα mAb.

Table 1. Comparison of FKN coating density of FKN in μ-slides with the FKN expression in resting and stimulated HUAECs

	Molecules FKN/μm²
μ-slides coated with 1 μg/mL FKN	418 ± 51
HUAECs resting	36 ± 1
HUAECs stimulated	212 ± 17

Data are the mean ± SEM of 3 independent experiments performed in triplicate.

Bead adhesion assay with glycocalicin-coupled microspheres

Platelet-rich plasma was separated from RBCs by centrifugation at 120*g* for 15 minutes. After washing 3 times, packed erythrocytes were supplemented with glycocalicin-coupled microspheres and diluted with Tyrode buffer 2 to obtain a final microsphere density of 5×10^6/mL and a hematocrit of 50%. This suspension was passed over protein-coated μ-slides and evaluated as described for reconstituted blood.

Human internal mammary arteries

Immediately after explantation, human internal mammary arteries obtained from coronary artery bypass graft surgery were placed in cold Dulbecco modified Eagle medium buffer (containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 50 U/mL heparin) and dissected free from surrounding fat and connective tissue. Prepared arteries were stored at 4°C in M199 medium (containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 50 U/mL heparin, and 10% pooled human serum) and used within 72 hours. The samples were stimulated with 50 ng/mL TNF-α and IFN-γ each (both from PeproTech) or left untreated for 20 hours at 37°C before the experiment.

To evaluate the adhesion of blood platelets to intact human arteries, artery segments were longitudinally opened and immobilized in the μ-slides to permit en face analysis of platelet interaction with the intima of the artery and immediately used for the blood perfusion experiments.

Results

The aim of the present study was to elucidate FKN′s role in platelet adhesion beyond its known platelet-activating capacity. More specifically, can adhesion to FKN be uncoupled from activation and what receptors are involved? Platelets were reconstituted with autologous RBCs and perfused through μ-slides coated with full-length FKN at various physiologic wall shear rates. Platelets that did not move more than 1 cell diameter during 10 seconds were scored adherent and included in the analyses.[12]

FKN enhances platelet adhesion to VWF

As shown in Figure 1A, platelets were captured from flowing blood by immobilized FKN at a wall shear rate of 150 s^{-1}. Although the adhesion of platelets was significant, it was modest, only 2-fold higher than the number of platelets adhering to antipolyhistidine-coated control slides (Figure 1A). However, elevating wall shear rate to 600 s^{-1} and 1800 s^{-1} prevented platelet adhesion to immobilized FKN (data not shown). The lack of platelet interaction with immobilized FKN at elevated shear rates was not the result of protein detachment from the μ-slides surface because platelets rapidly repopulated the slides after reducing the shear rate to 150 s^{-1} at the expected adhesion rate (data not shown). FKN mediated the rapid arrest of platelets without extensive rolling before firm arrest (supplemental Video), such as observed with VWF. FKN coating density in μ-slides was 418 ± 51 molecules FKN/μm² (comparable with the FKN coating density reported on glass[16]), and the calculated amount on human umbilical artery endothelial cells (HUAECs) was 36 ± 1/μm² before and 212 ± 17/μm² after stimulation (Table 1).

Table 2. Influence of FKN coimmobilization on VWF coating density and VWF conformation

	% before FKN addition
VWF coating density	104.1 ± 2.0
VWF glycocalicin binding	107.9 ± 5.8

Data are mean ± SEM of 3 independent experiments performed in triplicate. Coating density of VWF before and after FKN was measured by ELISA. VWF conformation was assessed by the ability of VWF to bind glycocalicin after FKN application by ELISA.

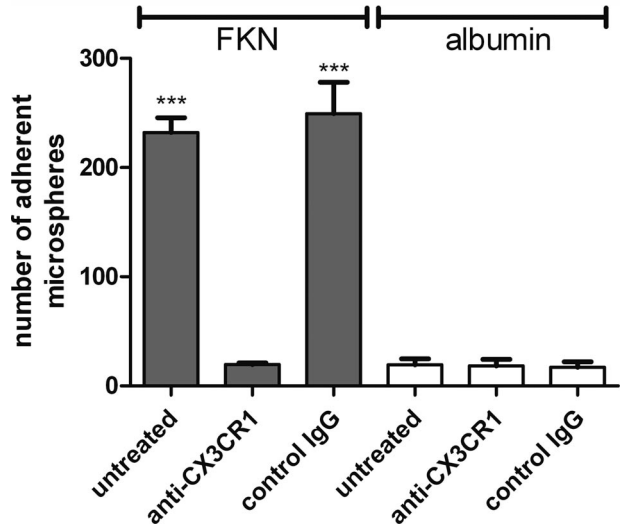

Figure 2. Adhesion of FKN-coated microspheres to platelets immobilized in μ-slides. Leukocyte-free platelets were immobilized in μ-slides. After removal of nonadherent platelets, surface-attached platelets were incubated with a CX3CR1 neutralizing antibody (anti-CX3CR1), matched isocontrol (control IgG), or vehicle alone (untreated) before perfusion with FKN-coated microspheres (FKN) or BSA-coated microspheres (albumin) suspended in PBS for 15 minutes at a wall shear rate of 150 seconds. After washing the μ-slides with PBS, the number of adherent microspheres was counted. Results are the mean ± SEM of 3 independent experiments with platelets from different leukocyte concentrates. ***$P < .001$ in 1-way analysis of variance with Bonferroni Multiple Comparison Test versus the number of adherent control microspheres perfused over identically treated platelets.

To examine whether FKN acts in conjunction with VWF, μ-slides were coated with VWF or a combination of FKN and VWF. Reconstituted blood was aspirated in parallel through μ-slides, and the interactions of flowing platelets with the surface were analyzed. Consistent with previous reports, platelet deposition onto VWF was dependent on the applied shear rate[1] and the number of adherent platelets increased with increasing shear rates from 150 s^{-1} to 1800 s^{-1} (Figure 1B). However, at low and medium shear, the presence of immobilized FKN induced additional recruitment of firmly attached platelets to VWF, and this effect was more than additive (Figure 1A-B). Adhesion of platelets to VWF coimmobilized with FKN was not influenced by a change in VWF coating density or a change in VWF conformation because of the presence of FKN (Table 2). The additional contribution of FKN in glycocalicin binding was not detectable under these circumstances because of the high VWF/FKN ratio and the smaller FKN compared with VWF glycocalicin binding signal strength (see Figure 6A). In all subsequent experiments, 1 μg FKN was used because this dose was within the linear dose-response curve (Figure 1C).

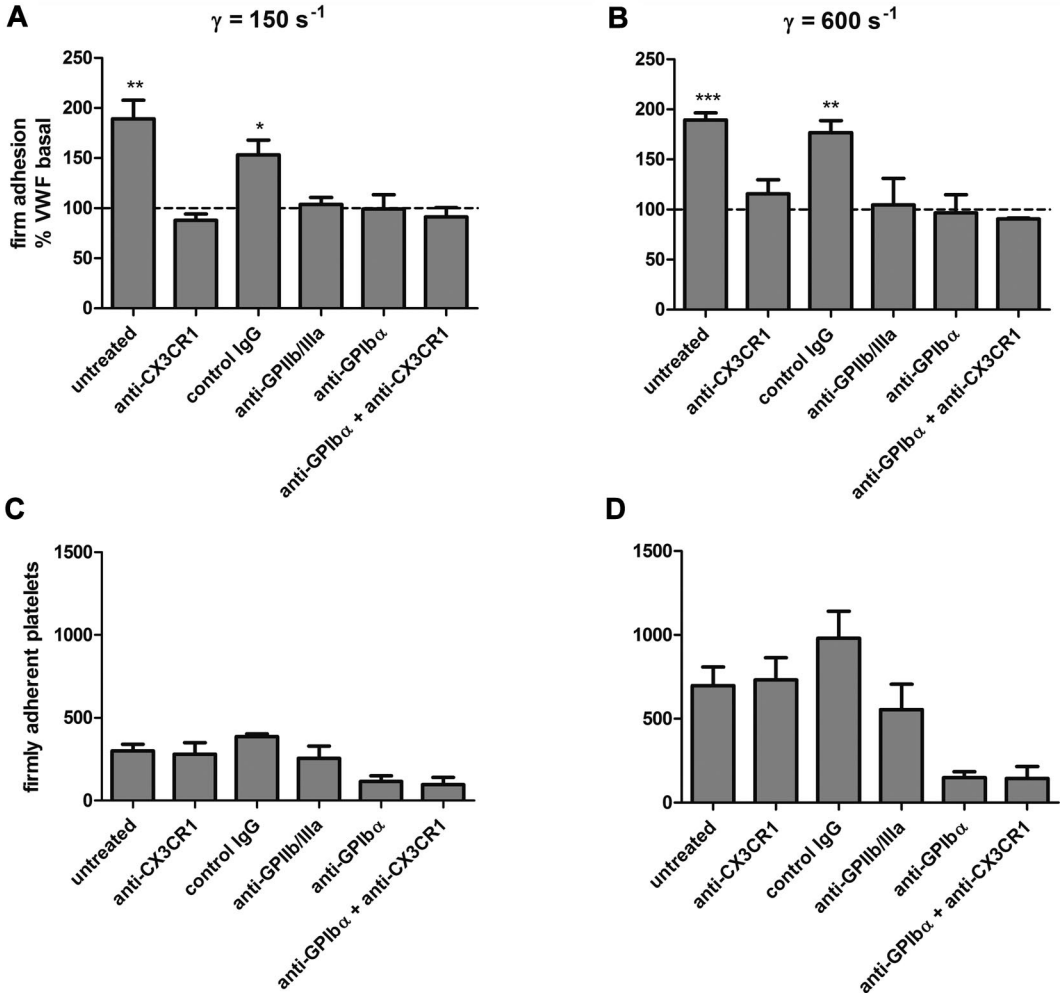

Figure 3. Effects of pretreating platelets with various inhibitors on FKN-mediated enhanced adhesion. Washed platelets were preincubated with vehicle alone (untreated), a neutralizing anti-CX3CR1 antibody, isotype-matched control (control IgG), the anti-GPIIb/IIIa antibody C7E3 (anti-IIb/IIIa), or the anti-GPIbα antibody HIP-1 as indicated for 30 minutes before reconstitution with autologous RBCs and perfused over VWF and FKN coimmobilized with VWF. (A-B) Perfusion was at a wall shear rate of 150 s^{-1} (A) and 600 s^{-1} (B). Shown is the percentage of platelets adhering to FKN coimmobilized with VWF, and the number of platelets adhering to VWF alone (C-D) was set to 100% for each treatment (dotted line). $*P < .05$, $**P < .01$, $***P < .001$ in one sample *t* test versus 100%, which represents the adhesion rate of platelets to VWF only surfaces. (C-D) The absolute number of platelets adhering to VWF-coated surface at the wall shear rate of 150 s^{-1} (C) and 600 s^{-1} (D). Results are the mean ± SEM of at least 4 independent experiments with blood from different donors.

Immobilization of FKN on slides and microspheres did not evoke major structural artifacts without a physiologic role because immobilization of heat-denatured FKN did not promote additional platelet adhesion or rolling to VWF (data not shown).

FKN-activated platelet adhesion to VWF via CX3CR1

CX3CR1 is highly expressed in certain subsets of circulating cells, mainly leukocytes and lymphocytes.[17] To determine whether differences in platelet adhesion rates to immobilized FKN were the result of leukocyte contamination in reconstituted blood, FKN coupled to microspheres was perfused over a monolayer of leukocyte-free platelets. Leukocyte-free platelets were obtained from platelet concentrates and formed a dense confluent monolayer after perfusion over VWF at 1800 s^{-1}. FKN-expressing microspheres accumulated on surface adherent CX3CR1-expressing platelets (Figure 2) and preincubating platelets with a neutralizing anti-CX3CR1 antibody before microsphere perfusion completely prevented microsphere accumulation, whereas isotype control treatment had no effect. Control microspheres, prepared in the same way as FKN microspheres, substituting BSA for FKN, did not interact significantly with immobilized platelets (Figure 2). This proves that the observed platelets' adhesion rates to FKN were not the result of leukocytes present in the reconstituted blood.

To probe the bonds involved in adhesion to FKN coimmobilized with VWF, platelets were incubated with various inhibitors before reconstitution with autologous RBCs. Platelet preincubation with a neutralizing anti-CX3CR1 antibody significantly reduced FKN-dependent enhanced platelet adhesion to the level seen on VWF alone (Figure 3A-B). Isotype antibody treatment did not have this effect, thus demonstrating the specific engagement of the FKN-CX3CR1 axis in mediating enhanced adhesion of flowing platelets to FKN coimmobilized with VWF. Furthermore, blocking either the main integrin GPIIb/IIIa on platelets with abciximab or GPIbα with HIP-1 not only significantly reduced platelet adhesion to VWF as expected (Figure 3C-D) but completely obliterated the enhancing effect of FKN on the adhesion to VWF.

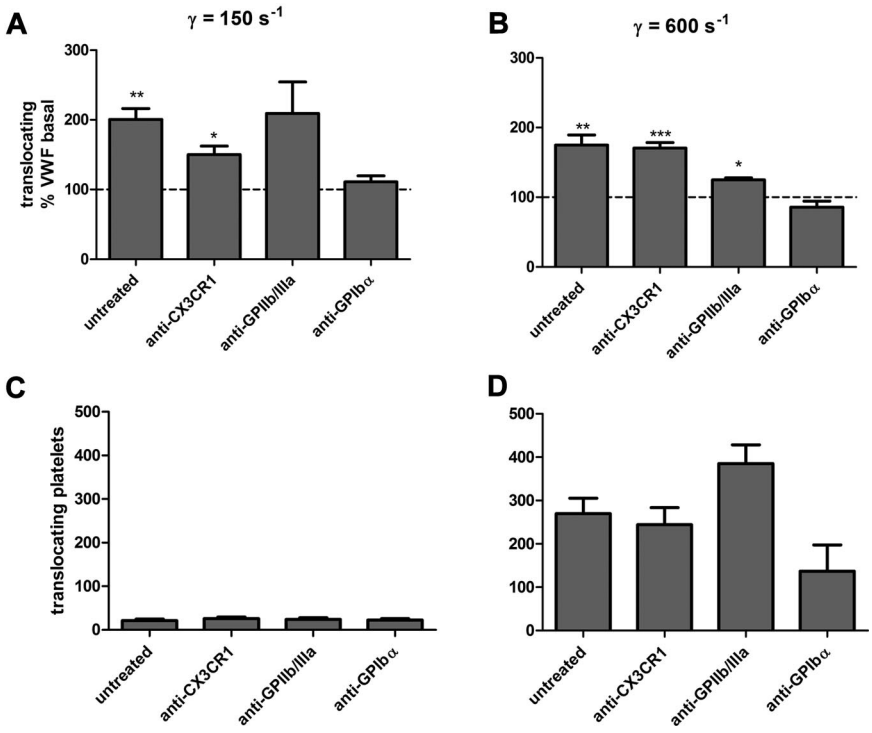

Figure 4. Quantification of the number of rolling platelets over VWF and FKN coimmobilized with VWF. Washed platelets were preincubated with modulators as described in Figure 3 and perfused over VWF and FKN coimmobilized with VWF. The number of rolling platelets was quantified after removing firmly adherent platelets by image processing. (A-B) Perfusion at a wall shear rate of 150 s^{-1} (A) and 600 s^{-1} (B). Shown is the percentage of platelets rolling on FKN coimmobilized with VWF, and the number of platelets rolling on VWF alone (C-D) was set to 100% for each treatment (dotted line). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ in one sample *t* test versus 100%, which represents the number of platelets rolling on VWF only surfaces. (C-D) The absolute number of platelets rolling on VWF-coated surface at the wall shear rate of 150 s^{-1} (C) and 600 s^{-1} (D). The results are the mean ± SEM of at least 4 independent experiments with blood from different donors.

FKN-stimulated platelet rolling over VWF independent of CX3CR1

The main role of the GPIb-V-IX complex is to capture platelets from flowing blood, reduce platelet velocity, and mediate platelet rolling on VWF. During rolling on VWF, platelets become activated, and firm and stable adhesion can be established via integrins.[1] Therefore, we counted the number of platelets moving on the protein surface, exhibiting a characteristic start-and-stop translocation movement with transient arrest up to several seconds. Platelets showing spatial displacement on the field of view greater than their own diameter during 1 second observation time were considered rolling and automatically counted after exclusion of stationary platelets by image processing.[12] The presence of FKN induced the rolling of additional platelets on VWF (Figure 4). Unexpectedly, this enhanced rolling was not mediated by the FKN receptor CX3CR1 because preincubating platelets with a neutralizing anti-CX3CR1 antibody was completely without effect. Abciximab only partially inhibited FKN-dependent additional platelet rolling, and these data indicate the participation of receptors other than CX3CR1 in augmented platelet rolling on FKN. However, blocking GPIbα function completely prevented enhanced platelet surface rolling on FKN immobilized in combination with VWF.

FKN interacts directly with GPIbα

To test whether GPIbα might interact with FKN under flow conditions, the extracellular domain of GPIbα, termed glycocalicin, was purified and coupled covalently to fluorescent microspheres. In the following, glycocalicin will be termed GPIbα, although glycocalicin is only the extracellular component of GPIbα, missing the transmembrane and cytoplasmatic domain.

Microspheres were supplemented with RBCs and perfused through protein-coated μ-slides. GPIbα-coupled microspheres interacted with immobilized VWF up to shear rates of 1800 s^{-1}, and interaction rates were significantly enhanced in the presence of FKN. Furthermore, GPIbα-coupled microspheres adhered to immobilized FKN at 150 s^{-1} and 600 s^{-1} in the absence of VWF (Figure 5A). The interaction of BSA-conjugated control microspheres with all surfaces was negligible (data not shown). The interaction of GPIbα with VWF and FKN was prevented by preincubating the microspheres with a neutralizing anti-GPIbα antibody (Figure 5B) to the same extent. Moreover, this treatment also inhibited the adhesion to FKN surfaces to levels of control surfaces (Figure 5C).

Enzyme-linked immunosorbent assay (ELISA) was used to determine the dissociation constant (k_D) of FKN binding to GPIbα. FKN bound to GPIbα with high nanomolar affinity (k_D was 4.9 ± 0.7nM; Figure 6B). Furthermore, FKN did not compete with VWF for GPIbα binding (Figure 6A). FKN appears to bind to the N-terminal domain of GPIbα because the antibodies 27A10, 12G1, and 12E4 abolished FKN binding, whereas 6B4 did not interfere with FKN binding but is reported to prevent VWF binding[18] (Figure 6C). The binding domains of the used mAbs are shown in Table 3.

Endothelial FKN contributes to platelet adhesion to HUAECs and human arteries

To extend these findings to a more physiologic model, the impact of FKN-dependent platelet adhesion to HUAECs was investigated. HUAECs were grown to confluence in μ-slides, and FKN expression was induced by TNF-α and IFN-γ. FKN expression was confirmed by immunofluorescence (data not shown).

Platelets interacted weakly with resting endothelium but strongly adhered to the activated endothelium up to wall shear rates of 600 s^{-1} (Figure 7A). This additional platelet adhesion to stimulated endothelium was, to some extent, the result of enhanced FKN expression because it was partially inhibited by pretreating the endothelial cells with a blocking anti-FKN antibody, whereas a

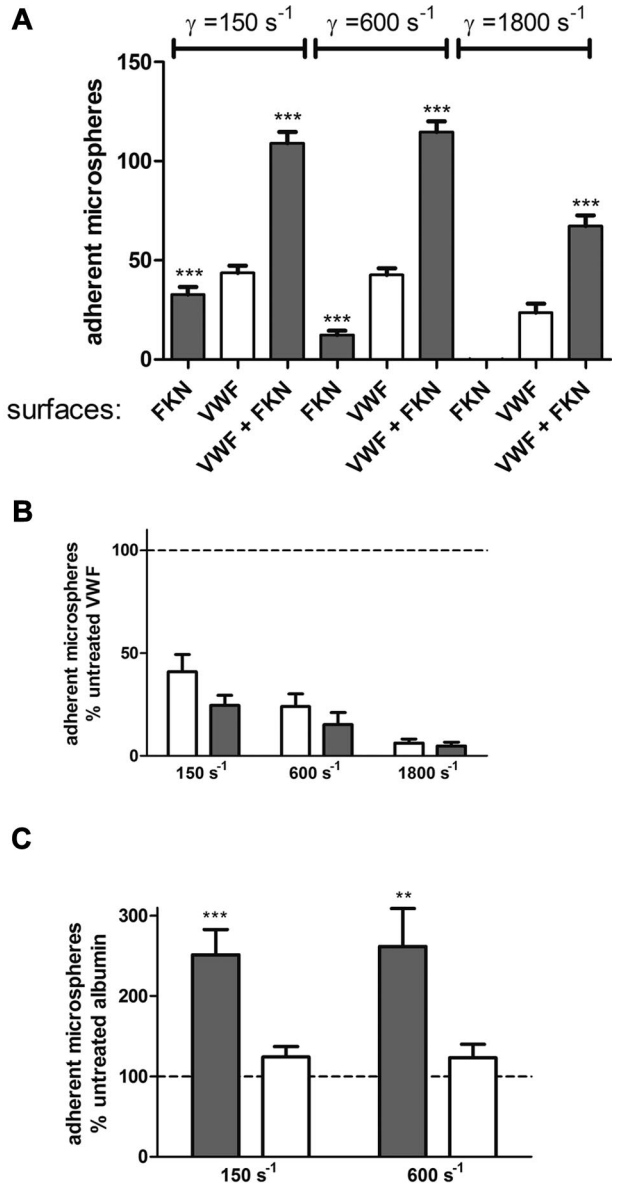

Figure 5. Interaction of GPIbα-coupled microspheres with FKN, VWF, and the combination under flow conditions. (A) Glycocalicin, the extracellular domain of GPIbα, was covalently coupled to fluorescent microspheres and combined with RBCs to obtain reconstituted blood and perfused through μ-slides coated with FKN, VWF, or FKN coimmobilized with VWF at a wall shear rate of 150, 600, or 1800 s^{-1}. The rate of nonspecific background adhesion to the control surface was subtracted from the presented adhesion rates. Results are the mean ± SEM of 3 independent experiments with blood from different donors. ***$P < .001$ by 1-way analysis of variance with Bonferroni Multiple Comparison Test versus control or VWF adhesion rates. (B) GPIbα-coupled microspheres were preincubated with the neutralizing anti-GPIbα antibody HIP-1 before perfusion over VWF (open bars) and FKN coimmobilized with VWF (closed bars). The adhesion of untreated microspheres to VWF surfaces was set to 100% (dotted line). Results are the mean ± SEM of 3 independent experiments with blood from different donors. (C) GPIbα-coupled microspheres were preincubated with the neutralizing anti-GPIbα antibody HIP-1 before perfusion over FKN and control surfaces. The adhesion of untreated microspheres to control albumin surfaces for each treatment was set to 100%, and the number of microspheres adhering to FKN for untreated (closed bars) and anti-GPIbα-treated microspheres (open bars) was evaluated. Results are the mean ± SEM of 3 independent experiments with blood from different donors. **$P < .01$, ***$P < .001$ in one sample *t* test versus adhesion to control surface with identical treatment.

treatment with isotype control had no influence (Figure 7B). These data prove that FKN-dependent adhesion played a role in this more physiologic model. Comparable results were obtained in experiments in which GPIbα-expressing microspheres were substituted for the platelets in flowing blood: endothelial FKN specifically induced the adhesion and accumulation of GPIbα-expressing microspheres (data not shown).

To approach human pathophysiology in vivo, we studied platelet adhesion to intact human arteries obtained from coronary artery bypass graft. FKN expression was induced by TNF-α and IFN-γ and was confirmed by immunocytochemistry (data not shown). Platelets adhered to the endothelial intima of activated arteries in an FKN-dependent manner (Figure 7C).

Discussion

FKN′s role in the process of platelet activation has recently been demonstrated. Both soluble and membrane-bound FKN on human umbilical vein endothelial cells induces platelet degranulation and surface expression of P-selectin, which in turn promotes adhesion of leukocytes to inflamed and FKN-expressing endothelium at high shear rates.[8] However, the function of FKN beyond platelet activation, particularly with regard to potentially mediating adhesion of CX3CR1-expressing platelets to immobilized FKN, remains unexplored to date.

In the present study, we demonstrate, for the first time, the adhesive capacity of membrane-bound FKN, not only via CX3CR1 expressed on platelets under physiologic flow conditions. Three additional lines of evidence are provided, pointing to the role of FKN in supporting enhanced platelet adhesion to VWF via direct GPIbα-FKN interactions using intact human platelets, GPIbα-expressing microspheres, and ELISA interaction studies.

Platelet adhesion to FKN was platelet specific and not because of contamination of reconstituted blood with leukocytes. However, FKN-dependent platelet adhesion was modest in the absence of additional adhesion ligands and occurred only at low shear, consistent with mechanisms involved in FKN-mediated leukocyte adhesion.[5,20]

However, when FKN was coimmobilized with VWF, the platelet adhesion revealed a substantial increase in the number of stationary adherent platelets compared with exclusively VWF-coated slides at shear rates up to 600 s^{-1}. Because this increase was more than additive, cooperating mechanisms beyond the mere FKN-CX3CR1 interaction are probably involved. Indeed, all combinations of inhibitory anti-GPIIb/IIIa, anti-GPIbα, and anti-CX3CR1 antibodies decreased the adhesion of flowing platelets to immobilized FKN plus VWF to levels of VWF by itself. This is consistent with the notion that FKN activates platelets and thus augments GPIIb/IIIa activity[7] and consistent with the concept of platelets using multiple adhesion pathways between FKN, in conjunction with VWF. Thus, these results demonstrate that adhesion of platelets to FKN and VWF is a multistage dynamic process. The competence of the endothelial VWF and platelet GPIbα axis in supporting platelet adhesion under high shear conditions has been well characterized.[21,22] VWF captures platelets through the interaction with GPIbα from the bloodstream and decelerates platelets below their hydrodynamic velocity. The slowing of platelets enables the establishment of low affinity but high strength bonds capable of mediating irreversible arrest as proven for GPIIb/IIIa ligands, such as fibrinogen. We here demonstrate that, after platelet deceleration and rolling on VWF, GPIbα-dependent additional platelet adhesion to FKN and CX3CR1-dependent activation by FKN takes place (supplemental Figure 1). The conversion of platelets' major integrin GPIIb/IIIa from a low to

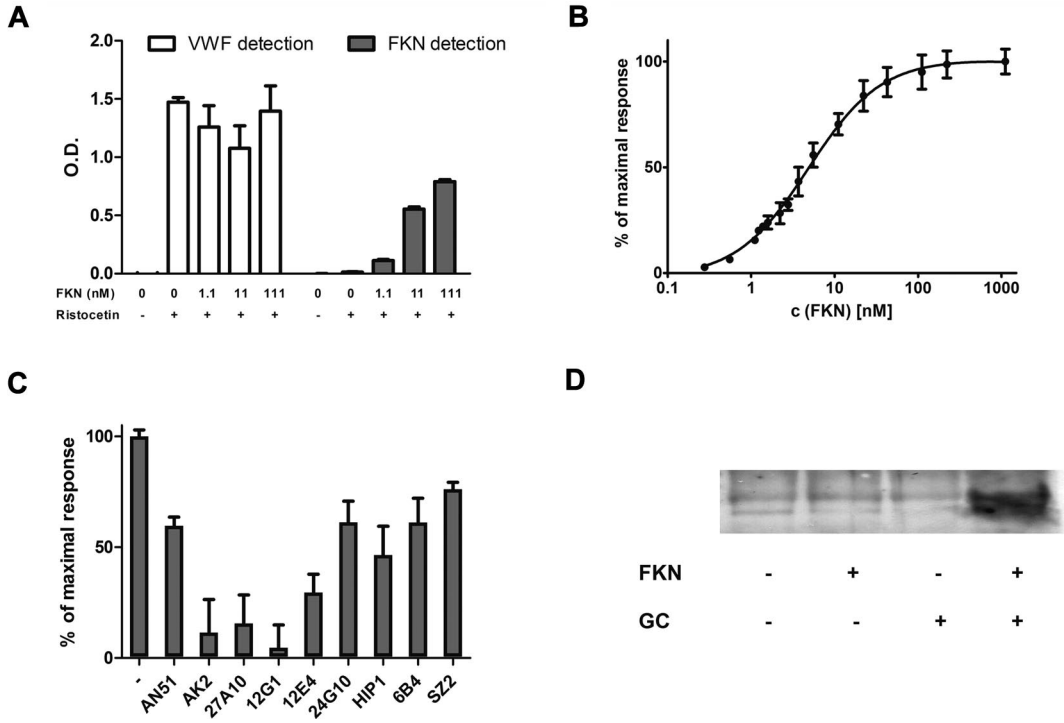

Figure 6. Detection of FKN and VWF binding to GPIbα by ELISA. (A) Glycocalicin was immobilized in microtiter plates, and FKN at the indicated concentration was allowed to bind before VWF and ristocetin addition. Bound VWF (open bars) or FKN (closed bars) was detected with the appropriate antibodies. No binding of VWF to GPIbα was seen in the absence of ristocetin. The experiment was performed twice, and results presented here are derived from one representative experiment and are presented as mean ± SEM for that experiment performed in triplicate. O.D. indicates optical density. (B) Concentration-dependent binding of FKN to GPIbα. Bound FKN was detected by ELISA as described in panel A. Shown is the mean ± SEM of 3 independent experiments. (C) Mapping of the FKN ligand binding site in GPIbα. Effect of diverse mAbs, with known GPIba binding site, on FKN binding. Untreated control (labeled) was set to 100%, and the inhibition of the corresponding mAb is shown. Results are shown as mean ± SEM of 3 independent experiments. (D) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of FKN and glycocalicin protein interaction pull-down experiments: recombinant his-tagged FKN was allowed to bind to Ni-NTA agarose and incubated with glycocalicin or buffer. Bound proteins were eluted with imidazole and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Western blot analysis with the anti-GPIbα mAb 27A10 to detect the presence of glycocalicin.

a high affinity receptor was recently demonstrated to be mediated by FKN[7] and confirmed in our study. Unexpectedly, we found that, in contrast to firm platelet adhesion, GPIbα-mediated enhanced platelet rolling on FKN coimmobilized with VWF was unaffected by antagonizing the FKN receptor CX3CR1. Platelet rolling, however, could be completely inhibited by neutralizing the GPIbα function. Based on these findings, 2 mechanisms might be hypothesized. First, blocking the first step in the adhesion cascade of flowing platelets to immobilized VWF or FKN coexpressed with VWF may also prevent all subsequent activation steps that take place downstream of GPIbα-dependent platelet capturing from flowing blood. Alternatively, a specific tethering interaction between immobilized FKN and platelet GPIbα may initiate enhanced platelet rolling in addition to VWF interacting with GPIbα.

To distinguish between these, we investigated the adhesive capacity of FKN to GPIbα under physiologic flow using the extracellular domain of GPIbα, purified and coupled to microspheres, as a simplified substitute for platelets in a flow-based adhesion assay. GPIbα interacted with immobilized FKN without requiring the participation of other adhesion ligands, and this interaction was prevented by preincubating the microspheres with an anti-GPIbα antibody. In the presence of FKN, significantly more GPIbα displaying microphores interacted with VWF at all shear rates tested, an effect that is specific because it was prevented by inhibition of GPIbα on the microspheres. Under static conditions, FKN interacted with GPIbα with nanomolar affinity and FKN did not compete with VWF for binding to GPIbα.

A growing number of GPIbα binding ligands beyond its main ligand VWF have been identified in the last decades, such as thrombin, protein C, Mac-1, P-selectin, thrombospondin, and some coagulation factors.[23] FKN can now be added as a new candidate to the list of physiologically relevant GPIbα binding ligands.

The FKN binding site in GPIbα appears to lie close to the N-terminal leucine-rich repeats, amino acids 1 to 59. This has been mapped to be binding sites of the mAbs 27A10, 12G1, and 12E4, which blocked FKN binding to GPIbα but did not interfere with VWF binding.[18] Blocking the C-terminal flanking region with 6B4 (amino acids 201-282) prevents VWF binding but did not influence FKN binding. Interestingly, 24G10 binds to amino acids 1 to 81, prevents VWF binding, yet does not interfere with FKN. This is consistent with the hypothesis that an intramolecular association between the N-terminal region (amino acids 1-81) and the C-terminal flank exists (amino acids 201-268). AN51 (amino acids 1-38) and SZ2 (amino acids 269-281),[19] on the other hand, had no influence on FKN binding. Therefore, we

Table 3. Binding sites of the mAbs used to characterize the FKN binding site in GPIba

Clone	Binding site, amino acids	Reference
AN51	1-35	19
AK2	36-59	19
27A10	35-59	18
12G1	1-59	18
12E4	1-59	18
24G10	1-81	18
HIP-1	59-81	19
6B4	201-268	18
SZ2	269-282	19

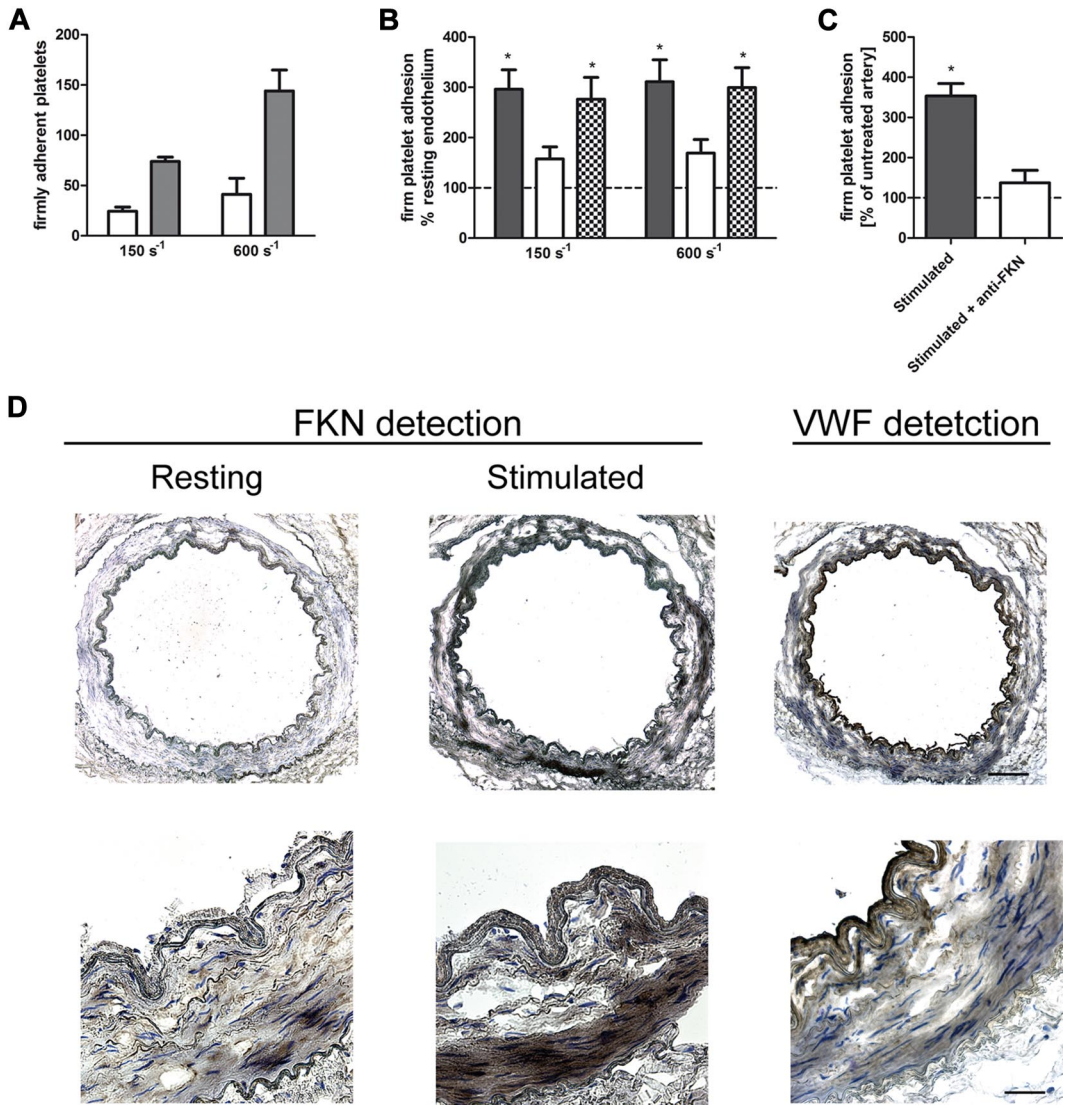

Figure 7. Adhesion of platelets to arterial endothelial cells under physiologic flow conditions. (A) Arterial endothelial cells were allowed to grow to confluence in μ-slides and stimulated (closed bars) or not (open bars) with TNF-α and IFN-γ for 20 hours. Human blood platelets in reconstituted blood were perfused at the indicated wall shear rate as described in Figure 1. The experiment was performed 3 times, and results presented here are derived from one representative experiment and are presented as mean ± SEM for that experiment performed in pentaplicate. (B) Endothelial cells were preincubated with vehicle alone (closed bars), a neutralizing anti-FKN antibody (open bars), or isotype-matched control (hatched bars) as indicated for 2 hours before perfusion initiation. Shown is the percentage of platelets adhering to stimulated endothelial cells at the indicated shear rate as percentage of the number of platelets adhering to resting endothelial cells with the respective treatments (dotted line). The results are the mean ± SEM of 3 independent experiments with blood from different donors.*$P < .05$ in one sample *t* test versus 100%, which represents the adhesion rate of platelets to resting endothelial cells. (C) Adhesion of platelets to the endothelium of intact human arteries. Blood was perfused at a wall shear rate of 600 s^{-1} over human artery, and the number of adherent platelets after 15-minute perfusion was recorded. Platelet adhesion rates to unstimulated arteries were set to 100% (dotted line). Shown are the relative adhesion rates to TNF-α- and IFN-γ-stimulated arteries with and without a neutralizing anti-FKN mAb. The results are the mean ± SEM of 3 independent experiments with blood and arteries from different donors.*$P < .05$ in one sample *t* test versus 100%, which represents the adhesion rate of platelets to resting arteries. (D) Immunohistochemical detection of FKN in sections of human internal mammary arteries. Representative photomicrographs of the endothelium immunostained for FKN (brown staining) in resting (left panel) and TNF-α- and IFN-γ-stimulated (middle panel) artery segments, counterstained blue with hematoxylin. Also shown is immunohistochemical detection of VWF (right panel). Bar represents 200 μm in the upper row and 50 μm in the lower row. Images were recorded with a Zeiss AxioObserver Z1 using a 5×/0.16 NA (top row) and a 20×/0.8 NA objective (bottom row) with an AxioCam MR3 camera (Zeiss) and processed in AxioVision LE 4.8.2.0 and ImageJ 1.45d software.

can conclude that the C-terminal domain is essential for VWF binding and that FKN binding occurs to the N-terminal domain amino acids 39 to 81.

We extended our in vitro findings to cell culture models and induced FKN expression on endothelial cells. Platelet adhesion to the endothelium was substantially increased after simulation, and FKN was partially responsible for stable platelet arrest on activated endothelial cells. Blocking FKN on the endothelium prevented platelet accumulation, and comparable results were obtained in blood in which GPIbα-expressing microspheres were substituted for platelets. The activated endothelium supported platelet adhesion, and endothelial FKN significantly contributed to stable platelet deposition to the inflamed endothelium. Finally, a similar FKN-dependent platelet adhesion was demonstrated in intact human arteries, a system we think is the model most closely approaching human pathophysiology in vivo. Induction of FKN in human arteries probably mimics the early stages of atherosclerosis. The FKN coating density in the μ-slides was in the same range of FKN expression on physiologic surfaces. These values are comparable with the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1[24] and P-selectin[16] on endothelial cells.

Thus, immobilized FKN seems to operate as a molecule capable of inducing GPIbα-dependent platelet rolling in cooperation with VWF at 600 s^{-1}, and this finally results in augmented stationary platelet adhesion. A comparable adhesion characteristic was observed in the FKN-promoted monocyte adhesion at elevated shear. The presence of additional adhesion-mediating molecules (eg, vascular cell adhesion molecule-1) was mandatory for initial leukocyte tethering and before FKN induced arrest at elevated shear.[17] Our results demonstrate an analogous situation in the FKN-mediated platelet arrest after tethering flowing platelets to VWF, deceleration, and CX3CR1 independent, but GPIbα-dependent, rolling of platelets on FKN, concomitant activation via CX3CR1 with increased avidity of GPIIb/IIIa and finally, enhanced firm platelet arrest. The function of endothelial FKN on platelets again was comparable with that on leukocytes: in both cell types, the activation by FKN is mediated by its cognate receptor CX3CR1. This is in contrast to the adhesive capacity: in leukocytes, FKN-dependent adhesion is mediated also by CX3CR1, whereas in platelets, the adhesive capacity was mediated by the VWF receptor GPIbα. In platelets, activation and adhesion by FKN were mediated by 2 distinct receptors, whereas in leukocytes CX3CR1 is the single receptor for FKN binding and mediates FKN-dependent adhesion and activation. The impact of GPIbα on the formation of atherosclerosis remains to be fully elucidated because previous studies have provided conflicting results: blocking GPIbα with mAb injection decreased plaque progression,[25] whereas GPIbβ knockout suggests that the GPIb-V-IX complex is not essential to the genesis of atherosclerosis.[26] However, GPIb ablation is associated with a more or less severe thrombocytopenia, so further studies are necessary to demonstrate a specific GPIbα involvement in atherosclerosis formation, independent of changes in platelet count or size.

Endothelial VWF[27-30] and endothelial FKN[9-11,31,32] are both implicated in the genesis of atherosclerosis, and it is tempting to speculate that our findings support the view that platelets interact with endothelial cells in an FKN-dependent manner in very early stages of that disease. Furthermore, TNF-α, among other inflammatory cytokines, stimulates the release of VWF from endothelial cells.[33]

Acknowledgments

The authors thank Marina Henke, Riad Haceni, and Bettina Picard-Willems for expert technical assistance, Dr Budde for VWF multimeric analysis, Dr Schneppenheim for providing the recombinant VWF, and Dr Anton Moritz, Dr Thomas Schmitz-Rixen, Dr Katleen Broos, Dr Myron Cybulsky, and Dr Kevin Wollard for fruitful discussions and helpful hints.

This work was supported by the Johann Wolfgang Goethe University Frankfurt (an institutional grant of Nachlass Schmelz).

Authorship

Contribution: S.M.d.S. designed research, performed experiments, analyzed data, and wrote the manuscript; U.K., K.S., K.N., K.K., and A.Z. designed research and performed experiments; N.M. provided human arteries; H.D. provided anti-GPIb mAbs and critically revised the manuscript; and S.H. supervised research and critically revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Sascha Meyer dos Santos, J. W. Goethe University Hospital Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany; e-mail: santos@med.uni-frankfurt.de.

References

1. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell.* 1996; 84(2):289-297.
2. Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. *J Clin Invest.* 1983;72(1):1-12.
3. Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature.* 1997;385(6617):640-644.
4. Imai T, Hieshima K, Haskell C, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell.* 1997;91(4):521-530.
5. Fong AM, Robinson LA, Steeber DA, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med.* 1998; 188(8):1413-1419.
6. Haskell CA, Cleary MD, Charo IF. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction: rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. *J Biol Chem.* 1999;274(15): 10053-10058.
7. Schafer A, Schulz C, Eigenthaler M, et al. Novel role of the membrane-bound chemokine fractalkine in platelet activation and adhesion. *Blood.* 2004;103(2):407-412.
8. Schulz C, Schafer A, Stolla M, et al. Chemokine fractalkine mediates leukocyte recruitment to inflammatory endothelial cells in flowing whole blood: a critical role for P-selectin expressed on activated platelets. *Circulation.* 2007;116(7):764-773.
9. Combadiere C, Potteaux S, Gao JL, et al. Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation.* 2003;107(7):1009-1016.
10. Lesnik P, Haskell CA, Charo IF. Decreased atherosclerosis in CX3CR1−/− mice reveals a role for fractalkine in atherogenesis. *J Clin Invest.* 2003;111(3):333-340.
11. Cheng C, Tempel D, van Haperen R, et al. Shear stress-induced changes in atherosclerotic plaque composition are modulated by chemokines. *J Clin Invest.* 2007;117(3):616-626.
12. Meyer Dos Santos S, Klinkhardt U, Schneppenheim R, Harder S. Using ImageJ for the quantitative analysis of flow-based adhesion assays in real-time under physiologic flow conditions. *Platelets.* 2010;21(1):60-66.
13. Meyer dos Santos SM, Weber CC, Franke C, Muller WE, Eckert GP. Cholesterol: coupling between membrane microenvironment and ABC transporter activity. *Biochem Biophys Res Commun.* 2007;354(1):216-221.
14. Hassenpflug WA, Budde U, Obser T, et al. Impact of mutations in the von Willebrand factor A2 domain on ADAMTS13-dependent proteolysis. *Blood.* 2006;107(6):2339-2345.
15. Borthakur G, Cruz MA, Dong JF, et al. Sulfatides inhibit platelet adhesion to von Willebrand factor in flowing blood. *J Thromb Haemost.* 2003;1(6): 1288-1295.
16. Haskell CA, Cleary MD, Charo IF. Unique role of the chemokine domain of fractalkine in cell capture: kinetics of receptor dissociation correlate with cell adhesion. *J Biol Chem.* 2000;275(44): 34183-34189.
17. Kerfoot SM, Lord SE, Bell RB, Gill V, Robbins SM, Kubes P. Human fractalkine mediates leukocyte adhesion but not capture under physiological shear conditions: a mechanism for selective monocyte recruitment. *Eur J Immunol.* 2003;33(3):729-739.
18. Cauwenberghs N, Vanhoorelbeke K, Vauterin S, et al. Epitope mapping of inhibitory antibodies against platelet glycoprotein Ibalpha reveals interaction between the leucine-rich repeat N-terminal and C-terminal flanking domains of glycoprotein Ibalpha. *Blood.* 2001;98(3):652-660.
19. Shen Y, Romo GM, Dong JF, et al. Requirement of leucine-rich repeats of glycoprotein (GP) Ibalpha for shear-dependent and static binding of von Willebrand factor to the platelet membrane GP Ib-IX-V complex. *Blood.* 2000;95(3):903-910.
20. Green SR, Han KH, Chen Y, et al. The CC chemokine MCP-1 stimulates surface expression of CX3CR1 and enhances the adhesion of monocytes to fractalkine/CX3CL1 via p38 MAPK. *J Immunol.* 2006;176(12):7412-7420.
21. Dopheide SM, Maxwell MJ, Jackson SP. Shear-dependent tether formation during platelet translocation on von Willebrand factor. *Blood.* 2002; 99(1):159-167.
22. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood.* 2006;108(6):1903-1910.

23. Clemetson KJ, Clemetson JM. Platelet GPIb complex as a target for anti-thrombotic drug development. *Thromb Haemost.* 2008;99(3):473-479.
24. Smith LA, Aranda-Espinoza H, Haun JB, Hammer DA. Interplay between shear stress and adhesion on neutrophil locomotion. *Biophys J.* 2007;92(2):632-640.
25. Massberg S, Brand K, Gruner S, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med.* 2002; 196(7):887-896.
26. Strassel C, Hechler B, Bull A, Gachet C, Lanza F. Studies of mice lacking the GPIb-V-IX complex question the role of this receptor in atherosclerosis. *J Thromb Haemost.* 2009;7(11):1935-1938.
27. Methia N, Andre P, Denis CV, Economopoulos M, Wagner DD. Localized reduction of atherosclerosis in von Willebrand factor-deficient mice. *Blood.* 2001;98(5):1424-1428.
28. De Meyer GR, Hoylaerts MF, Kockx MM, Yamamoto H, Herman AG, Bult H. Intimal deposition of functional von Willebrand factor in atherogenesis. *Arterioscler Thromb Vasc Biol.* 1999; 19(10):2524-2534.
29. Tull SP, Anderson SI, Hughan SC, Watson SP, Nash GB, Rainger GE. Cellular pathology of atherosclerosis: smooth muscle cells promote adhesion of platelets to cocultured endothelial cells. *Circ Res.* 2006;98(1):98-104.
30. Theilmeier G, Michiels C, Spaepen E, et al. Endothelial von Willebrand factor recruits platelets to atherosclerosis-prone sites in response to hypercholesterolemia. *Blood.* 2002;99(12): 4486-4493.
31. Damas JK, Boullier A, Waehre T, et al. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. *Arterioscler Thromb Vasc Biol.* 2005;25(12):2567-2572.
32. McDermott DH, Fong AM, Yang Q, et al. Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. *J Clin Invest.* 2003;111(8):1241-1250.
33. Bernardo A, Ball C, Nolasco L, Moake JF, Dong JF. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood.* 2004;104(1):100-106.