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An endothelial cell-dependent pathway of coagulation

(coagulation factor/fibrin/tissue factor)

DAVID STERN*, PETER NAWROTH*, DEAN HANDLEY†, AND WALTER KISIEL‡

*Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032; †Sandoz Research Institue, East Hanover, NJ 07936; and ‡Departments of Pathology and Biochemistry, University of New Mexico School of Medicine, Albuquerque, NM 87131

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ABSTRACT **Although the endothelial cell is considered antithrombogenic, endothelium has recently been shown to participate in procoagulant reactions. In this report cultured bovine aortic endothelial cells are shown to propagate a procoagulant pathway starting with factor XI_a, leading to activation of factors IX, VIII, X, and prothrombin, culminating in fibrinopeptide A cleavage from fibrinogen and formation of a fibrin clot. Electron microscopic studies demonstrated that fibrin strands are closely associated with the endothelial cells. Endotoxin-treated endothelial cells, having acquired tissue factor activity, generated fibrinopeptide A in the presence of factors VII_a, IX, VIII, X, prothrombin, and fibrinogen. Factor X activation by factor VII_a and tissue factor expressed by endothelial cells is 10 times greater in the presence of factors IX and VIII than in their absence. This indicates that on the perturbed endothelial cell surface, factors IX and VIII do have an important role in the activation of factor X. Addition of platelets (10^8 per ml) augmented thrombin formation seen in the presence of endothelium alone by about 15-fold. Anti-human factor V IgG decreased this enhanced thrombin formation in the presence of platelets, indicating that factor V from platelets was playing an important role in thrombin formation. These data lead us to propose that endothelial cells can actively participate in procoagulant reactions. Although platelets can augment thrombin formation by these endothelial cell-dependent reactions, endothelial cells alone can lead to formation of a cell-associated fibrin clot. The endotoxin-treated endothelial cell provides a model of the thrombotic state supplying tissue factor to initiate coagulation and propagating the reactions leading to fibrin formation. This endothelial cell-dependent pathway suggests a central role for factors VIII and IX consistent with their importance in hemostasis.**

The modern view of hemostasis and thrombosis was largely influenced by the publication 20 years ago of the waterfall (1) or cascade (2) theory of coagulation. This model has been enriched by addition of the platelet membrane, supplying a cellular surface promoting formation of the prothrombinase complex, which is a factor V_a–X_a complex promoting efficient prothrombin activation (3–5). Endothelium, forming the luminal vascular surface, is another cell surface continuously interacting with coagulation factors. Traditionally, endothelium has been felt to play a passive role in hemostatic events providing an inert barrier to prevent exposure of coagulation factors and platelets to extravascular tissues. Recent studies have indicated that rather than providing an inert surface for the blood to flow over, the endothelial cell can play an active role in preventing activation of the coagulation system (6–9). Anticoagulant heparin-like molecules have been localized to the vessel surface (6). Antithrombin III bound to this heparin-like material on the endothelial cell surface demonstrates enhanced inactivation of proteases (7). Thrombomodulin, another endothelial cell surface molecule, is a cofactor for activation of the protein C anticoagulant pathway (8). Elaboration of plasminogen activators, which promote clot lysis (9), and prostacyclin, which decreases platelet reactivity (10), is also important in the antithrombogenic nature of endothelium.

Endothelium is also ideally situated to be in the first line of defense in vascular injury and to promote thrombosis in pathologic states. Recently, participation of endothelial cells in procoagulant reactions has received more attention. Aortic endothelium, both cultured and native, can bind factors IX and IX_a (11–13). Cell-bound factor IX can be activated by both intrinsic and extrinsic pathways of coagulation and cell-bound factor IXa in the presence of factor VIII can activate factor X (14). Factor X_a incubated with endothelial cells can activate prothrombin (13, 15). This reaction is dependent on endogenous factor V (16) expressed by endothelium and can be blocked by preincubating the cells with antifactor V antibody (13, 15). Perturbation of endothelial cells by agents such as endotoxin results in induction of tissue factor activity (14, 17, 18). These studies suggest a role of the endothelial cell in coagulation.

The current studies were prompted by the hypothesis that a complete procoagulant pathway can occur on the endothelial cell surface. The results indicate that cultured endothelial cells can propagate activation of the coagulation pathway when factor XI_a is added, leading to activation of factors IX, X, and prothrombin and culminating in formation of fibrin strands that appear to be closely associated with the cells. Perturbed endothelium, with induced tissue factor activity (14, 17, 18), promotes factor VII_a-dependent activation of factors IX and X, leading to thrombin and fibrin formation. When coagulation is initiated by factor VII_a and perturbed endothelium, factor X_a formation is 10 times greater in the presence of factors IX and VIII than when only factor X is present, suggesting a central role for these factors in the endothelial cell-dependent tissue factor pathway. Based on these data we hypothesize that endothelial cells play an important role in the balance of anticoagulant and procoagulant mechanisms in hemostasis and thrombosis. Perturbed endothelial cells, being capable of localizing coagulation factors and promoting activation of the coagulation system up to formation of fibrin strands, provide a model of the thrombotic state. This pathway of coagulation is endothelial cell-dependent, since it requires expression of tissue factor and factor V as well as interaction of the vitamin K-dependent coagulation factors VII_a, IX, IX_a, X, and X_a with the endothelial cell surface.

METHODS

Coagulation Proteins and Antisera. Bovine factors VII [7110 units (u)/mg], IX (260 u/mg), and X (100 u/mg) were

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Abbreviations: BAEC, bovine aortic endothelial cells; u, unit(s); S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S-2238, D-Phe-Pip-Arg-*p*-nitroanilide (where Pip = pipecolyl).

purified to homogeneity as described (19–21). Factor VII was activated by using bovine factor XII_a (generously provided by K. Fujikawa, University of Washington, Seattle, WA) (22) coupled to CNBr-Sepharose. Factor IX was activated by human factor XI_a and factor X was activated by the protease from Russell's viper venom (23). In each case the activating enzyme was covalently linked to CH-Sepharose. Human factor XI_a was generously provided by P. Bajaj (University of California, San Diego), human α-thrombin was a gift of J. Fenton (New York State Department of Health, Albany), and purified human fibrinogen (24) was provided by J. Koehn of this department. Bovine prothrombin (1300 u/mg) and α-thrombin (2.5 NIH u/μg) were prepared as described (25, 26). Bovine factor VIII (factor $VIII_c$/von Willebrand factor) was prepared by the method of Newman *et al.* (27). It was then chromatographed on an antifibrinogen and antifibronectin affinity column. The final material was homogenous on reduced 5% $NaDodSO_4$/PAGE, showing one band with a molecular weight about 200,000 corresponding to the von Willebrand factor. The factor VIII preparations used in this work had a protein concentration of 1.1 mg/ml and a factor VIII coagulant activity of 70 u/ml. Factor VIII coagulant activity in this preparation was stable over 6 months of storage at −80°C, and no other coagulant activities (including factors VII, IX, X, XI, XII) were detected by clotting assay. This factor VIII preparation was 30- to 35-fold activatable by bovine α-thrombin [factor VIII (10 u/ml), thrombin (0.01 u/ml)].

Burro anti-bovine factor V IgG (28) and normal burro serum were generously provided by P. Tracy and K. Mann (Mayo Clinic, Rochester, MN). Normal burro IgG was prepared as described (29). Rabbit anti-bovine tissue factor IgG (30) was generously provided by R. Bach (Mt. Sinai School of Medicine, New York), and normal rabbit IgG was prepared by protein A-Sepharose CL-4B (31) (Pharmacia). Serum from a patient with an acquired antibody to factor V was generously provided by H. Glueck (Veteran's Administration, Cincinnati, OH) (32). Normal human IgG and anti-human factor V IgG were also prepared by using protein A-Sepharose CL-4B (31). Protein concentrations were determined colorimetrically (33), and concentrations of activated factors were determined by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (34).

Preparation of Endothelial Cells and Platelets. Bovine aortic endothelial cells (BAEC) were obtained from 3-month-old calves and grown in culture as described (11). They were characterized by the presence of von Willebrand factor antigen (35) and angiotensin-converting enzyme activity (36). All assays were carried out 24 hr after the cells reached confluence by washing the monolayer three times with calcium/magnesium-free Hanks' balanced salt solution and incubating the cells for 1 hr at 37°C with Dulbecco's modified Eagle's medium. Monolayers were then washed three more times with Hanks' balanced salt solution and incubation buffer [10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and 5 mg of bovine serum albumin (fatty acid free) per ml] was added.

Endothelial cells were treated with endotoxin by incubating them for 8 hr at 37°C with a phenolic extract of *Escherichia coli* serotype O26:B6 (Sigma) at 100 ng/ml in Dulbecco's modified Eagle's medium without serum. After endotoxin treatment, endothelial cell viability was about 85% and monolayers were prepared for activation studies identically to untreated cells.

For experiments using platelets, blood was drawn from healthy volunteers who had not taken any medication for 2 weeks by the protocol described by Van Rijn *et al.* (37). Venepuncture was carried out and 100 ml of blood was collected into 20 ml of acid/citrate/dextrose (52 mM citric acid/80 mM trisodium citrate/180 mM glucose). Platelet-rich plasma was obtained after centrifugation at 220 × *g* for 20 min and platelets were pelleted by centrifugation (1000 × *g* for 20 min). The platelet pellet was resuspended in buffer containing 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes (pH 6.7), 5 mM glucose, and 0.05% human serum albumin (fatty acid free). Platelets were washed three times in this buffer by adding 1/14th volume of acid/citrate/dextrose and centrifuging at 600 × *g* for 20 min. Finally, the platelets were resuspended in the above Hepes buffer with the pH adjusted to 7.5 and at a final concentration of 8×10^6 platelets per mm^3. Platelet preparations were carried out at room temperature over 90 min and used immediately. These platelet suspensions showed only minimal clumping by phase microscopy and did not support significant factor X activation in the presence of factors IX_a and VIII. After incubation with human α-thrombin (1.0 u/ml for 2–3 min at 37°C), the activated platelets readily supported factor X activation.

Activation Studies. Activation studies were carried out at room temperature by adding purified proteins to monolayers equilibrated previously with incubation buffer for 5 min. Factor X_a formation was determined from aliquots (0.1 ml) of the reaction mixture removed from contact with the cells at timed intervals and added to 0.5 ml of 50 mM Tris (pH 7.9), 175 mM NaCl, 10 mM EDTA, and 0.5 mg of ovalbumin per ml. Factor X_a activity was determined as described (11) by adding the entire 0.6-ml sample to a cuvette along with 0.1 ml of Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222; Helena Laboratories, Beaumont, TX) at 1.0 mM. Samples for determination of thrombin activity were obtained similarly and were as-

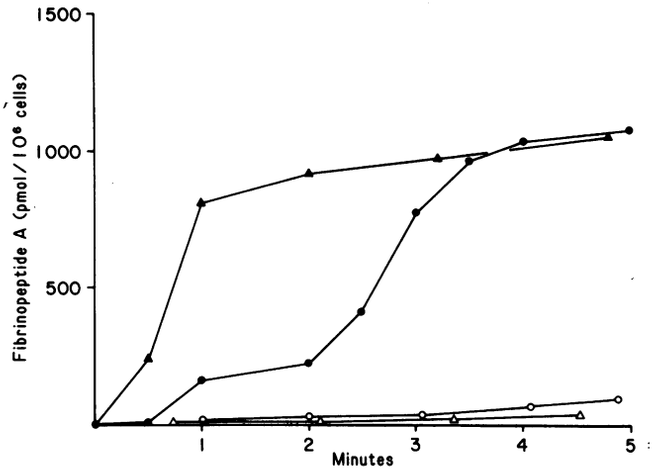

FIG. 1. Cleavage of fibrinogen with release of fibrinopeptide A by endothelial cell-dependent procoagulant reactions. Monolayers of BAEC (P_4; 1.3×10^6 cells) were incubated with 1.0 ml of incubation buffer and factors XI_a (4 nM), IX (88 nM), VIII (0.75 u/ml), X (181 nM), prothrombin (1.4 μM), and fibrinogen (2.3 μM), each in a volume of 5–12 μl (●). A solid fibrin clot formed after 183 sec. Endothelial cells were preincubated with burro anti-bovine factor V IgG (200 μg/ml) for 30 min and then the above mixture of coagulation proteins was added (○). Control burro IgG (200 μg/ml) did not have an effect on fibrinopeptide A generation. Endotoxin-treated endothelial cells (P_4; 1.0×10^6 cells) were incubated with 1.0 ml of incubation buffer containing factor VII_a (2 nM) and factors VIII, IX, X, prothrombin, and fibrinogen as above (▲). A solid fibrin clot formed after 54 sec. Endotoxin-treated endothelial cells were preincubated with rabbit anti-bovine tissue factor IgG (150 μg/ml) for 40 min and then factors VII_a, VIII, IX, X, prothrombin, and fibrinogen were added as above (△). Control rabbit IgG did not have an effect on fibrinopeptide A generation. When factor VII_a replaced factor XI_a in the reaction mixture with control BAEC, no significant fibrinopeptide A cleavage occurred after 10 min. In each case, 0.1 ml of the reaction mixture supernatant was withdrawn at the indicated time and processed in the fibrinopeptide A assay. The mean of duplicates is plotted.

sayed by the same protocol using D-Phe-Pip-Arg-*p*-nitroanilide (where Pip = pipecolyl) (S2238; Helena Laboratories) at 0.3 mM. Factor X_a and thrombin concentrations were determined from the linear portions of standard curves in which known amounts of factor X_a or thrombin were assayed under conditions identical to the experimental samples.

When fibrinogen was included in the reaction mixtures and fibrinopeptide A cleavage was determined, 0.1-ml samples were removed from contact with the monolayer at fixed times and added to 0.3 ml of ethanol at 4°C. Ethanol precipitation and fibrinopeptide A assay were then carried out by a radioimmunoassay (38).

Scanning Electron Microscopy. Samples were fixed in 3% glutaraldehyde (in 0.15 M sodium cacodylate buffer) and subjected to osmication, staining with uranyl acetate, dehydration, and critical point drying. They were then sputter-coated with gold palladium and viewed in an AMRAY-1600 scanning electron microscope.

RESULTS AND DISCUSSION

Cultured BAEC in the presence of factors XI_a, VIII, IX, X, prothrombin, and fibrinogen can promote a cell-dependent procoagulant pathway leading to fibrinopeptide A generation (Fig. 1) and fibrin formation (Fig. 2). In experiments using quiescent endothelial cells, the coagulation system was initiated by the addition of factor XI_a. In the absence of factor VIII, IX, X, prothrombin, or fibrinogen, little or no generation of fibrinopeptide A occurred. Also, the interaction of coagulation proteins with specific cellular binding sites on the endothelium was essential since in the absence of endothelial cells no fibrinogen cleavage resulted. When endothelial monolayers were preincubated with antibody to bovine factor V, subsequent fibrinopeptide A generation was blocked by >90%. This indicates that the endothelial cells were providing a factor V-like activity required for assembly of the prothrombinase complex (13, 15, 16). No exogenous source of phospholipid, such as the platelet, was necessary for factor X and prothrombin activation. The importance of continuous contact between endothelium and the coagulation factors was observed when aliquots of the reaction mixture supernatant were withdrawn from the dish with cells and then incubated in polystyrene test tubes. Under these conditions no additional factor X_a or thrombin activity was generated, as determined by chromogenic substrate assays. Finally, if endothelial cells were preincubated with factors IX and VIII for 5 min, washed to remove unbound protein, and then incubated with factors XI_a, X, prothrombin, and fibrinogen, the same amount of fibrinopeptide A formed as when monolayers were not washed. This indicates that only endothelial cell-associated factors IX and VIII participated in these reactions. Similar results concerning fibrinopeptide A formation were observed by using bovine aortic segments with a continuous layer of endothelium.

When endothelial cells treated with endotoxin were incubated with factors VII_a, IX, VIII, X, prothrombin, and fibrinogen, fibrinopeptide A generation occurred (Fig. 1). Endotoxin-treated endothelial cells have induced tissue factor activity (14, 17, 18) and the central role of this cofactor in starting the series of reactions leading to fibrinogen cleavage was demonstrated by the >90% inhibition of fibrinopeptide A generation in the presence of antibody to bovine tissue factor. Fibrin clot formation occurred when coagulation was initiated by factor VII_a in the presence of perturbed endothelial cells or by factor XI_a in the presence of quiescent endothelial cells. The electron micrograph (Fig. 2) demonstrated that the

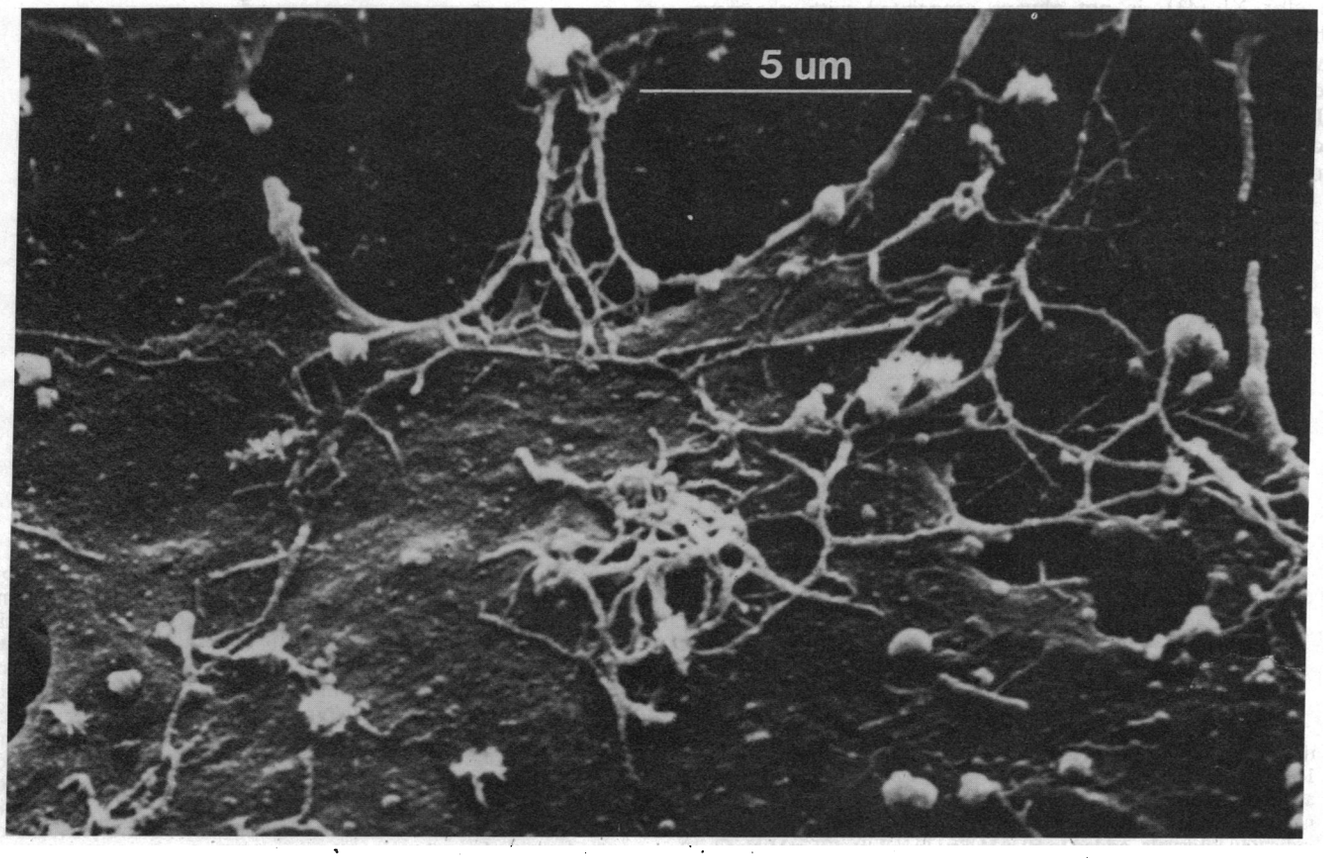

FIG. 2. Fibrin clot on endothelial cells. BAEC (P_3; 1.2×10^6 cells) were incubated with 1.0 ml of incubation buffer and factors XI_a, IX, VIII, X, prothrombin, and fibrinogen. When the first definite fibrin strands were seen, monolayers were washed four times with albumin-free incubation buffer and fixed for scanning electron microscopy in 3% glutaraldehyde in 0.15 M sodium cacodylate buffer.

fibrin strands formed were closely associated with the endothelial cells. The endothelial cells also appeared retracted, as exemplified by peripheral exposure of the underlying culture dish in contrast to the initially confluent monolayer. This is in agreement with previous studies demonstrating retraction of endothelial cells after formation of fibrin clots by thrombin (39). Interruption of the continuity of the monolayer with subsequent exposure of subendothelium may enhance the thrombogenic nature of a perturbed area of the vessel wall. The fibrin strands appear to originate and focally concentrate on the luminal side of the cells. This was not seen on exposed areas of the culture dish.

Previous studies from our laboratory have demonstrated factor VII_a activation of cell-bound factor IX on perturbed endothelial cells with induced tissue factor activity but not on quiescent endothelial cells (14). This suggested a possible sequence of hemostatic events on the endothelial cell surface starting with factor VII_a activation of factor IX and proceeding with factor IX_a activation of factor X in the presence of factor VIII. Further studies examining the role of factor IX in factor X activation in the presence of endothelial cells have been carried out (Fig. 3). In the presence of factors IX and VIII, factor X activation by factor VII_a with endotoxin-treated endothelial cells was considerably greater than in their absence. Preincubation of endothelial cells with rabbit anti-bovine tissue factor IgG (165 μg/ml) blocked factor X_a formation by >90%, whereas control IgG (171 μg/ml) had no significant effect regardless of the presence of factors IX and VIII. This indicates that the tissue factor VII_a complex was the activator of factors IX and X.

The tissue factor-initiated pathway of coagulation may well predominate *in vivo* for two reasons. First, the alternative activator of factor IX is factor XI_a, but severe deficiency of factor XI (40), in the absence of an acquired antibody to factor XI (41), is not always associated with a serious bleeding diathesis. Second, subendothelial layers of the vessel wall, fibroblasts, and smooth muscle cells as well as endothelial cells after perturbation all express tissue factor. Previous investigators (42, 43) have employed rather high concentrations of tissue factor to activate factor IX, and under these conditions factor X activation by factor VII_a readily occurs in the absence of factors VIII and IX. Under physiological conditions, however, these factors are essential for normal hemostasis and their deficiency results in the hemophilias, which are the most frequent severe congenital bleeding disorders. In order to examine this question in more detail, factor X activation by factor VII_a and endotoxin-treated endothelial cells was studied in the presence of factors VIII and IX. In the presence of factors VIII and IX, factor X activation occurred after a short lag phase (Fig. 3), whereas in their absence, the activation of factor X was limited. These experiments demonstrate that on the perturbed endothelial cell, which has acquired tissue factor activity, factors VIII and IX play an important role in factor X activation.

The interaction of the endothelial cell-dependent procoagulant pathway with the platelet was examined by adding unstimulated platelets to cell monolayers (Fig. 4). For these experiments bovine endothelial cells were incubated with factors IX_a and VIII and washed, and factor X and prothrombin were added then in the presence of human platelets. Platelets increased thrombin formation by about 15-fold. Similar results were observed with bovine platelets (data not shown). The inhibition of thrombin formation by an antibody to human factor V indicates that the platelet effect was due in large part to release of their endogenous factor V activity promoting rapid prothrombin activation by factor X_a. In the absence of platelets, anti-human factor V IgG decreased thrombin formation by endothelial cells only mini-

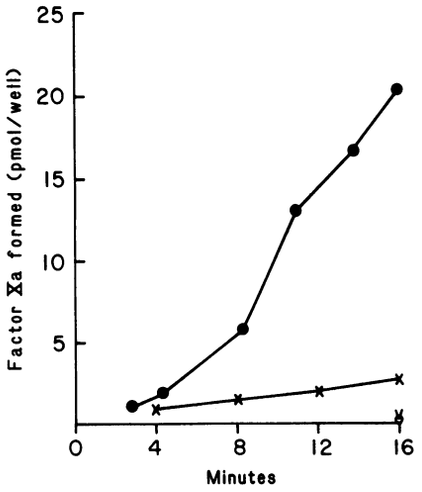

FIG. 3. Endotoxin-treated endothelial cells and factor X activation. Monolayers of endotoxin-treated BAEC (P_6; 1.1 × 10^6 cells) in 1.0 ml of incubation buffer were incubated with factors VII_a (1.8 nM) and X (182 nM) in the presence (●) or absence (×) of factors IX (68 nM) and VIII (1.1 u/ml). Aliquots of 0.1 ml were removed at the indicated intervals, added to 50 mM Tris (pH 7.9), 175 mM NaCl, 10 mM EDTA, and 0.5 mg of ovalbumin per ml, and assayed in the S2222 chromogenic substrate assay. The mean of duplicates is plotted and the experiment was repeated four times.

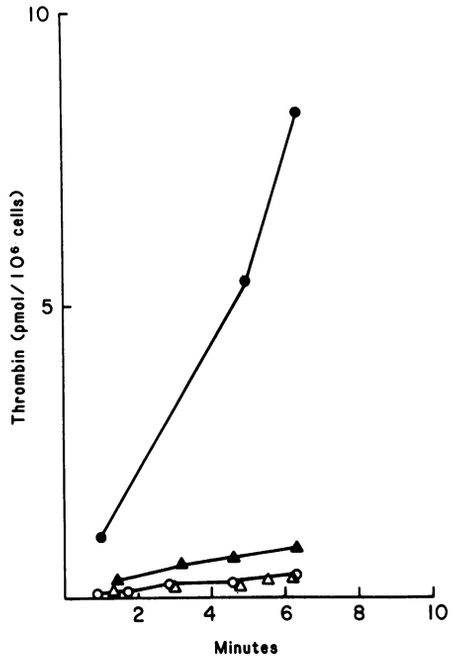

FIG. 4. Effect of platelets on the thrombin formation by endothelial cells. Monolayers of BAEC (P_6; 1.3 × 10^6 cells) in 1 ml of incubation buffer were incubated with factors IX_a (0.9 nM), VIII (1.2 u/ml), X (175 nM), and prothrombin (1.5 μM) in the presence (●) or absence (○) of 1.1 × 10^8 platelets per ml. Another set of monolayers was incubated with the same coagulation proteins and anti-human factor V IgG (100 μg/ml) in the presence (▲) or absence (△) of platelets. Addition of normal human IgG (100 μg/ml) had no effect on prothrombin activation in the presence or absence of platelets. Addition of human antifactor V IgG (100 μg/ml) did not effect factor X activation in the presence or absence of platelets in contrast to its effect on thrombin formation in the presence of platelets (▲). In each case, aliquots of 0.1 ml were removed at the indicated times and assayed in the chromogenic substrate assay. The mean of duplicates is plotted and the experiment was repeated four times.

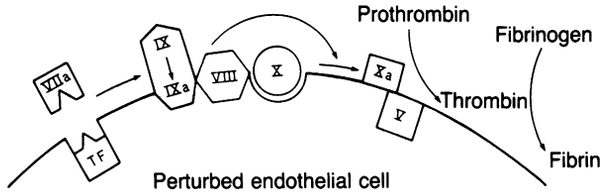

FIG. 5. Schematic depiction of the endothelial cell procoagulant pathway on the surface of a perturbed endothelial cell. TF, tissue factor.

mally. These experiments indicate that the antifactor V antibody blocked the activity of human factor V from platelets more efficiently than endothelial cell bovine factor V (16). The source of factor X_a in these experiments was probably the endothelial cell-dependent pathway since unstimulated platelets, in the absence of endothelial cells, did not promote significant factor IX_a–VIII-catalyzed factor X activation. Thus, endothelial cells can initiate a procoagulant pathway that results in platelet activation, recruiting them to augment the procoagulant response.

These data provide strong evidence to support the concept that endothelial cells actively initiate and propagate procoagulant reactions. When coagulation is initiated with factor XI_a, quiescent endothelial cells can readily propagate coagulation reactions. These reactions may well be involved in the generation of the baseline levels of thrombin and fibrinopeptide A measured *in vivo* (44, 45). Although platelets greatly augment thrombin formation by endothelial cell-dependent reactions, endothelial cells alone can initiate formation of a fibrin clot. The endotoxin-perturbed endothelial cell provides a model of the thrombotic state involving factor VII_a (Fig. 5). These perturbed endothelial cells have induced tissue factor activity and in the presence of factor VII_a they are capable of initiating a series of reactions in which factor VII_a activates cell-bound factor IX. Cell-associated factors IX_a and VIII can then activate factor X and the factor X_a formed interacts with endothelial cell factor V, promoting thrombin formation. Finally, thrombin cleaves fibrinogen, resulting in a fibrin clot. This model provides a simple, endothelial cell-dependent mechanism for initiation of coagulation at the site of an injured or pathological vessel wall. Further experiments are necessary, however, to clarify the mechanism of activation of factor XI and expression of tissue-factor activity that triggers these endothelial cell-dependent coagulation pathways.

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