The consequences of assembling the contact system of proteins on the surface of vascular cells has received little study. We asked whether assembly of these proteins on the surface of cultured human endothelial cells (HUVECs) results in the activation of prekallikrein (PK) and its dependent pathways. Biotinylated PK binds specifically and reversibly to HUVECs in the presence of high molecular weight kininogen (HK) (apparent $K_d$ of $23 \pm 11$ nmol/L, $B_{\text{max}}$ of $1.7 \pm 0.5 \times 10^5$ sites per cell [mean $\pm$ SD, $n = 5$ experiments]). Cell-associated PK is rapidly converted to kallikrein. Surprisingly, the activation of cell-associated HK-PK complexes is entirely independent of exogenous factor XII ($K_m = 30$ nmol/L, $V_{\text{max}} = 12 \pm 3$ pmol/L/min in the absence $v$, $K_m = 20$ nmol/L, $V_{\text{max}} = 9.2 \pm 2.1$ pmol/L/min in the presence of factor XII). Rather, kallikrein formation is mediated by an endothelial cell-associated, thiol protease. Cell-associated HK is proteolyzed during the course of prekallikrein activation, releasing kallikrein from the surface. Furthermore, activation of PK bound to HK on HUVECs promotes kallikrein-dependent activation of pro-urokinase, resulting in the formation of plasmin. These results indicate the existence of a previously undescribed, factor XII-independent pathway for contact factor activation on HUVECs that regulates the production of bradykinin and may contribute to cell-associated plasminogen activation in vivo.

The biologic function(s) of the plasma proteins of the contact system in hemostasis has been uncertain. Deficiencies of these proteins are not associated with clinical bleeding despite marked prolongation of in vitro surface-activated coagulation times. Paradoxically, studies suggest a role for contact system proteins in fibrinolysis. Patients deficient in individual contact factor proteins may be at increased risk for thrombosis.

Activation of the contact factor pathway promotes plasma fibrinolytic activity and antibodies to tissue-type and urokinase plasminogen activators neutralize only 75% of plasma fibrinolytic activity after stimulation with 1-desamino-8-D-arginine vasopressin.

However, direct plasminogen activator activity of kallikrein, activated factor XII, or factor XIa are present in the activation of prekallikrein (PK) and factor XI. HK binds to cells through sites present in domains 3, 4, and 5 and binds to PK through a site on domain 6. Alternatively, kallikrein has been reported to cleave pro-urokinase (Pro-UK) at a rate that suggests a potential physiologic role. Recent observations also indicate that binding of contact factor proteins to cell surfaces accelerates the formation of two-chain urokinase (tcuPA) and plasmin, suggesting a potential mechanism by which these reactions may occur in vivo.

The mechanism by which contact system proteins are assembled and activated on cell surfaces has received little attention. We and others have previously reported that platelets and endothelial cells express binding sites for high molecular weight kininogen (HK), a multidomain protein that is both a binding site and a cofactor for the activation of prekallikrein (PK) and factor XI. HK binds to cells through sites present in domains 3, 4, and 5 and binds to PK through a site on domain 6. Because endothelial cells also have the capacity to bind factor XII (FXII), it has been assumed that activation of the contact pathway on endothelium proceeds similarly to that which occurs in plasma on artificial surfaces. The present study indicates that assembly of the contact factor proteins on endothelial cells results in PK activation. Activation of PK results in proteolysis of HK with probable liberation of bradykinin and stimulation of plasminogen activator activity. However, to our surprise, PK activation on endothelial cells is independent of FXII or its activated forms.

Materials and methods

Proteins. HK was purified from plasma using sequential carboxymethyl-papain-Sepharose (CM-papain-Sepharose) and Blue-Sepharose affinity chromatography as previously reported. HK migrated as a 120-kD protein on sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE) after reduction with 2% β-mercaptoethanol. HK had a specific activity of 12 to 20 U/mg. Purified HK was iodinated with IODOGEN (Pierce, Rockford, IL) as previously reported.

Human PK was purchased from Enzyme Research Laboratories (South Bend, IN). The protein migrated as a doublet at 88 and 85 kD on 10% SDS-PAGE under reduced conditions and expressed approximately 1% to 3% of the amidolytic activity of kallikrein. No FXII or its activated forms were found in the HK or PK preparations by immunoblotting using a monospecific goat antiserum to human FXII. PK was also iodinated with IODOGEN using identical techniques previously reported for HK. Iodinated PK was a doublet at 88 and 85 kD on 10% SDS-PAGE under reducing conditions. FXII, purchased from Enzyme Research Laboratories, migrated predominantly as a single band at 80 kD on 10% SDS-PAGE under reduced conditions and expressed less than 1% of the amidolytic activity of activated FXII. Activated factor XII (aFXII) was purchased from Enzyme Research Laboratories. aFXII was validated as two bands at 50 and 28 kD on 10% SDS-PAGE under reduced conditions. Factor XIIa fragment (βFXIIa; a generous gift from Dr Robin Pixley, Temple University School of Medicine, Philadelphia, PA) migrated predominantly as a single band at 28 kD on 10% SDS-PAGE under reduced conditions. PK was converted to kallikrein by adding βFXIIa at a molar ratio of 1:74 (βFXIIa:PK). On reduced 10% SDS-PAGE, kallikrein migrated at three bands at 51, 37, and 34 kD.

Pro-UK and tcuPA were purchased from American Diagnostica.

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A NOVEL MECHANISM FOR CONTACT ACTIVATION 517

(Greenwich, CT) or were the generous gifts of Dr Jack Henkin (Abbott Laboratories, Abbott Park, IL). Pro-UK migrated as a single band at 56 kD on 10% SDS-PAGE under reduced conditions. Glu-plasminogen was purchased from American Diagnostica. Fibrinogen, factor XI, lys-plasminogen, and plasmin were purchased from Enzyme Research Laboratories. C1-inhibitor was purified from plasma and antibodies to it were purchased as previously reported.44 SDD31 (SDDWIPDQTD- PNGLSNPISDPFPDTSPK), a 31-amino acid peptide that constitutes the PK binding region on HK,35 was synthesized at the University of Michigan Protein and Carbohydrate Structure Facility. This peptide, which spans amino acids 565 to 595 in the mature sequence of HK, is named by using the first three letters of its sequence followed by the number of total amino acids in the peptide. Plasminogen activator inhibitor-1 and rabbit antiserum to it were generously supplied by Dr David Ginsburg (University of Michigan, Ann Arbor, MI). Monoclonal antibodies (MoAbs) HKL13, which is directed to domain 5 of HK; HKL16, which is directed to the PK binding site on HK; and PK6, which is directed to the HK binding site on PK, were generously provided by Dr Werner Müller-Esterl (Johannes Gutenberg University, Mainz, Germany).35,36 Goat antiserum to human FXII (generously provided by Dr Robin Pixley, Temple University) was adsorbed with FXII-deficient plasma to make it monospecific before use. Polyclonal antibodies to factor XII were also purchased from Enzyme Research Laboratories and Haematologica Technologies (Essex Junction, VT). Rabbit antiserum to human PK was prepared as previously reported,37 and antibodies were purified on kallikrein-Sepharose. Pooled normal human plasma and FXII-deficient plasma were purchased from George King Biochemicals, Inc (Overland Park, KS). Prekallikrein-deficient human plasma was from a patient who was characterized to have less than 1% prekallikrein activity and antigen levels.

Functional and immunochemical assays. HK and FXII procoagulant activities were measured using a one-stage, kaolin-activated coagulant assay38 with total kininogen-deficient and FXII-deficient (George King Biochemicals, Inc) plasmas as the substrate, respectively. Total kininogen-deficient plasma was donated by the late Mayme Williams (Philadelphia, PA). One unit of HK and FXII procoagulant activity is equal to that found in 1 mL of normal plasma. The amidolytic activity of plasma kallikrein and activated FXII were measured using 0.4-1 mmol/L chromogenic substrate, H-D-Pro-Phe-Arg-pNA · 2HCl (S-2302; Pharmacia, Franklin, OH) as previously reported.33 One unit of activity (40 µg/mL) is equal to 2.47 µmol of substrate hydrolyzed/min/mL plasma.33 Immunoblotting of HK, PK, FXII, plasminogen activator inhibitor-1, and C1 inhibitor was performed using the chemiluminescence system of Amersham (Arlington Park, IL). Prekallikrein-deficient human plasma was from a patient who was characterized to have less than 1% prekallikrein activity and antigen levels.

Biotinylation of purified PK. PK was biotinylated as previously reported for HK.25,26 The protein concentration in each fraction of gel-filtered biotinylated-PK (biotin-PK) was determined by its absorbance at 280 nm using an extinction coefficient of 11.7.40 Incorporation of biotin into PK was determined by adding 2-(4')-hydroxyazobenzene)benzolic acid41 according to the manufacturer’s instructions (Pierce). Each molecule of PK was labeled with 1 to 3 molecules of biotin without causing its activation as determined either by a change in migration on SDS-PAGE or by the development of amidolytic activity. Biotin-PK could be completely converted to biotin-kallikrein by βFXIIa.

Endothelial cell culture. Cultures of human umbilical vein endothelial cells (HUVECs) were established as previously described.21 Primary cultured cells were passaged twice and then frozen in liquid nitrogen at 1 to 3 × 10⁶ cells/mL. Frozen cells (1.0 mL) were thawed quickly and resuspended in 10.0 mL Endothelial Cell Growth Medium-Modified MCDB 131 (Clonetics, San Diego, CA), which contained 2% fetal calf serum and which was supplemented with Bovine Brain Extract (Clonetics) and centrifuged at 300g for 5 minutes at room temperature. The pellet was resuspended in 15.0 mL of the medium, and the cell suspension was grown to confluence on fibronectin (20 µg/mL) -coated flasks (Corning Inc, Corning, NY).

Binding of biotin-PK to HUVECs. All binding experiments were performed at 37°C, unless otherwise stated, on fibronectin-coated, 96-well microtiter plates (Nunclon; Thomas Scientific, Swedesboro, NJ) using cells passaged three or four times as previously published.25,26 HUVECs were always used within 24 hours of reaching confluence. Each well contained 3 to 4 × 10⁴ cells. All incubation and washing steps were performed using HEPES-Tyrode’s binding buffer [0.135 mol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO₃, 0.36 mmol/L NaH₂PO₄, 14.7 mmol/L HEPES (N-2-hydroxyethylpiperazine-N₂-2-ethanesulfonic acid)] containing 50 µmol/L Zn²⁺, 1 mmol/L Mg²⁺, 3.5 mg/mL bovine serum albumin, 3.5 mg/mL dextrose, pH 7.35, in the presence of 2 mmol/L Ca²⁺, unless otherwise stated. HUVECs were washed five times before performing all binding studies. In most experiments, HUVECs were incubated with 20 nmol/L HK in 100 µL for 1 hour, which is sufficient to saturate their specific binding sites,21,22 and the unbound HK was removed, whereas in other experiments the cells were washed three times. The cells were then incubated with various concentrations of biotin-PK in a 100 µL reaction volume for 1 hour at 37°C unless otherwise stated. Binding of biotin-PK to HUVECs was the same whether unbound HK was removed by aspiration alone or by washing. Nonspecific binding, unless otherwise stated, was determined by measuring binding in the presence of 50-fold molar excess unlabeled PK. Specific binding was determined by subtracting nonspecific binding from total binding. Cell-associated biotin-PK was measured using ImmunoPure streptavidin horseradish peroxidase conjugate (Pierce) and the peroxidase-specific fast-reacting substrate, turbo-3',5'-tetramethylbenzidine dihydrochloride (turbo-TMB; Pierce), as previously reported.25 Bound biotin-PK was measured by the absorbance at OD₄₅₀nm using a Microplate auto reader EL 311 (Bio-Tek Instrument, Winooski, VT).

In certain experiments, binding of ¹²⁵I-PK to HUVECs in suspension was measured. Briefly, HUVECs were removed from culture dishes with 0.05% trypsin, 0.53 mmol/L EDTA solution (Life Technologies, Grand Island, NY), which was immediately inactivated with trypsin neutralizing solution (Clonetics), and the cells were washed twice by centrifugation using HEPES-Tyrode’s binding buffer and brought to a final cell suspension of 3.5 × 10⁶ cells/mL. The suspended cells were preincubated with 20 nmol/L HK and ¹²⁵I-PK (20 nmol/L) was added in the absence or presence of 50-fold molar excess PK. After 5 to 40 minutes, 50-µL aliquots of the cell suspension were centrifuged at 10,000g for 2 minutes in a Beckman Microcentrifuge Model E (Fullerton, CA) over a 200-µL oil gradient consisting of 1 part Apiezon (Biddle Instruments, Blue Bell, PA) and 9 parts N-butyolphalathate (Fisher Scientific, King of Prussia, PA). The tips of the tapered centrifuge tubes were amputated and counted in a gamma counter. The amount of bound ¹²⁵I-PK was calculated based on the specific radioactivity of the ligand.

Additional studies were performed to evaluate the reliability of using biotin-PK to measure binding to cells. Although four additional wash steps were used when biotin-PK was used as the ligand than when ¹²⁵I-PK was used, the percentage of specific binding of biotin PK (0.5% ± 0.14%) and ¹²⁵I-PK (0.62% ± 0.12%) were not significantly different (P > 0.05). These data indicated that, like biotin-HK and ¹²⁵I-HK,25 biotin-PK and ¹²⁵I-PK bound with sufficient affinity to cell-associated HK to permit them to be used interchangeably.

Quantification of HUVEC-bound biotin-PK. To convert the color reaction of bound biotin-PK to pmoles PK bound, standard curves for each batch of biotin-PK were developed as previously reported for biotin-HK binding.25 Kaolin (200 mg/mL) in HEPES-Tyrode’s binding buffer without added divalent cations was preincubated with 60 nmol/L HK for 1 hour at 37°C with constant mixing. After removing unbound HK by centrifugation at 3,000g for 30 seconds, biotin-PK (0.02 to 3.0 pmol) was incubated with the HK-treated kaolin suspension in triplicate

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for 10 minutes at 37°C with constant mixing. The kaolin suspension was then blocked by adding 1% bovine serum albumin and then incubated with a 1:500 dilution of streptavidin horseradish peroxidase conjugate for 1 hour. The relative amount of streptavidin horseradish peroxidase conjugate bound to the centrifuged and resuspended kaolin was determined as described for biotin-HK binding to HUVECs. After subtracting the absorbance of kaolin-HK alone, a standard curve from three or more identical experiments was generated relating the absorbance at each amount of biotin-PK to its concentration by linear regression.

Characterization of PK binding to HUVECs.

The apparent affinity (Kp) and number of binding sites (Bmax) for specific biotin-PK binding to HUVECs, preincubated with HK (20 nmol/L), were determined by the method of Scatchard. The apparent affinity of biotin-PK binding for HUVECs was also assessed by determining the capacity of unlabeled PK to inhibit the binding of biotin-PK. Biotin-PK (20 to 40 nmol/L) was incubated with 0- to 100-fold molar excess PK for 1 hour with confluent HUVECs that had been preincubated in the presence (20 nmol/L) or absence of HK. The 50% inhibitory concentration (IC50) was determined, and the apparent Kp of the competitor was calculated by the technique of Müller as previously reported, using the formula Kp = 8/3 [(1 − T)] where [T] equals the molar concentration of the IC50 of the competitor and [T] is molar concentration of the added biotin-PK.

PK activation on endothelial cells.

Activation of PK bound to HUVECs was measured three ways. First, confluent monolayers of HUVECs in microtiter plate wells were preincubated with buffer containing 2% radioimmunoassay grade bovine serum albumin (Sigma, St Louis, MO) for 1 hour at 37°C. PK in HEPES-Tyrode’s binding buffer containing 50 µmol/L Zn+2 was incubated with cells preincubated with either saturating concentrations of HK (20 nmol/L) or buffer. Kallikrein activity was then measured as the hydrolysis of 0.4 nmol/L S2302 over 1 hour (Pharmacia). In certain experiments, FXII (20 nmol/L), αFXIIa (3.4 nmol/L), or βFXIIa (3.4 nmol/L) was added along with the chromogenic substrate. In other experiments, plasma kallikrein (20 nmol/L) was substituted for PK. In other experiments, the activation of PK bound to HK on HUVECs was measured in the presence of a twofold neutralizing concentration of an antibody to FXII. To determine this, 20 nmol/L αFXIIa was incubated with increasing concentrations of anti-factor XII IgG (1.0 to 1,000 µg/mL). It was found that 0.2 mg/mL antibody from two sources inhibited greater than 95% of the hydrolytic activity of αFXIIa for S2302. Therefore, 0.4 mg/mL of anti-FXII IgG was added to 4 × 10^4 HUVECs pretreated with 20 nmol/L HK in the presence of 20 nmol/L PK and the amydolitic activity was determined relative to the activity generated in the absence of antibody. In different experiments, endothelial cell-bound PK activation was measured when the source of PK was different plasmas. Briefly, HUVECs, preincubated with 20 nmol/L HK, were incubated with NHP, FXII-deficient plasma, or PK-deficient plasma for 1 hour at 37°C. After washing the cells with HEPES-Tyrode’s buffer containing 50 µmol/L Zn+2, 0.4 nmol/L S2302 was added and the extent of hydrolysis of the chromogenic substrate was measured over the next 1 hour.

Second, the kinetics of PK activation on HUVEC monolayers were determined in the absence or presence of exogenous FXII. The cells were preincubated with HK (20 nmol/L) for 1 hour and PK (1 to 100 nmol/L) was added for an additional 1 hour. The wells were washed three times with HEPES-Tyrode’s binding buffer containing 50 µmol/L Zn+2, after which FXII (20 nmol/L) and S2302 (0.4 nmol/L) were added and hydrolysis of the substrate was measured over the next 1 hour. In certain experiments, FXII was omitted. The amount of kallikrein formed was determined using a standard curve generated by adding known amounts of soluble plasma kallikrein under identical conditions. The Ka and Vmax of PK activation on HUVECs were determined from double reciprocal plots.

Third, experiments were performed to determine if cell-bound PK was activated to kallikrein or whether cell-associated PK expressed intrinsic activity. HUVECs were incubated for 1 hour with 20 nmol/L HK, unbound HK was removed, and the cells were incubated with 20 nmol/L biotin-PK in HEPES-Tyrode’s buffer containing 50 µmol/L Zn+2 for variable lengths of time. The reaction was stopped by washing the cells three times, after which the contents of the wells were solubilized by adding electrophoresis sample buffer containing 2% β-mercaptoethanol. The proteins were then boiled, separated on 10% SDS-PAGE, electroblotted onto a nitrocellulose membrane, and blocked with Blotto and streptavidin-horseradish peroxidase (1:500; Pierce) was added. Biotin-PK bound to the nitrocellulose was detected by measuring chemiluminescence (Amersham). The extent to which the biotin-PK was cleaved was determined by densitometer scanning of the blot using a transmittance/reflectance scanner (Model GS 300; Hoefer Scientific Instruments, San Francisco, CA) in the transmittance mode.

Determination of the protease class of the cell-associated PK activating enzyme.

Two sets of experiments were performed to determine the biochemical features of the protease(s) that activated PK bound to HK on HUVECs. First, studies were performed to determine if containing factor XIIa, kallikrein, or another serine protease could be responsible for PK activation. To do this, we determined if neutralizing concentrations (0.4 mg/mL) of two antibodies to FXII, phenylmethyl sulfonyl fluoride (PMSF; 1 mM), SBTI (1 mg/mL), Pro-Phe-Arg-chloromethylketone (10 µmol/L), or benzamidine (1 mmol/L) would block activation of HK-bound PK on HUVECs. Activation was determined by detecting cleavage of PK (88,85-kD doublet) to kallikrein on reduced 10% SDS-PAGE, which mostly appeared as the 51-kD heavy chain of kallikrein. Pro-Phe-Arg-chloromethylketone was the generous gift of Dr Charles Kettner (Dupont-Merck Pharmaceutical Co, Wilmington, DE). All other inhibitors were purchased from Sigma. Each of these protease inhibitors were titrated against 20 nmol/L factor XIIa or kallikrein to determine the minimal concentration that would abolish the enzymes’ hydrolytic activity against S2302. This inhibitory concentration was the one used in the experiments.

Second, studies were performed to determine the biochemical requirements of the membrane-associated, PK activating enzyme. In these experiments, the minimal concentration of the inhibitor that produced maximal inhibition was used. Furthermore, each inhibitor was examined for its ability to directly inhibit kallikrein itself. In these experiments, HK-saturated HUVECs were incubated with PK in the absence or presence of antipain (100 µmol/L), cysteine (10 µmol/L), HgCl2 (1 µmol/L), glutathione (100 µmol/L), calpain inhibitor (10 mmol/L; Calbiochem, La Jolla, CA), E64 (10 µmol/L), hydroxymercuribenzoic acid (10 µmol/L), iodoacetamide (10 µmol/L), iodoacetamide (10 µmol/L), dithiothreitol (DTT; 10 mmol/L), 2-mercaptoethanol (10 mmol/L), TIMP-1 and TIMP-2 (20 µg/mL; Calbiochem), BB94 (15 µmol/L), Cystatin (1 µmol/L), pepstatin A (10 µmol/L), EDTA (10 mmol/L), EGTA (10 mmol/L), 1,10 phenanthroline (10 mmol/L), phosphoramidon (10 µmol/L), zincov (100 µmol/L), enalapril (10 mmol/L), lisinopril (10 mmol/L), bathepanthrolamine (100 µmol/L), or sodium hydrosulphite (10 µmol/L). After incubation, S2302 (0.4 µmol/L) was added and the extent of hydrolysis of the substrate was determined. BB94 was a generous gift of Dr Steve Weiss (University of Michigan). Unless otherwise stated, the remaining inhibitors were purchased from Sigma.

Cleavage of HUVEC-bound HK by activated PK.

Experiments were performed to determine whether activation of PK was accompanied by cleavage of the HK to which it was bound. HUVECs were incubated with 20 nmol/L 125I-HK for 1 hour. Unbound ligand was removed and the cells were incubated with 20 nmol/L PK or its buffer for variable lengths of time. The reaction was stopped by washing the cells three times, after which the contents of the wells were solubilized by adding electrophoresis sample buffer containing 2% β-mercaptoethanol. The samples were boiled, separated on 10% SDS-PAGE, and
analyzed by autoradiography and scanning densitometry as described above.

Measurement of Pro-UK activation on endothelial cells. Pro-UK activation on HUVECs was measured. Activation of Pro-UK (20 nmol/L) on HUVECs preincubated with PK (20 nmol/L) or sequentially with HK (20 nmol/L) and PK (20 nmol/L) was determined. HUVECs were incubated with 20 nmol/L HK in 100 µL HEPES-Tyrode’s binding buffer containing 50 µmol/L Zn^{+2} for 1 hour at 37°C. Unbound HK was removed, 20 nmol/L PK was incubated for an additional 1 hour, and the cells were washed three more times. Pro-UK (20 nmol/L) and 0.6 mmol/L Glu-Gly-Arg-pNA HCL (S2444; Pharmacia) were added and the hydrolysis of the substrate was monitored for 75 minutes at 37°C. In certain of these experiments, a neutralizing concentration (0.4 mg/mL) of anti-FXII antibody was added. The kinetics of Pro-UK activation (5 to 1,000 nmol/L) on HUVECs in the presence or absence of HK (20 nmol/L), PK (20 nmol/L), or FXII (20 nmol/L) was studied as well. Formation of tcuPA on HUVECs was also determined with reference to a standard curve generated by adding known amounts of soluble tcuPA to S2444. The K_i and V_max of two-chain urokinase formation was determined from double reciprocal plots.

Measurement of plasminogen activation on endothelial cells. Plasminogen (1 µmol/L) activation in plastic wells and on confluent HUVECs in microtiter plate wells was determined by measuring the hydrolysis of 0.3 nmol/L Val-Leu-Lys-pNA HCl (S2251; Pharmacia). The contribution of Pro-UK (2 nmol/L) to plasminogen activation under the same condition was determined. In other experiments, HUVECs were preincubated with HK (20 nmol/L) for 1 hour, unbound HK was removed, PK or kallikrein (20 nmol/L) was added for second hour, and plasminogen (1 µmol/L) was added for a third hour, before Pro-UK (2 nmol/L) and S2251 (0.3 nmol/L) were added to start the reaction. In other experiments, Pro-UK was added to the wells in the presence of a twofold neutralizing concentration of antibody to FXII (0.4 mg/mL), 20 nmol/L FXII, or 3.4 nmol/L β-FXIIa. Under all experimental conditions, the hydrolysis of chromogenic substrate was monitored continuously for 210 minutes at 37°C. The amount of plasmin formed from plasminogen was determined using a standard curve generated by adding known amounts of soluble plasmin.

Statistics. Significant differences were measured using the t-test for groups of unpaired data.

RESULTS

Binding of biotin-PK to HUVECs. In view of the fact that PK circulates in plasma predominantly complexed with HK,29-31 initial experiments were performed to determine if PK bound to HUVECs through HK. Biotin-PK bound specifically to HUVEC monolayers preincubated with HK and Zn^{+2} (Fig 1A). Zinc ion alone did not support binding of biotin-PK in the absence of HK. The presence of 2 mmol/L Ca^{+2} in the buffers had no effect on binding (data not shown). Additional studies showed that 125I-PK also bound specifically to suspensions of HUVECs preincubated with HK (Fig 1B). These combined data indicated that the labeled PKs bound to HK on HUVECs, irrespective of whether the cells were in monolayers or suspension.

Biotin-PK binding to HUVECs in the presence of added HK. The specificity of biotin-PK binding to HK on HUVECs was shown several ways. First, biotin-PK binding to HUVECs preincubated with 20 nmol/L HK was completely inhibited by 50-fold molar excess PK, factor XI, and the peptide SDD31 (data not shown). In contrast, 100-fold molar excess Lys-plasminogen, C1-inhibitor, fibrinogen, and Glu-plasminogen inhibited biotin-PK binding by 34%, 21%, 15%, and 8%, respectively ($P \leq .05$) compared with the absence of inhibitor (data not shown). FXII appeared to increase biotin-PK binding, but this difference was not statistically significant. Second, binding of PK in the presence of added HK was 100% inhibited by 2.5 molar excess MoAbs HKL16, which is known to recognize the PK binding site on HK, and PK6, which is directed to the HK binding site on PK, whereas HKL13, which is directed to domain 5 on HK, had no effect (Fig 2A). Taken together, the data suggested that PK bound to a site on domain 6 of cell-associated HK that was shared with factor XI.31 PK also inhibited biotin-PK binding to HUVECs preincubated with HK in a concentration-dependent fashion with an IC_{50} of 80 nmol/L (apparent $K_i = 23$ nmol/L; Fig 2B), suggesting that biotin-PK and PK compete for the same site. When HUVECs were preincubated with biotin-PK for 60 minutes, 86% of the binding was reversed by adding 100-fold molar excess PK (data not shown).
Biotin-PK binding to HUVECs in the absence of added HK. The results of the binding experiments suggested that HUVECs expressed more binding sites for biotin-PK than the actual number of molecules of HK bound (1.0 \pm 0.02 \times 10^7 sites/cell). Several additional experiments were performed to address the possibility that there may be additional PK binding sites on HUVECs independent of those provided by added HK. Biotin-PK binding to HUVECs in the absence of added HK was blocked by PK with an IC_{50} of 800 nmol/L (apparent K_{i} = 285 nmol/L; Fig 3A). These data suggested that, in the absence of added HK, a lower affinity, specific binding site(s) for biotin-PK was present on these cells. SDD31, a peptide corresponding to the PK binding site on HK, also blocked biotin-PK binding to HUVECs in the absence of added HK with an IC_{50} of 40 nmol/L (Fig 3B). In support of this finding, MoAb PK6, which is directed to the region on PK that binds to HK, blocked binding completely (Fig 3C). Taken together, these data indicated that all of the binding of PK to HUVECs is mediated by the same region on PK. We then asked whether endogenous HUVEC HK could account for some of this biotin-PK binding in the absence of added HK. MoAb HKL16, which is directed to the PK binding site on HK, blocked biotin-PK binding to HUVECs in the absence of added HK (Fig 3C). This finding suggested that HUVECs did express endogenous HK that was available to bind PK. We then asked whether the PK binding site was human HK derived from the HUVECs or heterologous HK adsorbed from bovine serum. In the support of the former possibility, HKL16 did not recognize bovine plasma HK on immunoblot, but it did detect human plasma HK (data not shown). However, it was noted that binding of biotin-PK was inhibited only 75\% by 10-fold molar excess HKL16 in the absence of added HK. This finding indicated that about 25\% of PK binding may involve an additional, uncharacterized site(s) (Fig 3C). However, because virtually all plasma PK circulates as a complex with HK, we focused the remaining investigations on characterizing the biologic consequences of forming HK and PK complexes on the endothelial cells.

Activation of PK on HUVECs. Investigations were performed to determine if PK bound to HK on the surface of HUVECs could be activated through mechanisms similar to those known to occur in plasma and on artificial surfaces. HUVECs did not hydrolyze the plasma kallikrein chromogenic substrate in the absence of added contact proteins (data not shown). These data suggested that washed HUVECs have little if any kallikrein- and/or activated FXII-like activity tightly bound and nonexchangeable to be measured in this system. Further, little enzymatic activity was seen when HK, PK, or kallikrein alone was permitted to bind to HUVECs (Fig 4A). Indeed, when equal amounts of PK and plasma kallikrein were added to HUVECs preincubated with HK, significantly more activity (P < .001) was detected from the assembly of the HK-PK complex than from the HK-kallikrein complex. Furthermore, the addition of FXII, αFXIIa, or βFXIIa to the HK-PK complex on HUVECs did not increase the extent of chromogenic activity above that which could be accounted for by activating PK bound to HK on HUVECs alone. In fact, the addition of FXII, αFXIIa, or βFXIIa resulted in significantly

Figure 2. Specificity of biotin-PK binding to HUVECs in the presence of added HK (A) The effect of MoAbs to HK and PK on the binding of biotin-PK to HUVECs preincubated with 20 nmol/L HK. Binding of biotin-PK to HUVECs was measured in the presence of 1- to 10-fold molar excess of the MoAbs, HKL13 (○), HKL16 (●), or PK6 (□). Binding of biotin-PK in the presence of the antibodies is expressed as a percentage of the binding in their absence. The data are the mean ± SEM of three experiments. (B) PK and biotin-PK compete for binding to HUVECs. HUVECs pretreated with 20 nmol/L HK were incubated with 20 nmol/L biotin-PK in the presence of 10 to 2,000 nmol/L PK. Binding of biotin-PK in the presence of PK is expressed as a percentage of its binding in the absence of PK. The data shown are the mean ± SEM of three experiments. The absence of standard error bars at some points indicates that the variation was too low to indicate visually.
and another protein. This band did not increase over time and complex having formed between biotin-PK or biotin-kallikrein 85 and 88 kD (Fig 5A). However, a new band appeared at 116 kD within the first minutes, consistent with a SDS-stable 85 and 88 kD (Fig 5A). When biotin-PK was incubated with HUVECs in the absence of reduced conditions. When HK for up to 120 minutes, biotin-PK predominantly migrated at 51, 40, and 37 kD appeared at 1 to 5 minutes and increased in intensity over the ensuing 120 minutes (Fig 5B). By 60 minutes, the 51-kD band constituted 46% of the total protein. These data indicated that exogenous FXII did not contribute to the rate of PK activation when bound to HK on HUVECs.

Studies were next performed to determine if the chromogenic activity was attributable to the enzymatic conversion of PK to kallikrein, to a distinct enzyme with kallikrein-like activity, or to a conformational change in PK that occurred upon binding that exposed its catalytic site (Fig 5). Biotin-PK predominantly migrated as a doublet at 88 and 85 kD on SDS-PAGE under reduced conditions. When βFXIIa was added, biotin-PK was cleaved into a heavy chain of 51 kD and two light chains at 37 and 34 kD. A fourth band also was seen at 40 kD (Fig 5A and B). When biotin-PK was incubated with HUVECs in the absence of HK for up to 120 minutes, biotin-PK predominantly migrated at 85 and 88 kD (Fig 5A). However, a new band appeared at 116 kD within the first minutes, consistent with a SDS-stable complex having formed between biotin-PK or biotin-kallikrein and another protein. This band did not increase over time and constituted approximately 28% of the total biotin-PK (average of all lanes from 1 to 120 minutes) on densitometer scan.

Furthermore, threefold molar excess HKL16 did not block the formation of the 116-kD complex on HK-treated cells, indicating that this other PK-binding protein was not HK (data not shown). This higher molecular mass band also was not found to be a complex between kallikrein and plasminogen activator inhibitor-1 or C1-inhibitor on immunoblot (data not shown). By 60 to 120 minutes, small amounts of cleaved forms of biotin-PK (<5% of the total protein) were seen at 51 and 40 kD (Fig 5A). Thus, little cleavage of PK occurred when it was incubated with HUVECs in the absence of added HK. In contrast, when biotin-PK was incubated with HK prebound to HUVECs, changes in PK structure occurred more rapidly and extensively (Fig 5B). The 116-kD band consisted of only 5% of the total protein over 120 minutes. Cleaved products of biotin-PK at 51, 40, and 37 kD appeared at 1 to 5 minutes and increased in intensity over the ensuing 120 minutes (Fig 5B). By 60 minutes, the 51-kD band constituted 46% of the total protein. These data indicated that the chromogenic activity described in Fig 4 was due to the conversion of PK to kallikrein; second, the conversion occurred more rapidly in the presence of HK; and third, the generation of kallikrein on HUVECs did not require an exogenous source of activated FXII. In experiments not shown, the ability of PK bound to HK on HUVECs to become activated occurred regardless of whether the PK was incubated in plasma or buffer. Lastly, PK (or kallikrein) formed an SDS-stable 116-kD complex with an endogenous HUVEC protein other than HK.

Characterization of the FXII-independent, HUVEC-mediated PK activation. Investigations were then performed to determine the mechanism by which kallikrein was generated from
the cell-associated HK-PK complex. Studies were first performed to determine if residual tissue culture media, which contained 2% fetal calf serum, was a source of activated FXII. Tissue culture media contained less than 0.0001 U/mL activated FXII coagulant activity relative to pooled normal plasma. A second set of investigations was performed to determine the chemical class of protease inhibitor(s) capable of blocking the generation of kallikrein. Initial studies were directed at determining if factor XIIa, kallikrein, or another serine protease could be responsible for the PK activation seen when bound to HK on HUVECs. In view of the fact that serine protease inhibitors would directly block kallikrein amidolytic activity, these experiments determined if serine protease inhibitors could block the generation of kallikrein, as indicated by a change in the conversion of PK to kallikrein assessed by SDS-PAGE, an event that is independent of kallikrein amidolytic activity (Fig 6). Biotin-PK migrated a thick band between 88 and 85 kD (Fig 6A). aFXIIa alone produced multiple breakdown products of biotin-PK that migrated at 51, 40, 37, and 34 kD (Fig 6A). When biotin-PK was bound to HK on HUVECs, activated PK migrated predominantly at the 51-kD band with minor bands seen at 40, 37, and 34 kD (Fig 6A). No change in the migration of endothelial cell-associated biotin-PK was seen in the presence of neutralizing concentrations of two antibodies to FXII or IgG (Fig 6A). These data indicated that the PK activating enzyme(s) was not due to a protein antigenically related to plasma factor XIIa that might have been present either in the PK preparation or associated with endothelial cells.
Additional experiments were performed to exclude the possibility of factor XIIa, kallikrein, or another serine protease contaminating the preparations to account for PK activation. Biotin-PK migrated as a thick band at about 88-85 kD and contained a minor fragment at about 66 kD (Fig 6B). When the biotin-PK was activated with αFXIIa, greater than 95% of the labeled PK now migrated at the 51-kD band identical to the natural substrate, HK (Fig 7). Soluble¹²⁵I-HK migrated predominantly as a 120-kD protein under reduced conditions. When HK and biotin-PK (20 nmol/L), the intensity of the 120-kD band was reduced by 75% and new bands appeared at 64-55 and 46 kD within the first 75% and 14% of the protein, respectively (Fig 7A). These data are consistent with our previous findings that endothelial cell bound exogenous HK is not substantially cleaved upon binding.²¹

In contrast, when HUVEC-bound¹²⁵I-HK was incubated with PK (20 nmol/L), the intensity of the 120-kD band was reduced by 75% and new bands appeared at 64-55 and 46 kD within the first 7 minutes that constituted 61% and 14% of the protein, respectively (Fig 7B); by 30 minutes, the 120-kD band had disappeared completely and a new 40-kD HK band progressively increased in intensity ultimately accounting for 14% of the total protein. These data indicated that the kallikrein formed on HUVECs in the HK-PK complex can cleave its receptor and native substrate, HK.
The effect of PK activation on two-chain urokinase and plasmin formation. Because kallikrein is a potent activator of Pro-UK in vitro,13 the capacity of kallikrein formed on HUVECs to activate Pro-UK was examined (Fig 8A). Incubation of Pro-UK with HUVECs increased urokinase activity almost threefold over that seen with Pro-UK alone.45 The addition of HK and PK to HUVECs increased the level of urokinase activity an additional 1.6-fold (Fig 8A). In these experiments, no attempt was made to inhibit the synthesis of plasminogen activator inhibitor-1. The same amount of Pro-UK activation occurred in the presence of a neutralizing concentration of an antibody to FXII was present (Fig 8B). Moreover, the addition of HK and PK to Pro-UK and plasminogen was associated with a 4.3-fold increase in plasminogen activation. Furthermore, the same increase in plasmin formation occurred when a neutralizing concentration of antibody to FXII was present (Fig 8B). Moreover, the addition of FXII and βFXIIa did not potentiate plasmin formation above that seen with the HK and PK complex alone (data not shown). Furthermore, the substitution of kallikrein for PK resulted in a 1.7-fold decrease in plasmin formation (data not shown). These results indicated that optimal formation of measured plasmin on HUVECs did not require FXII or its activated forms.

**DISCUSSION**

These studies indicate that assembly of contact proteins, HK and PK, on cultured endothelial cells leads to the formation of kallikrein, which, in turn, cleaves HK, presumably liberating bradykinin, and promotes the activation of Pro-UK and the generation of plasmin. Kallikrein formation on HUVECs is critically dependent on the sequential binding of HK and PK and was independent of added or endogenous FXII or eFXII-like enzyme. Furthermore, the extent of kallikrein activity is autoregulated, because, once formed, the enzyme proteolyzes its receptor (HK) resulting in its liberation from the HUVEC surface. These results stand in stark contrast to the well-established role of FXII in the activation of PK in plasma and on artificial surfaces. These studies show for the first time contact system activation on a biologic surface in the absence of a negatively charged artificial surface. It is noteworthy to point out that the so-called elusive, physiologic negatively charged

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**Table 1. Inhibition Profile of Endothelial Cell Prekallikrein-Activating Enzyme(s)**

<table>
<thead>
<tr>
<th>Inhibitor Class</th>
<th>Inhibitor</th>
<th>[Inhibitor]</th>
<th>% Inhibition</th>
<th>% Kallikrein Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine protease inhibitors</td>
<td>Antipain</td>
<td>100 µmol/L</td>
<td>92 ± 7.1</td>
<td>±5%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>10 mmol/L</td>
<td>99 ± 3.4</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>HgCl₂</td>
<td>1 mmol/L</td>
<td>96 ± 3.6</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>10 mmol/L</td>
<td>99 ± 3.5</td>
<td>±5%</td>
<td>28.6%</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td></td>
<td>5%</td>
<td>98 ± 2.3</td>
<td>±5%</td>
</tr>
<tr>
<td>Glutathione</td>
<td>100 µmol/L</td>
<td>62 ± 4.5</td>
<td>10.4%</td>
<td></td>
</tr>
<tr>
<td>Calpain Inhibitor</td>
<td>10 mmol/L</td>
<td>46 ± 3.6</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>Z-Phe-OH</td>
<td>1 mmol/L</td>
<td>45 ± 2.4</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>E64</td>
<td>10 mmol/L</td>
<td>2.5 ± 3.8</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>10 mmol/L</td>
<td>2.5 ± 1.5</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>Iodoacetic Acid</td>
<td>10 mmol/L</td>
<td>3.1 ± 1.8</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>3 mmol/L</td>
<td>0.3 ± 0.1</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td>Cystatin</td>
<td>1 mmol/L</td>
<td>1.0 ± 0.3</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>OH-Mercuroibenico Acid</td>
<td>10 mmol/L</td>
<td>1.2 ± 0.1</td>
<td>±5%</td>
<td></td>
</tr>
<tr>
<td>Metalloprotease inhibitors</td>
<td>Zincov</td>
<td>100 µmol/L</td>
<td>20 ± 2.5</td>
<td>±5%</td>
</tr>
<tr>
<td></td>
<td>TIMP-1</td>
<td>20 µg/mL</td>
<td>15 ± 2.9</td>
<td>±5%</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td>20 µg/mL</td>
<td>0.4 ± 0.2</td>
<td>±5%</td>
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<tr>
<td></td>
<td>BB94</td>
<td>15 µmol/L</td>
<td>0.1 ± 0.0</td>
<td>±5%</td>
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<tr>
<td>Angiotensin converting enzyme inhibitors</td>
<td>Lisinopril</td>
<td>10 mmol/L</td>
<td>1.1 ± 0.4</td>
<td>±5%</td>
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<td>Enalapril</td>
<td>10 mmol/L</td>
<td>0.4 ± 0.2</td>
<td>±5%</td>
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<tr>
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<td>Phosphoramidon</td>
<td>10 mmol/L</td>
<td>16 ± 3.2</td>
<td>±5%</td>
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<tr>
<td></td>
<td>Pepstatin A</td>
<td>10 mmol/L</td>
<td>0.2 ± 0.1</td>
<td>±5%</td>
</tr>
</tbody>
</table>

* The values given represent the mean ± SD of inhibition of the prekallikrein-activating enzyme associated with HUVECs.

# The values given represent the percentage of inhibition of kallikrein itself by the protease inhibitor.
surface for contact protein zymogen activation may really be the assembly of contact proteins by binding to putative receptors on endothelial cells or other cell membranes. Recognition of a PK activating mechanism on HUVECs supports this notion.28

The initial requirement for kallikrein formation on HUVECs is the binding of HK to HUVECs. Our data indicate clearly that HK is the predominant binding site for PK. PK binding to HUVECs is inhibited by MoAbs that block the sites on PK and on HK necessary for them to form a complex, whereas an antibody to a neighboring domain of HK has no effect. Binding of PK is also inhibited by a peptide corresponding to its binding
Table 2. Activation of Pro-Urokinase by the Contact Proteins

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Km (nmol/L)</th>
<th>Vmax (nmol/L/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK + PK + FXII†</td>
<td>135 ± 81</td>
<td>14.5 ± 8</td>
</tr>
<tr>
<td>HK + PK</td>
<td>64 ± 5</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td>HK + FXII</td>
<td>61 ± 12</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>PK + FXII</td>
<td>86 ± 44</td>
<td>4 ± 1.4</td>
</tr>
</tbody>
</table>

*HUVECs were preincubated with 20 nmol/L HK for 1 hour at 37°C. Unbound HK was removed, HUVECs were incubated with 20 nmol/L PK for another 1 hour, and the cells were washed. The capacity of the cell-associated PK/HK complex to activate Pro-UK was determined by PK for another 1 hour, and the cells were washed. The capacity of the unbound HK was removed, HUVECs were incubated with 20 nmol/L PK, prekallikrein (PK), and factor XII (FXII), as indicated.

†The reactants added included high molecular weight kininogen (HK), prekallikrein (PK), and factor XII (FXII), as indicated.

The specific enzyme(s) responsible for the conversion of PK to kallikrein by HUVECs cannot be determined from our studies, but our experiments do provide some insights into its mechanism of action and cellular location. First, no serine protease inhibitor or the addition of neutralizing antibodies to FXII prevented cleavage of PK bound to HK on HUVECs. It is theoretically possible that a cell-bound form of αFXIIa that is protected from high concentrations of serine protease inhibitors and a neutralizing concentration of antibody to FXII could be responsible for this cleavage. However, the concentrations of these inhibitors exceed any possible cell-bound αFXIIa by many orders of magnitude and there is no precedent for this possibility. Second, the finding that PK activation is blocked by antipain, HgCl₂, cysteine, β-mercaptoethanol, and DTT indicates that kallikrein formation is by an enzyme that is not factor XIIa-like and not due exclusively to a conformational change upon binding. Third, at least one PK activating enzyme(s) has properties consistent with it being a thiol protease. It does not appear to be calpain itself because E64 does not inhibit kallikrein formation at all. Furthermore, HK is itself a potent cysteine protease inhibitor (Kᵢ = 0.71 nmol/L), but it clearly promoted rather than inhibited PK activation. However, it could be argued that the capacity of HK to function as a cysteine protease inhibitor was nullified by it being bound to endothelial cells, because its cellular binding site on domain 3 overlaps with the region that expresses cysteine protease inhibitory activity. Inhibition by certain metal ion chelating agents can be explained by these compounds removing Zn²⁺ from the complex of HK-PK, thus dissolving the protein-protein assembly necessary for the PK activating enzyme to function. Last, the PK activator(s) on HUVECs also appears to have different require-
ments than a Hageman factor activator, which has been found previously in homogenates of cultured rabbit endothelial cells.\textsuperscript{46}

Kallikrein formed on the surface of HUVECs from the HK-PK complex is positioned to exert several potentially important functions. First, cell-bound HK was cleaved by the formed kallikrein on HUVECs. This result may be an important control step in this pathway, releasing kallikrein from its binding site. Second, the pattern of cleavage of HK is identical to that seen in plasma and on cells by kallikrein when bradykinin is liberated.\textsuperscript{44,49} Bradykinin formed in proximity to the endothelium may be especially potent, because, theoretically, it could engage its receptors before inactivation by plasma kininases.\textsuperscript{50} Moreover, bradykinin liberation can result in potent tissue plasminogen activator release from endothelial cells in vivo.\textsuperscript{51,52}

Kallikrein formed from HK-PK complex also activated Pro-UK, which, in turn, caused a 4.3-fold increase in plasminogen activator on HUVECs. In that these experiments were performed without taking measures to inactive plasminogen activator inhibitor-1, they represent minimum estimates of urokinase activity and suggest that the kallikrein-dependent mechanism described here may be more important to cell surface plasminogen-activating activity than in plasma. The data may explain why mice genetically engineered to lack plasminogen have normal concentrations of tcuPA found in their urogenital tract.\textsuperscript{53} These investigations indicate a possible mechanism whereby a small amount of Pro-UK can be converted to tcuPA independent of plasin, fibrin, or tissue plasminogen activators. This pathway may potentiate Pro-UK activation associated with its binding to its receptor.\textsuperscript{54}

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High Molecular Weight Kininogen Regulates Prekallikrein Assembly and Activation on Endothelial Cells: A Novel Mechanism for Contact Activation

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