We have previously shown that the zymogen factor XI (FXI) binds to activated platelets but not to human umbilical vein endothelial cells (HUVEC). It has also been reported that activated factor XIa (FXIa) by factor XIIa (1992) J. Biol. Chem. 267, 19833–19839; Shariat-Madar, Z., Mahdi, F., and Schmaier, A. H. (2001) Thromb. Haemostasis 85, 544–551). It has also been reported that activated FXI (FXIa) binds to 1.5 × 10^6 sites per HUVEC and promotes the activation of factor IX by cell bound FXIa (Berrettini, M., Schleef, R. R., Heeb, M. J., Hopmeier, P., and Griffin, J. H. (1992) J. Biol. Chem. 267, 19833–19839). Therefore, the binding of FXIa to activated platelets was compared with FXI binding to HUVEC and HEK293 cells immobilized on microcarrier beads. Specific and saturable zinc-dependent FXI binding was demonstrated to 250 ± 48 sites per activated platelet (K_D = 1.7 ± 0.78 nM) and 6.5 ± 0.4 × 10^4 sites per HUVEC (K_D = 2.4 ± 0.5 nM), whereas no binding to HEK293 cells was detected. A titration with high molecular weight kininogen had no effect on high FXI binding to platelets, but revealed a concentration-dependent increase in the amount of FXIa bound to HUVEC. The rate of factor IXa generation catalyzed by FXIa was unaffected by the presence of surfaces; however only the activated platelet surface protected FXIa from inhibition by protease nexin 2. The results presented here confirm the conclusion that activated platelets are procoagulant while unstimulated endothelial cells are not.

Human factor XI (FXI) is a homodimeric protein that is essential for initiating the consolidation phase of blood coagulation. FXI is activated to factor XIa (FXIa) by factor XIIa (FXIIa), by thrombin, or by autotransglutamylation (1–3). The activation of FXI to FXIIa requires the presence of a negatively charged surface, which under physiological conditions is provided by activated platelets (4). The binding of FXI to platelets (and also to heparin) is mediated by the Apple 3 domain of FXI (5–7), whereas the binding of FXIa to heparin occurs solely through a cysteine constrained loop within the catalytic domain (8). The FXIa domain that mediates its binding to activated platelets has not been identified. The natural substrate for FXIa is factor IX (FIX), which is activated to FIXa, the enzyme component of the FX activating complex on activated platelets (9, 10).

The homodimeric nature of FXIa is thought to be necessary for the simultaneous binding of the enzyme to the activated platelet surface, mediated through one subunit and the binding of FIX by the second subunit (11). Alternatively, the activation of FIX, through proteolytic cleavage resulting in the release of the activation peptide by FXIa on activated platelets, may require the active sites of both subunits because monomer FXIa can activate FIX only in solution, not in the presence of activated platelets (11). Interestingly, FIX is activated by hydrolysis of two scissile bonds without release of an intermediate (12). The substrate binding site for FIX within FXIa has been postulated to reside within the Apple 2 domain from studies utilizing small peptide competition, whereas studies with chimeric proteins suggest that this site resides within the Apple 3 domain (13–17).

FXIa binds with high affinity (K_D = 3.25 nM) to ~225 sites on the activated platelet surface (18, 19). Binding of FXIa to activated platelets was shown to require the presence of high molecular weight kininogen (HK), but the rates of FIX activation in the presence and absence of activated platelets were similar (18, 19). This suggests either that binding of FXIa (18) and FIX (10) to the platelet surface do not increase rates of FIX activation or alternatively that an actual rate enhancement is obscured by simultaneous inhibition of FXIa by protease nexin 2 (PN2) secreted from platelets (20–22). It has also been reported that FXIa binds with high affinity (K_D = 1.5 nM) to sites (~1.5 × 10^6 per cell) exposed on unstimulated human umbilical vein endothelial cells (HUVEC) (23). Binding of FXIa to unactivated HUVEC was reported to require the presence of both HK and zinc ions, and HUVEC-bound FXIa was reported to activate FIX at reduced rates compared with solution phase FIX activation (23).

Previously (24), we have addressed the question of whether the initiation of the consolidation phase of blood coagulation occurs on platelets or on endothelium, since it had previously been shown that zymogen FXI binds both to activated platelets (~1500 sites/platelets; K_D = 10 nM) (4, 25) and to unactivated HUVEC (2.2–13 × 10^6 sites/cell; K_D = 4.5–6.9 nM) (23, 26). We demonstrated (24) that the reported interaction of FXI with resting HUVEC represented an artifact of the binding assay carried out in microtiter wells (23, 26). During the course of these previous studies we developed an assay for examining the
binding of FXI to HUVEC cultured on collagen-coated microcarrier beads, permitting direct comparison with FXI binding to activated platelets (24). In the present study, we have utilized this assay (24) to examine the binding of FXIa to both platelets and HUVEC. We have confirmed that FXIa binds specifically and reversibly with high affinity to sites that are distinct from FXI on both platelets and HUVEC. The binding of FXIa to HUVEC is unlikely to occur physiologically, since plasma concentrations of HK abrogate FXIa binding to HUVEC. Furthermore, the rate of FIX activation by FXIa is not enhanced by physiological surfaces; however, the activated platelet surface protects the catalytic activity of FXIa from inhibition by PN2. This report supports the hypothesis that the activated platelet surface is procogulant and the endothelium is non-thrombogenic.

MATERIALS AND METHODS

Proteins—FXIIa, FXIa, FXI, FIX, FXIIa, FX, FX, prekallikrein, kal-likrein, and goat anti-human FXIIa IgG (α-FXIIa) were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). HK and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). PN2 was a kind gift from Dr. William Van Nostrand, State University of New York, Stony Brook, NY. The thrombin receptor activation peptide (TRAP, SFLRRN-amide) was synthesized at the Protein Chemistry Laboratory at the University of Pennsylvania (Dr. John Lambris, Director).

Preparation of 125I-FXIIa—125I-FXIIa was prepared by the activation of radiolabeled FXI. FXI was radiolabeled with 125I using the IODO-GEN method (27). Approximately 100 μg of protein was incubated with 1 μCi of carrier-free Na125I (Amersham Biosciences) for 20 min in an IODO-GEN-coated vial and centrifuged through a 1-cc Sephadex G-50 0.001N HCl. The antibody generated by incubation with CNBr-activated Sepharose (1 g, Sigma) in 1 mCi of carrier-free Na 125I (Amersham Biosciences) for 20 min in an IODO-GEN-coated vial and centrifuged through a 1-cc Sephadex G-50 0.001N HCl. The antibody α-FXIIa was diluted to 1 mg/ml in sodium bicarbonate buffer (pH 8.3) and an equal volume of beads was added overnight at 4 °C with constant stirring. Ethanolamine (1 M final concentration) was added to the reaction mixture for 2 h with constant stirring. The resin was then washed three times in alternating sodium acetate and sodium bicarbonate. Optical density was measured for each eluate wash for the absorbance at wavelength 280 nm to detect non-cross-linked antibody. The resin was stored at 4 °C in sodium bicarbonate buffer with 0.03% sodium azide. After final purification and removal of FXIIa 125I-FXIIa was visualized using SDS-PAGE analysis for the appearance of the 30- and 50-kDa enzyme bands under reducing conditions. Specific radioactivity (1.1 104 cpm/mg) and biological activity (246 uM) were determined as reported previously (44a).

Measurement of Amidolytic Activity—The substrate pyro-Glu-Pro-Arg-pNA (S-2366, Chromogenix, Malmö, Sweden) was used to determine FXIa activity. The rate of cleavage of S-2366 (250 μM) was determined by the absorbance at 405 nm and compared with a standard curve generated from FXIa.

Cell Culture—Normal pooled HUVEC were purchased from Cascade Biologicals (Portland, OR) and cultured in low serum growth supplement (2% fetal bovine serum) in a humidified atmosphere of 5% CO2 at 37 °C. Medium was changed after 24 h and then on alternate days until the cells reached confluence. Cells were passaged and no longer used after the fifth passage. Cells (1 × 106 passage 1) were frozen in 1 ml of MeSO Freeze buffer and stored in liquid nitrogen. HEK293 cells and Chinese hamster ovary cells were cultured similarly in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. HUVEC or HEK293 cells (1 × 105 cells/ml) were added to 5 ml (1.5 × 108 beads) of swelled Cytoxid-3 collagen-coated microcarrier beads (Amersham Biosciences) that were washed in complete endothelial cell media. After 30 min at room temperature the beads and cells were transferred to a powder flask (Whelan Scientific, Millville, NJ) and incubated under constant stirring (60 rpm) in a humidified atmosphere of 5% CO2 at 37 °C. Microcarrier bead cultures (0.5 ml) were mixed with 0.5 ml of trypan blue (Invitrogen) to determine the extent of confluence, since the microcarrier beads, but not cell-coated beads, retained trypan blue staining (24).

Platelet Isolation—Platelets were prepared as described (28, 29).

Briefly, platelets were obtained from platelet rich plasma after centrifugation of whole blood collected into acid-citrate-dextrose. Platelet-rich plasma was centrifuged through a bovine serum albumin (BSA) gradient and gel-filtered through a 50-ml Sarophase 2B column. Platelet pellets were counted electronically using a particle counter (Coulter Electronics, Hialeah, FL) and then washed in 10 ml Hepes containing 5 mM EDTA once, and three times in 10 ml Hepes buffer without EDTA. Activation of platelets was measured by shape change, aggregation, and release of ADP using a Lumi-Aggregometer (Chrono-Log Corp., Haverton, PA).

RESULTS

FXIa Binding to Platelets and HUVEC—Either HUVEC grown to confluence on microcarrier beads or platelets isolated from fresh whole blood and activated with the thrombin receptor (PAR-1) activation peptide (SFLRRN-amide or TRAP) were incubated with increasing concentrations of 125I-FXIIa. After centrifugation through silicone oil and separation of cell-bound from free protein, binding curves were generated as shown in Fig. 1. Specific and saturable binding was detected to both platelets (Fig. 1A) and HUVEC but not HEK293 cells (Fig. 1B). Specific binding was calculated by subtracting non-specific binding (i.e. the amount of 125I-FXIIa not displaced by Hepes containing 5 mM EDTA or a molar excess of non-labeled FXIa) from the total amount of 125I-FXIIa bound. FXIa binding to 250 ± 48 high affinity sites per platelet (Kd = 1.7 ± 0.78 nm) and to 6.5 ± 0.4 × 104 high affinity sites per HUVEC (Kd = 2.4 ± 0.5 nm). Both studies were carried out in the presence of 2 mM calcium ions and 25 μM zinc ions (divalent metal concentrations determined to be optimal).
timal for FXI binding to platelets (4, 25) but in the absence of HK. No binding sites were detected on either HUVEC or platelets when experiments were performed in the absence of zinc ions (data not shown).

**Effect of HK**—HK had previously been reported to be essential in the binding of FXIa to both HUVEC (23) and activated platelets (18, 19); however, we were able to detect binding sites on both activated platelets and HUVEC in the absence of HK (Fig. 1). To examine the role of HK in the binding of FXIa to both activated platelets and HUVEC, HK was titrated into the binding reaction. A saturating concentration of FXIa (5 nM) was incubated with increasing concentrations of HK in the presence of zinc ions (25 μM) and either TRAP-activated platelets (Fig. 2A) or HUVEC (Fig. 2B). FXIa binding to activated platelets was unaffected by the presence of HK. In contrast, increasing concentrations of HK decreased the amount of FXIa bound to HUVEC, suggesting that under physiological conditions, FXIa would not bind to HUVEC.

**Specificity of FXIa Binding**—To determine the specificity of FXIa binding to both the activated platelets and to the HUVEC surface, we attempted to compete FXIa binding sites from the surface of platelets and HUVEC using different proteins at 100× molar excess (Fig. 3). The FXIa receptor on platelets is distinct from that of the FXI receptor, because FXI was incapable of displacing FXIa. Furthermore, FXI did not displace FXIa binding to HUVEC, most likely because there are no FXI binding sites on HUVEC (24). The proteins, prekallikrein, FIX, and the IgG α-FXII, were also incapable of displacing FXIa binding to either HUVEC or platelets when experiments were performed in the absence of zinc ions (data not shown).

**Activation of FIX by FXIa**—The activation of FIX by FXIa is the only reaction within the intrinsic or consolidation phase of the coagulation cascade where the rate appears to be unaffected by the presence of activated platelet surface (9, 11, 30–33). However, evidence exists that suggests activated platelets protect the activity of FXIa from inactivation by protease inhibitors (34) including PNG, which is secreted from activated platelets. No binding sites were detected on either HUVEC or platelets when experiments were performed in the absence of zinc ions (data not shown).
platelets (22). To elucidate the role of both platelets and HUVEC in the activation of FIX by FXIa, studies were performed in the presence and absence of PN2 (Fig. 4). In the presence of platelets (Fig. 4, B and C), HUVEC (Fig. 4, D and E) or a buffer control (Fig. 4A), no change was observed in rate of FIXa formation in the absence of PN2. The activation of FIX by FXIa was virtually completely inhibited by PN2 in solution or in the presence of unstimulated platelets and HUVEC (Fig. 4, A and B–E). However, in the presence of activated platelets, the activation of FIX by FXIa was preserved in the presence of exogenously added PN2 (Fig. 4B). Thus the activated platelet surface had little or no direct effect on the rate of FIX activation by FXIa and the reaction proceeded at uninhibited rates when PN2 was present. In contrast, FXIa-catalyzed FIX activation was completely inhibited by PN2 in solution or in the presence of HUVEC.

DISCUSSION

The central biological question addressed by the present studies relates to the identification of the cellular locus of coagulation reactions involved in the initiation of the consolidation phase of blood coagulation. Cellular surfaces have previously been suggested to participate in this important physiological process by binding coagulation FXI, promoting its proteolytic activation, binding the enzyme, FXIa, and facilitating the activation of FIX to FIXa and subsequent coagulation reactions leading to generation of sufficient amounts of thrombin to effect normal hemostasis. Two of these cellular surfaces

**Fig. 4.** FXIa-catalyzed FIX activation: effect of activated platelets, HUVEC, and PN2. FXIa (2 nM) was incubated in HT buffer alone (A) or with platelets (B), TRAP-activated platelets (C), HUVEC (D), or TRAP-activated HUVEC (E) for 5 min prior to the addition of FIX (1 μM). In some cases PN2 (20 nM) was added 5 min prior to the addition of FIX. Aliquots were removed at 0, 5, 10, 15, 20, 25, 30, 35, and 100 min and examined by Western blotting as described under “Materials and Methods.” Densitometry analysis of the blots performed in triplicate are represented in the presence (○) and absence (●) of PN2.
are the surface membranes of activated platelets (35) and resting endothelial cells (23, 26). We have previously compared these two cellular surfaces for their capacity to bind FXI and promote its activation to FXIIa (24). This comparison required the development of a novel method for immobilizing endothelial cells (HUVEC) on microcarrier beads because of the very high level of nonspecific binding of HK to microtiter plates that resulted in the artificial appearance of a very large number of FXI binding sites originally, which were erroneously interpreted as FXI receptors on resting endothelial cells (23, 24, 36). Our studies demonstrated that activated platelets exposed a discrete number of saturable, specific, high affinity FXI binding sites and promoted the activation of FXII preferentially by thrombin (24, 37) and also by FXII and FXI (37). In contrast HUVEC, either in the resting state or after activation with thrombin or the thrombin receptor (PAR1) activation peptide (SFLRNR-amide), were unable to bind FXI (24).

FXI binding to activated platelets did not require the presence of added HK (Figs. 1A and 2A). This result differs from a previous report of studies carried out in the absence of added zinc ions that demonstrated a requirement for added HK to promote FXI binding to activated platelets (19). However that is bound to platelets either from plasma or released from platelets may explain this discrepancy. HK did, however, abrogate the binding of FXIIa to HUVEC (but not activated platelets, Fig. 2A) in a concentration-dependent manner (Fig. 2B). FXI and HK may compete for the same receptor on HUVEC; proposed HK receptors include the uPAR, C1qR, cyto-keratin-1 receptor complex (38–40), the glycoprotein Ib-IX-V receptor complex (41), and heparan sulfate glycosaminoglycans located at cell junctions (42). Another potential explanation is that HK does not compete with FXI for the same binding site, but rather the FXI/HK complex remains sequestered in solution and does not associate with the HUVEC surface. It can be concluded from these observations that under physiological conditions in vivo, where the concentration of HK in blood plasma is ~650 nm, FXI would fail to bind to HUVEC, but would bind to the surface of activated platelets.

The presence of the activated platelet surface in both the thrombin-generating and FXA-generating complexes increases the overall catalytic efficiency by many orders of magnitude. In contrast, the rate of FXII activation by FXIa is unaffected by the presence of activated platelets or phospholipids (9, 11, 30, 31, 33) even though both enzyme (FXIIa) and substrate (FXI) bind to activated platelets with high affinity (10, 19). The fact that activated platelets release protease inhibitors such as PN2, which potently inhibits FXIa (KC ~ 500 pm) (20, 43) suggests that the activated platelet surface could theoretically increase the rate of FXI activation and simultaneously, through the release of PN2, inhibit FXIa, thereby limiting the detection of an increased rate of FIX generation.

Studies by Scandura et al. (22) using a small fluorescent peptide substrate (BOC-EAR-AMC) determined that activated platelets release 1–1.5 nM PN2/10^7 platelets and that in the presence of HK the surface of activated platelets protects FXIIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIa from inactivation. Platelets activated by TRAP secreted enough HK to protect FXIIa and FXI from complete inactivation, but not fully protect FXIa in solution and localize subsequent coagulation reactions to the platelet hemostatic thrombus. These observations are consistent with the concept that the primary phenotype of the endothelium is anticoagulant and non-thrombogenic, whereas the major function of activated (but not resting) platelets is to participate in the initiation of the consolidation pathway of blood coagulation leading to the local explosive generation of thrombin at sites of vascular injury.

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The Interaction of Factor XIa with Activated Platelets but Not Endothelial Cells Promotes the Activation of Factor IX in the Consolidation Phase of Blood Coagulation

T. Regan Baird and Peter N. Walsh

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