Endothelium and Regulation of Coagulation

David M. Stern, MD
Ciro Esposito, MD
Herwig Gerlach, MD
Marlene Gerlach, MD
Jane Ryan, PhD
Dean Handley, PhD
Peter Nawroth, MD

Endothelial cells form the luminal vascular surface and thus have a central role in the regulation of coagulation. One important way in which endothelial cells control the clotting system is by regulating the expression of binding sites for anticoagulant and procoagulant factors on the cell surface. In the quiescent state, endothelial cells maintain blood fluidity by promoting the activity of numerous anticoagulant pathways, including the protein C/protein S pathway. After activation, as can be brought about by cytokines, the balance of endothelial properties can be tipped to favor clot formation through coordinated induction of procoagulant and suppression of anticoagulant mechanisms. Tumor necrosis factor suppresses the endothelial anticoagulant cofactor thrombomodulin and induces expression of the procoagulant cofactor tissue factor. Working in concert, these changes can allow fibrin formation to proceed in an inflamed focus but maintain blood fluidity in the surrounding area of normal vasculature. Recent studies suggest that similar changes in endothelial coagulant properties can be induced by advanced glycosylation end products, proteins modified by glucose that accumulate in the vasculature at a rapid rate in diabetic subjects, indicating the potential relevance of these mechanisms in diabetic vascular disease. *Diabetes Care* 14 (Suppl. 1):160–66, 1991

The coagulation mechanism, as it appears from kinetic studies of purified proteins in solution, is a complicated maze of enzyme-substrate reactions. Our work has focused on the role of cellular receptors in regulating the coagulation mechanism. We have used endothelium as a model system, because as the cells forming the luminal vascular surface, endothelial cells are strategically positioned to play a central role in the regulation of both anticoagulant and procoa-

gulant pathways. The schematic depiction of endothelial cell coagulant properties in Fig. 1 demonstrates the capacity of endothelium to support mechanisms promoting and inhibiting clot formation. The procoagulant cofactor tissue factor can prime the procoagulant reactions leading to thrombin formation, and the anticoagulant cofactor thrombomodulin can bind that thrombin, initiating the antithrombotic protein C/protein S pathway. This leads to the hypothesis that regulation of receptor expression controls a unique balance of procoagulant and anticoagulant reactions on the endothelial cell surface. In the quiescent state, the balance includes a predominance of anticoagulant mechanisms with little evidence of procoagulant activity. After endothelial activation, the balance can be tipped to facilitate formation of coagulation proteases and fibrin. This review will first examine several coagulant properties of endothelium and then describe their modulation by a mediator of the host response, tumor necrosis factor (TNF).

PROTEIN C/PROTEIN S PATHWAY AND ENDOTHELIUM

The protein C/protein S pathway is an important natural anticoagulant mechanism protecting against thrombosis (1; Fig. 1). The clinical significance of this pathway is evident from thrombotic diatheses observed in patients deficient in protein C or protein S. Proteins C and S are the vitamin K–dependent plasma coagulation proteins
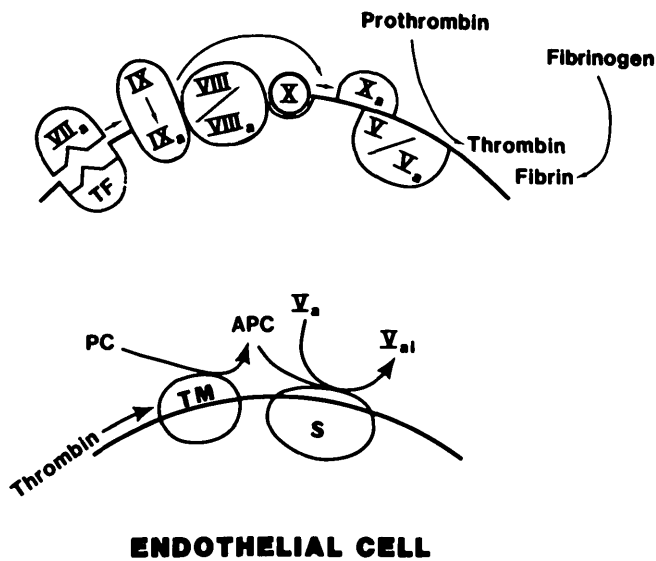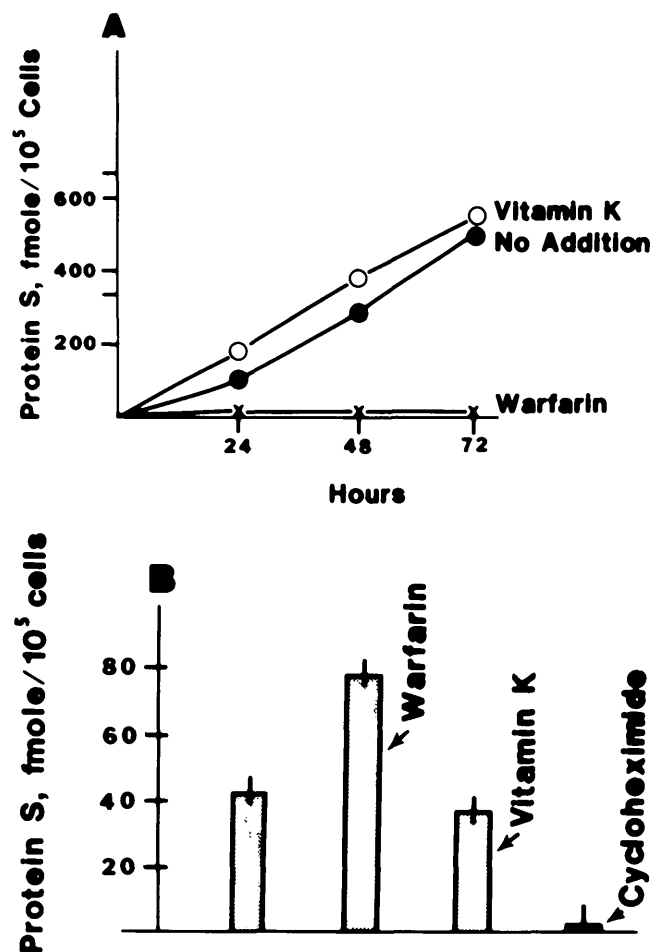
ENDOTHELIAL CELL

FIG. 1. Schema of endothelial cell procoagulant pathway (*top*) and protein C/protein S pathway (*bottom*). TF, tissue factor; TM, thrombomodulin; PC, protein C; APC, activated protein C; S, protein S. Roman numerals correspond to blood-coagulation factors.

that, in addition to vessel wall cofactors, comprise this anticoagulant mechanism. The protein C/protein S pathway is initiated when thrombin interacts with the endothelial cell receptor thrombomodulin, facilitating activation of protein C. Activated protein C can inactivate the cofactors essential for blood-coagulation factor X and prothrombin activation: blood-coagulation factors VIIIa and Va, respectively. For example, for blood-coagulation factor IXa to effectively promote the formation of activated factor X, the factor VIII/VIIIa is required as a cofactor. The anticoagulant protein C/protein S system promotes selective inactivation of factor VIIIa, downregulating this central procoagulant mechanism. The same situation exists for activated protein C/protein S–mediated inactivation of factor Va in prothrombin inactivation; inactivation of factor Va blocks thrombin formation.

For activated protein C, the enzyme in this anticoagulant pathway, to function effectively, it must form a complex with protein S on an appropriate cellular surface. Studies have indicated that the activated protein C/protein S complex can form on endothelium (2). Kinetic studies in the presence of endothelium have indicated that factor Va inactivation occurs rapidly with low concentrations of protein S and activated protein C; the half-maximal rate is achieved at a protein S concentration of 0.2 nM and an activated protein C concentration of 0.06 nM. Comparison of radioligand-binding studies with radioiodinated activated protein C and protein S and parameters from kinetics experiments suggest the following model for assembly of the activated protein C/protein S complex on endothelium: protein S binds to a specific cell surface site and facilitates activated

protein C–endothelial cell interaction. The activated protein C/protein S complex appears to assemble in response to lower concentrations of reactants on endothelium than platelets or phospholipids, suggesting the vessel wall may be the physiological locus on which this reaction takes place.

In addition to supplying vessel wall-binding sites for the protein C/protein S complex, endothelium synthesizes protein S (3). Endothelial cell monolayers maintained in serum-free medium steadily release protein S into the culture fluid (Fig. 2). Addition of the warfarin derivative 3(α-acetonyl benzyl)-4-hydroxycoumarin (1 mg/ml) considerably decreases protein S release and results in its accumulation intracellularly. Cycloheximide decreases both release of protein S and the normal

FIG. 2. Protein S released from and remaining associated with endothelial cells. *A*: released protein S. Endothelial cell monolayers maintained in serum-free medium were washed and incubated in serum-free medium alone (●) or medium supplemented with either vitamin K (O) or warfarin (x). Supernatant was assayed for protein S content. *B*: intracellular protein S. Cell-associated protein S was assayed after 72 h in serum-free medium alone (*no label*) or serum-free medium containing vitamin K or cycloheximide.
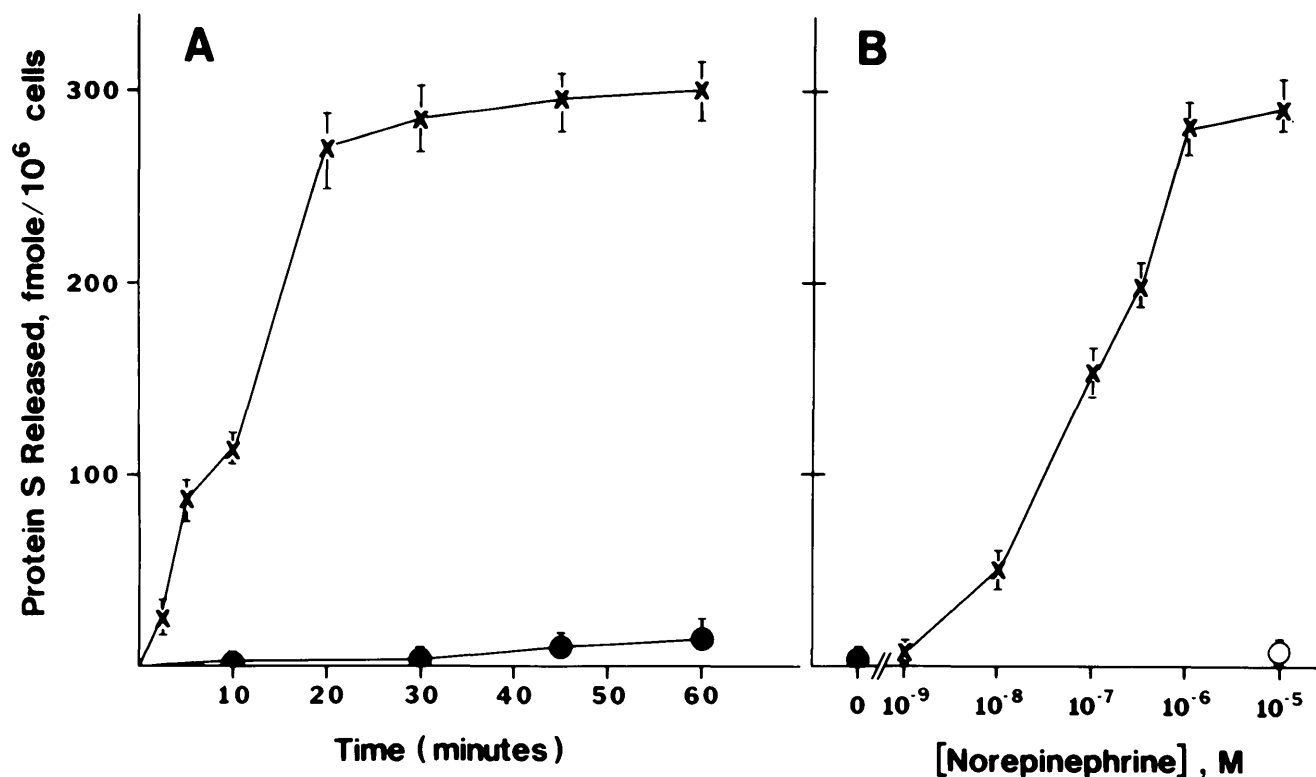
FIG. 3. Norepinephrine-induced release of endothelial cell protein S. *A*: time course. Endothelial cultures were incubated in serum-free medium alone (●) or in medium containing norepinephrine (x), and aliquots were assayed for released protein S. *B*: dose response. Endothelial cells were incubated in serum-free medium alone or serum-free medium containing indicated concentration of norepinephrine. Protein S antigen in supernatant is plotted versus added concentration of norepinephrine.

level of intracellular protein S. These data, and results of immunoprecipitation experiments with metabolically labeled endothelium and functional studies (3), indicate that endothelium is an extrahepatic site of protein S synthesis. The possible clinical significance of these observations derives from studies indicating that in patients with hepatic disease, although the levels of other vitamin K–dependent coagulation factors decrease dramatically, protein S levels are only minimally decreased (4). Another important consequence of a storage pool of protein S in the endothelium involves mechanisms of stimulated release (Fig. 2*B*). Incubation of endothelium with norepinephrine leads to a time-dependent release of protein S into culture medium (5). The rapid phase of norepinephrine-induced protein S release is complete within ~20 min (Fig. 3*A*). Studies with a wide range of norepinephrine concentrations indicate that protein S release is half maximal at 10^{-7} M and maximal by 10^{-5} M (Fig. 3*B*). Furthermore, studies with adrenergic antagonists indicate that norepinephrine-induced protein S release occurs exclusively by an α_1-adrenergic mechanism; propranolol has no effect, but α-adrenergic antagonists are inhibitory with a hierarchy indicating α_1-adrenergic–receptor subselectivity. These studies delineate a novel mechanism by which the protein C/protein S system can respond to stimulation by the autonomic nervous system.

Taken together, these data indicate that the protein

C/protein S pathway is closely linked to endothelium. The receptor thrombomodulin binds to thrombin, promoting formation of activated protein C. The functional unit of this anticoagulant pathway, the activated protein C/protein S complex, also assembles effectively on the endothelial cell surface propagating the reactions leading to factor Va and VIIIa inactivation. In addition, endothelium synthesizes and releases protein S. Thus, the endothelium has an active role in promoting function of the powerful antithrombotic protein C/protein S pathway. This mechanism can halt procoagulant reactions on the vessel surface.

It is evident that thrombin, the final enzyme in the procoagulant pathway, must be formed for the protein C/protein S pathway to be initiated. One effective mechanism by which thrombin formation could occur would be if small amounts of this enzyme were produced on the endothelial cell surface in the immediate vicinity of receptors for the protein C/protein S pathway. This led us to consider the potential of endothelium to promote activation of coagulation.

ENDOTHELIAL CELL PROCOAGULANT PATHWAY

Although quiescent endothelium has a paucity of procoagulant activity, stimulation of endothelial cells by
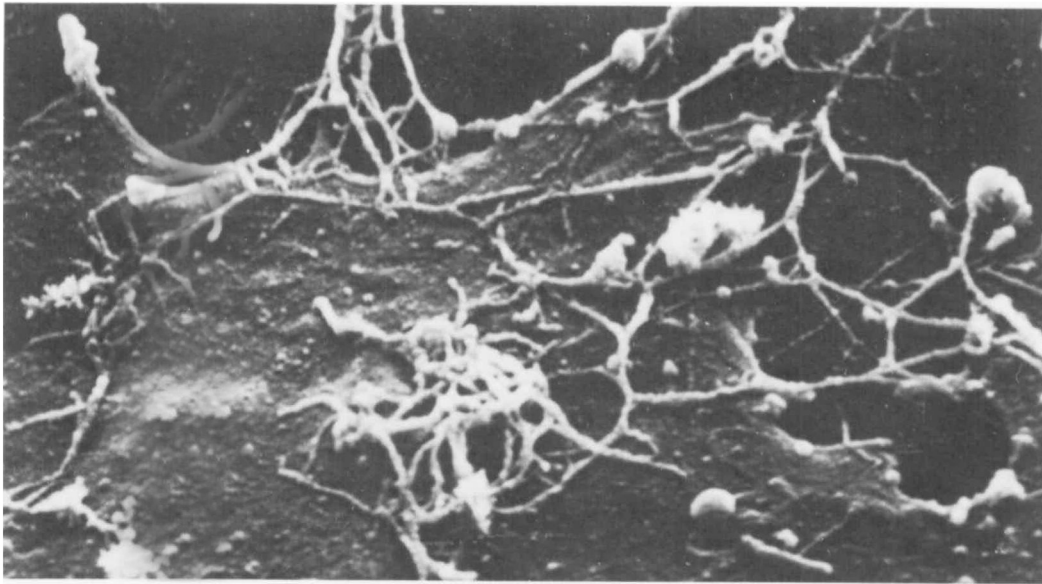
FIG. 4. Fibrin clot on endothelial cells. Endothelial cells were incubated with 1 ml of incubation buffer and factors XIa, IX, VIII, X, prothrombin, and fibrinogen. When first definite fibrin strands were seen, monolayers were washed 4 times with albumin-free incubation buffer and fixed for scanning electron microscopy in 3% glutaraldehyde in 0.15 M sodium cacodylate buffer (×3500).

various mediators (see below) leads to induction of the procoagulant cofactor tissue factor (6,7). Tissue factor promotes factor VIIa–mediated activation of factors IX and X, initiating activation of coagulation, and is the major physiological activator of the clotting mechanism. This led us to examine whether endothelium could propagate the reactions leading to thrombin formation, i.e., factor X and prothrombin activation. When endothelium was incubated with the components of the factor X activation complex (factors IXa, VIII, and X), factor Xa formation occurred in response to low concentrations of factor IXa (the half-maximal rate of factor Xa formation was achieved at factor IXa concentrations of ~0.2–0.4 nM) (8). Further studies demonstrated that factor IXa interacts with a specific cell surface cofactor, which promotes assembly of the factor IXa-VIII-X complex on the endothelial surface (9). This trypsin-sensitive endothelial cell–derived membrane protein appears to be a distinct receptor for factor IX/IXa.

In addition to promoting the activation of factor X, the prothrombinase complex can assemble on the surface of native and cultured endothelium and generate thrombin (10–12). The efficiency of thrombin formation on the surface of quiescent endothelium, however, may be less than that for platelets or other cellular surfaces (11). This would suggest that, in homeostasis, prothrombin activation may occur on other cellular surfaces, not the endothelium. In pathological states, the situation may be quite different.

These studies indicate the procoagulant potential of endothelium and suggest that, if endothelial cells were incubated with the appropriate coagulation factors, activation of coagulation with fibrin formation would occur (13). Under these conditions, fibrinogen cleavage did occur, and the fibrin strands that formed were closely associated with the endothelial cells (Fig. 4). The endothelial cells, which were initially confluent, exhibited a retracted profile consistent with the results of previous studies on fibrin-endothelial cell interaction. This allows for further augmentation of the procoagulant response as plasma constituents are exposed to the basement membrane.

If one considers the balance of endothelial cell coagulant properties (Fig. 5), in the quiescent state, anticoagulant properties would predominate with little procoagulant activity. Under these conditions, only small amounts of thrombin would form, and these could bind to thrombomodulin, initiating the protein C/protein S pathway. In an activated state, however, this balance could be shifted to favor procoagulant mechanisms, and the larger amounts of thrombin formed would then lead to fibrin deposition.

CYTOKINE-MEDIATED REGULATION OF ENDOTHELIAL CELL COAGULANT PROPERTIES

To test the hypothesis that the balance of coagulant properties on the endothelial cell surface could be shifted to favor activation of coagulation, we considered the host response in inflammation. The Shwartzmann re-

FIG. 5. Schema of balance of anticoagulant and procoagulant mechanisms on surface of quiescent and perturbed (or activated) endothelium.
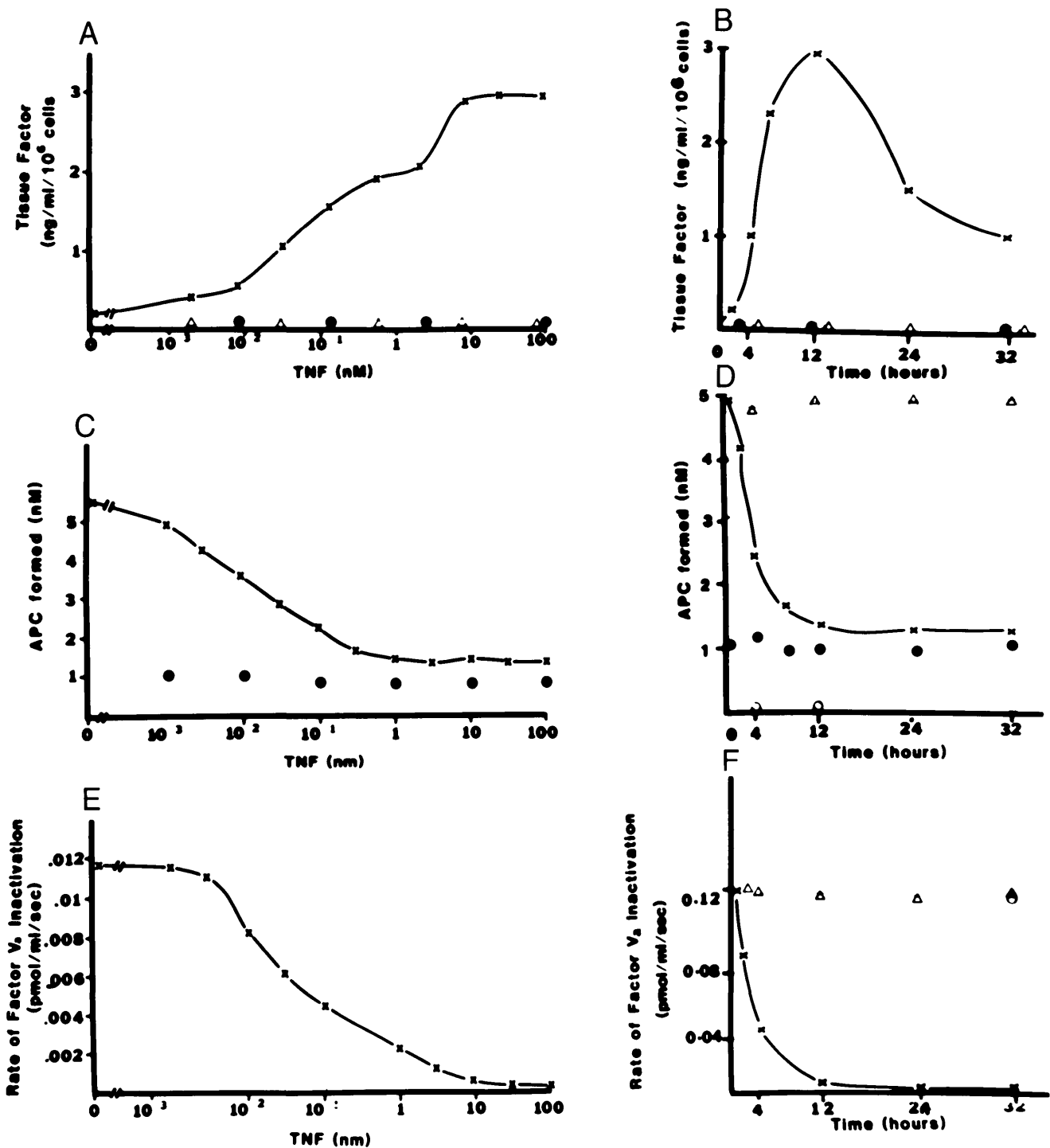
FIG. 6. Effect of tumor necrosis factor (TNF) on endothelial cell hemostatic properties. Endothelial cells (ECs) were incubated with TNF, and effect on EC tissue factor (A, B), activated protein C formation (C, D), and factor Va inactivation (E, F) was assessed. A: dependence of tissue factor induction on dose of TNF. ECs were incubated with TNF (x) alone or in presence of cycloheximide (●). Where indicated (△), assay mixture was with factor VII–deficient plasma in place of normal plasma. B: time course of tissue factor induction. ECs were incubated in serum-free medium (△) or serum-free medium containing TNF (x), heat-treated TNF (○), or TNF and cycloheximide (●). C: dependence of decreased protein C activation on dose of TNF. ECs were incubated with TNF (x), and activated protein C (APC) formation was assessed. ●, Indicated APC formation in presence of blocking antibody to thrombomodulin. D: time course of decreased APC formation. ECs were incubated with TNF (x) or serum-free medium alone (△). Where indicated (●), blocking antibody to thrombomodulin was present. E: dependence of decreased factor Va inactivation on dose of TNF. ECs were incubated with TNF (x), and then APC–protein S-mediated factor Va inactivation was studied. F: time course of decreased factor Va inactivation. ECs were incubated with TNF (x) or serum-free medium alone (△), and factor Va inactivation was studied.

action, a basic phenomenon in the biology of bacterial infections includes prominent thrombotic pathology in addition to tissue infiltration by leukocytes. This led us to examine whether TNF, a central mediator of the host response in Gram-negative sepsis, could modulate endothelial cell coagulant properties by promoting induction of procoagulant and suppression of anticoagulant activity.

First, we examined the binding of TNF to endothelium (14). The results of a series of radioligand binding studies demonstrate high-affinity specific sites on the endothelial cell surface. The affinity of TNF for these sites is comparable to that observed on other cells with TNF receptors. These findings led us to carry out a second set of studies examining the effects of TNF–endothelial cell interaction on cellular coagulant properties (Fig. 6) (15). When TNF is incubated with endothelium, induction of tissue factor activity is evident within 1–2 h (15,16; Fig. 6, A and B). Concomitant with the enhancement of tissue factor activity is suppression of thrombomodulin activity (Fig. 6, C and D). In addition to suppression of protein C activation, endothelial cell–dependent activated protein C/protein S–mediated factor Va inactivation is attenuated (Fig. 6, E and F). Together, these data indicate that a coordinated and unidirectional shift in endothelial cell coagulant properties has occurred: a procoagulant tissue factor is induced, whereas anticoagulant cofactor activity for the protein C pathway is suppressed. Studies from other laboratories have confirmed this shift in endothelial coagulant properties in response to TNF and have extended these observations to other systems, such as the fibrinolytic mechanism. These changes in vessel wall coagulant properties could underlie TNF-induced fibrin accumulation in tumor vasculature and the ubiquitous fibrin deposition in inflammatory lesions.

CONCLUSIONS

Evidence has been presented that a class of receptors located on the endothelial cell surface can play an important role in regulation of the coagulation mechanism. Recognition of this role of endothelium leads to a broader concept of vascular injury in which endothelial cell denudation is only a later part of the pathological picture. Events can be initiated on a morphologically intact but functionally altered endothelium. This approach provides new insights into the pathogenesis of thrombotic disorder in the setting of modulation of vessel wall receptors that regulate coagulation. Modulation of these receptors by host-response mediators can target activation of coagulation to an inflammatory focus, potentially providing a mechanism underlying other types of fibrin deposition in the Shwartzmann reaction and vascular pathology. Thus, whereas anticoagulant mechanisms are suppressed and procoagulant expression is enhanced in an inflammatory focus, the integrity of antithrombotic pathways is preserved in the normal vasculature, pre-

venting activation of coagulation from spilling of the inflamed area. This cellular approach to coagulation draws the clotting mechanism into a broader biologic context. For example, the presence of advanced glycosylation end products, proteins modified by glucose that accumulate in the vasculature at an accelerated rate in diabetic subjects, could modulate expression of endothelial coagulation receptors (17). Recent results support this suggestion; advanced glycosylation end products can induce endothelial tissue factor and suppress thrombomodulin, indicating the potential relevance of these receptors to the pathogenesis of vascular lesions in diabetes (18).

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