

# Activation of Rabbit Hageman Factor by Homogenates of Cultured Rabbit Endothelial Cells

ROGER C. WIGGINS, DAVID J. LOSKUTOFF, CHARLES G. COCHRANE, JOHN H. GRIFFIN,  
and THOMAS S. EDGINGTON, *Departments of Immunopathology and Molecular  
Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037*

**ABSTRACT** Rabbit Hageman factor was proteolytically cleaved and activated by a homogenate prepared from cultured rabbit endothelial cells. Cleavage of radiolabeled Hageman factor was monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Endothelial cell-mediated cleavage of Hageman factor was demonstrated both in a purified system and in plasma, was time and concentration dependent, and was associated with formation of the characteristic 28,000  $M_r$  form of active Hageman factor. The rate of cleavage of Hageman factor was not affected by Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa.), hexadimethrine bromide (Polybrene, Aldrich Chemical Co., Inc., Milwaukee, Wis.), hirudin, soybean trypsin inhibitor, or antisera to plasminogen or prekallikrein. However, cleavage was enhanced by kaolin, and was inhibited by diisopropylfluorophosphate. The enzyme responsible for cleavage of Hageman factor was localized to the 100,000- $g$ -sedimentable, subcellular fraction of the endothelial cell homogenate and was relatively specific, because neither radiolabeled rabbit Factor XI nor rabbit prekallikrein were themselves proteolytically cleaved by the endothelial cell homogenate. However, when these molecules were incubated with the homogenate in the presence of Hageman factor, both Factor XI and prekallikrein were cleaved, demonstrating that Hageman factor had been activated by the endothelial cell homogenate. Furthermore, the kallikrein generated by endothelial cell homogenate-activated Hageman factor was capable of liberating kinin from high molecular weight kininogen as measured by bioassay. Cultured rabbit endothelial cells, therefore, possess the capacity

to activate Hageman factor by proteolysis. This may be one mechanism for Hageman factor activation in vivo.

## INTRODUCTION

The blood is enveloped by a continuous layer of endothelial cells lining the complex ramifications of the vascular system. These cells represent the anatomical barrier and an apparently important interface between blood and contiguous tissues. The compatibility of the intact endothelial cell surface with blood and its constituent procoagulant proteins is a central and necessary condition for maintenance of vascular homeostasis. Although the mechanism of this compatibility has not been fully elucidated, it is clear that the production by endothelial cells of fibrinolytic enzymes (1, 2), prostacyclin (3), and other factors that influence platelet function (4, 5) or vascular tone (6) may play a significant role in ensuring vessel patency. The endothelial cell surface is equally important in hemostasis and the induction of thrombus formation inasmuch as damage to this layer is associated with attachment of platelets and the initiation of coagulation (7). Although it is tacitly accepted that coagulation is initiated by contact with constituents of the subendothelium, the possibility that the endothelial cell per se participates in the initiation or modification of procoagulant events should not be overlooked.

In our study, we have employed cultured rabbit endothelial cells as a model to explore the role of the endothelium in the initiation of the intrinsic procoagulant pathway. Here we provide evidence that these cells possess an enzyme that can activate rabbit Hageman factor (HF, blood coagulation Factor XII)<sup>1</sup> by

This is publication 1731 from the Departments of Immunopathology and Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.

A preliminary report of this work was presented at the Meeting of the American Hematology Society, New Orleans, La. 1978. *Blood*. 52(Suppl. 1): 371. (Abstr.)

Received for publication 25 April 1979 and in revised form 5 September 1979.

<sup>1</sup>*Abbreviations used in this paper:* EC, endothelial cell; HF, Hageman factor; HF<sub>a</sub>, activated HF;  $\alpha$ -HF<sub>a</sub>, the 82,000  $M_r$  form of HF<sub>a</sub>;  $M_r$ , molecular weight; SBTI, soybean trypsin inhibitor; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TBS, Tris-buffered saline.

proteolysis, and thereby lead to activation of the coagulation, kallikrein, and kinin-forming pathways.

## METHODS

### *Purified proteins from rabbit plasma*

**Hageman factor.** HF was purified from rabbit plasma either by the method of Griffin and Cochrane (8, 9) or by affinity chromatography using anti-rabbit HF immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and subsequent elution of HF with 5 M guanidine followed by DEAE- and SP-Sephadex (Pharmacia Fine Chemicals) ion exchange chromatography.<sup>2</sup> HF prepared by both techniques was the same with respect to specific clotting activity (86 clotting U/mg protein in human HF-deficient plasma), migration on polyacrylamide gels in the presence of sodium dodecyl sulfate (a single-chain polypeptide with an apparent  $M_r$  of 82,000), cleavage fragments generated in kaolin-activated rabbit plasma (52,000, 50,000, and 28,000  $M_r$ ), and half-life of radiolabeled HF in rabbits (28–31 h). The 82,000  $M_r$  form of activated Hageman factor ( $\alpha$ -HF<sub>a</sub>) preparation<sup>2</sup> was a single polypeptide chain of 82,000  $M_r$  on sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) under nonreducing conditions, but 82% of the protein dissociated into fragments of 50,000 and 28,000  $M_r$  in the presence of reducing agents. The specific clotting activity was 1.6-fold greater than that of single-chain HF. Prekallikrein (kindly provided by Dr. Richard Ulevitch, Research Institute of Scripps Clinic), Factor XI, and high  $M_r$  kininogen were purified from citrated rabbit plasma as previously reported (10–12). Factor XI and prekallikrein migrated as homogeneous single-protein bands on SDS-PAGE in the presence and absence of reducing agents. The high  $M_r$  kininogen preparation was heterogeneous by this criterion, with major protein bands observed at 110,000 and 70,000  $M_r$  in the presence of reducing agents. The specific clotting activity of high  $M_r$  kininogen preparation assayed in high  $M_r$  kininogen-deficient plasma (Fitzgerald trait) was 9 cu/mg of protein. (1 cu is defined as the amount of activity present in 1 ml of normal citrated rabbit plasma.)

**Cell culture.** Endothelial cells (EC) were isolated from intact rabbit vena cava as previously described (1), and grown on Falcon disposable plasticware (Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif.) in modified Eagle's medium (with Earle's salts) supplemented with fetal bovine serum (10%), streptomycin (100  $\mu$ g/ml), and penicillin (100 U/ml). All culture media and supplements were from Grand Island Biological Co., Grand Island, N. Y. Cultures were maintained at 37°C in a 95% air, 5% CO<sub>2</sub> atmosphere. EC were routinely subcultured once a week (1:5), after trypsinization (Grand Island Biological Co., 0.25% for 5–10 min) of confluent monolayers. All cells used in these studies had been subcultured 8–20 times. Confluent cultures were well contact inhibited, could not be stimulated to divide with fresh serum, and continued to express morphological and biochemical properties of the intact endothelium (13). Rabbit intimal cells were kindly provided by Dr. B. Bounassisi, University of California at San Diego, and rabbit aortic smooth muscle cells were isolated as described (14).

**Preparation of cell homogenates and subcellular fractions.** Cultures were washed three times with cold phosphate-buf-

fered saline and removed from the surface of the Petri dish with a rubber policeman. The cells were washed again by centrifugation at 800 g for 5 min, and the cell pellets were resuspended in cold hypotonic homogenization medium (0.25 M sucrose, 1 mM EDTA, 0.01 M Tris-HCl, pH 8.1) at 4°C and homogenized with a tight-fitting, Teflon pestle tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa.) until 90–95% of the cells were disrupted. The nuclei were removed by centrifugation at 600 g for 10 min, and the supernate was centrifuged at 100,000 g for 60 min to yield a cytosol and a particulate fraction.

**SDS-PAGE and autoradiography.** SDS-PAGE was performed according to Laemmli (15). The stacking and running gels contained 4 and 10% acrylamide, respectively. The samples were boiled for 4 min in the presence of 2% sodium dodecyl sulfate and 2.5%  $\beta$ -mercaptoethanol and then applied to the gel. After electrophoresis, the gels were fixed and stained in 50% trichloroacetic acid and 0.1% Coomassie Brilliant Blue R250 (Bio-Rad Laboratories, Richmond, Calif.), destained in 10% glacial acetic acid, dried with a Slab Gel Drier (Hoefer Scientific Instruments, San Francisco, Calif.), and placed on Kodak X-omatic film (Eastman Kodak Co., Rochester, N. Y.) to localize the radiolabeled protein. Molecular weights of radiolabeled proteins were determined from the relative migration of internal standard proteins analyzed on the same gel. The radioactivity on the gel was quantitated by slicing the gel and counting the radioactivity in each slice. The percent of proteolytic cleavage of radiolabeled proteins was calculated by determining the proportion of total radioactivity in the gel that remained at the native molecular weight after treatment and comparing it with that in the control. In all cases, at least 70% of the radioactivity in control gels was at native molecular weight.

**HF-depleted rabbit plasma.** HF-depleted rabbit plasma was prepared using an insolubilized IgG fraction of monospecific goat anti-rabbit HF as described elsewhere (11). HF-depleted plasma was reconstituted by adding sufficient purified HF back to the depleted plasma to normalize the clotting time.

**Kinin bioassay.** 200-g, virgin, female Brown Norway rats were injected intramuscularly with diethylstilbestrol (100  $\mu$ g in mineral oil) 18 h before assay. The uterus was removed, and a single uterine horn used in a Schultz-Dale constant-temperature apparatus for kinin assay. Synthetic bradykinin (Sandoz Inc., East Hanover, N. J.) was used as a standard. The results were quantitated by measuring the time from stimulus to contraction. A linear relationship was observed between the log of the bradykinin concentration and the log of the time between stimulus and contraction. The results were expressed as bradykinin equivalents in nanograms.

**Miscellaneous.** All chemicals and reagents used were of the highest grade available. Protein determinations were made by the method of Lowry et al. (16) using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. Radiolabeling of protein with <sup>125</sup>I was performed by the chloramine-T method (17) to a specific activity of 1–3  $\mu$ Ci/ $\mu$ g. After radiolabeling, the protein solutions were dialyzed against 0.01 M sodium acetate buffer, pH 5.0, and then stored at –20°C. After radiolabeling, as much as 7% of the radiolabel migrated at the 28,000  $M_r$  position on SDS-PAGE. Radioactivity was measured in a Packard Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Human HF-deficient plasma was purchased from George King Biomedical, Inc., Overland Park, Kansas. Human  $\alpha$ -thrombin was a generous gift of Dr. J. Fenton, Division of Laboratories and Research, New York State Department of Health, Albany. Hirudin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Diisopropylfluorophosphate was purchased from

<sup>2</sup> Wiggins, R. C., and C. G. Cochrane. Rabbit Hageman factor. Activation of prekallikrein and Factor XI by  $\alpha$ -HF<sub>a</sub>. The role of the negatively charged surface. Submitted for publication.

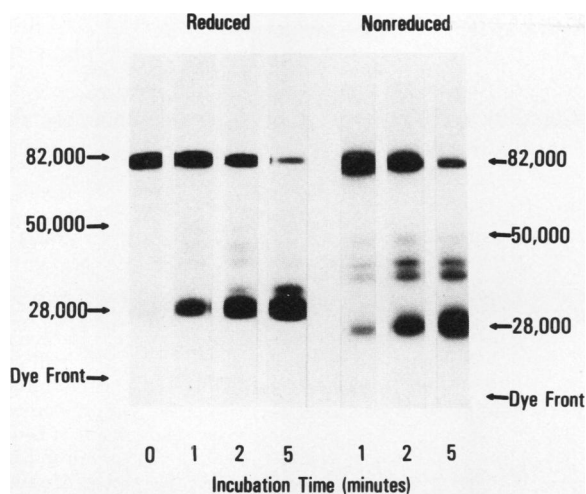


FIGURE 1 SDS-PAGE autoradiograms of  $^{125}\text{I}$ -HF exposed to EC homogenate analyzed under reducing and nonreducing conditions.  $^{125}\text{I}$ -HF (1.3  $\mu\text{g}$ ) was incubated at  $37^\circ\text{C}$  with EC homogenate (480  $\mu\text{g}$ ) in the presence of 0.01 M Tris, pH 7.4, and bovine serum albumin (40  $\mu\text{g}$ ) in a final reaction volume of 126  $\mu\text{l}$ . At the times shown, a 20- $\mu\text{l}$  subsample was removed and boiled in the presence of 20  $\mu\text{l}$  5% sodium dodecyl sulfate for 4 min. 20  $\mu\text{l}$  of this mixture was then removed and boiled for 2 min in the presence of  $\beta$ -mercaptoethanol. The reduced and nonreduced mixtures were analyzed separately by SDS-PAGE and autoradiography as described in Methods.

Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. The angiotensin-converting enzyme inhibitor SQ 20881 was generously provided by Dr. W. B. McDowell, The Squibb Institute for Medical Research, Princeton, N. J.

## RESULTS

### *Cleavage of HF in the presence of EC homogenates.*

Incubation of  $^{125}\text{I}$ -HF with the EC homogenate prepared from cultured cells resulted in proteolytic cleavage of the native 82,000  $M_r$   $^{125}\text{I}$ -HF molecule as shown in Fig. 1. In the presence and in the absence of reducing agents, the dominant cleavage fragment had an estimated  $M_r$  of about 28,000 and in independent experiments was observed to comigrate with the 28,000  $M_r$  fragment generated in kaolin-activated, citrated, normal rabbit plasma. This finding is compatible with the concept that the 28,000  $M_r$  fragment ( $\beta$ -HF<sub>a</sub>) is being generated under these conditions. Further support for this concept came from using preparations of  $^{125}\text{I}$ -HF with low specific activity (0.2  $\mu\text{Ci}/\mu\text{g}$ ), in which the  $^{125}\text{I}$  was predominantly (>72%) attached to the 28,000  $M_r$  fragment when assessed in kaolin-activated, normal rabbit plasma. In the presence of the EC homogenate, the appearance of the major band of cleaved  $^{125}\text{I}$ -HF at the 28,000  $M_r$  position indicated that this fragment originated from that portion of the HF molecule known to contain the active site serine. Additional cleavage

fragments of  $^{125}\text{I}$ -HF were observed at 46,000 and 41,000  $M_r$  (Fig. 1). If the  $^{125}\text{I}$ -HF was first preincubated with kaolin to permit surface binding and was then incubated with the cell homogenate, a greater proportion of the 50,000  $M_r$  cleavage fragment was observed on reduced gels (data not shown). This fragment migrated in the same position as the heavy chain HF fragment generated in kaolin-activated, normal rabbit plasma.

The effect of the EC homogenate upon  $^{125}\text{I}$ -HF added to rabbit plasma in the presence or absence of kaolin is shown in Fig. 2. In two separate experiments, EC homogenate alone induced cleavage of only 3–5% of the  $^{125}\text{I}$ -HF (lane 2). Addition of kaolin alone resulted in 10% cleavage (lane 3). However, in the presence of both the EC homogenate and kaolin, >35% of the molecules were cleaved (lane 4) over the same time interval. The cleavage fragments of  $^{125}\text{I}$ -HF observed under these conditions were at 50,000 and 28,000  $M_r$ . The difference in the patterns of cleavage observed in Figs. 1 and 2 may have been the result of the EC homogenate enhancing reciprocal activation between HF and prekallikrein in kaolin-activated plasma.

*Rate of cleavage of HF by the EC homogenate.* The rate of conversion of native  $^{125}\text{I}$ -HF (82,000  $M_r$ ) to the 28,000  $M_r$  fragment were measured during a 20-min incubation at  $37^\circ\text{C}$  (Fig. 3). The disappearance of native HF was rapid (50% in 2–3 min). There was a concomitant increase in radioactivity in the 28,000  $M_r$  region of the gel, indicating that the  $^{125}\text{I}$ -HF was undergoing time-dependent proteolytic cleavage to yield the 28,000  $M_r$  fragments. Other fragments, as shown in Fig. 1, were also generated during the incubation.

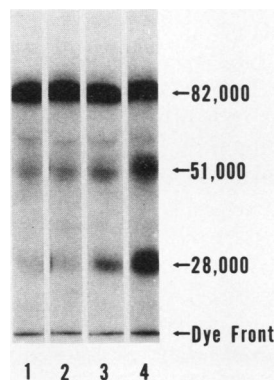


FIGURE 2 SDS-PAGE autoradiogram of  $^{125}\text{I}$ -HF in rabbit plasma exposed to EC homogenate for 10 min at  $37^\circ\text{C}$ .  $^{125}\text{I}$ -HF (150 ng) in citrated rabbit plasma (15  $\mu\text{l}$  of 25% plasma in Tris-buffered saline [TBS]) was incubated in the presence of TBS alone (lane 1), the EC homogenate (130  $\mu\text{g}$  of cellular protein) (lane 2), kaolin (40  $\mu\text{g}$ ) (lane 3), or kaolin with EC homogenate (lane 4). The mixtures were analyzed by SDS-PAGE and autoradiography.

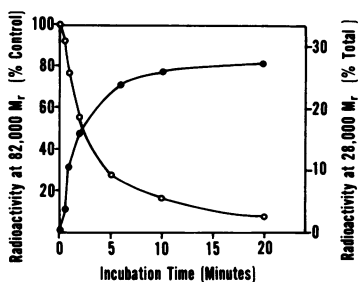


FIGURE 3 Rate of cleavage of HF by EC homogenate.  $^{125}\text{I}$ -HF (1.2  $\mu\text{g}$ ) in 100  $\mu\text{l}$  0.1 M Tris buffer, pH 7.4, containing bovine serum albumin (1 mg/ml) was incubated with EC homogenate (450  $\mu\text{g}$  of cellular protein). Aliquots (10  $\mu\text{l}$ ) were removed at the times shown and analyzed by SDS-PAGE. The bands visualized by autoradiography were excised and counted for radioactivity. The disappearance of radioactivity from the 82,000  $M_r$  band (○) was associated with the appearance of radioactivity at 28,000  $M_r$  (●).

*Comparative effects of EC homogenate on radio-labeled HF, prekallikrein, and Factor XI.* To assess the substrate specificity of the HF-cleaving activity, the effect of EC homogenate on prekallikrein and Factor XI was examined.  $^{125}\text{I}$ -Prekallikrein (280 ng),  $^{125}\text{I}$ -Factor XI (200 ng), and  $^{125}\text{I}$ -HF (200 ng) were incubated separately with the EC homogenate (430  $\mu\text{g}$ ) for 20 min at 37°C in a final volume of 52  $\mu\text{l}$  and in the presence of 0.1 M Tris, pH 7.4, and bovine serum albumin (10  $\mu\text{g}$ ). Aliquots (10  $\mu\text{l}$ ) were removed at intervals for analysis by SDS-PAGE. After 1, 5, and 20 min of incubation, the relative proportion of molecules cleaved were: Factor XI—0, 0, 0%; prekallikrein—0, 0, 0%; and Hageman factor—22, 59, 78%, respectively. Thus, under these conditions, only the  $^{125}\text{I}$ -HF underwent proteolytic cleavage. The  $^{125}\text{I}$ -prekallikrein and Factor XI (which were present at the same molar concentrations as HF) remained at their native position. In control experiments, the  $^{125}\text{I}$ -prekallikrein and  $^{125}\text{I}$ -Factor XI were shown to be cleaved normally in kaolin-activated rabbit plasma. In experiments using higher concentrations of the EC homogenate (>20 mg/ml cellular protein) no more than 5% of the  $^{125}\text{I}$ -Factor XI or  $^{125}\text{I}$ -prekallikrein molecules were cleaved in 20 min. This specificity of the EC homogenate contrasts sharply with that of trypsin.  $^{125}\text{I}$ -HF and  $^{125}\text{I}$ -prekallikrein, under conditions similar to those described above, were equally sensitive to cleavage by trypsin over a wide dose range.  $^{125}\text{I}$ -Factor XI was ~twofold more resistant to trypsin (data not shown). Thus, the proteolytic activity of the EC homogenate, unlike that of trypsin, appears to be relatively specific for HF.

*Subcellular localization of the HF-cleaving activity.* To determine whether the HF-cleaving activity was present in the cytosol or was associated with a sedimentable or particulate fraction of the cells, EC were

disrupted by homogenization and subjected to differential centrifugation. A sedimentable particulate fraction and supernatant cytosol were isolated after centrifugation at 100,000 g for 60 min and analyzed for their capacity to cleave HF. As shown in Fig. 4, all HF-cleaving activity present in the whole homogenate (lane 2) was recovered in the particulate fraction (lane 4), where total cleavage of  $^{125}\text{I}$ -HF into fragments of 28,000 and 31,000  $M_r$  were observed. No HF-cleaving activity was detected in the cytosol (lane 3). Thus, the activity would appear to be predominantly associated with the microsomal fraction of the cells (e.g., plasma membrane, subcellular organelles, and other vesicles or secretion granules).

#### *Evidence that HF is activated by EC homogenate*

*Prekallikrein cleavage and activation by activated HF (HF<sub>a</sub>) generated by the EC homogenate.* To demonstrate that the EC homogenate not only proteolytically cleaved but also activated HF molecules, the EC homogenate was incubated with native single-chain HF. This HF was then assessed for its ability to cleave prekallikrein. As shown in Fig. 5, the HF alone (lane 1) or EC homogenate alone (lane 2) caused no detectable proteolytic cleavage of prekallikrein. However, when both the EC homogenate and HF were present, extensive proteolytic cleavage of  $^{125}\text{I}$ -prekallikrein was observed (lane 3). The resulting fragments of 33,000 and 48,000  $M_r$  were similar to those observed when  $^{125}\text{I}$ -prekallikrein was present in kaolin-activated plasma.

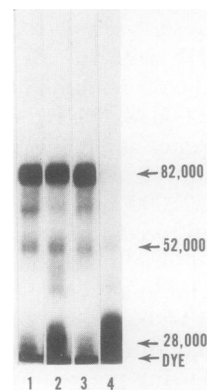


FIGURE 4 Autoradiogram demonstrating subcellular localization of HF-cleaving activity.  $^{125}\text{I}$ -HF (100 ng) was incubated in a final volume of 21  $\mu\text{l}$  for 10 min at 37°C in the presence of 0.1 M Tris buffer, pH 7.6, containing bovine serum albumin (1 mg/ml) (lane 1), buffer and the whole EC homogenate (31  $\mu\text{g}$ ) (lane 2), buffer with the 100,000-g supernatant (12  $\mu\text{g}$ ) (lane 3), or buffer with the 100,000-g pellet (45  $\mu\text{g}$ ) (lane 4). The mixtures were analyzed by SDS-PAGE and autoradiography.

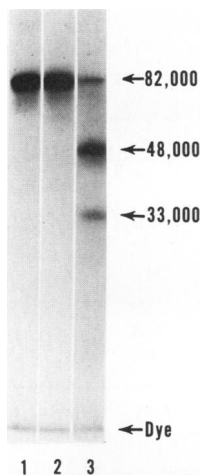


FIGURE 5 SDS-PAGE autoradiogram of  $^{125}\text{I}$ -prekallikrein incubated in the presence of HF and EC homogenate.  $^{125}\text{I}$ -prekallikrein (160 ng) in  $5\ \mu\text{l}$  of 0.1 M Tris buffer, pH 7.4, containing bovine serum albumin (1 mg/ml) was incubated for 10 min at  $37^\circ\text{C}$  in the presence of HF (300 ng) alone (lane 1), the EC homogenate alone (45  $\mu\text{g}$  of cellular protein) (lane 2), or both HF and the EC homogenate (lane 3). The reaction volumes (22  $\mu\text{l}$ ) were equalized by addition of TBSaline and analyzed by SDS-PAGE and autoradiography.

A similar experiment was conducted in a plasma system (Fig. 6). When the EC homogenate was incubated with normal rabbit plasma to which  $^{125}\text{I}$ -prekallikrein had been added, 18% of the  $^{125}\text{I}$ -prekallikrein was cleaved, resulting in the formation of fragments of 33,000 and 48,000  $M_r$  (lane 3). In rab-

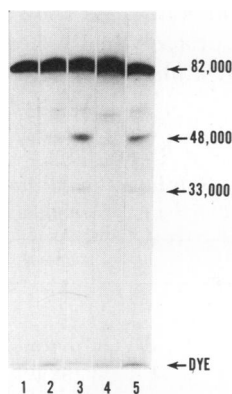


FIGURE 6 SDS-PAGE autoradiograms of  $^{125}\text{I}$ -prekallikrein in plasma.  $^{125}\text{I}$ -prekallikrein (160 ng) was incubated for 10 min at  $37^\circ\text{C}$  in various rabbit plasmas (12  $\mu\text{l}$  of 50% rabbit plasma in TBS) in the presence or absence of the EC homogenate (100  $\mu\text{g}$  of cellular protein).  $^{125}\text{I}$ -Prekallikrein was incubated in normal rabbit plasma (lane 1) or HF-depleted rabbit plasma reconstituted with purified HF (lane 2). EC homogenate was incubated with  $^{125}\text{I}$ -prekallikrein in the normal rabbit plasma (lane 3), in the HF-depleted plasma (lane 4), and in HF-depleted plasma reconstituted with purified HF (lane 5).

bit plasma from which the HF had been specifically removed by immunoabsorption (see Methods),  $^{125}\text{I}$ -prekallikrein remained uncleaved after incubation with the EC homogenate (lane 4). However reconstitution of the HF-depleted plasma with purified HF resulted in cleavage of 25% of the  $^{125}\text{I}$ -prekallikrein (lane 5).  $^{125}\text{I}$ -Prekallikrein incubated in the normal rabbit plasma or reconstituted HF-depleted plasma alone resulted in no detectable cleavage (lanes 1 and 2). Thus, both in a partially purified system and in plasma, the EC homogenate converted HF to a form that proteolytically cleaved prekallikrein.

To confirm that the observed cleavage of radio-labeled molecules was indeed associated with the acquisition of activity, the ability of kallikrein (generated by EC homogenate-HF<sub>a</sub>) to release kinin from purified rabbit high  $M_r$  kininogen was measured by bioassay (Table I). No detectable kinin activity was generated by single-chain HF incubated with prekallikrein and high  $M_r$  kininogen. In contrast, the addition of the EC homogenate (with 60  $\mu\text{g}$  of angiotensin-converting enzyme inhibitor) caused contraction of the rat uterus, with a response time equivalent to that of 18 ng of bradykinin. Preliminary studies had shown that bradykinin was destroyed when incubated with the EC homogenate alone, and that this effect could be inhibited by a 1,000-fold M excess of the angiotensin-converting enzyme inhibitor SQ 20,881. Furthermore, the inhibitor itself, at this dose, had no agonistic effect on the rat uterus, and incubation of the rat uterus in the presence of the inhibitor did not subsequently alter the sensitivity of the uterus to bradykinin. We, therefore, conclude from this study that the EC homogenate was capable of activating HF to activate prekallikrein to kallikrein, which then released kinin from high  $M_r$  kininogen. These findings complement the cleavage data described above.

**Factor XI cleavage by HF<sub>a</sub> generated by the EC homogenate.** To further characterize the activity associated with EC-cleaved HF, the ability of a mixture of HF and EC homogenate to cleave another HF substrate, Factor XI, was evaluated (Fig. 7). Native  $^{125}\text{I}$ -Factor XI (lane 1) was cleaved slightly in the presence of high  $M_r$  kininogen and a kaolin surface (lane 2), and the addition of the EC homogenate to this mixture had no effect (lane 3). Addition of HF to the  $^{125}\text{I}$ -Factor XI-high  $M_r$  kininogen-kaolin mixture resulted in cleavage of only 9% of the  $^{125}\text{I}$ -Factor XI molecules (lane 4). However, when the EC homogenate was added to the latter mixture in the presence of HF, as much as 46% of the  $^{125}\text{I}$ -Factor XI molecules were cleaved (lane 5). The cleavage fragments generated at 50,000 and 33,000  $M_r$  migrated at the same position as those observed when  $^{125}\text{I}$ -Factor XI was present in kaolin-activated rabbit plasma. Removal of high  $M_r$  kininogen from the above

TABLE I  
Kinin Generation by Purified Proteins of the HF-System in the Presence of EC Homogenate

$\alpha$ -HF <sub>a</sub> (1.5 $\mu$ g)	HF (1.6 $\mu$ g)	EC homogenate (220 $\mu$ g)	Prekallikrein (2 $\mu$ g)	High M <sub>r</sub> kininogen (5 $\mu$ g)	Response time	Bradykinin equivalent
-	+	+	+	+	3.5	18
-	+	-	+	+	NR	<4
-	-	+	+	+	NR	<4
-	+	+	-	+	NR	<4
-	-	-	+	+	NR	<4
+	-	-	+	+	3.0	22

HF,  $\alpha$ -HF<sub>a</sub>, and the EC homogenate (with angiotensin-converting enzyme inhibitor SQ 20881) were separately preincubated in the presence of 10  $\mu$ l 1 M Tris, pH 7.4, in a final volume of 50  $\mu$ l in a polypropylene tube for 5 min at 37°C. Prekallikrein (or 10  $\mu$ l 0.1 M Tris buffer, pH 7.4) was added to each tube for 3 min at 37°C. Finally, high M<sub>r</sub> kininogen (50  $\mu$ l) was added and incubated for 10 min at 37°C. The reaction was stopped by boiling for 4 min, and the kinin generated was measured by bioassay using an estrogen-sensitized rat uterus (see Methods). NR, no response.

reaction mixture reduced the proteolytic cleavage to 12% (lane 6), a proportion only slightly greater than that seen with HF in the absence of EC homogenate (lane 4). The EC homogenate, therefore, contains a molecule(s) that can convert HF to a form capable of cleaving both prekallikrein and Factor XI.

#### Effect of protease inhibitors in HF-cleaving activity of EC homogenate

**Diisopropylfluorophosphate.** The addition of diisopropylfluorophosphate (2 mM) to the EC homog-

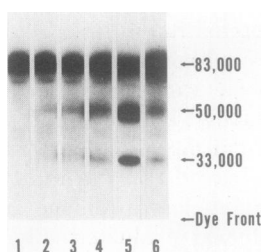


FIGURE 7 SDS-PAGE autoradiograms of <sup>125</sup>I-Factor XI incubated with various agents. <sup>125</sup>I-Factor XI (80 ng) was added to 5  $\mu$ l of 0.1 M Tris, pH 7.4, containing bovine serum albumin (1 mg/ml) and kaolin (50  $\mu$ g TBS). To this mixture was added HF (300 ng), high M<sub>r</sub> kininogen (400 ng), or EC homogenate (55  $\mu$ g of cellular protein). The final reaction volume was made up to 40  $\mu$ l with TBS. After 15 min at 37°C, the samples were analyzed by SDS-PAGE and autoradiography. <sup>125</sup>I-Factor XI was incubated in the presence of kaolin alone (lane 1), kaolin and high M<sub>r</sub> kininogen (lane 2), kaolin, high M<sub>r</sub> kininogen, and the EC homogenate (lane 3). HF was added to kaolin and high M<sub>r</sub> kininogen in the absence (lane 4) and presence of EC homogenate (lane 5). The effect of the absence of high M<sub>r</sub> kininogen from the above reaction mixture (HF, kaolin, and EC) is shown in lane 6.

enate for 5 min at 22°C abolished the cell-associated HF-cleaving activity (Table II, A), suggesting that the enzyme in the EC homogenate responsible for HF cleavage is a serine protease.

**Soybean trypsin inhibitor (SBTI).** Because the HF-cleaving activity could have been the result of contamination of the homogenate by residual tissue culture trypsin, the sensitivity of the EC enzyme to SBTI was assessed. As shown in Table II (B) the EC enzyme was insensitive to SBTI, whereas trypsin, at a dose that cleaved 99% of the <sup>125</sup>I-HF molecules, was completely inhibited by SBTI.

**Thrombin and hirudin.** As shown in Table II (C) thrombin did not proteolytically cleave <sup>125</sup>I-HF, even at a relatively high final concentration of 10 U/ml.

TABLE II  
Effect of Protease Inhibitors on Cleavage of Hageman Factor by EC Homogenate

Inhibitor	Activator	HF cleaved %
A DFP* (2 mM)	EC (11 mg/ml)	0
	EC (11 mg/ml)	62
B SBTI (50 $\mu$ g/ml)	EC (11 mg/ml)	61
	EC (11 mg/ml)	62
	Trypsin (25 $\mu$ g/ml)	3
	Trypsin (25 $\mu$ g/ml)	99
C Hirudin (20 U/ml)	EC (8 mg/ml)	94
	EC (8 mg/ml)	96
	Thrombin (10 U/ml)	4
	Thrombin (10 U/ml)	6

\* Diisopropylfluorophosphate.

Hirudin also did not block the EC-mediated HF cleavage. Thus, the responsible enzyme in the EC homogenate does not appear to be thrombin.

#### *Effect of antisera to HF, kallikrein, and plasmin on HF-cleaving activity*

As shown in Table III, cleavage of  $^{125}\text{I}$ -HF by the EC enzyme derived from cells cultured for 3 d in rabbit serum was not inhibited by the IgG fraction of antisera raised against rabbit kallikrein or rabbit plasmin, nor by the IgG fraction of preimmune goat serum. However, the IgG fraction of anti-rabbit HF blocked HF cleavage. As a positive control for these experiments, we demonstrated that cleavage of HF by kallikrein was completely inhibited by antikallikrein IgG. In other experiments (not shown), the antiplasmin antiserum has been shown to inhibit plasmin activity. These results indicate that the EC enzyme is neither kallikrein nor plasmin, which is consistent with the finding that the EC enzyme was not inhibited by SBTI (Table II, B), a molecule that inhibits both kallikrein and plasmin (18).

#### *Effect of Triton X-100, hexadimethrine bromide (Polybrene), and kaolin on HF cleavage by the EC homogenate*

Incubation of the EC homogenate with Triton X-100 or Polybrene for 10 min at 22°C had no effect upon the extent of HF cleavage. (EC homogenate alone and EC homogenate with Triton X-100 [0.5%] cleaved 90 and 86% of  $^{125}\text{I}$ -HF, respectively; EC homogenate alone and EC homogenate with Polybrene cleaved 23 and 25% of  $^{125}\text{I}$ -HF, respectively.) Thus, cleavage by EC homogenate does not appear to be simply the result of a surface effect. Furthermore, preincubation of the  $^{125}\text{I}$ -HF with kaolin enhanced the cleavage of HF in the presence of the EC homogenate ~10-fold. (EC homogenate [1.0 mg/ml] alone, EC homogenate [1.0 mg/ml] with kaolin [2.5 mg/ml], and EC homogenate [0.125 mg/ml] with

kaolin [2.5 mg/ml] cleaved 20, 77, and 15% of  $^{125}\text{I}$ -HF, respectively.)

#### *HF-cleaving activity associated with other cultured vascular cells*

Cell homogenates prepared from two other cultured cell types from rabbit aorta were assessed for their ability to proteolytically cleave  $^{125}\text{I}$ -HF. Under the conditions under which the EC homogenate at 3 mg/ml cellular protein concentration cleaved 43% of the  $^{125}\text{I}$ -HF, a homogenate prepared from cells with the cytological features of smooth muscle cells (14) at a concentration of 3.4 mg/ml cleaved 33% of the  $^{125}\text{I}$ -HF molecules. A second cell line derived from rabbit aorta and thought to represent rabbit intimal cells cleaved 30% of the  $^{125}\text{I}$ -HF molecules when present at a concentration of 2.8 mg/ml. The relative HF-cleaving activities of the two-cell homogenates was, therefore, similar to that of the endothelial cell homogenate, and similar cleavage fragments were generated, suggesting that the HF-cleaving enzyme is not restricted to endothelial cells.

## DISCUSSION

Activation of HF can initiate coagulation via activation of Factor XI (19–22) or Factor VII (23–25). HF<sub>a</sub> proteolytically converts prekallikrein to kallikrein, leading to plasmin generation (26, 27), the release of the vasoactive peptide bradykinin from high M<sub>r</sub> kininogen (28), and activation of the renin–angiotensin system (29). The mechanism by which this potentially powerful HF-dependent mediator system in activated *in vivo* remains to be elucidated. Artificial, negatively charged substances such as glass, kaolin, or celite activate the plasma system *in vitro*. Some collagen and basement membrane preparations (30, 31), bacterial endotoxins (32), and urate crystals (33) may serve as activating surfaces in physiological or pathophysiological situations. However, the manner by which HF is activated *in vivo* and the role of the negatively charged surface in this process remain conjectural.

The results summarized in this report indicate that cultured EC contain an enzyme that cleaves and activates HF. The EC activity appears to be relatively specific for HF because, under the experimental conditions explored, neither prekallikrein nor Factor XI were readily cleaved when separately incubated with the EC homogenate. Because diisopropylfluorophosphate abolished HF cleavage by the EC homogenate, it appears that a serine protease is responsible for this activity. Surface binding of HF renders it much more susceptible to cleavage by a number of enzymes (34). Because neither Triton X-100 nor hexadimethrine bromide (Polybrene) diminished the

TABLE III  
*Effect of Antisera on Cleavage of Hageman Factor by EC Homogenate*

IgG fraction	Activator	HF cleaved
		%
Normal goat serum	EC (5 mg/ml)	57
Anti-HF	EC (5 mg/ml)	0
Antiplasmin	EC (5 mg/ml)	69
Antikallikrein	EC (5 mg/ml)	63
Antikallikrein	Kallikrein (5 μg/ml)	0
Normal goat serum	Kallikrein (5 μg/ml)	48

HF-cleaving activity of the cell homogenate, and because kaolin enhanced this activity, it seems unlikely that the presence of negatively charged material in the cell homogenate rendered HF more susceptible to enzymatic cleavage. Proteolysis of HF by the enzyme was demonstrated both in a purified system and in plasma. A principle cleavage fragment generated from HF by the EC enzyme was the 28,000  $M_r$  fragment. This appears to be the same fragment that is generated during activation of HF in kaolin-activated plasma, and is probably the fragment that has been shown to contain the active site of HF (35). That the HF is activated as well as proteolytically cleaved by the EC homogenate is evident from the ability of HF to subsequently cleave its substrate molecules—Factor XI and prekallikrein—at cleavage sites apparently identical to those observed when these molecules are cleaved and activated in purified reaction mixtures and in kaolin-activated rabbit plasma (10, 22). Furthermore,  $HF_a$  generated by the EC homogenate was also shown to activate prekallikrein to kallikrein and, thus, to release kinin from high  $M_r$  kininogen as measured by a rat uterus bioassay system. Finally, activation of HF was not only demonstrated in the partially purified system, but also in rabbit plasma. We conclude from these observations that cultured rabbit EC possess the capability to activate HF by proteolysis.

In preliminary studies, a similar HF-cleaving enzyme was detected in cultured aortic smooth muscle and intimal cells. Thus, it would appear that this enzyme is not restricted to endothelial cells, but like tissue factor (36), is present in other types of vascular cells.

The capacity of the EC-activated HF to cleave both prekallikrein and Factor XI is of interest with respect to the observed cleavage fragments of HF. Studies of the human HF system suggest that the free 28,000  $M_r$  form of  $HF_a$  ( $\beta$ - $HF_a$ ) is much more capable of activating prekallikrein than Factor XI, whereas the 82,000  $M_r$  form of  $HF_a$  ( $\alpha$ - $HF_a$ ), which binds to negatively charged surfaces, is capable of activating both prekallikrein and Factor XI (37, 38). In the present studies of the cleavage of HF in solution by the EC homogenate, the dominant form of  $HF_a$  generated was the 28,000  $M_r$  form  $\beta$ - $HF_a$ . It was therefore expected, and confirmed, that this material could indeed cleave prekallikrein. The finding that, in the presence of kaolin and high  $M_r$  kininogen, Factor XI could also be activated by EC-generated  $HF_a$  is consistent with our observation that kaolin-bound HF was cleaved by the EC extract to yield the 52,000 and 28,000  $M_r$  fragments of the surface-bound  $\alpha$ - $HF_a$ . The marked requirement for high  $M_r$  kininogen for Factor XI activation emphasizes the role of this cofactor mole-

cule in placing the Factor XI on an appropriate surface where it can be activated by  $\alpha$ - $HF_a$  (12, 39).

The possibility that the cells contain a trypsin-like enzyme or were contaminated by trypsin from prior passage was excluded by showing that the relative sensitivity of HF, Factor XI, and prekallikrein to the EC enzyme was quite different from that of trypsin. In addition, it was shown that the EC enzyme, unlike trypsin, was not inhibited by SBTI. Several other possible HF-cleaving enzymes were considered as sources of the observed HF-cleaving activity. Kallikrein, plasmin, or thrombin (from serum used in the growth medium or produced *de novo* by the cultured cells) might conceivably have caused the observed HF cleavage. However, thrombin was shown not to cleave HF, and kallikrein and plasmin were excluded both by using specific antisera against these components and by the resistance of cleavage to an inhibitor (SBTI) that inhibits kallikrein and plasmin (18). Thus, not only were extraneous kallikrein or plasmin excluded as the causal agent of HF cleavage, but also the possibility that the cultured EC might themselves synthesize these enzymes was excluded.

The HF-cleaving enzyme appeared to be associated with some structural element of the cell because it was recovered in the particulate fraction after centrifugation of cell homogenate at 100,000  $g$ . Studies are at present directed toward establishing whether the enzyme is associated with cell membrane, cellular organelles, or subendothelial structures. A plasminogen activator has been shown to be produced by EC cells (1). It is also inhibited by diisopropylfluorophosphate, and can be sedimented with the particulate fraction of the cells. These observations raise the possibility that the HF-cleaving and plasminogen-activating enzymes might be identical. Further studies are in progress to investigate the relationship between these activities.

Endothelial cells are known to produce enzymes that activate plasminogen (1, 2), procoagulant molecules such as tissue factor (36), and factors that influence platelet function and vascular tone (3–7). We now report the capacity of this cell type to activate HF. Whether this activity can be released from stimulated or damaged EC into the surrounding medium, or whether it remains associated with the corpse of the injured cell must be determined. However, if the HF-cleaving activity is exposed when EC or deeper layers are damaged, HF activation should occur. Furthermore, damage to the EC layer might expose subcellular structures with a sufficient density of negative charges to facilitate assembly of the surface-bound HF–high  $M_r$  kininogen–Factor XI–prekallikrein complexes similar to the one demonstrated for kaolin (12, 39, 40). Under these circumstances, the



EC enzyme could initiate HF cleavage, an essential step for the reciprocal activation between HF and prekallikrein (41). This may be one mechanism by which HF is activated in vivo.

#### ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of B. Loskutoff and David Revak and the willing secretarial assistance of Margaret Stone and Betty Goddard.

This work was supported in part by training grant HL-07195 and research grants HL-16411, AI-07007 from the National Institutes of Health, the Council of Tobacco Research, and Research Career Development Award HL-00192 to J.H.G.

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