Comparative Antiplatelet Efficacy of a Novel, Nonpeptide GPIIb/IIIa Antagonist (XV454) and Abciximab (c7E3) in Flow Models of Thrombosis

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Abstract—Glycoprotein (GP) IIb/IIIa is pivotal in homotypic platelet aggregation and may also be involved in the heterotypic adhesion of leukocytes and tumor cells to platelets. This study was primarily undertaken to compare the antiplatelet efficacy of a novel, nonpeptide GPIIb/IIIa antagonist, XV454, to that of abciximab in 2 flow models of platelet thrombus formation: (1) direct shear-induced platelet aggregation imposed by a cone-and-plate rheometer and (2) platelet adhesion onto von Willebrand factor (vWF)/collagen I followed by aggregation in a perfusion system. XV454 inhibited platelet aggregation in a concentration-dependent manner in both experimental models. Maximal inhibition of aggregation was achieved by XV454 at ≈70% receptor occupancy, which is lower than the ≥85% previously reported for abciximab. At similar levels of receptor blockade (≈45%), XV454 appeared to be relatively more effective than abciximab in suppressing platelet aggregation. Neither XV454 nor abciximab inhibited platelet adhesion to collagen. Pretreatment of surface-adherent platelets with either XV454 or abciximab inhibited the attachment of monocytic THP-1 cells under flow. In contrast, the rapidly reversible GPIIb/IIIa inhibitor orbofiban failed to suppress these heterotypic interactions. These findings demonstrate that XV454 is a potent GPIIb/IIIa antagonist with a long receptor-bound lifetime like abciximab and may be beneficial for the treatment/prevention of thrombotic complications. **(*Arterioscler Thromb Vasc Biol*. 2001;21:149-156.)**

Key Words: platelets ■ adhesion ■ aggregation ■ shear stress ■ XV454, abciximab

Intravascular thrombosis is 1 of the most frequent pathological events and a major cause of morbidity and mortality in Western countries. Critical steps in the development of acute coronary syndromes are the disruption or erosion of atherosclerotic plaque and the formation of partially or completely occlusive thrombi. Arterial platelet thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and von Willebrand factor (vWF), with subsequent platelet activation and aggregation. In particular, platelet adhesion to immobilized, exposed vWF associated with collagen I under simulated abnormal arterial flow conditions is mediated by the platelet glycoprotein (GP) Ib/IX complex.[1,2] This adhesion step is followed by aggregation predominantly mediated by the binding of fibrinogen and vWF to GPIIb/IIIa in the presence of ADP.[1–4] In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels,[5] may be important. Shear-induced platelet aggregation is dependent on the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation, which also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.[6,7] Platelet activation and aggregation have been implicated in a number of vascular diseases such as unstable angina, myocardial infarction, transient ischemic attack, and stroke.[8,9] Thus, pharmacological modulation of platelet function is of clinical importance.

The final common step in homotypic platelet aggregation, regardless of the stimulus, involves the interaction of adhesive proteins such as fibrinogen and vWF with platelet GPIIb/IIIa. Several studies have identified the pivotal role of GPIIb/IIIa receptors in coronary thrombosis. Hence, this platelet integrin receptor has emerged as a rational therapeutic target in the management of acute coronary syndromes.[10] Various large-scale phase III clinical trials have illustrated the usefulness of GPIIb/IIIa antagonists in the treatment and prevention of acute ischemic syndromes. Intravenous admin-

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istration of abciximab (c7E3) in high-risk patients undergoing angioplasty has been shown to reduce the composite incidence of major ischemic events at the 30-day primary end point.[10] In other clinical studies, abciximab demonstrated efficacy when given in combination with thrombolytic therapy and in refractory unstable angina patients before angioplasty.[11–13] Additionally, several other selective GPIIb/IIIa antagonists, including eptifibatide (Integrilin, Cor Therapeutics), tirofiban, and lamifiban, are in advanced stages of clinical development and aimed primarily for intravenous use in the treatment and prevention of acute ischemic heart diseases.[13–15] Current intravenously administered small-molecule GPIIb/IIIa antagonists, such as Integrilin or tirofiban, in clinical trials have a faster rate of dissociation from human platelets, reflecting their short duration of antiplatelet effects compared with that of abciximab.[3,16,17] Sustaining antiplatelet efficacy levels with intravenous GPIIb/IIIa antagonists can be achieved with intravenous bolus and infusion regimens. Clinical studies with orally active, rapidly reversible, small-molecule GPIIb/IIIa antagonists, including orbofiban, xemilofiban, sibrafiban, and lefradafiban, have demonstrated variable antiplatelet activity in humans on administration 2 or 3 times per day.[16,18–21] These factors prompted us to develop a potent, highly specific GPIIb/IIIa antagonist, XV454,[22] with a relatively slow platelet dissociation rate for the treatment of different thromboembolic disorders.

The primary goal of this study was to assess the antiplatelet efficacy of XV454 in in vitro flow models of platelet thrombus formation and compare it with that of abciximab. A rheometric/flow cytometric method was used to monitor direct shear-induced platelet aggregation in the bulk phase.[17,23,24] In addition, we combined a parallel-plate perfusion chamber with a computerized epifluorescence video microscopy system to visualize in real time and quantify the adhesion and subsequent aggregation of human platelets in whole blood flowing under conditions of abnormally elevated shear stress (1500 s^{-1}) over type I fibrillar collagen.[3,4]

GPIIb/IIIa may also be involved in the heterotypic adhesion of leukocytes and tumor cells to platelets.[25–27] These cell-cell interactions have been implicated in the pathogenesis and/or progression of inflammatory, metastatic, and thrombotic disorders.[25,27] Consequently, we evaluated the effects of GPIIb/IIIa antagonists on the attachment of monocytic THP-1 cells to surface-adherent platelets under dynamic flow conditions (1.5 dyne/cm^2), thus simulating events encountered at sites of vascular injury in postcapillary venules. In this in vitro flow assay, any unbound GPIIb/IIIa antagonist is removed by a brief washing step after platelet preincubation and before perfusion of the monocytic cells, thereby enabling us to distinguish agents with slow platelet off-rates such as XV454 and abciximab from those with relatively faster off-rates such as orbofiban.

Methods

Reagents

XV454 and YZ202 (the active, free acid form of orbofiban) were synthesized at DuPont Pharmaceuticals Co. Abciximab was obtained from Centocor, Inc, and conjugated to FITC by standard techniques.[24] The fluorescein-to-protein molar ratio was ≈2. XL086-FITC was prepared by reaction of cyclic [D-Lys-N_2-methyl-L-arginylglycyl-L-aspartyl-3-(aminomethylbenzoic acid)] with FITC and purified by reverse-phase high-performance liquid chromatography.[28] XL086 has 1 fluorescein coupled at the D-Lys position.[28] CD42a (anti-GPIX)-FITC and CD42b (anti-GPIb)-FITC were purchased from Becton Dickinson and Pharmigen, respectively.

Blood Collection

Venous blood was collected from healthy, aspirin-free volunteer donors into polypropylene syringes containing either porcine heparin (Elkins-Sinn Inc; heparin sodium, 10 U/mL final concentration)[1,29] or sodium citrate (0.38% final concentration). Specimens were transferred to plastic tubes, stored at room temperature, and used within 4 hours of collection in flow assays.

Rheometric/Flow Cytometric Experiments

Citrated blood in the presence or absence of GPIIb/IIIa antagonists was subjected to a pathological shear rate of 4000 s^{-1} for 60 seconds by the use of a cone-and-plate rheometer (RS150, Haake). Controls included blood specimens that had been introduced into the rheometer but were not exposed to shear stress. Aliquots of control and sheared blood were immediately fixed with 1% formaldehyde in Dulbecco's phosphate buffered saline (PBS, Sigma) and incubated with saturating concentrations of CD42-FITC for 30 minutes in the dark. Subsequently, specimens were diluted with 2 mL of 1% formaldehyde and analyzed in a FACScan (Becton-Dickinson) flow cytometer as described elsewhere in detail.[17,23,24]

Binding of fluoresceinated CD42b (anti-GPIb), XL086 (an analogue of XV454),[28] or abciximab to platelets was calculated by measuring the mean FITC fluorescence intensity of at least 5000 platelets, which were identified on the basis of their characteristic forward- and side-scatter profiles in citrated blood specimens in the presence or absence of GPIIb/IIIa antagonists. At each experimental state, the FITC fluorescence values were normalized by using as a reference the pre-XV454/preabciximab incubation (control specimen) fluorescence levels. Nonspecific XL086 binding was determined by the addition of a 5-fold excess of XV454 and accounted for <15% of the total binding.[28] Prior work has demonstrated that binding of fluoresceinated monoclonal antibodies (CD42b or abciximab) or cyclic RGD peptide (XL086) detected by flow cytometry and expressed as mean fluorescence intensity is highly correlated with that detected by a radiometric method.[28] Because treatment of blood specimens with XV454 or abciximab inhibits the subsequent binding of XL086-FITC or abciximab-FITC, respectively, to platelets, the decrease in FITC fluorescence values after drug incubation is correlated with the percentage of GPIIb/IIIa receptors occupied by the drug.[17,28]

Preparation of Collagen-Coated Surfaces

Collagen suspensions of type I acid-insoluble fibrils (1 mg/mL final concentration) were prepared from bovine Achilles' tendon (Sigma) in 0.5 mol/L acetic acid, pH 2.8.[30] Glass coverslips (24×50 mm, Corning) were coated with 200 μL of fibrillar collagen (coated area=12.7×23 mm^2)[3,4] and placed in a humid environment for 45 minutes, and the excess collagen was rinsed off with 10 mL of Dulbecco's PBS before assembly into the flow chamber. The collagen density on the glass surfaces was estimated to be ≈2 $\mu g/cm^2$.[29]

Perfusion Studies

Platelet deposition onto collagen was quantified under dynamic flow conditions by using a parallel-plate flow chamber. A coverslip coated with type I collagen was assembled in a chamber (205 μm channel depth, 1.26 cm channel width) and mounted on the stage of an inverted microscope (Nikon TE300) equipped with an epifluorescence illumination attachment, an X60 fluor objective, an X1 projection lens, and a silicon-intensified target video camera (VE-1000, Dage) connected to a VCR and a TV monitor. Heparinized blood containing the fluorescent dye mepacrine (quinacrine dihydro-

chloride, Sigma) at a final concentration of 10 μmol/L[3,4,29] was perfused through the chamber for 1 minute at a wall shear rate of 1500 s^{-1}, thereby simulating flow conditions in partially constricted arteries.[5] The accumulation of mepacrine-labeled platelets to collagen was monitored in real time and recorded on videotape. Before the perfusion experiments, blood was incubated with either XV454, abciximab, or vehicle (control) at 37°C for 10 minutes. The microscope stage, flow chamber, and blood were maintained at 37°C by an incubator heating module and incubator enclosure during the experiment.

Evaluation of Platelet Adhesion and Subsequent Platelet Aggregation

Images from videotapes were digitized by using a Scion frame grabber and a personal computer and processed with the use of OPTIMAS image processing software (Agris-Schoen Vision Systems). Images were analyzed by computer at 5, 15, and 60 seconds for each perfusion experiment. The number of adherent individual platelets in the microscopic field of view (3.2×10^4 μm^2) during the initial 15 seconds of flow was used as a measure of platelet adhesion that initiates platelet thrombus formation.[3,4] The number of platelets in each individual thrombus was calculated as the total thrombus intensity (area×average intensity) multiplied by a value determined by dividing the number of single platelets in the 5-second images by the sum of the total intensities of these single platelets.[3,4] The extent of platelet aggregation (platelet-platelet cohesion) that occurred subsequent to platelet adhesion was represented by the number of platelets in each individual thrombus over the perfusion period of 1 minute.

Preparation of Immobilized Platelet Layers

Platelet-rich plasma was prepared by centrifugation of citrated whole blood at 160*g* for 15 minutes. The platelet count was adjusted to 2×10^8 per milliliter before being bound to 3-aminopropyltriethoxysilane-treated glass slides for 60 minutes.[26] Under these conditions, a confluent layer of platelets (17 500 platelets/mm^2) was formed, as evaluated by light microscopy for each experiment.[26] Nonspecific binding was blocked with 0.1% bovine serum albumin for 10 minutes at 37°C.

THP-1–Platelet Adhesion Assays

The monocytic THP-1 cell line was obtained from the American Type Culture Collection (Manassas, Va) and cultured in the recommended medium. THP-1 cells were washed once, resuspended in serum-free media containing 0.1% bovine serum albumin at a concentration of 10^6 cells/mL, and used within 4 hours in the adhesion flow assay.

THP-1 cell adhesion to immobilized platelets was visualized by phase-contrast video microscopy (Nikon TE200 microscope and a CCD100 camera [Dage-MTI]).[26] Surface-adherent platelets were then incubated with thrombin (1 U/mL) for 10 minutes at 37°C. After the platelet layer was washed with Dulbecco's PBS/0.1% bovine serum albumin for ≈2 minutes, THP-1 cells were perfused through the chamber for 3 minutes at a wall shear stress of 1.5 dyne/cm^2, thereby mimicking the fluid-mechanical environment of the postcapillary venules.[5] A single field of view (×20, 0.20 mm^2) was monitored during the 3 minutes of the experiment, and at the end, 5 fields of view (×20) were monitored for 15 seconds each. Three parameters were quantified in the analysis[26]: (1) the number of total interacting cells during the entire 3-minute experiment; (2) the number of firmly adherent cells after 3 minutes of shear flow; and (3) the average rolling velocity. Interacting cells were defined as those that tethered to the platelet layer for at least 2 seconds and included both firmly adherent and rolling cells. Their number was determined manually by reviewing the videotapes. Firmly adherent cells were considered those that remained stationary for at least 10 seconds at the end of the 3-minutes run, as evidenced by image processing. Rolling velocity was computed as the distance traveled by the centroid of the THP-1 cell divided by the time interval by using OPTIMAS 6.5. For inhibition studies, surface-adherent platelets were preincubated with abciximab (3.5 μg/mL), XV454 (100 nmol/L), or orbofiban (100 nmol/L) for 10 minutes during the thrombin incubation.

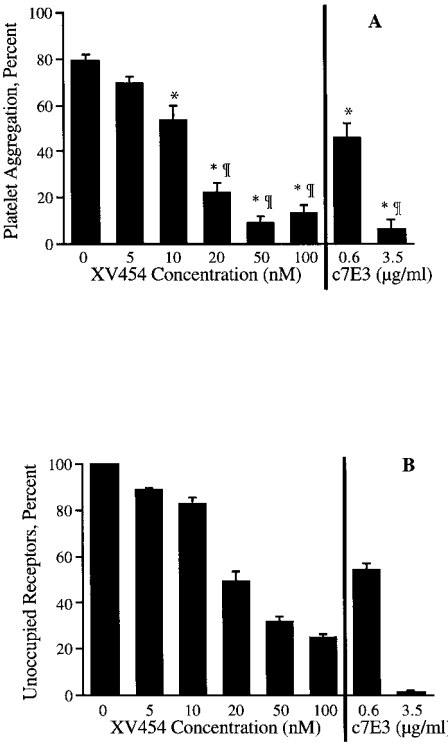

Figure 1. Effects of GPIIb/IIIa antagonists on (A) shear-induced platelet aggregation and (B) receptor availability. Citrate-anticoagulated blood specimens were incubated with the indicated concentrations of XV454, abciximab, or vehicle for 10 minutes at room temperature. A, Blood samples were exposed to a pathological stress level of 4000 s^{-1} for 60 seconds in a cone-and-plate rheometer. The percent platelet aggregation is defined in terms of the disappearance of single platelet-sized particles identified in a flow cytometer by an FITC-conjugated antibody to platelet membrane GPIX (CD42a-FITC). Values are mean±SEM (n=5). *$P<0.01$ vs control, ¶$P<0.01$ vs 0.6 μg/mL abciximab. B, Platelets were distinguished from other blood cells on the basis of their characteristic forward- and side-scatter profiles. Receptor availability was calculated by measuring the mean fluorescence intensity of XL086-FITC (a fluorescent analogue of XV454) and abciximab-FITC of at least 5000 platelets in blood specimens. Values are mean±SEM (n=5).

Statistical Analysis

The data are expressed as mean±SEM. Statistical significance of differences between means was determined by single-factor ANOVA. When means were shown to be significantly different, multiple comparisons by pairs were performed by Tukey's test. Probability values <0.05 were selected to indicate statistical significance.

Results

Shear-Induced Platelet Aggregation: Rheometric/Flow Cytometric Studies

XV454 inhibited, in a concentration-dependent manner, shear-induced (4000 s^{-1} for 60 seconds) platelet aggregation in citrate-anticoagulated blood specimens (Figure 1A). Max-

TABLE 1. Effects of GPIIb/IIIa Antagonists on GPIb Expression Levels and Platelet Adhesion to the Collagen I/vWF Surface

GPIIb/IIIa Antagonists	GPIb Expression Levels (% of Control)	Platelet Adhesion to Collagen I (% of Control)
Control	100	100
XV454		
10 nmol/L	100.8±0.7	ND
20 nmol/L	99.3±1.6	93.9±7.2
50 nmol/L	100.2±1.3	ND
100 nmol/L	98.1±1.9	99.9±8.5
Abciximab		
0.6 μg/mL	99.1±3.3	106.6±8.9
3.5 μg/mL	98.4±4.5	104.8±5.6

ND indicates not determined. Blood specimens were incubated with XV454, abciximab, or vehicle (control) for 10 minutes at 37°C before use in flow cytometric or perfusion assays. GPIb expression was calculated by measuring the mean fluorescence intensity of CD42b-FITC antibody for at least 5000 platelets. Data are expressed as percent of FITC fluorescence relative to control (mean±SEM, n=3). Platelet adhesion is defined as the percentage of the number of adherent platelet thrombi on the collagen I surface (3.2×10^4 μm^2) after 15 seconds of flow relative to control samples (mean±SEM, n=5–9).

imal inhibition of platelet aggregation was achieved by XV454 at 50% to 75% platelet GPIIb/IIIa receptor occupancy (Figures 1A and 1B). High concentrations of XV454 (50 to 100 nmol/L) demonstrated similar potency in suppressing direct shear-induced platelet aggregation to that of abciximab when used at a concentration of 3.5 μg/mL (76 nmol/L) (Figure 1A). This abciximab concentration corresponds to the theoretical maximal blood level produced by injecting the recommended dose of 0.25 mg/kg into a patient with a blood volume of 72 mL/kg[31] and resulted in ≥97% receptor blockade in all donors (Figure 1B). The extensive GPIIb/IIIa receptor blockade caused by either abciximab or XV454 did not interfere with the binding of anti-GPIb monoclonal antibody on the platelet surface (Table 1).

We next evaluated the efficacy of both XV454 and abciximab in inhibiting shear-induced platelet aggregation when similar levels of receptor blockade were achieved by both drugs in vitro. At a ≈45% GPIIb/IIIa receptor occupancy (Figure 1B), XV454 (20 nmol/L) demonstrated a relatively higher potency than abciximab (0.6 μg/mL, or 13 nmol/L) in inhibiting platelet aggregation induced by high shear in the absence of any exogenously added chemical agonist (Figure 1A).

Perfusion Experiments

Heparinized whole blood from healthy donors, treated in vitro with XV454, abciximab, or vehicle, was perfused at 1500 s^{-1} for 1 minute over collagen I. The resulting platelet adhesion, measured by the number of adherent platelet thrombi on the collagen I surface after 15 seconds of flow,[3,4] is shown in Table 1. The data indicate that there was no significant change in the extent of GPIb-mediated platelet adhesion to collagen I/vWF between GPIIb/IIIa antagonist-treated and control (vehicle-treated) blood specimens at all concentrations tested. This finding is in agreement with flow cytometry results showing that the GPIb receptors remained available for binding of vWF, as probed by an anti-GPIb monoclonal antibody (Table 1).

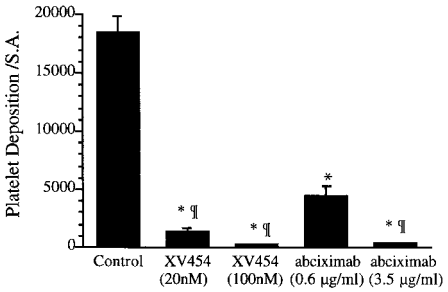

Figure 2. Effects of GPIIb/IIIa antagonists on platelet deposition to a collagen I/vWF surface from heparinized blood perfused for 1 minute at a shear rate of 1500 s^{-1}. Blood samples were incubated with XV454 (20 or 100 nmol/L), abciximab (3.5 or 0.6 μg/mL), or vehicle for 10 minutes at 37°C before perfusion. Values are expressed as mean±SEM (n=5–9). *$P<0.05$ vs control, ¶$P<0.05$ vs 0.6 μg/mL abciximab.

As individual platelet thrombi merge into larger clumps in control specimens after 60 seconds of flow, the number of thrombi decreases concomitantly (data not shown).[3,4] The platelet aggregation that occurs subsequent to platelet adhesion to collagen I is mediated by vWF and fibrinogen binding to platelet GPIIb/IIIa.[1,3,4] At a shear rate of 1500 s^{-1}, 18 444±1473 platelets were deposited on a 3.2×10^4-μm^2 collagen I surface area after 60 seconds of blood perfusion (Figure 2). Also at this time point, large thrombi containing ≥500 platelets accounted for ≥80% of all platelets on the surface (Table 2). Both GPIIb/IIIa antagonists, XV454 and abciximab, significantly reduced total platelet accumulation and the number of platelets per thrombus (platelet aggregation) in a concentration-dependent fashion (Figures 2 and 3). High concentrations of either XV454 (100 nmol/L) or abciximab (3.5 μg/mL) essentially eliminated the formation of platelet aggregates (Table 2). At a similar GPIIb/IIIa receptor occupancy level, XV454 (20 nmol/L) was relatively more effective than abciximab (0.6 μg/mL) in inhibiting platelet deposition onto a collagen type I surface (Figures 2 and 3).

THP-1 Cell Attachment to Immobilized Platelets Under Flow Conditions

In this flow assay, surface-anchored platelets were pretreated with the GPIIb/IIIa antagonists for 10 minutes, and any unbound drug was removed by a brief washing step (≈2 minutes) before perfusion of the THP-1 monocytes. We therefore reasoned that agents with slow platelet off-rates, such as XV454 ($t_{1/2}$ of dissociation=110 minutes, K_d=1 nmol/L)[22,32] and abciximab ($t_{1/2}$ of dissociation=40 minutes, K_d=9.0 nmol/L)[22,32] that are distributed predominantly as receptor-bound entities with little unbound in the plasma,[33] could effectively block these heterotypic interactions. In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation=0.2 minutes, K_d >110 nmol/L),[32] whose antiplatelet efficacy depends on the plasma concentration of the active drug, would not exhibit any

TABLE 2. Platelet Thrombus Size Distribution

	No. of Thrombi Containing These Amounts of Platelets					
Treatment Group	1–5	6–20	21–150	151–500	501–1000	>1000
Control	4 (0.1)	6 (0.4)	2 (1.3)	10 (15.0)	2 (6.7)	7 (76.5)
XV454						
20 nmol/L	71 (11.5)	31 (17.9)	23 (70.6)	0	0	0
100 nmol/L	141 (100)	0	0	0	0	0
Abciximab						
0.6 μg/mL	25 (1.2)	40 (6.3)	42 (39.8)	14 (52.7)	0	0
3.5 μg/mL	162 (100)	0	0	0	0	0

The number of thrombi in each platelet number category on the collagen surface (3.2×10^4 μm^2) was counted after heparinized blood was perfused at 1500 s^{-1} for 1 minute. Values in parentheses indicate the percentages of total accumulated platelets in thrombi of various sizes in the area of observation. The size distribution of platelet thrombi was shifted from large (>500) platelets to smaller thrombi (≤500) in samples incubated with GPIIb/IIIa antagonists. These are results from a representative perfusion experiment.

inhibitory effects. Therefore, this assay could allow us to distinguish GPIIb/IIIa antagonists with slow platelet off-rates from those with relatively faster off-rates.

Our data indicate that immobilized platelets supported extensive THP-1 cell adhesion (1525±483 interacting THP-1 cells/mm^2, n=4). At a wall shear stress of 1.5 dyne/cm^2, THP-1 cells tethered and rolled stably along the platelet surface for some distance, which was variable but sometimes quite long (the entire path of a field of view), with an average velocity of 14.0±1.9 μm/s. A significant number of interacting THP-1 cells became firmly adherent within a 1- to 5-second interval after tethering to the platelet substrate (Figure 4). XV454 (100 nmol/L) and abciximab (3.5 μg/mL) significantly reduced the stable adhesion of THP-1 cells to immobilized platelets (Figure 4) while simultaneously increasing the number of rolling THP-1 cells (data not shown).[26] Furthermore, blockade of the platelet GPIIb/IIIa receptor with either agent significantly increased the rolling velocity of THP-1 cells (44.9±3.3 and 43.3±3.3 μm/s for XV454- and abciximab-treated platelets, respectively). This effect is similar to that previously reported for leukocyte rolling over stimulated endothelium in the presence of blocking antibodies against integrins.[34] In distinct contrast, the short-acting GPIIb/IIIa antagonist orbofiban (100 nmol/L) failed to suppress these heterotypic adhesive interactions (Figure 4) and to affect the average rolling velocity of interacting THP-1 cells (17.8±2.3 μm/s). It should be noted

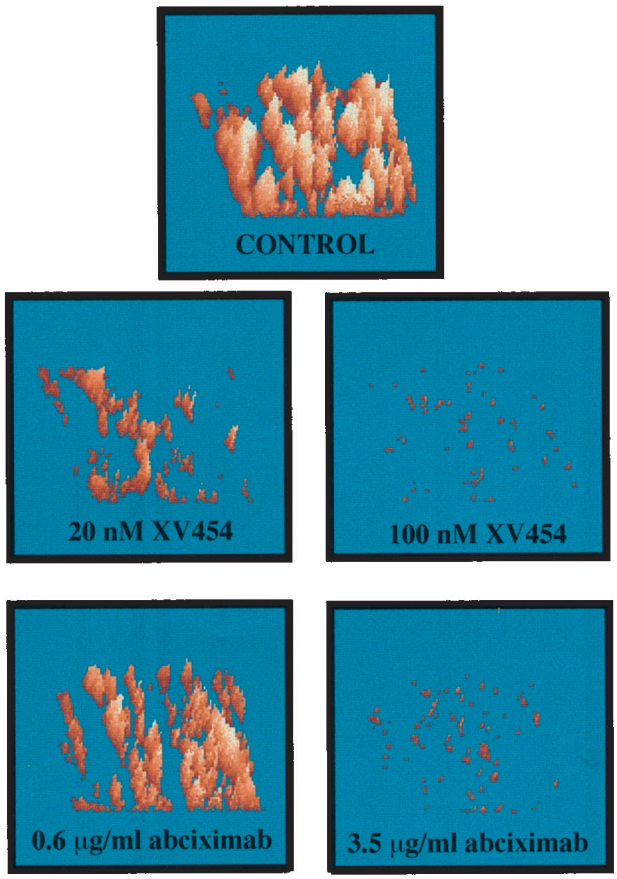

Figure 3. Three-dimensional computer-generated color representations (from videotape of real-time data) of platelet adhesion and subsequent aggregation on collagen I/vWF from normal heparinized blood perfused at 37°C for 1 minute at 1500 s^{-1}.

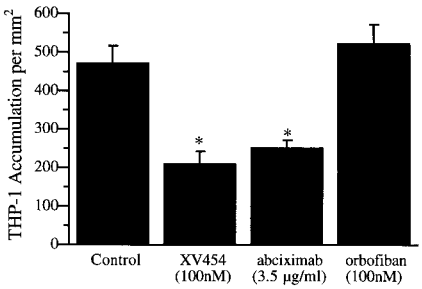

Figure 4. Firm adhesion of THP-1 monocytic cells on immobilized platelets after 3 minutes at a wall shear stress of 1.5 dyne/cm^2. Platelets were pretreated with XV454 (100 nmol/L), abciximab (3.5 μg/mL), orbofiban (100 nmol/L), or vehicle for 10 minutes before perfusion of the THP-1 cells. Values are expressed as mean±SEM (n=4; different donors). *$P<0.001$ vs control.

that orbofiban (100 nmol/L) is effective in eliminating the formation of large platelet aggregates, defined as ≥300 platelets per thrombus, onto collagen type I surfaces when this concentration (100 nmol/L) is maintained in the suspension medium during the perfusion process (data not shown).

Discussion

In this study, we evaluated the efficacy of a novel, nonpeptide, small-molecule platelet GPIIb/IIIa antagonist, XV454, in inhibiting platelet responses under dynamic flow conditions. Two different in vitro models of thrombosis, cone-and-plate rheometry and perfusion, were tested. The rheometry model simulates the elevated levels of fluid shear stress generated in stenosed coronary arteries, which can directly cause platelet activation and aggregation in the absence of any exogenously added chemical agonist.[6,17] The extent of shear-induced platelet aggregation is dependent on both the magnitude of shearing stress and shear exposure time.[24] The perfusion system represents a model of blood flowing over injured, exposed arterial subendothelium and allows us to monitor and quantify both platelet adhesion and subsequent aggregation in real time in the presence of controlled levels of elevated shear stress.[1–4] In both models, XV454 consistently inhibited platelet aggregation in a concentration-dependent fashion. The inhibition of aggregation was correlated with the percentage of GPIIb/IIIa receptor occupancy. Maximal inhibition was achieved by XV454 at ≈75% GPIIb/IIIa receptor blockade, which appears to be lower than the ≥85% occupancy required by abciximab.[4,10] Furthermore, when similar extents of GPIIb/IIIa receptor blockade were achieved by XV454 and abciximab in vitro, XV454 appeared to be relatively more effective than abciximab in suppressing platelet aggregate formation. This finding is in concert with previous data showing that XV459, a nonpeptide, small molecule similar to XV454, had a relatively lower IC_{50} than abciximab in reducing platelet aggregation in response to chemical agonist stimulation.[35]

The ≈75% receptor occupancy achieved by 100 nmol/L XV454 was sufficient to eliminate platelet aggregate formation under flowing conditions regardless of the choice of anticoagulant. Previous work has demonstrated comparable IC_{50} values for both XV459 and abciximab in inhibiting platelet aggregation irrespective of the heparin or citrate choice.[22] This result is in clear contrast to the significant shift in the IC_{50} values of Integrilin[13] and orbofiban in inhibiting platelet aggregation to a greater extent in citrate (relatively lower IC_{50}) than in heparin (relatively higher IC_{50}) collected blood, which is due to the partial calcium-chelating effect of citrate that results in artificial enhancement of ex vivo or in vitro Integrilin antiplatelet efficacy.

Despite the extensive GPIIb/IIIa receptor blockade caused by high concentrations of either XV454 or abciximab, the GPIb receptors remained available for binding ligand, as evidenced by flow cytometry. This result is in accord with our perfusion experiments that showed that treatment of whole-blood specimens with either drug did not affect platelet adhesion onto a collagen I surface under conditions of abnormally high arterial flow, a situation that is entirely dependent on GPIb-vWF interaction.[1,2]

Evidence suggests that GPIIb/IIIa may also mediate leukocyte attachment to immobilized platelets under flow,[27] although other reports have failed to confirm this finding.[36,37] Nevertheless, GPIIb/IIIa has been consistently reported to play a pivotal role in the adhesive interactions of platelets with a variety of tumor cell lines, a process that appears to be critical to the hematogenous dissemination of tumor cells.[25,26] Along these lines, our data clearly demonstrate the involvement of GPIIb/IIIa in the stable adhesion of monocytic THP-1 cells to surface-anchored platelets under dynamic flow conditions. In this flow assay, any unbound GPIIb/IIIa antagonist was removed by brief washing (≈2 minutes) after platelet preincubation and before perfusion of the monocytic THP-1 cells, thereby enabling us to distinguish agents with markedly distinct affinities and receptor-bound lifetimes. We reasoned that agents with slow platelet off-rates would effectively block these heterotypic interactions, whereas rapidly reversible agents would not exhibit any inhibitory effects. Our results indeed show that XV454 and abciximab, but not orbofiban, significantly inhibited THP-1 cell attachment to surface-bound platelets, a finding that correlates with their respective platelet off-rates.[32] These observations suggest a possible extended duration of the in vivo antiplatelet efficacy of XV454 compared with that of other short-acting, small-molecule GPIIb/IIIa antagonists.

Nearly all GPIIb/IIIa antagonists, including abciximab, induce conformational changes within the GPIIb/IIIa complex that can be probed by using ligand-induced binding site antibody reagents.[33,38] It has been suggested that ligand-induced binding site epitope expression may report a potential intrinsic activating property of certain GPIIb/IIIa antagonists and that this effect should be considered when evaluating these agents.[38,39] However, the currently available clinical data do not indicate that GPIIb/IIIa antagonist–induced expression of ligand-induced binding site epitopes is associated with platelet stimulatory events in vivo,[33] possibly because receptor-activating properties of GPIIb/IIIa antagonists may be masked by the sustained blockade of the receptor.

GPIIb/IIIa inhibitors, when administered at suboptimal doses, may transiently bind to the GPIIb/IIIa complex, induce the active conformational change, and allow increased fibrinogen binding to occur after their dissociation from the receptor.[19,38] It was recently shown that oral administration of orbofiban enhanced platelet reactivity with respect to fibrinogen binding and P-selectin expression.[19] Although in vitro studies suggest that low concentrations of certain GPIIb/IIIa antagonists with either slow or fast off-rates induce platelet-stimulatory events,[38] it is likely that these responses are more pronounced for short-acting inhibitors whose antiplatelet efficacy depends on the plasma concentration of the active drug and rapidly declines after discontinuation of infusion. In contrast, drugs with slow off-rates such as XV454 and abciximab are distributed predominantly as receptor-bound entities, and their platelet-inhibitory effects can be measured for several days after drug administration has been terminated.[17] Therefore, the number of GPIIb/IIIa receptors per

platelet that are left in an activated state after drug dissociation occurs is likely to be lower for GPIIb/IIIa antagonists with slow dissociation rates than those with relatively faster off-rates. Consequently, an oral version of a high-affinity antagonist (low K_d) like XV454 might be expected to have a higher chance of success than rapidly reversible GPIIb/IIIa antagonists, although this aspect still lies in the future. However, the clinical benefits demonstrated by the long-acting agent abciximab and the short-acting inhibitors eptifibatide and tirofiban, which are known to induce ligand-induced binding site epitope expression, suggest that these agents, when administered at optimal doses and for the appropriate period of time, could effectively block platelet-stimulatory responses and mask any potential intrinsic receptor-activating properties. Taken altogether, we conclude that XV454 is a potent, small-molecule GPIIb/IIIa antagonist with a long receptor-bound lifetime like abciximab, and it may have a beneficial effect on the treatment and/or prevention of thrombotic disorders.

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