Assembly and Expression of an Intrinsic Factor IX Activator Complex on the Surface of Cultured Human Endothelial Cells*

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Endothelial cells expose specific receptors for blood clotting factors and, upon perturbation, can initiate and propagate the reactions of the extrinsic pathway of blood coagulation leading to fibrin formation on the cell surface. The existence of an intrinsic mechanism of Factor IX activation on cultured human umbilical vein cells (HUVECs) was investigated by studies of the interaction between HUVECs and two proteins of the contact activation system, the cofactor high molecular weight kininogen (H-kininogen) and the zymogen Factor XI. In the presence of zinc ions (10–300 μM), 125I-labeled H-kininogen bound to HUVECs in a time-dependent, reversible, and saturable manner, with calcium ions exerting an inhibitory effect on the zinc-dependent binding. Analysis of the binding data by the LIGAND computer program indicated that HUVECs, in the presence of 2 mM CaCl₂ and 100 μM ZnCl₂ at 37°C, bound 1.14 × 10⁵ H-kininogen molecules per cell with an apparent dissociation constant of 55 nM. HUVEC-bound H-kininogen functions as the cell surface receptor for both 125I-labeled Factor XI and 125I-labeled Factor XIa, since HUVECs cultured in contact with the depleted serum do not detectably bind either the zymogen or the enzyme in the absence of H-kininogen and zinc ions. In the presence of saturating concentrations of H-kininogen, 2 mM CaCl₂ and 100 μM ZnCl₂, the binding of 125I-labeled Factor XI and Factor XIa to HUVECs was time-dependent, reversible, and saturable, with apparent dissociation constants of 4.5 and 1.5 nM, respectively. HUVEC-bound complexes of H-kininogen and Factor XI generated Factor XIa only after the addition of purified Factor XIa, and cell-bound Factor XIa in turn activated Factor IX, as documented by a 125I-labeled activation peptide release assay for 3H-Factor IX activation. The results indicate that cultured HUVECs provide a surface for the assembly and expression of an intrinsic Factor IX activator complex that may participate in the initiation of blood coagulation at sites of vascular injury.

The endothelial cell layer forming the luminal surface of blood vessels is actively involved in the regulation of the hemostatic system both in normal conditions and after vascular injury. On the one side, quiescent endothelial cells represent a major source of potent antithrombotic mechanisms, including prostacyclin (1) which inhibits platelet activation, plasminogen activators (2) which initiate fibrinolysis, heparin-like molecules (3), and thrombomodulin (4) which support distinct anticoagulant pathways aimed to neutralize thrombin activity. All these systems are thought to be essential to modulate the growth of the hemostatic plug and to prevent thrombus formation.

On the other hand, injured endothelial cells participate in normal hemostasis and thrombus formation also by providing a variety of localized procoagulant mechanisms. Perturbed endothelial cells can initiate blood coagulation by expressing tissue factor activity on their surface (5–7), and they can propagate the reaction of the extrinsic pathway of blood coagulation through the activation of F.XI (8) and prothrombin (9). Endothelial cells also possess specific, high affinity binding sites for F.XI and F.XIIa, and cell-bound F.XIa can activate F.X in the presence of Factor VIII (10, 11). Fibrin can be generated on the surface of quiescent cultured endothelial cells incubated with Factor VIII, F.XI, Factor X, and prothrombin after the addition of purified F.XIa (12), suggesting that endothelial cells can propagate both extrinsic and intrinsic coagulation pathways.

However, mechanisms of activation of F.XI on the surface of endothelial cells have not been studied, and little is known about the possible interaction of the endothelial cells with the factors of the contact system which initiate the intrinsic pathway of blood coagulation. In early studies, rabbit endothelial cells have been shown to contain an activator of F.XII (13). More recently, cultured human umbilical vein endothelial cells (HUVECs) have been found to contain H-kininogen and to express this protein on their surface where unoccupied binding sites for H-kininogen are also available (14, 15). H-kininogen functions "in vitro" as an essential cofactor for the activation of the contact system by facilitating the association of two protein zymogens, F.XI and plasma prekallikrein, with artificial negatively charged surfaces where they are transformed into the respective enzymes, the serine proteases F.XIa and plasma kallikrein, by the action of F.XIIa (16, 17). It is not known if the same function is exerted by H-kininogen.

* This work was supported in part by Grant HL-21544 from the National Institutes of Health. Part of the results have been presented in abstract form elsewhere (Berrettini, M., Schleef, R. R., Heeb, M. J., Hopmeier, P., and Griffin, J. H. (1991) Thromb. Haemostasis 65, 693). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: F.X, Factor X; F.XII, Factor XII; F.XIIa, activated Factor XII; F.XI, Factor XI; F.Xla, activated Factor XI; F.IX, Factor IX; F.IXa, activated Factor IX; H-kininogen, high molecular weight kininogen; HUVEC, human umbilical vein endothelial cell; SDS-PAGE, sodium-dodecyl sulfate polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; cu, clotting unit, defined as the amount present in 1 ml of pooled citrated normal plasma.
also "in vivo." In plasma, H-kininogen circulates in noncovalent complexes with F.XI (18) and plasma prekallikrein (19), but the "physiologic" activating surface, where the factors of the contact system can be assembled, has not yet been clearly identified. There are data suggesting that cells can provide such a surface; in particular, blood platelets (20, 21) and neutrophils (22) have been shown to specifically bind H-kininogen in the presence of zinc ions, and this cofactor is essential for the association of F.XI on the platelet surface.

The present study was intended to ascertain whether endothelial cells can provide a surface for the assembly and activation of the contact system of blood coagulation. We here present data indicating that HUVECs, besides having binding sites for H-kininogen, also specifically bind F.XI and F.Xa in the presence of H-kininogen. Furthermore, cell-bound F.XI can be activated by F.XIIa, and cell-bound F.Xa in turn activates F.XIII.

**MATERIALS AND METHODS**

**Reagents—**All chemicals were the best grade commercially available. Fetal calf serum, trypsin, penicillin, and streptomycin were from Gibco, Grand Island, N. Y. M. A. Products, tissue culture plastic ware from Corning, endothelial cell growth factor from Biomedical Technologies, Inc., and human fibrinogen (grade L) and oligopeptide substrates (S-2302, H-D-Pro-Phe-Arg-p-nitroanilide; S-2366, pyro-Glu-Pro-Arg-p-nitroanilide) from Helena Laboratories. The specific F.XIII inhibitor, a bovine extract from popcorn (popcorn inhibitor) was a kind gift of Dr. Francisco Espafia, Hospital "La Fe" Research Center, Valencia, Spain.

**Proteins—**Published procedures were used to purify H-kininogen (23), F.XII (24), F.XI (25), and prekallikrein (26). All these proteins appeared >95% pure when analyzed by SDS-PAGE, and had the following specific activities: H-kininogen, 15 cu/mg F.XI, 55 cu/mg F.XII, 248 cu/mg; and plasma prekallikrein, 18 cu/mg. F.XIX was prepared by barium citrate adsorption and elution of fresh plasma as previously described (27). The dialyzed barium eluate was chromatographed on QAE-Sepharose (Pharmacia LKB Biotechnology Inc.) and then on heparin-Sepharose (Pharmacia) as described (28), except that the buffer for the heparin-Sepharose step was 0.05 M MES, 2 mM CaCl₂, 1 mM benzamidine, 0.02% NaN₃, pH 6.0, and the linear gradient was 0–0.6 M NaCl in that buffer. The final preparation had a specific procoagulant activity of 180 cu/mg and was greater than 95% homogeneous by SDS-PAGE.

**Enzymes—**F.XIIa was prepared by incubation of purified F.XII with 5 μg/ml dextran sulfate at 37 °C for 90 min as previously described (29) and consisted of disulfide-linked heavy and light chains (α-F.XIIa) as judged by SDS-PAGE of reduced and nonreduced samples. This preparation had a specific activity of 16.6 μM⁻¹ min⁻¹ when tested against the substrate S-2302, 0.4 mM in 0.09 M Tris-HCl, 0.09 M NaCl, 1 mg/ml BSA, pH 8.3, at room temperature. F.XIIa was prepared as previously described (20). The enzyme had a specific activity of 72.5 μM⁻¹ min⁻¹ against S-2366, when the substrate was used at 0.4 mM in 0.09 M Tris-HCl, 0.09 M NaCl, 1 mg/ml BSA, pH 8.3, at room temperature.

**Radiolabeling of Proteins—**F.XI and H-kininogen were radioiodinated with carrier free Na¹²⁵I (Amersham) by the IODO-GEN (Pierce) method (31). The two radioiodinated proteins, >95–98% precipitable in 5% trichloroacetic acid, had specific activities of 2–4 and 0.5–1.1 μCi/μg, respectively, and retained at least 90–95% of their original clotting activity. ¹²⁵I-Labeled F.XIIa was obtained by activation of radiolabeled F.XI using the same procedure employed to obtain F.XIa, and it retained at least 90% of its intrinsic enzymatic activity against the substrate S-2366. F.XIIa was triturated as previously described (30).

H-F.XIX contained 6.6 mol of triiodide per mol of F.XIX (267,000 cpm/μg) and retained full coagulant activity, and the radioactivity was 98% precipitable in cold 5% trichloroacetic acid.

To compare the binding affinity of native and ¹²⁵I-labeled H-kininogen and F.XI for HUVEC, endothelial cells were incubated (see below under "Binding Studies") with a mixture of labeled and nonlabeled proteins at various relative ratios (5–95%) but constant total concentrations. A 95–98% correlation was found between the percentage of added and bound labeled proteins, indicating that there was no difference in binding affinity between labeled and nonlabeled ligands.

**RESULTS**

**Binding of ¹²⁵I-Labeled H-Kininogen to Endothelial Cells—**In the presence of 100 μM ZnCl₂ and 2 mM CaCl₂, ¹²⁵I-labeled H-kininogen bound to HUVECs in a time-dependent manner with maximal binding after 60 min (Fig. 1A). Zinc ions were an essential requirement for binding, since in their absence the binding of ¹²⁵I-labeled H-kininogen to HUVEC was similar to the nonspecific binding measured in the presence of zinc ions plus a 100-fold molar excess of nonlabeled H-kininogen.
puter analysis of the binding data (LIGAND program) showed that the binding of 3.2 pg/ml (26 nM) 125I-labeled H-kininogen at 37 °C for 30 min (closed circles, squares) reached saturation when 65 nM radiolabeled ligand was added. Com-puterbased radioactivity was dissociated from the cells by 60 min (open circles). The reversibility of the binding was examined by measuring the dissociation of bound 125I-labeled H-kininogen from the cells at several time points after the addition of 100-fold molar excess of nonlabeled H-kininogen. In parallel experiments, the binding of 125I-labeled H-kininogen was initiated after an incubation of 60 min (data not shown). The reversibility of the binding was tested by adding a 100-fold molar excess of nonlabeled H-kininogen to endothelial cells previously incubated with 125I-labeled H-kininogen for 30 min at 37 °C (as indicated by the arrow), and by measuring cell-associated radioactivity after further incubation for 30 and 60 min (squares). B, saturability. Endothelial cells were incubated at 37 °C for 60 min with various concentrations of 125I-labeled H-kininogen in incubation buffer. The points represent the mean of duplicate values of specific binding obtained by subtracting the nonspecific binding determined from total binding. Nonspecific binding was measured in the presence of a 100-fold molar excess of nonlabeled H-kininogen. The insert shows the bound/free versus bound plot obtained by analyzing the data by the LIGAND program.

Other tested divalent metal ions (Mg++, Cu++) did not substitute for zinc. In the presence of 2 mM CaCl2, the binding of H-kininogen to HUVECs increased with increasing concentrations (10 to 300 μM) of ZnCl2, reaching a maximum at 100 μM ZnCl2. It was also observed that Ca++ ions (0.2 to 5 mM) exerted an inhibitory effect on the binding so that lower concentrations of zinc were required for maximal binding when the Ca++ concentration was decreased (data not shown). Since for some of the following experiments a buffer containing 2 mM CaCl2 was required, we further analyzed the binding of 125I-labeled H-kininogen to HUVECs using a buffer containing 2 mM Ca++ and 100 μM Zn++. The reversibility of the binding was measured after the addition of 100-fold molar excess of nonlabeled H-kininogen. Fig. 1A shows the results of the experiments where the displacement of 125I-labeled H-kininogen was initiated after 30 min of incubation with HUVECs. About 70% of the bound radioactivity was dissociated from the cells by 60 min after the addition of a 100-fold molar excess of nonlabeled H-kininogen. The same results were obtained when displacement of 125I-labeled H-kininogen was initiated after an incubation of 60 min (data not shown). The number and affinity of H-kininogen binding sites on HUVECs were then determined at equilibrium as a function of H-kininogen concentra-tion (Fig. 1B). In the presence of 2 mM CaCl2 and 100 μM ZnCl2, specific binding of H-kininogen to HUVECs reached saturation when 65 nM radiolabeled ligand was added. The results of the computer analysis of the binding data (LIGAND program) showed that HUVECs express a single class of binding site for 1.14 ± 0.32 × 10^7 molecules/cell having an apparent dissociation constant of 55 ± 9 nM.

Binding of 125I-labeled Factor XI and Factor Xla to Endothelial Cells—Since H-kininogen is known to mediate the association of F.XI to either artificial (16) or natural (39) surfaces, we investigated whether H-kininogen is also required for the binding of F.XI to the surface of endothelial cells. In parallel experiments, the binding of F.Xla to HUVECs was studied. When purified 125I-labeled F.XI (Fig. 2, A) or F.Xla (Fig. 2, B) were incubated with HUVECs in the presence of zinc ions and saturating concentrations of H-kininogen, a time-dependent binding of both ligands to HUVECs was observed reaching a maximum after 30–60 min. In the absence of added H-kininogen, the binding of 125I-labeled F.XI/F.Xla to the cells was greatly reduced, but it was still greater than the nonspecific binding measured in the presence of added H-kininogen plus a 100-fold molar excess of nonla-beled F.XI/F.Xla. The question arose of whether HUVECs grown in fetal calf serum exposed a limited number of H-kininogen independent binding sites for F.XI/F.Xla. We first investigated whether HUVECs grown in 20% fetal calf serum expressed HMWK on the surface. Using a radioimmunoassay with mouse monoclonal antibodies directed against the light chain of H-kininogen, HUVECs grown in fetal calf serum were found to bind about 15% of the radioactivity measured in the same cells after incubation with saturating (100 nM) concentrations of added H-kininogen. When HUVECs were grown in contact factor-depleted human serum, the radioac-

FIG. 1. Binding of 125I-labeled H-kininogen to HUVECs. A, time course, ion dependency, and reversibility. HUVECs were incubated with 3.2 pg/ml (26 nM) 125I-labeled H-kininogen at 37 °C for increasing amounts of time in incubation buffer. At the indicated times, the cells were washed four times with the appropriate buffer, and the radioactivity associated with the cells was counted as described under “Materials and Methods.” Each point represents the mean of two observations. Closed circles, total binding; triangles, nonspecific binding determined by measuring bound 125I-labeled H-kininogen in the presence of a 100-fold molar excess of nonlabeled H-kininogen. In parallel experiments, ZnCl2 was omitted from the incubation buffer (open circles). The reversibility of the binding was tested by adding a 100-fold molar excess of nonlabeled H-kininogen to endothelial cells previously incubated with 125I-labeled H-kininogen for 30 min at 37 °C (as indicated by the arrow), and by measuring cell-associated radioactivity after further incubation for 30 and 60 min (squares). B, saturability. Endothelial cells were incubated at 37 °C for 60 min with various concentrations of 125I-labeled H-kininogen in incubation buffer. The points represent the mean of duplicate values of specific binding obtained by subtracting the nonspecific binding determined from total binding. Nonspecific binding was measured in the presence of a 100-fold molar excess of nonlabeled H-kininogen. The insert shows the bound/free versus bound plot obtained by analyzing the data by the LIGAND program.

FIG. 2. Time course, requirements, and reversibility of the binding of 125I-labeled Factors XI and Xla to HUVECs. HUVECs were incubated with 1.5 μg/ml (or 9.3 nM) 125I-labeled F.XI (A) or with 1 μg/ml (or 6.2 nM) 125I-labeled F.Xla (B) at 37 °C in incubation buffer. The radioactivity associated with the cells was measured at the indicated times as described under “Materials and Methods.” Each point represents the mean of two observations. Different experimental conditions are represented. HUVECs grown in 20% fetal calf serum were used in some experiments and the binding of 125I-labeled F.XI or F.Xla was measured in the presence or absence of contact factors, and the binding of 125I-labeled F.XI or F.Xla was measured either in the presence (closed squares, dashed lines) or in the absence (open squares, dashed lines) of added H-kininogen. The arrows indicate parallel experiments performed to study the reversibility of the binding of 100-fold molar excess of nonlabeled ligand was added to HUVECs grown in 20% fetal calf serum after 30-min incubation with 125I-labeled ligands. Dissociation of 125I-F.XI or F.Xla from the cells was then measured after further incubation for 30 and 60 min (closed circles and dashed lines).
tive signal was reduced to less than 2%. The binding of 125I-labeled F.XI/F.XIa to HUVECs was then reevaluated using cells which had been grown in contact factor-depleted human serum (Fig. 2). No significant association of the two ligands to HUVECs was detected in the absence of added H-kininogen, while in the presence of saturating concentrations of H-kininogen the amount of bound radioligands was higher than that measured using HUVECs grown in 20% fetal calf serum. When zinc ions were not included in the incubation buffer, 125I-labeled F.Xi/F.XIa did not bind to HUVECs in the presence of H-kininogen (data not shown). All these observations suggest that HUVECs do not have H-kininogen independent binding sites for F.XI/F.XIa and that H-kininogen is an essential requirement for the association of both F.XI and F.XIa to the surface of endothelial cells.

The binding of both 125I-labeled F.XI and F.XIa to HUVECs was shown to be reversible, since more than 90% of the radiolabeled ligands could be displaced from the cells by 60 min after the addition of a 100-fold molar excess of nonlabeled protein (Fig. 2). The specificity of the binding was then evaluated by competition experiments where 125I-labeled F.XI/F.XIa were incubated with HUVEC in the presence of H-kininogen and a 100-fold molar excess of various proteins (Table I). Nonlabeled F.XI greatly reduced the binding of 125I-labeled F.XIa, while nonlabeled F.XIa completely suppressed the binding of 125I-labeled F.XI. The binding of both 125I-labeled Factor XI and Factor Xla was significantly reduced by plasma prekallikrein, but not by other proteins. Since F.XI and human plasma prekallikrein share a common binding domain on the H-kininogen molecule (40), our observations further support the concept that H-kininogen functions on the surface of HUVECs as a receptor for F.XI and F.XIa.

The binding of 125I-labeled F.XI/F.XIa to HUVECs in the presence of H-kininogen was found to be saturable. HUVECs were grown in culture medium containing 20% fetal calf serum and were incubated with various concentrations of 125I-labeled F.XI or F.XIa in the presence of 100 nM H-kininogen. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabeled ligands and was subtracted from total binding. In a typical experiment shown in Fig. 3, binding sites for both F.XI and F.XIa were apparently saturated when the corresponding 125I-labeled ligand was added at concentrations of 3–6 nM and 1–6 nM, respectively (Fig. 3A). Analysis of the specific binding curves using the LIGAND program (Fig. 3B) yielded an apparent dissociation constant for F.XI of 4.5 ± 0.8 (standard error, SE) nM with 2.7 ± 0.6 (SE) × 10^10 molecules bound to a single class of sites per cell. For Factor Xla, the apparent dissociation constant was calculated to be 1.5 ± 0.2 (SE) nM with 1.5 ± 0.3 (SE) × 10^10 molecules bound to a single class of sites per cell.

Functional Activity of HUVEC-bound F.XIa—Further studies were performed to evaluate the functional significance of F.XIa association to the surface of HUVEC. Initial experiments were carried out to ascertain whether upon prolonged incubation with buffer HUVEC-bound F.XIa remained associated with the cells or spontaneously dissociated from their surface. HUVECs were incubated with 100 nM H-kininogen and 6.2 nM 125I-labeled F.XIa in incubation buffer (0.05 M Tris, 0.138 M NaCl, 2 mM CaCl2, 100 μM ZnCl2, 0.5% BSA, pH 7.35) for 60 min at 37 °C. The cells were washed four times and cell-associated radioactivity was measured either immediately or after further incubation of the cell with incubation buffer. More than 90% of the initial radioactivity was retained by the cells when the incubation was prolonged up to 90 min.

The activity of HUVEC-bound F.XIa as a F.IX activator was then studied using a highly specific activation peptide release assay for F.IX activation (Fig. 4). After incubation with saturating concentrations of H-kininogen and F.XIa, HUVECs were washed to remove all the nonbound reactants. l2H-labeled F.IX was added to the cells and its activation was monitored over time by measuring the release of tritium-labeled activation peptide. Under the experimental conditions described in Fig. 4, cell-bound F.XIa activated l2H-labeled F.IX in proportion to the amount added to HUVECs. No activation of l2H-labeled F.IX was detected when the cells were preincubated with buffer without added H-kininogen and F.XIa, suggesting that HUVECs by themselves did not directly activate l2H-labeled F.IX. In solution, in the absence of HUVECs and H-kininogen, an amount of F.XIa estimated to be equivalent to that bound to HUVECs at saturation activated F.IX approximately twice as fast as F.XIa bound to cells (Fig. 4). In other experiments (data not shown), the apparent Km for l2H-F.IX activated by F.XIa was determined and found to be 0.05 μM for F.XIa in each state. This suggests that F.XIa bound to cells is approximately (within two-fold) as active as F.XIa in fluid phase.
tion of F.XIa activity on the surface of endothelial cells could be a relevant mechanism for the initiation and propagation of cell-associated coagulation reactions, the ability of HUVEC to support the activation of cell-bound F.XI was studied. H-kininogen-F.XI complex was formed on the surface of HUVECs and generation of F.XIa was assessed by the appearance of "H-F.IX cleavage activity, as measured by activation peptide release assay under different experimental conditions (Fig. 5). When both H-kininogen and F.XI were present and presumably bound to HUVECs grown in 20% fetal calf serum (Fig. 5, A), the addition of purified F.XIIa resulted in "H-F.IX cleavage. The activation of "H-F.IX was dependent on F.XI since it could not be detected in the absence of cell-bound F.XI. However, partial activation of "H-F.IX occurred even in the absence of added F.XIIa, suggesting that HUVECs might possess F.XI activating mechanisms not dependent on F.XIIa. This hypothesis did not find support in the results of subsequent experiments. First, the activation of "H-F.IX observed in the absence of added F.XIIa was completely suppressed when HUVECs were incubated with H-kininogen, F.XI and the specific popcorn inhibitor of F.XIIa (Fig. 5, A). Second, when the experiments were repeated using HUVECs grown in 20% contact factor-depleted human serum, the activation of "H-F.IX was found to be strictly dependent on the addition of F.XIIa (Fig. 5, B), since no activation of "H-F.IX occurred if F.XIIa was omitted or replaced by F.XIII.

**DISCUSSION**

Taken together, the data collected in the present study provide us with an experimental basis to construct a model for the assembly of an intrinsic F.IX activator complex on the surface of cultured HUVECs. In the model proposed here, cultured HUVECs specifically bind the cofactor, H-kininogen, which in turn serves as the cell-surface receptor for F.XI. The zymogen F.XI can be locally activated by F.XIIa to generate F.XIa on the cell surface where this enzyme activates F.IX,

**FIG. 4. Activation of "H-F.IX by HUVEC-bound F.XIa.** HUVECs were incubated with 100 nM H-kininogen and F.XIa at 10 nM (closed circles) or 5 nM (triangles) for 60 min at 37 °C in incubation buffer. After washing the cells four times with buffer, "H-F.IX was added (5.8 μg/ml in the same buffer), and the release of trichloroacetic acid-soluble tritium was measured at intervals as described under "Materials and Methods." In control experiments (open circles) H-kininogen and F.XIa were omitted from the initial incubation. To compare HUVEC-bound F.XIa with the same enzyme in solution, an amount of F.XIIa (80 ng) presumably equivalent to that bound to HUVEC was added simultaneously with "H-F.IX to separate wells not coated with HUVEC and preincubated with buffer for 60 min at 37 °C (squares). The results are expressed as percent of the total radioactivity added to the wells, and the points represent the mean of two observations.

**FIG. 5. Activation of F.XI on the surface of HUVECs.** HUVECs were incubated for 60 min at 37 °C with 100 nM H-kininogen and 20 nM F.XI in incubation buffer. After washing the cells four times with incubation buffer, "H-F.IX was added (5.8 μg/ml in incubation buffer), and the release of trichloroacetic acid-soluble tritium was measured at the indicated times as described under "Materials and Methods." The results are expressed as cpm of trichloroacetic acid-soluble tritium, and the points represent the mean of duplicate observations. A illustrates the experiments performed with HUVECs grown in 20% fetal calf serum, while B illustrates the results of experiments using HUVECs which had been grown in 20% contact factor-depleted human serum (see "Materials and Methods"). In both instances, activation of "H-F.IX was studied in the absence (triangles) or in the presence (closed circles) of 1 μg/ml F.XIIa added to HUVECs simultaneously with "H-F.IX. In control experiments, F.XIIa and "H-FIX were added to HUVECs previously incubated with buffer, but without H-kininogen and F.XI (open circles). In separate wells, HUVECs grown in 20% fetal calf serum (A) were preincubated with H-kininogen, F.XI, and 1 μg/ml of popcorn inhibitor (diamonds), and the activation of "H-F.IX was monitored without adding F.XIIa. In parallel experiments using HUVEC which had been grown in contact factors-depleted human serum and preincubated with H-kininogen and F.XIa, F.XIIa was substituted by an equal amount of F.XII (B, diamonds).

thus establishing a cell-mediated intrinsic pathway mechanism for the initiation of blood coagulation.

Apparently, the physical event critical to the formation of a H-kininogen-F.XI complex on HUVECs is the binding of the cofactor to the surface of the cells, a process which has been studied in detail by two groups of investigators (14, 15). Here we confirm that: (a) HUVECs possess specific binding sites to which 125I-labeled H-kininogen binds with high affinity in the presence of zinc ions and (b) calcium ions exert an inhibitory effect on the zinc-dependent interaction of 125I-labeled H-kininogen with HUVECs. In the presence of 2 mM CaCl2, a concentration chosen because it was required for some of the studies of F.IX activation, optimal binding of 125I-labeled H-kininogen to HUVECs occurred at a concentration of 100 μM zinc ions which is somewhat higher than physiologic zinc concentration (25 μM). Under our experimental conditions (2 mM CaCl2, 100 μM ZnCl2 at 37 °C), the estimated dissociation constant (55 nM) was found to be very similar to that reported by van Iwaarden et al. (15) (35–50 nM in the presence of 25–50 μM zinc at 37 °C). However, the calculated number of H-kininogen molecules bound per cell at saturation was considerably higher in our study (1.14 × 108) than in the previous two studies (1.8–3.2 × 106 (14), 9.3 × 106 (15)). The discrepancy could possibly be explained, at least in part, by the different experimental conditions used to study the binding of 125I-labeled H-kininogen to HUVECs. First, we used a higher concentration of zinc ions (100 μM), and it has been shown that the number of binding sites for H-kininogen on HUVECs is directly proportional to the zinc ion concentration (14). Second, at 37 °C HUVECs internalize H-kininogen (41), and this process makes the calculations of bound molecules per cell somewhat approximate. Indeed, a lower number of binding sites was calculated when the binding of 125I-labeled
H-kininogen to HUVECs was studied at 4 °C (15), a condition that prevents internalization of molecules into cells. Based on the estimated dissociation constants and the plasma concentrations of H-kininogen, van Iwaarden et al. (14) estimated that under physiological conditions (37 °C, 10−25 mM zinc, 1 mM CaCl2) HUVECs at saturation express 1.6-2.8 × 10^6 molecules of H-kininogen per cell, i.e. a sufficient number of endothelial cell receptors for F.XI and plasma prekallikrein.

The question of whether H-kininogen mediates the association of F.XI to the surface of HUVECs, as it does on artificial surfaces (16) or on the surface of platelets (39), was specifically addressed here in various experiments. The concept that H-kininogen is indeed the receptor for F.XI on the surface of HUVECs is supported by several lines of evidence. First, when the cells were grown in human serum which had been depleted of contact factors, specific and saturable binding of 125I-labeled F.XI to HUVECs was detected only in the presence of added zinc ions and H-kininogen. The partial binding of 125I-labeled F.XI to HUVECs grown in fetal calf serum in the absence of added H-kininogen is probably still dependent on a limited number (approximately 15% of the total binding capacity) of H-kininogen molecules on the surface of cells. Although it is reported that HUVECs can synthesize H-kininogen (15), it is likely that the H-kininogen exposed on HUVECs cultured in fetal calf serum derives from the culture medium, since H-kininogen could not be detected in HUVECs grown in serum which had been depleted of H-kininogen (41). Second, the apparent dissociation constant for 125I-labeled F.XI binding to HUVECs is very similar to that reported for the binding of the same molecule to activated platelets in the presence of H-kininogen, i.e. 10 nM (39). Such, purified plasma prekallikrein was able to compete with 125I-labeled F.XI for binding to HUVECs in agreement with the knowledge that F.XI and plasma prekallikrein share a common binding site on the light chain of H-kininogen (40).

Furthermore, our results indicate that the same domain in the H-kininogen molecule represents the receptor for binding of both F.XIa and F.XI to HUVECs. Excess of nonlabeled enzyme completely blocked the association of 125I-labeled zymogen to HUVECs and, vice versa, excess of nonlabeled zymogen reduced the binding of 125I-labeled enzyme by 70%. The inability of F.XI to compete fully with F.XIa for binding to HUVECs is probably related to the fact that the affinity of the enzyme for binding sites is three times higher than that of the zymogen. However, the presence on HUVECs of additional binding sites for F.XIa, distinct from those for F.XI, cannot be absolutely excluded. For example, cultured aortic bovine endothelial cells have been shown to expose a selective high affinity binding site for F.XIa in the presence of both the cofactor, Factor VIII, and the substrate, Factor X (11). Similarly, based on the observation that F.XIa binds to the surface of activated platelets in the absence of added zinc ions and that F.XIa fails to block F.XIa binding to platelets, the existence of a distinct, specific F.XIa receptor on the platelet surface has been proposed (42).

In the presence of saturating concentrations of H-kininogen, both 125I-labeled F.XI and 125I-labeled F.XIa bound to HUVECs with high affinity (Kd of 1.5-4.5 nM). Since the plasma concentration of F.XI is about 25 nM (43), the binding sites for F.XI on endothelial cells should be saturated. Activation of 10% of plasma F.XI would generate F.XIa in concentrations (2.5 nM) that could compete with F.XI for a common binding site on endothelial cells, so that a significant number of enzyme molecules could become associated with the cell surface. Alternatively, we have shown that cell-bound F.XIa can be generated by activation of cell-associated F.XI. Under the experimental conditions described in this paper (nonstimulated HUVECs grown in contact factor depleted serum), the activation of HUVEC-bound F.XI was found to be strictly dependent on the addition of purified F.XIa. The possibility that a F.XII-independent pathway on the surface of HUVECs similar to that reported on the surface of activated platelets (44), was initially considered because HUVECs grown in fetal calf serum were apparently able to activate F.XI in the absence of exogenous F.XIa. This activation, however, was still dependent on F.XIa, probably adsorbed to the cells from the culture medium, as indicated by its disappearance in the presence of a specific F.XIa inhibitor or in HUVECs cultured in contact factor-depleted serum. Further studies of HUVEC grown in normal human serum will be required to establish optimal F.XI activation conditions and to ascertain whether HUVEC can interact with F.XII and generate F.XIIa on their surface, either in the quiescent state or after perturbation. Rabbit endothelial cells have been shown to contain an activator of F.XII apparently associated with the particulate fraction of cell homogenates (13). It is not known if a similar activator is contained also in human endothelial cells, but, if this were the case, it should not be exposed on the surface of quiescent HUVECs since, in the presence of added F.XII, H-kininogen, and F.XI, cultured HUVECs did not produce any F.XIIa activity. The hypothesis that such an activation could occur on the cell surface after perturbation of the endothelial cell is reasonable but remains to be proven. Regardless of mechanisms that lead to its generation, HUVEC-bound F.XIa retains its ability to activate F.XI in a purified system. The rate of activation of 3H-F.XI by F.XIa was lower for the cell-bound enzyme than for the same enzyme in solution. However, the amount of cell-bound F.XIa was not directly measured by us but simply inferred from the binding curve for F.XIa at saturation. Furthermore, on the surface of HUVEC the F.XIa may also undergo degradation or inactivation processes with partial loss of activity. The Kd for 3H-F.XI activation by F.XIa was apparently the same when the reaction was studied either in the presence or in the absence of HUVEC. Further detailed studies are needed to evaluate the kinetics of F.XI activation by HUVEC bound F.XIa, either in purified systems or in the presence of plasma. In platelet-depleted, functional activity of platelet-bound F.XIa as a F.XI activator was shown to be fully retained on the platelet surface (45).

According to the model proposed here, the ability of HUVEC to provide a surface for the assembly and expression of an intrinsic F.XI activator complex establishes new mechanisms for the localization of a potent clot-promoting activity to the surface of the vessel wall which may be important for hemostasis and thrombosis. Indeed, endothelial cell-associated procoagulant mechanisms might not be entirely dependent on the expression of tissue factor and activation of the extrinsic pathway of blood coagulation. Moreover, since the activation of the contact system in vitro triggers several body defense mechanisms, including fibrinolysis, kinin generation, and complement activation, localization of all these actions to the surface of endothelial cells may have relevant pathophysiologic consequences. For example, F.XIa has been recently shown to be a potent inactivator of type 1 plasminogen activator inhibitor (47). The activation of the contact system at sites of endothelial cell injury could locally generate F.XIa in amounts that may significantly reduce the concentration of type 1 plasminogen activator inhibitor, thus liberating tissue plasminogen activator from the control of its primary inhibitor.
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