

MODULATION OF ENDOTHELIAL CELL HEMOSTATIC
PROPERTIES BY TUMOR NECROSIS FACTOR

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Recent studies (1, 2) have indicated that endothelium provides a focal point for the interaction of mechanisms inhibiting and promoting activation of coagulation. Perturbation of endothelium can induce procoagulant activity, allowing these cells to initiate and propagate an entire coagulation pathway leading to the deposition of fibrin (2). Thus, modulation of endothelial cell coagulant properties could play a role, comparable to the traditionally accepted leukocyte procoagulants, in promoting fibrin deposition in response to inflammatory and neoplastic stimuli.

Prompted by this hypothesis and the recently described (3) identity between tumor necrosis factor (TNF) and cachectin, a mediator of the host response in gram-negative sepsis (4), we have examined the effect of TNF on the coagulant properties of endothelium. The results indicate that cultured bovine aortic endothelial cells incubated with recombinant TNF have enhanced procoagulant activity, tissue factor, and concomitant suppression of the protein C pathway, an antithrombotic mechanism that functions on the surface of quiescent endothelial cells. This unidirectional shift in endothelial cell hemostatic properties promoting clot formation provides insight into the intercellular signalling by which a monokine generated in response to sepsis can modulate the role of endothelium in coagulation.

Materials and Methods

Cell Culture. Bovine aortic and human umbilical vein endothelial cells were cultured and characterized as previously described (5).

Treatment of Endothelial Cells with TNF. Experiments were carried out 24 h after endothelial cells reached confluence using primary cultures and serially passaged cells (passages 1–8). Monolayers were washed three times with HBSS (Gibco Laboratories, Grand Island, NY), serum-free medium was added along with TNF, and cells were incubated for the indicated times at 37°C. After the incubation period, cultures were washed three times with incubation buffer and assayed for tissue factor activity (9.6-cm² wells), the ability to promote thrombin-mediated protein C activation (0.32-cm² wells), or the ability to promote activated protein C–protein S–mediated Factor Va inactivation (0.79-cm² wells).

Reagents and Assays. Recombinant human TNF, generously provided by BASF (Ludwigshafen, Federal Republic of Germany), was used for all studies. This TNF preparation, which was free of detectable endotoxin at the concentrations used in these experiments, migrated as a single band with an M_r of 17,000 under both reducing and nonreducing conditions on SDS-PAGE. This material has identical properties to previously described

(6) preparations of TNF (a manuscript concerning the detailed characterization of this TNF is in preparation by BASF). Heat treatment of TNF was carried out at 70°C for 1 h, and resulted in loss of cytolytic activity and loss of its effect on endothelial cell coagulant parameters (Fig. 1).

All coagulation factors were of bovine origin. The synthetic substrate tissue factor assay (Table I) (7) was carried out by adding purified Factors VIIa (8 nM) (generously provided by Dr. W. Kisiel, University of New Mexico, Albuquerque, NM) and X (1.3 μ M) (2) to monolayers in incubation buffer (1 ml) for 10 min at 21°C. Where indicated, rabbit anti-tissue factor IgG was added (200 μ g/ml) (8) (generously provided by Dr. R. Bach, Mt. Sinai School of Medicine, New York) for 30 min before the assay. One sample (0.1 ml) was removed from each reaction mixture and assayed for Factor Xa by monitoring (at 405 nm) hydrolysis of the chromogenic substrate benzo-Ile-Glu-Gly-Arg-*p*-nitroanilide (Helena Labs, Beaumont, TX) (0.1 ml; 0.05 nM) (2). Factor Xa formation was linear, only limited by the amount of tissue factor. Tissue factor activity was also assessed using a one-stage coagulant assay as previously described (7). The amount of tissue factor present was determined by comparison of the clotting time with a standard curve using the same coagulant assay and purified bovine tissue factor inserted into phospholipid vesicles (8) (generously provided by Dr. R. Bach).

Thrombin-mediated protein C activation was studied, as described previously (9), by incubating samples with thrombin (0.1 U/ml) and protein C (85 μ g/ml) for 40 min at 37°C in incubation buffer (0.1 ml). Antithrombin III (0.2 mg/ml) was then added to neutralize residual thrombin, the reaction mixture was assayed for activated protein C by monitoring (at 405 nm) hydrolysis of the chromogenic substrate D-Phe-Pip-Arg-*p*-nitroanilide (Helena Labs; 0.05 ml; 2 mM). Activated protein C formation over endothelial cell monolayers was linear over the time of the assay.

Protein S and Factor Va were purified to homogeneity as described (10). Activated protein C–protein S–mediated Factor Va inactivation was studied by adding activated protein C (1 nM), protein S (2 nM) and Factor Va (70 nM) for 1.5 min at 21°C to monolayers in incubation buffer (0.5 ml). Samples were removed, one from each reaction mixture, and assayed for residual Factor Va activity using one-stage clotting assay (10). Rates of Factor Va inactivation were determined from the slope of the linear initial portion of a plot of Factor Va activity versus incubation time.

Results

In contrast to the cytotoxic effect of TNF reported for tumor cells in vitro (3, 6), TNF did not exert its effect on cultured endothelial cells only via direct cytotoxicity. Rather, TNF specifically modulated endothelial cell hemostatic properties. Tissue factor, a cofactor initiating coagulation (8), is not normally expressed on the surface of endothelial cells. Incubation of cultured bovine aortic endothelial cells with TNF led to a dose-dependent induction of tissue factor (Fig. 1A). The identity of this procoagulant activity as tissue factor was confirmed by the Factor VIIa–dependence of Factor X activation, which could be prevented by anti-tissue factor IgG (Table I). Although neoplastic tissue has been reported (11) to have a direct Factor X activating enzyme, TNF did not induce significant amounts of this activity in endothelium (Fig. 1A, and Table I). Destruction of TNF cytolytic activity by heat treatment also prevented the induction of endothelial cell tissue factor, indicating a requirement for the functionally active molecule (Fig. 1). Similar heat-treatment of endotoxin had no effect on the induction of endothelial cell tissue factor (data not shown). Tissue factor activity induced by TNF was evident after a 2-h lag, increased steadily up to 10 h, and thereafter slowly declined (Fig. 1B). This is similar to the decay of IL-1-induced endothelial cell tissue factor activity (12), though the decline appears more

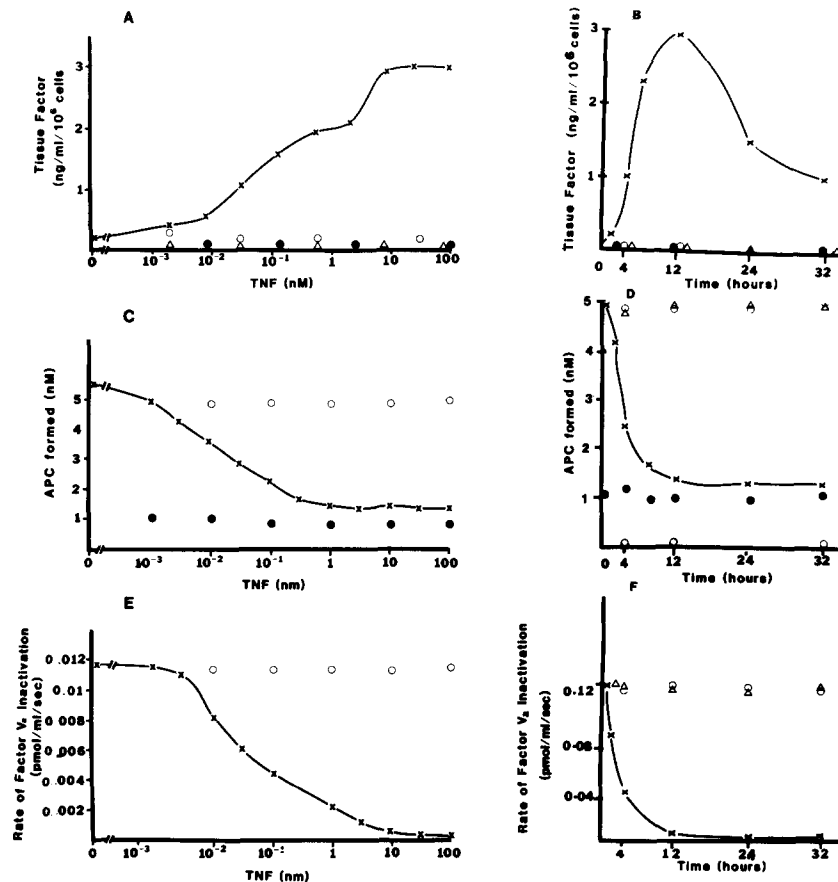


FIGURE 1. Effect of TNF on endothelial cell hemostatic properties. Cultured bovine aortic endothelial cells were incubated with TNF and the effect on endothelial cell tissue factor (A, B), thrombin-mediated activated protein C formation (C, D) and activated protein C–protein S–mediated Factor Va inactivation (E, F) was assessed. (A) Dependence of tissue factor induction on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (x) for 12 h. Tissue factor activity of endothelium was assessed by a coagulant assay as described in the text. Where indicated, Factor VII–deficient plasma replaced normal plasma (Δ). Heat-treated TNF (O) or cycloheximide (2 $\mu\text{g}/\text{ml}$) (\bullet) were added to certain cultures. (B) Time course of tissue factor induction. Monolayers were incubated in serum-free medium alone (Δ) or in the presence of TNF (10 nM) (x). Cycloheximide (\bullet) or heat-treated TNF (O) was added where indicated. Tissue factor was assayed as described in Materials and Methods. Where indicated, Factor VII–deficient plasma was used (\blacktriangle). (C) Dependence of decreased thrombin-mediated protein C activation on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (x) or heat-treated TNF (O). Endothelium was then incubated with thrombin and protein C, as described in the text, to assess its ability to promote thrombin-mediated protein C activation. Where indicated, goat anti–rabbit thrombomodulin IgG (150 $\mu\text{g}/\text{ml}$) was preincubated with endothelium for 30 min (\bullet). Control IgG from nonimmune animals had no effect on the assay. Results are expressed as activated protein C (APC) formed per 40 min per 10^5 cells. (D) Time course of decreased thrombin-mediated protein C activation. Monolayers were incubated with TNF (10 nM) (x), heat-inactivated TNF (O), or with serum-free medium alone (Δ) for the indicated times, and washed. Where indicated, goat anti–rabbit thrombomodulin IgG (150 $\mu\text{g}/\text{ml}$) (generously provided by Dr. N. Esmon, Oklahoma Medical Research Foundation) was preincubated with endothelium for 30 min (\bullet). Endothelium was then incubated with thrombin and protein C as described in the text to assess its ability to promote thrombin-mediated protein C activation. Results are expressed as activated protein C (APC) formed per 40 min per 10^5 cells. (E) Dependence of decreased

TABLE I
Activation of Factor X by TNF-treated Endothelial Cells

Cell treatment	Assay reaction mixture	Factor Xa formed (pmol/10 ⁵ cells)
None	VIIa, X	3 ± 1
TNF	VIIa, X	108 ± 17
TNF	X	4 ± 1
TNF	VIIa, X, anti-tissue factor IgG	11 ± 1

Endothelial cell monolayers were incubated with or without TNF (10 nM) for 12 h in serum-free medium and washed with incubation buffer. The tissue factor assay was then carried out using Factors VIIa and X as described in the text. Values are expressed as Factor Xa formed within 10 min.

gradual in the case of TNF. Procoagulant activity on the endothelial cell surface was not due to expression from a preformed pool, since lysates of control endothelial cells did not have significant tissue factor activity. Furthermore, cycloheximide (2 µg/ml) blocked the induction of tissue factor by TNF, indicating a requirement for de novo protein synthesis (Fig. 1B). Thus, TNF induces endothelium to synthesize and express tissue factor. Similar results were observed with cultured human umbilical vein endothelial cells (data not shown).

In view of the potent anticoagulant mechanisms operative on the endothelial cell surface, induction of procoagulant activity may not be sufficient for the vessel surface to play a role in the pathogenesis of a prethrombotic state. In this context, the importance of endothelial cell participation in the regulation of coagulation is exemplified by the thrombotic diathesis observed (13) in kindreds deficient in protein C or protein S, since function of the protein C anticoagulant pathway is dependent on cofactors present on the endothelial cell surface. Endothelium provides both a cell surface protein, thrombomodulin (25), enhancing thrombin-mediated formation of the anticoagulant enzyme-activated protein C, and cofactor activity promoting assembly of activated protein C-protein S complex, which is the functionally effective anticoagulant unit of the protein C pathway. Activated protein C formation was in large part dependent on the presence of endothelial cell thrombomodulin, as indicated by the 75% inhibition of protein C activation in the presence of antithrombomodulin IgG (Fig. 1C). TNF resulted in a dose-dependent decrease in endothelial cell-dependent thrombin-mediated protein C activation. At a TNF concentration of 30 pM, thrombomodulin-dependent protein C activation was decreased by ~50%. Decreased activated protein C formation was also dependent on the

activated protein C-protein S-mediated Factor Va inactivation on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (x) or heat-treated TNF (o) for 12 h. Endothelium was then assayed for the ability to promote activated protein C-protein S-mediated Factor Va inactivation after incubation with activated protein C, protein S, and Factor Va, as described in the text. Results are expressed as the rate of Factor Va inactivation per 10⁵ cells. (F) Time course of decreased activated protein C-protein S-mediated Factor Va inactivation. Monolayers were incubated with TNF (10 nM) (x), heat-inactivated TNF (o), or with serum-free medium alone (Δ) for the indicated times, and washed. Endothelium was then assayed for the ability to promote activated protein C-protein S-mediated Factor Va inactivation after the addition of activated protein C, protein S, and Factor Va, as described in the text. Results are expressed as the rate of Factor Va inactivation per 10⁵ cells.

incubation time of endothelium with TNF, with an effect evident by 1 h and maximal after 6 h (Fig. 1D). Controls indicated that decreased protein C activation was due to decreased formation of activated protein C rather than inactivation of the thrombin added or the activated protein C formed.

Recent studies (10) have shown effective assembly of functional activated protein C–protein S complex on the endothelial cell surface promoting Factor Va inactivation, and thereby regulating thrombin formation. Activated protein C is the enzyme and protein S functions as the cell surface cofactor (10). In contrast to the rapid Factor Va inactivation observed with control monolayers, after incubation with TNF, the rate of Factor Va inactivation was reduced (Fig. 1E). This effect was half-maximal at a TNF concentration of 40 pM. The rate of Factor Va inactivation was attenuated in a time-dependent manner after the addition of TNF to cultures with negligible rates after 8 h (Fig. 1F).

Discussion

The results reported here indicate that TNF induces endothelial cell tissue factor while suppressing endothelial cell-dependent protein C activation and anticoagulant function on the cell surface. Comparison of the time course and dose-response curve for TNF modulation of these distinct cellular coagulant properties indicates striking similarities in all cases (Fig. 1). This suggests that expression of these hemostatic properties may be under coordinate control at the intracellular level. Since a concerted change in the endothelial cell surface promoting the activation of coagulation involves both anticoagulant and procoagulant properties, such a linked control mechanism seems logical. The result of these changes is indicated by previous studies (2) showing that the induction of tissue factor in endothelial cells initiates a procoagulant pathway leading to the deposition of fibrin. Loss of effective function of the protein C anticoagulant pathway further enhances the propagation of procoagulant reactions on the endothelial cell surface. The net result of TNF–endothelial cell interaction is a unidirectional shift in the balance of anticoagulant and procoagulant mechanisms on the endothelial cell surface from the quiescent state in which anticoagulant mechanisms predominate, to a stimulated state in which procoagulant activities are dominant.

Fibrin deposition and activation of macrophages around malignant tissues, potentially resulting in local TNF release, is commonly observed by pathologists. Furthermore, the histologic description of the effect of TNF on tumors *in vivo* is often referred to as hemorrhagic necrosis. This leads to the hypothesis that local effects of TNF on endothelial cell coagulant properties could result in a coagulopathy, interrupting normal blood flow to the tumor and leading to necrosis. This suggests a model in which monokines alter endothelial cell hemostatic properties promoting clot formation, potentially limiting pathologic processes such as infection and tumors.

Summary

Tumor necrosis factor/cachectin (TNF) is a mediator of the septic state, which involves diffuse abnormalities of coagulation throughout the vasculature. Since

previous studies have shown that endothelial cells can play an active role in coagulation, we wished to determine whether TNF could modulate endothelial cell hemostatic properties. Incubation of purified recombinant TNF with cultured endothelial cells resulted in a time- and dose-dependent acquisition of tissue factor procoagulant activity. Concomitant with enhanced procoagulant activity, TNF also suppressed endothelial cell cofactor activity for the anticoagulant protein C pathway; both thrombin-mediated protein C activation and formation of functional activated protein C–protein S complex on the cell surface were considerably attenuated. Comparable concentrations of TNF (half-maximal affect at ~50 pM) and incubation times (half-maximal affect by 4 h after addition to cultures) were required for each of these changes in endothelial cell coagulant properties. This unidirectional shift in cell surface hemostatic properties favoring promotion of clot formation indicates that, in addition to leukocyte procoagulants, endothelium can potentially be instrumental in the pathogenesis of the thrombotic state associated with inflammatory and malignant disorders.

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