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A Pathway of Coagulation on Endothelial Cells

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Although the endothelial cell is considered antithrombogenic, endothelium has recently been shown to participate in procoagulant reactions. Factor IX bound to specific endothelial cell sites can be activated by the intrinsic and extrinsic pathways of coagulation. Perturbation of endothelium results in induction of tissue factor which promotes factor VII_a-mediated activation of factors IX and X, thus initiating procoagulant events on the endothelial surface. Cell bound factor IX_a, in the presence of factor VIII, promotes activation of factor X. The factor X_a formed can interact with endothelial cell factor V/V_a, resulting in prothrombin activation. Thrombin then cleaves fibrinogen and a fibrin clot closely associated with the endothelial cell forms. The perturbed endothelial cell thus provides a focus of localized procoagulant events. This model suggests a simple endothelial-cell-dependent mechanism for initiation of coagulation at the site of an injured or pathological vessel.

Key words: coagulation, endothelial cell, thrombosis

As the cells forming the luminal vascular surface, endothelial cells are strategically located to play an important role in the regulation of coagulation. 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 extra-vascular tissues. Recent studies, however, have indicated that rather than forming an inert surface, the endothelial cell can play an active role in preventing activation of the coagulation system. [1-5]. Anticoagulant heparinlike molecules have been localized to the vessel surface [1]. Antithrombin III bound to this heparinlike material on the endothelial cell surface demonstrates enhanced inactivation of proteases [2]. Thrombomodulin, another endothelial cell-surface molecule, is a cofactor for activation of the protein C anticoagulant pathway [3]. Plasminogen activators, which promote clot lysis [4], and prostacyclin, which decreases platelet reactivity [5], are also components of 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

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endothelial cells in procoagulant reactions has received more attention. Aortic endothelium, both cultured and native, can bind factors IX and IX_a [6–8]. Cell-bound factor IX can be activated by both intrinsic and extrinsic pathways of coagulation, and cell-bound factor IX_a in the presence of factor VIII can activate factor X [9]. Factor X_a incubated with endothelial cells can activate prothrombin [8,10]. This reaction is dependent on endogenous factor V [11] expressed by endothelium and can be blocked by pre-incubating the cells with antifactor V antibody [8,10]. Perturbation of endothelial cells by agents such as endotoxin results in induction of tissue factor activity [9,12–15]. These studies suggest a new role of the endothelial cell in initiating, promoting, and localizing procoagulant events.

This report will review studies concerning the interaction of factors IX, IX_a, X, and X_a with endothelium. These findings prompt the hypothesis that a complete procoagulant pathway can occur on the endothelial cell surface.

FACTORS IX AND IX_a AND ENDOTHELIUM

Our studies of coagulation factor-endothelial cell interaction began with factor IX. Although factor IX, along with factor VIII, has a crucial role in normal hemostatis [16], questions concerning the activation of the zymogen and the role of factor IX_a in the physiology of coagulation remain. The classical cascade pictures factors VIII and IX as part of the intrinsic activation system, suggesting that factor XI_a is an important activator of factor IX [17,18]. However, patients with severe factor XI deficiency and no clinical hemostatic consequences are well known [19]. Factor IX can also be activated by the tissue factor pathway [20], though factor X activation is considerably more efficient [21,22]. This suggests that direct factor X activation by tissue-factor VII_a should provide a physiologic bypass of factor IX_a-VIII-catalyzed factor X activation. This does not explain why deficiency of factor VIII or factor IX leads to such a severe bleeding diatheses and has resulted in a dichotomy between in vitro observations and clinical findings. Prompted by these questions, and knowing that factor IX has a central role in coagulation, we set out to determine if the study of factor IX-vessel wall interaction might provide some insights. At the beginning of this work no cellular sites localizing factor IX were known. A cellular site selectively localizing factor IX_a, the enzyme, would provide a powerful focus of procoagulant events. There were two pieces of evidence, however, which suggested the possibility of factor IX/IX_a-vessel wall interaction. First, factor IX infusion studies demonstrated an initial rapid clearance phase consistent with the possibility of intravascular binding sites [23–25]. Second, in the Wessler stasis model, an in vivo model of thrombogenesis [26], factor IX_a was the most potent coagulation factor as a thrombotic agent [27].

Radioligand binding studies carried out with purifed, radio-labelled factors IX and IX_a, and native bovine aortic endothelium, indicated the presence of specific and time-dependent binding [6–8]. Experiments in which increasing concentrations of radiolabelled factor IX or factor IX_a were incubated with the native endothelium of vessel segments demonstrated that binding was saturable, with half-maximal binding at 3–5 nM (Fig. 1). This is considerably below the plasma level of factor IX (70–100 nM), suggesting these sites should be saturated under normal conditions. Consistent with this hypothesis, experiments employing bovine aortic vessel segments have indicated elution of endogenously bound factor IX [8]. The levels of factor IX present

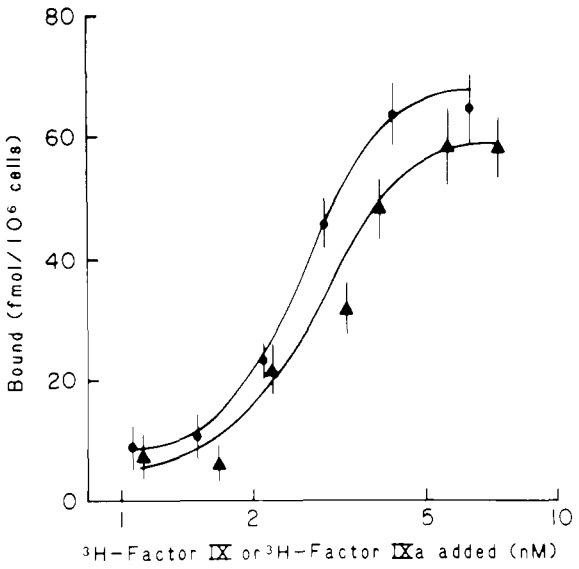

Fig. 1. Binding of radiolabelled factors IX and IX_a to bovine aortic vessel segments with a continuous layer of endothelium (0.79 cm^2 wells). Semilogarithmic plot in which specifically bound ligand is plotted versus the concentration of added ligand. Binding assays were carried out at room temperature employing a 30-min incubation time, as described [8]. The mean ± 1 SD is shown and the experiments were each repeated four times. ^{3}H-factor IX (●) and ^{3}H-factor IX_a (▲).

in the plasma of patients with factor IX deficiency and a serious bleeding diathesis would result in decreased occupancy of these sites. The data in Figure 1 indicate that the binding of the enzyme, factor IX_a, was comparable to that of the zymogen. Competitive binding studies have shown that factors IX and IX_a are equipotent competitors for the same sites [7]. However, other vitamin-K-dependent coagulation factors, including factors X and XII, prothrombin, and protein C, each present at a hundred-fold molar excess, did not inhibit factor-IX-endothelial cell binding (Fig. 2). Thus, although initially it seemed likely that the observed factor IX/IX_a-endothelial cell interaction represented interaction of factor IX with phospholipid (using synthetic phospholipids the dissociation constant is 2 μM [28]), the nanomolar affinity constant and specificity of factor IX binding suggest that more than phospholipid is involved. This hypothesis is supported by preliminary experiments demonstrating loss of specific factor IX binding sites after gentle treatment of endothelial cells with trypsin (1 mg/ml for 5 min at room temperature) (unpublished observation).

To complement these radoligand studies, factor IX-endothelial cell interaction was also studied, using factor IX labelled with colloidal gold (Fig. 3). The factor IX-colloidal gold conjugates [29] were distributed evenly over all the endothelial cells, closely apposed to the luminal surface. Thus, factor IX is associated with the endothelial cell membrane.

The demonstration of specific endothelial cell binding sites for factor IX was one step suggesting a coagulation factor can be localized to the vessel wall. The crucial issue in these binding studies, however, conerns the effect of surface binding on the coagulant properties of the proteins. Factor IX_a bound to the endothelial cell

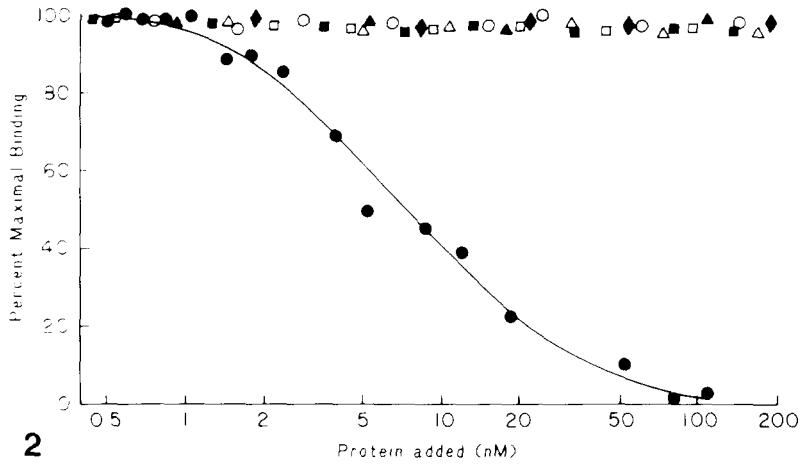

Fig. 2. Inhibition of ^{3}H-factor IX binding to native endothelium by unlabelled factors IX_a and X, prothrombin, and protein C. Aortic segments (0.79−cm^2 wells) were incubated with ^{3}H-factor IX (3 nM) alone or with increasing concentrations of unlabelled factor IX (●), prothrombin (△), factor X (▲), protein C (■), protein S (□), factor VII (○), or protein Z (♦) for 30 min at 21°C. Maximal specific binding was defined as the difference between binding in wells incubated with tracer alone and binding observed in the presence of unlabelled factor IX (1,500 nM). Nonspecific binding was 22% of the total binding. The mean of duplicates is plotted and the experiment was repeated twice.

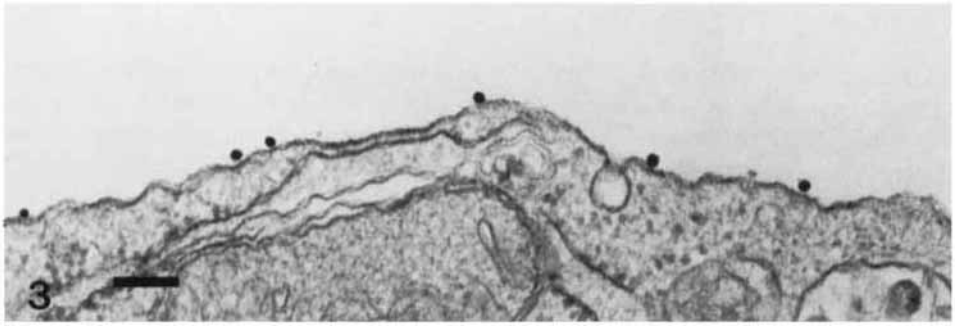

Fig. 3. Binding of factor IX-gold conjugates to bovine aortic endothelial cells. Cultured monolayers of endothelium (1.1×10^6 cells) were incubated for 2 hr at 4°C with factor IX bound to colloidal gold conjugates [28]. Gold probes are seen randomly distributed at the membrane surface. Marker bar: 0.1 nm. Addition of a 100-fold molar excess of free factor IX displaced 90% of the bound gold particles.

promotes the activation of factor X in the presence of factor VIII (Fig. 4A). This reaction is dependent on continuous contact between the reaction mixture and the endothelial cells but not on the presence of exogenous phospholipid. Removal of an aliquot of reaction mixture supernatant, followed by incubation in a test tube, resulted in no further factor X_a formation. Although cell-bound factor IX_a can promote factor X activation, there is a central problem with the concept that a shared factor IX/IX_a binding site could play a role in factor X_a formation. The problem arises from the observation that it is unlikely that the concentration of factor IX_a approaches that of

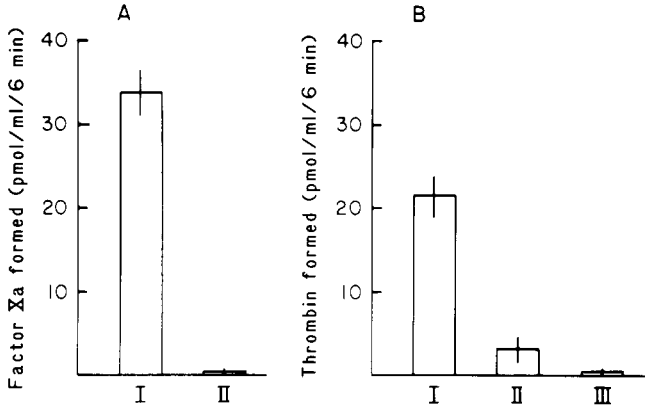

Fig. 4. Activation of factor X and prothrombin in the presence of endothelial cells. A) Factor X activation: factors IX_a (0.22 pmol/ml), VIII (2.4 U/ml), and X (300 pmol/ml) were mixed and incubated over cultured bovine aortic endothelial cells (I) or alone, in the absence of cells (II). At 6 min, an aliquot of reaction mixture was removed and assayed for factor X_a amidolytic activity using the Bz-Ile-Glu-Gly-Arg-pNA synthetic substrate assay [45]. B) Prothrombin activation: factors X_a (0.4 pmol/ml) and prothrombin (1.2 nmol/ml) were mixed and incubated over cultured endothelial cell monolayers (I) or alone (III). In II, the monolayer was pre-incubated for 45 min at 37°C with antibovine factor V IgG (200 μg/ml) (generously provided by Drs. Tracey and Mann). At 6 min an aliquot of each reaction mixture was withdrawn and assayed for thrombin amidolytic activity using the H-D-Phe-pip-Arg-pNA synthetic substrate assay [46]. The mean and ± 1 SD are shown.

the zymogen under physiologic conditions. This would suggest that factor IX_a-endothelial cell interaction would never occur, unless there was a specific factor IX_a binding site. The first experiments suggesting the presence of a class of selective factor IX_a-endothelial cell site were kinetic studies of factor X activation. Although factor IX_a-endothelial cell binding was half-maximal at 3–5 nM (Fig. 1), the initial rate of factor X_a formation (in the presence of saturating amounts of factors VIII and X) was half-maximal at about 130 pM [30]. Furthermore, factor IX was not an effective inhibitor of factor X activation whereas active site-blocked factor IX_a (dansyl-glu-gly-arg-factor IX_a) [31] was a potent inhibitor with K_i of 140 pM [30].

This data indicates that endothelial cells can bind factors IX and IX_a with equal affinity in the absence of other coagulation factors. When factors VIII and X are added, however, there is a selective factor IX_a-endothelial cell interaction. Initially it seemed possible that the selective factor IX_a cellular binding site might be due to formation of a factor IX_a-$VIII_a$ complex on the cell surface. This would be analogous to the binding of factors X_a and V_a to the platelet surface [32–34]. Recent studies, though, have demonstrated that factor X is required for induction of the specific factor IX_a site even after the factor VIII is activated [30]. This would suggest a model in which the substrate enhances the affinity of an enzyme-cell surface interaction. An ordered addition model similar to this in which the substrate enhances the affinity of factor VII_a for tissue factor has been proposed and studied extensively for the extrinsic pathway [35]. This promotes a view of coagulation in which formation of complexes on cellular surfaces rather than a cascadelike activation of single coagulation factors in the fluid phase are the crucial steps. Supporting this view, factor IX_a in its complex with factors VIII and X effectively catalyzes factor X activation.

At this point in our studies it seemed possible that the intrinsic factor X activation complex ("Tenase") could assemble on the endothelial cell surface. Therefore, cell-bound factor IX_a, in its selective cellular binding site including factors VIII and X, could potentially function as a vessel-localized focal point for promoting activation of the coagulation system. Our central question concerned whether formation of the selective factor IX_a binding site on endothelial cells was one component of a series of coagulation factor-vessel wall interactions. This prompted us to examine the interaction of factor X and its activated form with endothelium.

FACTORS X AND X_a AND ENDOTHELIUM

Factor X also binds to endothelium in a saturable manner [6,9]. In contrast to factor IX, factor X binding is of considerably lower affinity, being half-maximal at 300 nM, which is slightly above the plasma concentration. This is comparable to the observed dissociation constant, 0.25 μM, reported by Nelsestuen et al [28] for factor X-phospholipid binding. This suggested that factor X-endothelial cell binding might be in part mediated by the interaction of endothelial cell sites with the γ-carboxyglutamic acid residues of factor X. Consistent with this hypothesis, modified factor X from which the γ-carboxyglutamic acid residues had been selectively removed [35,36,37], (Gla-domainless-factor X) did not bind in a comparable fashion to endothelium. Although this suggests that cellular binding of factor X may be due to γ-carboxyglutamic acid interaction with phospholipid in the membrane, factor X binding was specific since neither factors IX and IX_a, nor prothrombin, nor protein C, were competitive inhibitors [6,9]. This result implies that other domains of the factor X molecule besides the γ-carboxyglutamic-acid-containing domain, might be involved in the binding. Alternatively, the factor X cellular binding site might be composed of more than phospholipid, accounting for its specificity for the factor X molecule among the vitamin-K-dependent coagulation factors. From our studies it remains unclear if this is also the binding site for factor X required for the formation of the selective factor IX_a binding site.

When factor X_a and prothrombin are incubated with endothelial cells, thrombin formation occurs (Fig. 4B). No exogenous factor V/V_a or phospholipid is required. This was first observed by Rodgers and Shuman using nonconfluent cultured aortic endothelial cells [10], and we have observed it with native endothelium as well [8]. Prothrombin activation is dependent on an endothelial cell-dependent factor V-like molecule, as indicated by inhibition when the cells are pre-incubated with an antibody to factor V (Fig. 4B). Recently, synthesis of factor V by cultured endothelial cells has been reported by Cerveny and colleagues [11]. These observations suggested that factor X_a-endothelial cell interaction would consist of factor X_a binding to endothelial cell factor V/V_a, analogous to that described on the platelet surface [32–34]. Studies of factor X_a binding to endothelium [8,38] have indicated that the situation is considerably more complex. Using the native endothelium of bovine aortic segments, factor X_a binding was seen to consist of at least two components [8]. A rapid phase of reversible binding (K_d = 1 nM) was followed by irreversible binding which involved formation of a covalent bond between factor X_a and a cell-associated protein. Factor X_a irreversibly bound to the endothelial cell surface had no coagulant activity. These protease nexinlike sites [39] were on the surface of native endothelium.

The demonstration of factor X and prothrombin activation on the endothelial cell surface suggested the possiblity of an entire procoagulant pathway. To test this hypothesis, endothelial cells were incubated with factors IX_a, VIII, and X, prothrombin, and fibrinogen. Clot formation occurred (Fig. 5) and was dependent on the presence of endothelium. The electron micrograph demonstrates that the fibrin strands formed are closely associated with the endothelial cells. 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. 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 [40] demonstrating retraction of endothelial cells after formation of fibrin clots by thrombin. Procoagulant events on the endothelial cell surface can thus interrupt the continuity of the monolayer with subsequent exposure of subendothelium, a substrate for platelet deposition. This perturbed area of the vessel wall could potentially provide a focus of thrombotic events.

PERTURBATION OF ENDOTHELIAL CELLS

The experiments discussed thus far indicate that once factor IX_a and/or factor X_a is formed, endothelial cells can support assembly of coagulation factors in a

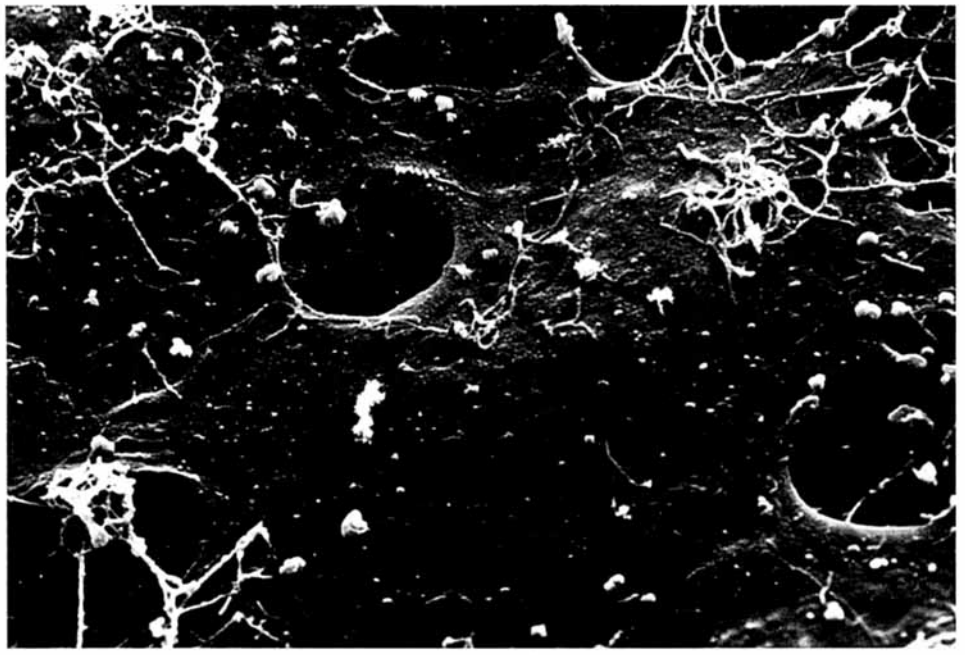

Fig. 5. Fibrin clot on endothelial cells. Bovine aortic endothelial cells (1.2×10^6 cells) were incubated with 1.0 ml incubation buffer and factors XI_a, IX, VIII, and X, prothrombin, and fibrinogen. When the first definite fibrin strands were seen, monolayers were washed four times with incubation buffer (HEPES balanced salt solution) and fixed for scanning electron microscopy using 3% glutaraldehyde in 0.15 M sodium cacodylate buffer as described [47]. Fibrin strands are seen at the cell surface. Marker bar = 5.0 nm.

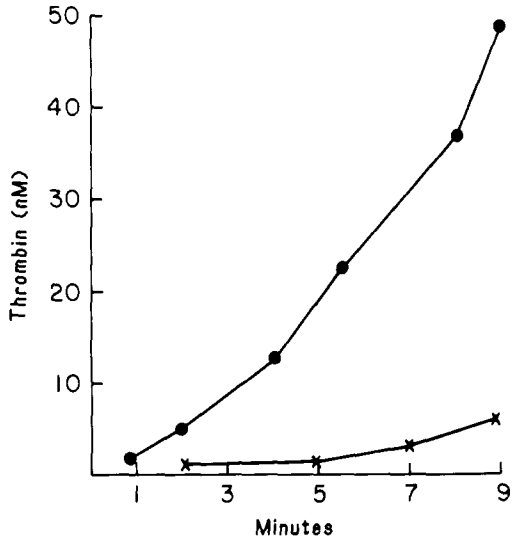

Fig. 6. Endotoxin-treated endothelial cells and factor X activation. Bovine aortic endothelial cells were incubated for 14 hr with endotoxin (100 ng/ml). Monolayers were washed and incubated with factors VII_a (1.8 nM) and X (182 nM) in the presence (●) or absence (X) of factors IX (68 nM) and VIII (1.1 U/ml). Aliquots of reaction mixture were removed at the indicated intervals, added to 50 mM tris (pH 7.9), 175 mM NaCl, 10 mM EDTA, 0.5 mg/ml ovalbumin, and assayed in the Bz-Ile-Glu-Gly-Arg-pNA chromogenic substrate assay as described [42,45]. The mean of duplicates is plotted.

manner which promotes propagation of the reactions leading to thrombin formation. The next question concerned initiation of coagulation by endothelium. Initiation of coagulation by cells is generally thought to involve exposure of tissue factor on a cellular surface [35]. Although tissue factor is an ubiquitous component of a large variety of cells [41], there are only small amounts of tissue factor expressed in quiescent endothelium. Recently, induction of significant tissue factor activity in cultured endothelium has been demonstrated by multiple agents including endotoxin, phorbol ester, and interleukin I [12–15]. Tissue factor is then available on the cell surface to promote activation of factors IX and X in the presence of factor VII_a. 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 [9]. 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-VIII activation of factor X. Further studies [42] examining the role of factor IX/IX_a in factor X activation on the perturbed endothelial cell surface have been carried out. Endotoxin-treated endothelial cells incubated with factor VII_a formed ten times more factor X_a in the presence of factors VIII and IX than in their absence (Fig. 6). Pre-incubation of endothelial cells with rabbit anti-bovine tissue factor IgG (165 μg/ml) (generously provided by Drs. Bach and Nemerson) [43] 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-factor VII_a complex was the activator of factors IX and X. Furthermore, on the perturbed endothelial cell surface, factors VIII and IX do play an important role in factor X activation by the tissue factor pathway. One might

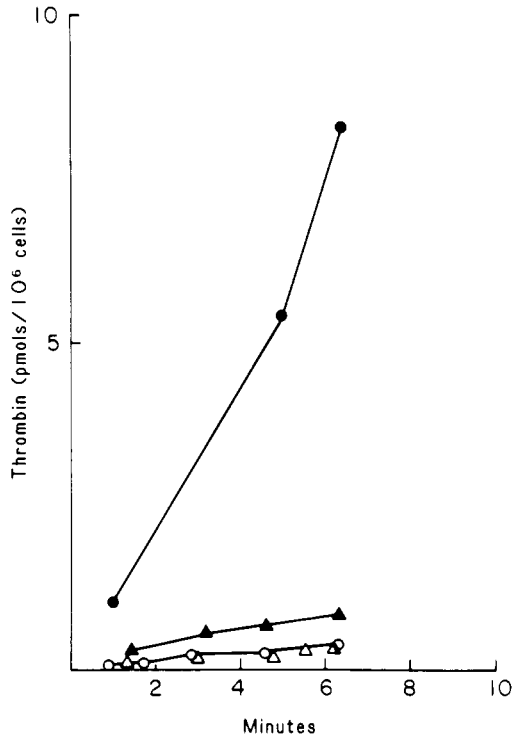

Fig. 7. Effect of platelets on the thrombin-formation by endothelial cells. Monolayers of bovine aortic endothelial cells (1.3×10^6 cells) in 1 ml of incubation buffer (HEPES buffered salt solution) were incubated with factors IX_a (0.9 nM), VIII (1.2 U/ml) and X (175 nM), and prothrombin (1.5 μM) in the presence of (●) or absence (○) of 1.1×10^8 platelets/ml. Another set of monolayers was incubated with the same coagulation proteins and anti-human factor V IgG (100 μg/ml) (generously provided by Dr. H. Glueck) in the presence of (▲) 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 anti-factor V IgG (100 μg/ml) did not affect 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.

speculate that on the perturbed endothelial cell surface either factor VII_a activation of factor IX is favored or, alternatively, the factor IX_a-VIII-X complex effectively promotes activation of factor X.

THE ROLE OF PLATELETS IN THE AMPLIFICATION OF THE ENDOTHELIAL CELL PROCOAGULANT PATHWAY

The presence of multiple inhibitory mechanisms preventing activation of coagulation on endothelium has been extensively studied. As briefly outlined in the beginning of this paper these mechanisms are diverse, ranging from enhancement of antithrombin III action by anticoagulant heparinlike molecules [1,2] to elaboration of prostacyclin [48,5]. This indicated that mechanisms leading to augmentation of the endothelial cell-dependent procoagulant reactions would be important to study. Our

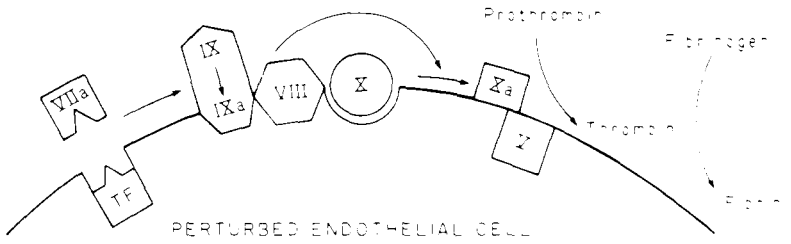

Fig. 8. Schematic depiction of the endothelial cell procoagulant pathway on the surface of a perturbed endothelial cell. TF, tissue factor.

first experiments [43] in this context were carried out by adding unstimulated human platelets to endothelial cell monolayers (Fig. 7). For these experiments bovine endothelial cells were incubated with factors IX_a and VIII, washed, and factor X and prothrombin were then added 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 (generously provided by Dr. Glueck) [44] indicates that the platelet effect was due in large part to release of their endogenous factor V which, after activation, promotes rapid factor X_a-mediated thrombin formation. In the absence of platelets, antihuman factor V IgG decreased thrombin formation by bovine endothelial cells only minimally. These experiments indicate that the anti-factor V antibody blocked the activity of human factor V/V_a from platelets more efficiently than endothelial cell bovine factor V/V_a [8,10,11]. 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 which results via thrombin formation in platelet activation, recruiting them to augment the procoagulant response.

SUMMARY:HYPOTHESIS

These data provide strong evidence supporting the concept that endothelial cells can actively participate in the initiation and propagation of procoagulant reactions. When coagulation is initiated with factor XI_a, procoagulant events start with activation of cell-bound factor IX and precede with factor X and prothrombin activation. Perturbation of endothelial cells can lead to the induction of tissue factor, which functions as a cofactor in the initiation of coagulation. The perturbed endothelial cell thus provides a focus of localized procoagulant events promoting factor VII_a-mediated activation of factors IX and X (Fig. 8). The predominant sequence of reactions on the endothelial cell surface includes factor VII_a activation of factor IX, followed by factor IX_a–VIII mediated activation of factor X. Once factor X_a is formed, it can interact with endothelial cell factor V, promoting thrombin formation. This thrombin can cleave fibrinogen, resulting in the formation of a fibrin clot which is closely associated with the endothelium. This model is a simple endothelial cell-dependent mechanism for initiation of coagulation at the site of an injured or pathological vessel. On the quiescent endothelial cell surface, procoagulant reactions are balanced by anticoagu-

lant mechanisms. In contrast, on the surface of perturbed endothelial cells procoagulant mechanisms would predominate, resulting in promotion of clot formation.

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