Investigations determined the relative preference of prekallikrein (PK) or factor XI/XIa (FXI/FXIa) binding to endothelial cells (HUVECs). In microtiter plates, biotinylated high molecular weight kininogen (biotin-HK) or biotin-FXI binding to HUVEC monolayers or their matrix proteins, but not fibronectin-coated plastic microtiter plate wells, was specifically blocked by antibodies to each of the receptors of HK, uPAR, gC1qR, or cytokerinat 1. Fluorescein isothiocyanate (FITC)-PK specifically bound to HUVEC suspensions without added Zn$^{2+}$, whereas FITC-FXI or -FXIa binding to HUVEC suspensions required 10 μM added Zn$^{2+}$ to support specific binding. Plasma concentrations of FXI did not block FITC-PK binding to HUVECs in the absence or presence of 10 μM Zn$^{2+}$. In the absence of HK, the level of FITC-FXI or -FXIa binding was half that seen in its presence. At physiologic concentrations, PK (450 nM) abolished FITC-FXI or -FXIa binding to HUVEC suspensions in the absence or presence of HK in the presence of 10 μM Zn$^{2+}$. Released Zn$^{2+}$ from 2–8 × 10$^6$ collagen-activated platelets/ml supported biotin-FXI binding to HUVEC monolayers, but platelet activation was not necessary to support biotin-PK binding to HUVECs. At physiologic concentrations, PK also abolished FXI binding to HUVECs in the presence of activated platelets, but FXI did not influence PK binding. PK in the presence or absence of HK preferentially bound to HUVECs over FXI or FXIa. Elevated Zn$^{2+}$ concentrations are required for FXI, but not PK binding, but the presence of physiologic concentrations of PK and HK also prevented FXI binding. PK preferential binding to endothelial cells contributes to their anticoagulant nature.

Current understanding of the role of platelets and endothelial cells in hemostasis and thrombosis suggests that activated platelets are loci upon which significant hemostatic reactions occur and endothelial cells contribute to the constitutive anticoagulant nature of the intravascular compartment. Both prekallikrein (PK$^1$) and factor XI (FXI$^2$) bind to endothelial cells (1). Prekallikrein activation on HUVECs contributes to single-chain urokinase activation, and FXI activation on platelets contributes to thrombin formation. We sought to determine if there is a preference for one or the other of these proteins to assemble on cultured endothelial cell membranes under physiologic zinc ion concentrations and platelet activation states.

The critical protein that serves as the receptor for PK and FXI on endothelial cells is high molecular weight kininogen (HK) (1, 2). HK specifically binds to endothelial cell membranes (3, 4). When cultured endothelial cells (HUVECs) are chilled to 4 °C, there are 0.93–2.6 × 10$^5$ HK binding sites/endothelial cell on confluent monolayers in microtiter plate wells (3–5). Alternatively, when HUVECs are maintained at 37 °C, there are 1 × 10$^5$ HK binding sites/HUVEC monolayers (5, 6). The specificity of HK binding to endothelial cells grown to confluence in microtiter plate wells or proteins related to the cultured cells has been shown by a number of techniques. First, treatment of endothelial cells with Pronase completely eliminates HK binding to microtiter plate wells (5). Second, treatment of endothelial cells with the metabolic inhibitors of aerobic metabolism (antimycin A), anaerobic metabolism (2-deoxy-d-glucose), and d-glucosic acid δ lactone completely abolishes HK binding to cultured endothelial cells (5). Third, antibodies to three of the HK endothelial cell membrane-binding proteins, gC1qR, urokinase plasminogen activator receptor, or cytodakinat 1, but not their corresponding non-immune IgG, block biotin-HK binding to HUVEC microtiter plates (7). Last, when labeled HK binds to confluent monolayers of HUVECs in microtiter plates, at least 66% of the total amount of binding seen is associated with the matrix of the cultured HUVECs and not the cells themselves (8). PK specifically binds to endothelial cell monolayers or suspensions in both the presence and absence of HK (1). PK binding to HK on HUVECs is completely blocked by antibody to the HK binding region and a peptide of PK binding region on HK (1). Similarly, FXI or factor XIa (FXIa) specifically binds to endothelial cell monolayers mostly in the presence of HK (2). Moreover, antibodies to the HK-binding proteins on the external membrane of endothelial cells and the FXI binding region on HK also completely block FXI binding to HK on cultured endothelial cells monolayers (2, 9). FXI or FXIa binding to HUVECs in the absence or presence of HK requires >7 μM free Zn$^{2+}$, unlike HK binding alone, which only requires 0.3 μM free Zn$^{2+}$ in buffer that contains gelatin, a carrier protein that does not bind zinc (2, 10). Although the total Zn$^{2+}$ concentration in plasma is 15–25 μM, the free Zn$^{2+}$ concentration in plasma, i.e. nate; HCG, Hepes carbonate gelatin buffer; turbo-TMP, 3,3,5,5'-tetramethylbenzidine dihydrochloride; uPAR, urokinase plasminogen activator receptor; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; 5-Br-PAPS, 2-(5-bromo-2-pyridylazo)-5-(propyl-N-sulfopropylamino)phenol sodium salt.
the Zn²⁺ concentration that is not protein bound, is only 0.3 μM. These data suggest that, under physiologic circumstances, FXI does not bind to HUVECs (11–13). Recent studies, however, question the validity of the above-cited HK and FXI binding studies to HUVECs (14, 15). Because HK itself has the ability to bind to plastic microtiter plates, a negatively charged surface, these authors claim that the extent of HK and, subshearly, FXI binding to HUVEC monolayers is really an artifact of HK binding to the plastic wells (14, 15). The present investigations address this interpretation of the work cited above (1–10). It also examines the relative likelihood of PK or FXI binding to HUVEC suspensions under simulated physiologic conditions where the free zinc ion concentration is manipulated between 0.3 to 10 μM. These studies indicated that HK binding is specific to cells and its matrix and PK is the predominant protein ligand of HK on endothelial cells regardless of the ambient Zn²⁺ concentration.

EXPERIMENTAL PROCEDURES

Materials—Human factor XI (FXI) (188–200 units/mg), single chain high molecular weight kinogenin (HK) (13 units/mg), human prekallikrein (PK) (22 units/mg), and human factor Xa (FXa) (265–341 units/mg) were purchased from Bioresearch Laboratories, Inc. (South Bend, IN) or Haematologic Technologies (Essex Junction, VT). All preparations were characterized by non-reduced and reduced SDS-PAGE, coagulant or amodicytic activity, and protein concentration prior to use. Biotinylation kits, ImmunoPure streptavidin horseradish peroxidase, and peroxidase-specific fast reacting substrate, 3,3',5,5'-tetramethylbenzidine dihydrochloride (turbo-TMP) were obtained from Pierce Chemical Co. (Rockford, IL). Fluorescein isothiocyanate (FITC) protein labeling kits were purchased from Molecular Probes (Eugene, OR). Human umbilical vein endothelial cells (HUVECs), endothelial cell growth medium, trypsin-EDTA, and trypsin-neutralizing solutions were purchased from Clonetics (San Diego, CA). Antibodies—Monoclonal antibody to gC1qR (clone 74.5.2) was purchased from Covance Research Products, Inc. (Richmond, CA). Monoclonal antibody against uPAR (SB10FC) was generously provided by Dr. Robert F. Todd III at the University of Michigan (Ann Arbor, MI). A goat anti-human cytokeratin 1 antibody (anti-GPV20) to the HK binding region on cytokeratin 1 was prepared and characterized as previously reported (7, 16, 17).

Labeling of Factors XI, Xla, PK, or HK—Biotinylation: FXI, FXla, PK, or HK in 4 ml sodium acetate-HCl and 0.15 M NaCl, pH 5.3, were dialyzed against sodium phosphate, 0.15 M NaCl, pH 7.4, and biotinylated according to the procedure of Pierce Chemical Co. (Rockford, IL) (5). Briefly, 1–2 mg of FXI, FXla, PK, or HK in 200-μl volume in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS), were incubated with sulfo-NHS-LC-biotin in 5-fold molar excess of Sulfo-NHS-LC-biotin to each protein. After incubation for 1 h at room temperature, the samples were then loaded onto 10-ml Econo-Pac 10 DG columns (Bio-Rad, Richmond, CA) and collected in 1-ml fractions. Biotinylated-FXI, -FXla, -PK, or -HK (biotin-FXI, -FXla, -PK, or -HK) were quantified by absorbance at 290 nm using their extinction coefficient and a protein assay (Bio-Rad). The specific activity of each labeled protein was determined using coagulant and protein assays, and the integrity of each protein after labeling was examined by reduced 10% SDS-PAGE. Biotin-FXI, -FXla, -PK, or -HK had a specific activity of 196, 19, or 11 units/mg, respectively, indicating that the proteins were labeled without loss of activity.

FITC Labeling—FXI, FXla, and PK were fluorescein-labeled according to the procedure of Molecular Probes (Eugene, OR). Briefly, 2 mg of FXI, FXla, or PK in 200 μl was dialyzed into 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS). The dialyzed proteins were transferred into the reaction tube, and 20 μl of 1 M sodium bicarbonate at pH 9 was added to the protein. The fluorescein isothiocyanate was then added in 5-fold molar excess of each of the fluorescein proteins. The reaction was stirred at room temperature for 1 h while protected from light. At the conclusion of the incubation, 7 μl of the hydroxylamine was added to the conjugation reaction and was stirred at room temperature for an additional 30 min. The labeled FXI, FXla, or PK was then loaded dropwise to the center of the spin column and allowed to bind to the bed of the spin column. FITC-HK was added to the reaction mixture in 5-fold molar excess of each of the fluorescein proteins. The reaction was stirred at room temperature for 1 h while protected from light. At the conclusion of the incubation, 7 μl of the hydroxylamine was added to the conjugation reaction and was stirred at room temperature for an additional 30 min. The labeled FXI, FXla, or PK was then monitored by absorbance at 280 nm using the extinction coefficient of each protein. The specific activity of FITC-FXI, -FXla, or -PK was measured to be 180, 196, or 18 units/mg, respectively, using a clotting or amidolytic assay, and the integrity of each labeled protein was examined on reduced 10% SDS-PAGE (1, 2). No activation of the sym- gens occurred during the labeling procedure.

Endothelial Cell Culture—Human umbilical vein endothelial cells (HUVECs) were obtained and cultured according to the distributor’s recommendations. Cells between the 1st and 5th passages were subcul- tured onto fibronectin-treated (1 μg/ml) 96-well microtiter plates 24 h prior to the start of the experiment. Cell viability on culture plates or in suspension was determined using trypsin blue exclusion. Cell numbers were determined by counting on a hemocytometer.

Isolation of Human Platelets—Fresh whole blood was collected from individual normal volunteers into one-tenth volume of 1 μM Phe-Pro-Arg-chloromethyl ketone in saline (Calbiochem, San Diego, CA) and centrifuged at 180 × g for 10 min at room temperature. Theuffy coat was removed, and its pH was adjusted to 6.5, and prostaglandin I2 (Sigma, St. Louis, MO) at 1 μM final concentration, was added. The platelet-rich plasma was then centrifuged at 1200 × g for 10 min at room temperature. After removing the plasma, the platelet pellet was re-suspended in Heps-carbonate gelatin buffer (HCG) (137 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 14.7 mM Hepes, 5.5 mM dextrose containing 0.1% gelatin, 2 mM CaCl₂, and 1 mM MgCl₂), pH 7.5, containing 250 μM Gly-Asp-Ser-Ser (Sigma) and centrifuged again at 1200 × g for 10 min. The final platelet pellet was re-suspended in HCG buffer, pH 7.4, and the platelets were counted in a Z1-series Coulter counter (Beckman Coulter, Miami, FL) (17). In all studies, the washed platelets were examined for their ability to aggregate (Chronolog Aggregometer, Hat- borow, PA) to 5 μg/ml collagen (Nycomed Arzneimittel, Munich, Germany).

Preparation of the Extracellular Matrix—Confluent monolayers of HUVECs in 96-well microtiter plates (PF6 CERT MAXISORB, #439454, Nunc Immunoplate, Fisher Scientific, Chicago, IL) were washed with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (PBS), and then removed after 15-min treatment with 0.5% Triton X-100 in PBS. The plate was then incubated with 0.025 M NH₄OH for 10 min. The matrix was washed five times with 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, containing 0.05% Tween 20 followed by washing five times with HCG buffer, pH 7.4 (6, 18). All the incubations were performed under the same conditions and in an incubator with 5% CO₂ at 37°C. In all cases after this treatment, no cells were detected by light microscopy of the microtiter plates.

HK Binding to Endothelial Cell Monolayers, Plastic, or Matrix—Biotin-HK binding was performed on confluent monolayers of HUVECs in 96-well microtiter plates, microtiter plate wells treated with 1 μg/ml fibronectin (plastic), or HUVEC-generated matrix in 96-well microtiter plates. After washing the cells, empty plastic wells, or the matrix with HCG, pH 7.4, the microtiter plate wells were incubated with a 100-μl reaction mixture containing 10 μl biotin-HK in the absence or presence of 1 μM HK or antibodies to uPAR, gC1qR, human cytokeratin (anti- GPV20), mouse IgG, or goat IgG for 60 min at 37°C. The relative amount of biotin-HK binding to the cells, plastic, or matrix was deter- mined using ImmunoPure streptavidin horseradish peroxidase conjugate (Pierce) and peroxidase-specific fast reacting substrate, turbo-TMP (Pierce), as previously described (5). Bound biotin-HK was quantified by measuring the absorbance of the reaction mixture at 450 nm using a microplate auto reader EL 311 from Bio-Tek Instruments (Winooski, VT).
exclusion. Initial experiments determined the optimal concentration for specific FITC-PK binding to HUVECs in suspension and the requirement for added Zn\(^{2+}\). Binding experiments were performed in 200-μl reaction mixtures containing 100 nm FITC-PK and 154 nm HK in the absence or presence of 5 μM PK, 30 nm FXI, and/or 10 μM Zn\(^{2+}\). These HUVEC (4 × 10\(^{4}\) cells/well) suspensions in HCG, pH 7.4, in a 96-well filtration plate (Millipore, Bedford, MA) were incubated on a pre-wetted hydrophilic polycylinlene fluoride (PVDF) membrane (pore size, 1.2 μm) with low protein binding capacity for 0–120 min at 37 °C as described previously (17, 19). At the end of the incubation, the cells were washed by vacuum absorption and filtered using multiScreen Separation System Vacuum Manifold from Millipore (Bedford, MA). The fluorescence of cell-bound FITC-PK to HUVECs bound to the PVDF membrane of the filtration plate was measured on a CytoFluor 4000 fluorescence plate reader from PerSeptive Biosystems, Inc. (Framingham, MA) with 485-nm excitation and 530-nm emission filters.

**FITC-FXI or FITC-FXIIa Binding to Endothelial Cell Suspensions**—FITC-FXI or FITC-XIa binding to HUVECs suspensions was also established. HUVECs were detached from culture plates with a trypsin-EDTA solution (Invitrogen) and washed with HCG, pH 7.4, followed by re-suspension in the same buffer. Initial experiments determined the optimal concentration for specific FITC-FXI or FITC-XIa binding to HUVECs suspensions. The binding was performed in 200-μl reaction mixtures containing 80 nM FITC-FXI or FITC-XIa in the presence or absence of 600 nm HK and the absence or presence of 10 μM Zn\(^{2+}\) and/or 450 nM PK. In certain experiments, in a 96-well filtration plate (Millipore, Bedford, MA) for 0–120 min at 37 °C as described previously (17, 19). At the end of the incubation, the cells were washed by vacuum absorption and filtered using multiScreen Separation System Vacuum Manifold from Millipore (Bedford, MA). The fluorescence of cell bound FITC-FXI or FITC-XIa to HUVECs bound to the PVDF membrane of the filtration plate was measured on CytoFluor 4000 fluorescence plate reader from PerSeptive Biosystems, Inc. with 485-nm excitation and 530-nm emission filters.

**FXI and Prekallikrein Binding to HUVECs in the Presence of Non-activated or Activated Platelets**—Confluent monolayers of HUVECs were washed three times with HCG, pH 7.4. Biotin-FXI (10 nm) in 100 μl was incubated with HUVECs monolayers in the presence of 40 nm HK and an increasing concentration of platelets (2–8 × 10\(^{5}\)/ml) in the absence of Zn\(^{2+}\) and in the absence or presence of 5 μg/ml collagen. The cells were incubated for 90 min at 37 °C on a rotating mixer maintained at 50 rpm. In control experiments, 10 nm biotin-FXI was incubated with HUVECs in HCG, pH 7.4, containing 40 nm HK in the absence or presence of 10 μM Zn\(^{2+}\). In other experiments, 10 nm biotin-FXI in the presence of 40 nm HK was incubated with HUVECs and activated platelets in the absence or presence of 450 nM PK. In similar experiments, biotin-PK (40 nm) and HK (600 nm) in 100-μl reaction mixtures were added to HUVEC monolayers with increasing concentration of platelets (2–8 × 10\(^{5}\)/ml) in the absence of Zn\(^{2+}\) and in the absence or presence of 5 μg/ml collagen. The cells were incubated for 60 min at 37 °C on a rotating mixer maintained at 50 rpm. In control experiments, 40 nm biotin-PK and 600 nm HK were directly incubated with HUVEC monolayers in the absence or presence of 10 μM Zn\(^{2+}\). In other experiments, 40 nm biotin-PK was incubated with HUVECs and collagen-activated platelets in the presence of 600 nM HK in the absence or presence of 30 nm FXI. After completion of all incubations, all cells were washed three times with HCG, and the relative amount of biotin-FXI or biotin-PK bound to the adherent HUVECs was determined using ImmunoPure streptavidin horseradish peroxidase conjugate (Pierce) and peroxidase-chromogenic 4-β-D-thiogalactoside substrate, turbo-TMB (17). Bound biotin-FXI or -PK was measured by the absorbance of the reaction mixture at 450 nm using a microplate auto reader EL 311 from Bio-Tek Instruments.

**Measurement of Zn\(^{2+}\) after Platelet-Endothelial Cell Interactions**—The concentration of the Zn\(^{2+}\) in the collagen-treated platelet releasates was determined by colorimetric assay (20). A Zn\(^{2+}\) standard curve was determined by adding 10 μl of known amount of Zn\(^{2+}\) (0.1–30 μM) or samples of collagen-treated platelet releasates with 90 μl of 20 μM 5-Br-PAPS (Wako Chemicals Inc., Richmond, VA) in 0.01 M Tris, 0.15 M NaCl, pH 7.4. The complex formation of Zn\(^{2+}\) with 5-Br-PAPS was monitored at 552 nm absorbance.

**RESULTS**

**Specificity of HK Binding to HUVECs in Microtiter Plates**—Recent investigations claimed that HK binding to endothelial cells in microtiter plates is an artifact of the HK binding to plastic alone (14, 15). Investigations were performed to examine those comments (14, 15). Antibody to uPAR, gC1qR, or cytokeratin 1 blocked biotin-HK binding to monolayers of HUVECs in microtiter plate cuvette wells 85, 87, and 79%, respectively, after nonspecific binding, as measured by inhibition by unlabeled HK, was subtracted (Fig. 1A) (7). Alternatively, antibodies to each of the HK-binding proteins present on HUVECs did not inhibit biotin-HK binding to fibronectin-coated plastic microtiter plate cuvette wells (Fig. 1A). Because the HUVEC matrix accounted for at least two-thirds of the total amount of biotin-HK binding to cultured HUVECs seen in microtiter plate cuvette wells (8), investigations were performed to determine if antibodies to the HK-binding proteins also influenced HK binding to cell matrix (Fig. 1A). Antibody to uPAR completely blocked biotin-HK binding to matrix (Fig. 1A). Alternatively, antibodies to gC1qR or cytokeratin 1 blocked biotin-HK binding to matrix by 89 or 55%, respectively, after nonspecific binding determined by unlabeled HK was removed (Fig. 1A). These combined studies indicated that the sum of biotin-HK binding to cultured HUVECs in microtiter plates under these conditions consisted of cell and matrix protein binding and not just an interaction with plastic surfaces.

**Specificity of FXI Binding to HUVECs in Microtiter Plates**—Additional investigations were performed to determine the specificity of FXI binding to HUVECs in microtiter plates (Fig. 1B). Because previous investigations showed that the majority of FXI binding to HUVEC monolayers required the presence of HK (2), these experiments were performed in the presence of added HK. Similar to what has been seen with HK binding alone, antibodies to uPAR, gC1qR, or cytokeratin 1 blocked biotin-FXI binding to HUVEC monolayers in the presence of HK by 82, 76, or 64%, respectively (Fig. 1B). These same antibodies had no influence on biotin-FXI binding to fibronectin-coated plastic microtiter plate wells in the presence of HK (Fig. 1B) (10). Alternatively, antibodies to uPAR, gC1qR, or cytokeratin 1 blocked biotin-FXI binding to HUVECs matrix in the presence of HK 62, 64, or 46%, respectively. These data suggested that previous studies on FXI binding to monolayers of HUVECs cultured in microtiter plates characterized cell and matrix protein binding and not an artifact of binding to plastic (2, 9). Furthermore, the sum of FXI binding to cultured endothelial cells had a substantial contribution from HK.

**Characterization of PK Binding to HUVEC Suspensions**—To obviate any concerns about nonspecific binding to artificial surfaces, suspension binding assays, as previously reported for factor XII, were utilized to characterize the relative importance of PK and FXI binding to HUVECs (17). Initial studies examined PK binding to HUVEC suspensions. Specific FITC-PK binding to HUVEC suspensions, similar to what had been previously reported for \(^{125}\)I-PK binding, were noted at 100 nm added FITC-PK (data not shown) (1). Further studies determined if added Zn\(^{2+}\) was necessary for FITC-PK binding to HUVEC suspensions. Similar to HK binding to HUVECs in HCG buffer (10), the level of FITC-PK binding to HUVECs was independent of the ambient Zn\(^{2+}\) concentration from no added to 100 μM (data not shown). FITC-PK binding to HUVEC suspensions reached a plateau in the level of binding at about 90 min in the absence or presence of 10 μM Zn\(^{2+}\) (Fig. 2, A and B, respectively). This binding was inhibited by the presence of 50-fold molar excess of unlabeled PK. Plasma concentrations of FXI (30 nm) in the absence or presence of 10 μM Zn\(^{2+}\) had no influence on FITC-PK binding to HUVECs (Fig. 2, A and B). These data suggested that FXI had no influence on PK binding to endothelial cells regardless of the ambient Zn\(^{2+}\) concentration.
Investigations next determined the characteristics of FITC-FXI binding to HUVEC suspensions (Fig. 3). 80 nM FITC-FXI in the presence of 10 μM Zn²⁺ was required for specific FXI binding in both the presence or absence of added HK (data not shown). Using 80 nM FITC-FXI in the presence of 600 nM HK and 10 μM Zn²⁺, there was a plateau in the level of binding at 90 min (Fig. 3A). In the absence of added zinc ion, the level of binding was inhibited about 3-fold. Furthermore, using only a 5.5-fold molar excess PK (450 nM, a concentration equal to that seen in normal plasma), the level of FITC-FXI binding was experiments, the wells were washed three times and the relative biotin-HK or biotin-FXI binding to wells with cells, plastic, or matrix was determined using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxidase-specific fast reacting substrate, turbo-TMP, as previously described. Bound biotin-HK or biotin-FXI was relatively quantified by measuring the absorbance of the reaction mixture at 450 nm using a microplate auto reader EL 311 (Bio-Tek Instruments). The results presented are the means ± S.E. of three independent experiments.

Characterization of FXI and FXIa Binding to HUVEC Suspensions—Investigations next determined the characteristics of FITC-FXI binding to HUVEC suspensions (Fig. 3). 80 nM FITC-FXI in the presence of 10 μM Zn²⁺ was required for specific FXI binding in both the presence or absence of added HK (data not shown). Using 80 nM FITC-FXI in the presence of 600 nM HK and 10 μM Zn²⁺, there was a plateau in the level of binding at 90 min (Fig. 3A). In the absence of added zinc ion, the level of binding was inhibited about 3-fold. Furthermore, using only a 5.5-fold molar excess PK (450 nM, a concentration equal to that seen in normal plasma), the level of FITC-FXI binding was...
inhibited to a level below that of the binding seen in the absence of added Zn$^{2+}$ (Fig. 3A).

When 80 nM FITC-FXI binding to HUVEC suspensions in the presence of 10 μM Zn$^{2+}$ was examined in the absence of added HK, a plateau in the level of binding was seen at 60 min (Fig. 3B). The level of FITC-XI binding seen was about 50% of that seen in the presence of HK. There was only a 33% decrease in FITC-FXI binding to HUVEC suspensions in the absence of added Zn$^{2+}$ (Fig. 3B). The presence of 450 nM PK blocked the level of FITC-FXI binding to that seen in the absence of no added zinc ion (Fig. 3B). This finding suggested that, in the absence of added HK, FITC-FXI bound to endogenous HUVEC HK or some other structure that also interacted with unlabeled FXI. Furthermore, plasma concentrations of PK were able to completely block this interaction (2, 3).

80 nM FITC-FXIIa was also shown to have specific binding to HUVEC suspensions in the presence of the 10 μM Zn$^{2+}$ and in the presence or absence of 600 nM HK (data not shown). When incubating 80 nM FXIIIa in the presence of 600 nM HK and 10 μM Zn$^{2+}$ with HUVEC suspensions, there was a plateau in binding at 60–90 min of incubation (Fig. 4A). The presence of 450 nM PK reduced the level of binding below that seen in the absence of zinc ion (Fig. 4A). When 80 nM FXIIa were incubated with HUVEC suspensions in the absence of added HK but in the presence of 10 μM Zn$^{2+}$, there also was a plateau in the level of binding at 90 min, but the total level of binding was also about half that seen in the presence of HK (Fig. 4B). Furthermore, the presence of 450 nM PK reduced the level of binding in the absence of added Zn$^{2+}$ (Fig. 4B). These data indicated that FXIIIa binding to HUVECs, regardless as to whether it is mediated by exogenous HK, was neutralized by plasma concentrations of PK. These combined data indicated that FXI and FXIIa binding to HUVECs in the absence of HK were...
Fig. 5. Collagen-stimulated platelets promote FXI binding to HUVECs. A, washed human platelets (2–8 × 10⁸ platelets/ml) (bottom three pairs of data) in HCG, pH 7.4, with no added Zn²⁺ were incubated over washed HUVEC monolayers in microtiter plate cuvette wells in 100-μl reaction mixtures containing 10 nM biotin-FXI and 40 nM HK in the absence (lined bar graph) or presence (checkered bar graph) of 5 μg/ml collagen. The numbers to the left of the graph indicate platelet concentrations in the experiment. When platelets were absent (biotin-FXI + HK), the cells were incubated with 10 nM biotin-FXI and 40 nM HK in the absence (lined bar graph) or presence (checkered bar graph) of 10 μM Zn²⁺ for 90 min at 37 °C on a rotating shaker. B, washed human platelets (2–8 × 10⁸ platelets/ml) (bottom three pairs of data) in HCG, pH 7.4, with no added Zn²⁺ were incubated over washed HUVEC monolayers in microtiter plate cuvette wells in 100-μl reaction mixtures containing 20 nM biotin-PK and 20 nM HK in the absence (lined bar graphs) or presence (checkered bar graph) of 5 μg/ml collagen and for 60 min at 37 °C on a rotating shaker. When the platelets were absent (biotin-PK+HK), the cells were incubated with 20 nM biotin-PK and 20 nM HK in the absence (lined bar graph) or presence (checkered bar graph) of 10 μM Zn²⁺ for 60 min at 37 °C on a rotating shaker. The numbers to the left of the graph indicate platelet concentrations in the experiment. At the completion of the incubation, the microtiter plate cuvette wells were washed, and the amount of biotin-FXI in the presence of 40 nM HK and collagen-activated platelets was determined using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxidase-specific fast reaction substrate, turbo-TMB, as previously described (5). Bound biotin-FXI or biotin-PK was relatively quantitated by measuring the absorbance of the reaction mixture at 450 nm using a microplate autoreader EL 311 (Bio-Tek Instruments). The results presented are the means ± S.E. of three independent experiments.

equal and that plasma concentrations of PK inhibited their binding (Figs. 3 and 4).

Influence of Platelet Activation on FXI or PK Binding to HUVEC Monolayers—Because zinc ion is an essential requirement for FXI or FXIa binding to HUVECs, investigations were performed to determine if activated platelets contributed sufficient Zn²⁺ to support FXI binding to HUVECs. When 2–8 × 10⁸ non-activated washed human platelets were incubated with 10 nM biotin-FXI in the presence of 40 nM HK, the level of binding seen to HUVEC monolayers was the same as that of incubating 10 nM biotin-FXI in the presence of 40 nM HK in the absence of added Zn²⁺ (Fig. 5A, lined bar graphs). Alternatively, when these same washed platelets are activated with 5 μg/ml collagen in the presence of 10 nM biotin-FXI and 40 nM HK, the level of biotin-FXI binding to HUVECs was substantially increased (Fig. 5A, checkered bar graphs). The amount of binding seen was similar to that produced by 10 nM biotin-FXI in the presence of 40 nM HK and 10 μM added Zn²⁺ (Fig. 5A, biotin-FXI+HK (checkered bar graphs)). These data indicated that activated platelets contributed sufficient Zn²⁺ to support FXI binding to the HUVECs (19–21). In support of that interpretation, the platelet supernatant zinc ion concentrations after collagen activation ranged from 3.2 ± 0.95 to 8.3 ± 2.7 μM (mean ± S.E.) for 2–8 × 10⁸ activated platelets, respectively. These values approach the 7 μM Zn²⁺ necessary to support FXI or FXIa binding to HUVECs without activated platelets (2).

Alternatively, collagen stimulation of washed platelets had less influence on PK binding to HUVECs than FXI (Fig. 5B). The level of 20 nM biotin-PK binding to HUVECs in the presence of 20 nM HK was not substantially influenced by the absence or presence of activated 2–8 × 10⁸ platelets/ml (Fig. 5B). When platelets were activated by 5 μg/ml collagen, there was a slight increase in the level of biotin-PK binding to HUVECs in the presence of HK over a range of physiologic concentrations of platelets (Fig. 5B, compare the checkered bar graphs to the lined bar graphs). However, the level of biotin-PK binding to HUVECs in the absence of added Zn²⁺ (Fig. 5B, biotin-PK+HK (lined bar graph)) was the same as biotin-FXI binding in their presence (Fig. 5B, biotin-PK+HK (checkered bar graph)). These combined data suggested that, under physiologic conditions, PK was the preferred ligand to bind to HUVECs in the absence or presence of platelet activation. However, when platelets were activated, a sufficient level of ambient Zn²⁺ became present to support FXI binding to endothelial cells.

Investigations were next performed to determine how physiologic concentrations of PK or FXI influenced the binding of the other ligand to endothelial cells. Initial studies showed that 10 nM biotin-FXI in the presence of 40 nM HK and 10 μM Zn²⁺ showed specific binding to HUVEC monolayers (Fig. 6, biotin-FXI+HK (checkered bar graph)). The level of 450 nM PK, the biotin-PK was reduced (Fig. 6, biotin-FXI+HK (lined bar graph)). The influence of PK at a concentration similar to that seen in plasma on FXI binding to HUVECs was next determined in the presence of collagen-activated platelets (Fig. 6). In the presence of 450 nM PK, 10 nM biotin-FXI binding to HUVECs in the presence of 40 nM HK and collagen-activated platelets was at the level of binding seen with non-activated platelets (Fig. 6, compare the level of binding of the lined versus checkered bar graphs). These data suggested that, in the presence of nearly plasma concentrations of PK, FXI did not bind to HUVECs, indicating that even after platelet activation, FXI was blocked from binding to HUVECs by the presence of plasma concentrations of PK.
Studies were next performed to determine if factor XI influenced PK binding to HUVECs (Fig. 7). In control experiments, the level of binding of 40 nM biotin-PK in the presence of 600 nM HK to HUVEC monolayers was the same in the presence of 30 nM FXI alone or 30 nM FXI and 10 μM Zn²⁺ (Fig. 7A). These data are consistent with the findings seen in Fig. 2 using a suspension binding system. In simultaneous binding experiments to that performed in Fig. 7A, the binding of 40 nM biotin-PK in the presence of 600 nM HK was examined in the presence of non-activated or activated platelet suspensions at three physiologic concentrations (Fig. 7B). As shown in Fig. 7B, the level of biotin-PK binding from suspension of non-activated platelets (dark bar graphs) was not significantly different than that seen with non-activated platelet suspensions containing 30 nM FXI (striped bar graphs) or collagen-activated platelet suspension also containing 30 nM FXI (open bar graphs). These data suggested that FXI did not influence PK binding to cultured EC regardless of the ambient Zn²⁺ concentration. These combined studies indicated that, at plasma concentrations of all reactants, FXI did not influence biotin-PK binding to HUVECs in the presence of HK.

**DISCUSSION**

The present investigation indicates that PK rather than FXI preferentially binds to endothelial cells membranes. PK specifically binds to HUVEC monolayers or suspensions in the presence of HK and physiologic concentrations of free zinc ion (1, 11–13, 21). The presence of physiologic concentrations of FXI has no influence on PK binding to HUVECs in the presence or absence of 0.3–10 μM free Zn²⁺. Alternatively, FXI or FXIa requires at least 23-fold higher concentrations of ambient zinc concentration for specific binding to HUVECs (2, 10). Furthermore, all of FXI or FXIa binding to HUVECs in the presence or absence of HK is inhibited by physiologic concentrations of PK even in the presence of 10 μM Zn²⁺. When plasma concentrations of all reactants are present, there is virtually no FXI binding to HUVECs and its matrix in the presence of PK. Thus, in the intravascular compartment, the ambient Zn²⁺ concentration and the plasma concentration of PK restrict FXI or FXIa from binding to endothelial cells. These findings are similar to those recognized to modulate factor XII binding to endothelial cells (17). Although factor XII binds to the same binding proteins, putative receptors, such as HK, the ambient zinc ion concentration, and the plasma concentration of HK are each sufficient to restrict factor XII binding under resting conditions (17). In the present studies, factor XI or XIa binding to endothelial cells occurs only if there is a sufficient activation of platelets liberating their intracellular Zn²⁺ to support binding. Platelets and, perhaps, other cells serve as zinc repositories that when activated in the intravascular compartment can support factor XI or XIa binding. However, our present data also suggest that elevation of the local Zn²⁺ concentration alone is not sufficient to overcome the inhibitory activity of plasma PK on plasma FXI.
FXI binding to platelets, it has been proposed that HK is not domains 2, 4, and 1, and peptides to Apple domain 3 compete investigations claiming that HK does not compete FXI a binding may yield different results subject to interpretation. Initial FXI main 3 platelet-binding site, although the physical evidence for that HK binds to FXI, resulting in exposure of the Apple domain 3 platelet-binding site, has been proposed that HK is not the platelet receptor for FXI/FXIIa (25–27). It has been proposed that HK binds to FXI, resulting in exposure of the Apple domain 3 platelet-binding site, although the physical evidence for that interpretation has not been presented (26, 28). Because HK is always present in these reaction mixtures, it is difficult to know what HK is actually contributing in these experiments (25–27). Alternatively, FXI alone was not demonstrated to bind to HUVECs in the absence of HK by a novel technique of growing endothelial cells on microcarrier beads (14). These latter results do not follow from our previous and present investigations that indicate that there is some FXI binding to HUVEC suspensions and monolayers in the absence of added HK (2). PK has also been recognized to bind to HUVECs in the absence of added HK (1). The finding that plasma concentrations of FXI completely block FXI or FXIIa binding to HUVECs in the absence of added HK suggests that these proteins bind to HUVEC HK or another protein that can bind both PK and FXI or FXIIa.

The significance of these studies overall is as follows. The fact that ambient Zn2+ concentration under quiescent physiologic conditions and plasma PK concentrations prevent FXI or FXIIa binding to endothelial cells indicates that FXI or FXIIa assembly on endothelial cells is not a primary event for the generation of thrombin. This interpretation is consistent with other investigators' findings using different techniques and data sets (6, 14, 15). Moreover, because plasma HK is present in molar excess (700 nm) to both PK (450 nm) and FXI (30 nm) and virtually all plasma PK and FXI circulate in complex with HK, FXI or FXIIa binding to cells in the absence of HK is physiologically irrelevant. It remains to be determined under what pathophysiologic circumstances FXI or FXIIa actually binds to endothelial cell membranes. Alternatively, in the intravascular compartment, PK predominately binds to HK. The physiologic role of PK assembling on endothelial cells has not been completely elucidated, but recent experimental results suggest a new understanding. When PK assembles on HK on endothelial cells, it is activated to plasma kallikrein by the prolylcarboxypeptidase, an enzyme that is constitutively present on endothelial cell membranes and matrix (1, 8, 18, 29).

Two substrates of formed kallikrein contribute to the constitutive anticoagulant nature of the intracellular compartment. It autodigests its receptor HK to liberate bradykinin, a potent vasodilator that stimulates tissue plasminogen activator, NO, and prostacyclin release from endothelial cells. Recent studies on the C1 inhibitor knockout mice indicate that bradykinin is constitutively formed to produce angioedema (30). Because C1 inhibitor inhibits plasma kallikrein and not tissue kallikrein, plasma kallikrein must be constitutively formed in the intravascular compartment. Formed kallikrein also leads to kinetically favorable single-chain urokinase activation to two-chain urokinase (1, 31, 32). Thus interference with the endothelial cell prekallikrein activating system may indirectly contribute to a change in the anticoagulant nature of the intravascular compartment. This interpretation along with other studies suggests that PK activation contributes to the constitutive anticoagulant nature of the intravascular compartment (5, 28, 33). These hypotheses are currently being examined in animal models.

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