TUMOR NECROSIS FACTOR/CACHECTIN-INDUCED INTRAVASCULAR FIBRIN FORMATION IN METH A FIBROSARCOMAS

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TNF/cachectin has widespread effects in various tissues (1–6). Endothelium is an important target of TNF. Occupancy of endothelial cell TNF receptors leads to activation of procoagulant function on the cell surface; to cell shape and cytoskeletal modulation; and to alterations in other cell functions (1–6). Generalized, disseminated hemostatic abnormalities follow infusion of larger concentrations of TNF into healthy animals (1–3, 6), but more localized activation of coagulant mechanisms might well occur in particular microvascular beds in response to lower or limiting concentrations of this cytokine in pathologic states. One such target may be the neovascularature of angiogenic tumors, whose vessels are under the influence of factors within the tumor microenvironment that potentially distinguishes their endothelium from that of other vascular beds.

The studies reported here demonstrate that such altered responses can be observed in the tumor vasculature in meth A sarcomas in mice, for within 30 min of TNF infusion, fibrin formation occurred throughout the tumor vascular bed in close relation to the endothelial cell surface and ultimately led to defective perfusion. In contrast, fibrin deposition and platelet aggregates were not observed in the normal vasculature. We have shown that the altered state of endothelium in the tumor vasculature appears to be due, at least in part, to factor(s) elaborated by cultured meth A cells that enhance the procoagulant response of endothelium to TNF. These findings provide a model system in which locally acting stimuli, by predisposing the endothelial cells to procoagulant response, can target the action of a circulating mediator to a particular vascular bed leading to selective thrombus formation.

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Materials and Methods

**In Vivo Infusion and Morphologic Studies.** For studies examining the effect of TNF on meth A sarcomas in vivo, BALB/c mice were injected intradermally with meth A sarcoma cells (10^5 cells/animal; generously provided by Drs. Hoffmann and Old, Memorial Sloan-Kettering Cancer Center, NY [7]). After 7-10 d, when tumors reached a size of ~1 cm in diameter, animals were injected intravenously via a tail vein with TNF alone (3 µg/animal) or TNF in the presence of either human fibrinogen (100 µg/animal) or 125I-fibrinogen (7.5 µg/animal). Human TNF (~10^8 U/mg) was generously provided by BASF (Ludwigshafen, Federal Republic of Germany), and this preparation was homogeneous on SDS-PAGE and distinct from lymphotoxin, as described previously (8). TNF was heat inactivated as described previously (8). Highly purified human fibrinogen, provided by Dr. J. Weitz (Hamilton University, Ontario, Canada), was radiolabeled by the lactoperoxidase method (9) (150 µCi/mg) and migrated as three bands of unequal intensity on reduced SDS-PAGE corresponding to the α, β, and γ chains. At the indicated time after the TNF infusion, fibrin deposition/accumulation of radioactivity in the tumor was assessed as follows. For morphologic studies, mice were anesthetized and subjected to whole-body beating heart perfusion fixation (90-110 mm Hg). Incorporation of radioactivity into the mouse tissue was determined after infusion of 125I-fibrinogen by removing a piece of tissue, weighing it and, counting the sample in a gamma counter. The presence of fibrin in tumor tissue was studied by excising tumor tissue, cutting it up finely with a scalpel, and then extracting it with buffer containing Triton X-100 (2%) and protease inhibitors (1.5 mM PMSF, 0.5 mM leupeptin, 20 µg/ml soybean trypsin inhibitor, 500 U/ml Trasylol). The extract was reacted with an equal volume of rabbit anti-mouse IgG immunobeads (Bio-Rad Laboratories, Richmond, CA) for 2 h at 37°C to remove mouse Ig. Control experiments in which a trace of radioiodinated mouse IgG was added to tissue extracts indicated that 99% of the IgG was adsorbed by the beads. The extract was then made 9 M in urea and 10 mg/ml in dithiothreitol, boiled for 3 min, and added to an equal volume of Laemmli SDS-PAGE (10). After boiling for 3 min, the mixture was centrifuged (10,000 RPM; 5 min), and the supernatant was subjected to SDS-PAGE (10%) and Western blotting. In each case, the same amount of tumor tissue was processed and the total protein loaded per lane of the gel was about the same. After Western blotting, nitrocellulose membranes were reacted with mAb specific for human fibrin (11) followed by 125I-affinity-purified goat anti-mouse IgG by the general method of Johnson et al. (12) as described previously (13). The blot was then dried and exposed to Kodak X-Omat (XAR5) film (Eastman Kodak Co., Rochester, NY) in the presence of a Cronex intensifying screen (Dupont Co., Wilmington, DE). Previous work has shown this antibody recognizes a polypeptide with M_r ~59,000 after SDS-PAGE and Western blotting of fibrin-containing samples (14). Mice were infused with human fibrinogen in these studies to visualize fibrin in the tumor bed, since it appeared to show considerably greater immunoreactivity with the fibrin-specific mAb than murine fibrin based on control studies under these conditions (also see Fig. 1 inset, lanes 2 and 3). Lack of visualization of band(s) corresponding to the heavy and light chains of mouse IgGs (Fig. 1 inset, lanes 1 and 2) indicates that removal of mouse Ig using the above procedure with anti-mouse IgG immunobeads was effective.

In certain experiments, mice were maintained on drinking water supplemented with the warfarin derivative 3(α-acetonylbenzyl)-4-hydroxycoumarin (0.7 mg/l) for 3 d before the TNF infusion (~7 d after the meth A cells were injected). Tumors grew to the same size in warfarin-treated and control animals. Before carrying out an experiment with the anticoagulated animals, a Factor X assay on the mouse plasma was performed (15). Only animals with Factor X levels of <1% were used.

**Cell Culture and Tissue Factor Assay.** Endothelial cells derived from human umbilical cord veins were prepared by the method of Jaffe (16) as modified by Thornton et al. (17). Experiments were carried within 24 h of the cells achieving confluence in 9.6-cm² wells (passages 1-5). Cells were characterized as endothelial based on the presence of von Willebrand Factor antigen (18), as described previously, and thrombomodulin activity (19). Meth A sarcoma cells, provided by Drs. Hoffman and Old (Memorial Sloan-Kettering Cancer Center), were grown in RPMI 1640 containing 10% FCS. Cultured medium was obtained from meth A cells by placing them in serum-free medium (RPMI containing 10 mM HEPE, pH 7.4,
20 μg/ml transferrin, 10 μg/ml insulin, 1 μg/ml polymyxin B, and 5 mg/ml BSA) for 48 h. Normal BALB/c mouse dermal fibroblasts were obtained from explant cultures of skin/subcutaneous tissue. They were maintained and used to prepare conditioned medium as described for meth A cells.

The tissue factor activity of endothelial cell monolayers was assayed using purified human Factors VIIa (8 nM) (generously provided by Dr. R. Bach, Mount Sinai Medical School, NY) and X (1.5 μM) in serum-free medium at 23°C. At 8 min, a sample (0.2 ml) of the reaction mixture was removed and assayed for Factor Xa activity by monitoring hydrolysis of the chromogenic substrate benz-Ile-Glu-Gly-Arg-p-nitroanilide (20). A mAb that blocks human tissue factor coagulant activity was generously provided by Dr. R. Bach.

Results

When normal and tumor-bearing mice were infused with high concentrations of TNF, 30 μg/animal or more, most of the animals died with thrombi in multiple organs, especially lung and liver, consistent with previous studies indicating the severe toxicity of TNF at these concentrations (21, 22). A TNF concentration of 10 μg/animal resulted in less marked systemic toxicity and thrombus formation. At 3 μg/animal, most animals survived without gross lesions in the normal vasculature, but hemorrhagic changes were observed in the tumors, indicating that this lower dose of TNF was triggering hemostatic abnormalities in the vascular bed of the meth A sarcoma, without widespread thrombohemorrhagic phenomena in other tissues.

Fibrin deposition in the tumor vasculature after infusion of TNF (3 μg/animal) was assessed by measuring accumulation of radioactivity in the tumor in the presence of 125I-fibrinogen (Fig. 1). About 10 times more radioactivity accumulated in the tumor bed of animals coinfused with TNF than in the meth A sarcomas of saline-

![Image](image-url)
BALB/c mice bearing meth A sarcomas were infused with 125I-fibrinogen alone (control) or in the presence of TNF (TNF) as described for Fig. 1. After 1 h, the indicated organ was excised, weighed, and counted. Data shown are the mean and SEM of triplicates.

| Organ  | Control (cpm/mg) | TNF (cpm/mg) |
|--------|-----------------|--------------|
| Heart  | 30 ± 20         | 40 ± 25      |
| Lung   | 75 ± 40         | 180 ± 60     |
| Skin   | 120 ± 60        | 160 ± 50     |

These data indicate that activation of the coagulation mechanism leading to fibrin deposition in the tumor bed occurs in response to the TNF infusion, and fibrin could be visualized in the tumor vascular bed after TNF infusion (Fig. 2). In animals infused with saline alone, there was no fibrin in the patent vessels of the tumors (Fig. 2a and b). 30 min after the infusion of TNF, however, fibrin was visible within the intravascular space closely associated with the endothelial surface (Fig. 2c and d). Fibrin was identified by the usual morphologic criteria (23), and the characteristic ultrastructural periodicity of 21.0 nm (Fig. 2e). The scanning electron micrograph (Fig. 2f) demonstrates fibrin strands apposed to the luminal endothelial cell surface, a situation never observed in control animals. Fibrin deposition was limited to the vessels in the tumor bed. At these early times, adherence of platelets and white cells on the vessel wall did not occur, and platelet thrombi were not seen in the spleen or other organs. This is consistent with the occurrence of localized activation of coagulation within the tumor vasculature presumably initiated by endothelium. 2 h after the TNF infusion, occlusive thrombi with a prominent fibrin component were observed throughout the tumor (Fig. 2f, g, and h). Concomitant with the appearance of these thrombi, unperfused areas were demonstrated in parallel studies carried out with Evans blue to visualize blood flow. At earlier times, 1 h after TNF infusion, unperfused areas were focal, whereas 1–2 h later, large areas, up to 80% of the tumor, could not be reached by the dye. The presence of thrombi within the tumor vessels...
FIGURE 2. TNF-induced changes in tumor vasculature as a function of time after injection. Mice bearing meth A sarcomas were infused with TNF and subjected to perfusion fixation at the indicated times. Light (a) and transmission (b) EM of tumors from saline-injected controls showing patency of tumor vessels (a, arrowheads), which are delimited by an intact luminal endothelium (E in b). Scanning (c) and transmission (d and e) EM 30 min after the injection of TNF. There is evidence of fibrin (F) accumulation at the endothelial cell surface. Fibrin strands (in c) are seen to be adherent to the endothelial cell surface and appear to exhibit direct endothelial cell interactions (d). The fibrin (both cell associated and intraluminal) exhibits a characteristic 21-nm periodicity (e). Light (f) and transmission (g) EM 2 h after TNF injection. Large areas of the tumor showed extensive vascular accumulation of red cells (f) resulting from distal areas of vascular thrombosis, one of which is delineated by brackets (g). Magnification: a, ×600; b, ×6,000; c, ×4,000; d, ×25,000; e, ×48,000; f, ×600; and g, ×2,000.

provides a potential link to in vitro studies demonstrating a procoagulant shift in endothelial cell hemostatic properties (1-6).

Activation of coagulation with fibrin formation after the infusion of TNF was not unexpected, based on the results of previous in vitro studies showing that TNF could
induce modulation of endothelial cell coagulant properties to favor clotting (1-6). However, the localization of fibrin deposition to the tumor vascular bed was unexpected. One mechanism that could account for localized clot formation in response to TNF would be a vessel wall-dependent process accentuated in the tumor bed. The diffuse nature of fibrin deposition in the tumor vascular bed and its close association with the endothelial cell surface supported the hypothesis that a tumor-endothelial cell interaction might be involved. To examine this, supernatants of cultured meth A sarcoma cells (which had no intrinsic procoagulant activity) generated under serum-free conditions, were incubated with endothelial cell monolayers (Fig. 3 A) and induction of tissue factor, a central initiator of coagulation (24), was examined. Exposure of cultured endothelium to tumor-conditioned medium alone led to at most a small, probably insignificant, increase in procoagulant activity. TNF, at a submaximal dose (0.1 nM), induced an increase in endothelial cell procoagulant activity, as previously reported (4, 5). Addition of dilutions of tumor-conditioned medium, along with TNF, to endothelial cell cultures greatly increased the procoagulant response in a dose-dependent manner. The procoagulant activity was identified as tissue factor based on the Factor VIIa dependence of Factor X activation and the inhibitory effect of anti-tissue factor antibody (Fig. 3 A, darkened and cross-hatched bars, respectively). Similar enhancement of tissue factor activity by tumor-conditioned medium was observed at several TNF concentrations. The experiments in Fig. 3 involve exposure of endothelium to TNF and tumor-conditioned medium for 7 h, sufficient time for the maximal procoagulant response to develop. Enhanced tissue factor activity of endothelial cell monolayers was first noted at considerably earlier times. In contrast to the effect of medium derived from meth A cells, medium conditioned by nontransformed BALB/c (the same strain from which the meth A sarcoma was derived) fibroblasts had no effect on endothelial cell coagulant activity in the presence or absence of TNF.

Preliminary characterization of the meth A-derived activity that augments the procoagulant response of endothelium to TNF indicates that it was nondialyzable (mol wt cut off; 3,500), heat sensitive (100°C for 10 min), and trypsin sensitive (50 μg for ½ h at 37°C). Gel filtration on Sephadex G150 (Fig. 3 B) demonstrated a broad peak of activity corresponding to a mol wt range of ~1-3 x 10^4. Although the identity of the factor(s) responsible for the TNF-enhancing effect of tumor-conditioned medium is not clear, sensitivity to heat treatment and the presence of polymyxin in all media (omission of polymyxin actually did not influence the results) make endotoxin contamination unlikely to account for the observed changes in endothelial cell TNF response. In this context, no IL-1, fibroblast growth factor, TNF, IFN-γ, or transforming growth factor α (TGFα) activity was detectable in tumor-conditioned medium. Since TGFβ has been found in most mammalian tissues and cell lines studied (31-33), it was important to determine whether TGFβ1 or TGFβ2

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1 Abbreviation used in this paper: TGF, transforming growth factor.
2 IL-1 assays were carried out in the laboratory of Dr. P. Lomedico, Hoffmann-LaRoche, Nutley, NJ; fibroblast growth factor was assayed in the laboratory of Dr. Dan Rifkin, New York University, New York, NY; TNF was assayed using the L cell assay (25) in our laboratory; IFN-γ was assayed at Hoffmann-LaRoche; transforming growth factor α was assayed in the laboratory of R. Derynck, Genentech, San Francisco, CA.
Figure 3. Enhanced induction of endothelial cell tissue factor in response to TNF in the presence of tumor-conditioned medium. (A) Conditioned medium from meth A sarcoma cells at the indicated dilution was incubated with endothelial cells in serum-free medium (RPMI containing 10 mM Hepes, pH 7.4, 20 µg/ml transferrin, 10 µg/ml insulin, 1 µg/ml polymyxin B, and 5 mg/ml human serum albumin). Cultures contained either serum-free medium alone (0), TNF (0.1 nM) alone (TNF), conditioned medium alone undiluted (CM), or TNF in the presence of conditioned medium at the indicated dilution (TNF + CM). Each addition was in a volume of 10 µl and the volume of serum-free medium was 1 ml. After 7 h of incubation at 37°C, monolayers were washed, and fresh serum-free medium (0.5 ml) was added along with Factors VIIa and/or X. After 8 min, the reaction was stopped and Factor Xa formation was assessed. The cross-hatched bar corresponds to cultures exposed to tumor-conditioned medium (½ dilution) in the presence of TNF followed by incubation for 1 h at 37°C with purified mAb (10 µg/ml) to human tissue factor. Then, Factors VIIa and X were added. Purified mouse IgG (10 µg/ml and 100 µg/ml) had no effect on endothelial cell coagulant activity as measured in this assay. The darkened bar corresponds to cultures incubated with tumor-conditioned medium (½ dilution) in the presence of TNF in which the coagulant assay was carried out in the presence of only Factor X. Results shown are the mean and SEM of triplicate determinations. Factor Xa generation was the same as untreated controls when tumor-conditioned medium was added directly to the Factor VIIa-X incubation mixture in the absence of endothelial cells. (B) Tumor-conditioned medium (obtained as described in A above) (1 ml) was applied to a Sephadex G150 column (0.9 x 55 cm), eluted with 10 mM Hepes (pH 7.4), 0.1 M NaCl, and fractions (1.3 ml) were collected. Aliquots of the indicated column fractions, at a ¼ dilution, were then incubated with endothelial cell monolayers in the presence of TNF (0.1 nM) for 7 h at 37°C. The tissue factor assay was carried out as described above (A) and Factor Xa formed over 7 min is shown (mean ± SEM). TNF denotes cells incubated with TNF alone (0.1 nM); B denotes cells incubated with column buffer alone; and TNF + B denotes cells incubated with TNF (0.1 nM) and column buffer. Column fractions 1-9 were inactive in this assay. The gel filtration column was calibrated with standard proteins including ribonuclease (1.37 x 10⁴), chymotrypsinogen A (2.5 x 10⁴), OVA (4.3 x 10⁴), and albumin (6.7 x 10⁴).

had the capacity to enhance the endothelial cell procoagulant response to TNF. These studies used human and porcine TGFβ because of the availability of purified material and the considerable sequence homology between TGF from different species (murine, porcine, and human) (31-33). Neither TGFβ₁ nor TGFβ₂ (at concentrations up to 500 pM) enhanced endothelial cell procoagulant activity in the presence of TNF, suggesting that the activity in meth A culture supernatants was distinct from TGFβ.

Discussion

Although thrombosis is a major cause of morbidity and mortality, mechanisms involved in the pathogenesis of localized intravascular clot formation are largely uncharacterized. Models of intravascular clot formation in which rapid thrombus formation occurs after contact of plasma factors and cellular elements of the blood
with subendothelial cell components (26) present a picture of thrombosis in which endothelial cell denudation is the critical initiating step. The results presented here indicate that TNF, an endogenously produced mediator of the host response, can selectively induce intravascular clot formation in the tumor vasculature of mice bearing meth A sarcomas in the presence of a viable endothelial monolayer and delineate another model of localized thrombosis. After infusion of TNF, radioiodinated fibrinogen/fibrin accumulated in the tumor (Fig. 1). The frank deposition of fibrin in the tumor vascular bed (Fig. 1, inset and Fig. 2) indicates that activation of coagulation with clot formation was clearly involved. In support of this hypothesis, coumadin, an anticoagulant that prevents γ-carboxylation of the vitamin K-dependent coagulation proteins and thereby down regulates their effective interaction with membrane surfaces (27), considerably decreased accumulation of radiolabel and appearance of the fibrin-specific epitope in the tumor bed. Furthermore, the intravascular localization of fibrin deposition with evidence of occlusive thrombi was noted in all animals studied up to 2 h after the TNF infusion (Fig. 2). In contrast, neither consistent focal uptake of 125I-fibrinogen/fibrin comparable in magnitude to that in the tumor bed nor thrombus formation was seen in the normal vasculature of tumor-bearing or normal mice. This indicates that the thrombogenic effects of TNF were being targeted to the tumor vascular bed.

A central question that evolves from these studies concerns possible mechanisms through which host and tumor-mediated processes could locally enhance the effects of systemically infused TNF. Elaboration of cytokines/angiogenic agents and/or other factors, such as changes in blood flow in the tumor bed, could certainly be involved. The experiments presented in Fig. 3 suggest that tumor cells elaborate a distinct mediator(s) that potentiates the coagulant response of endothelium to TNF. Since TNF has been shown to induce a shift in endothelial cell coagulant properties favoring activation of coagulation (1–6), enhancing this effect locally in a particular vascular bed could constitute a potent thrombogenic stimulus. The mechanism(s) through which tumor factor(s) modulate the response of endothelium to TNF remains unclear. Pilot studies examining the affinity and number of TNF binding sites on endothelium after exposure to tumor-conditioned medium have not demonstrated a change in the binding parameters of 125I-TNF. This suggests that the effect of tumor cell products on endothelial cell reactivity to TNF is mediated distal to ligand-receptor interactions.

Locally induced clot formation, occurring rapidly (initiated within 30 min of cytokine infusion), is only one example of a focal TNF effect in tumors. By 4–8 h after TNF infusion, tumor vessels are packed with leukocytic cells. Well-described toxic morphologic changes in the tumor cells and hemorrhagic necrosis also take place at later times (21, 28). Some of these local changes, such as adhesion of leukocytes to endothelium (29), may also be selectively enhanced in the tumor bed due to the effect of factor(s) in the tumor microenvironment such as that which enhances the TNF-induced procoagulant response. Although the relationship between early activation of coagulation in the tumor bed and tumor necrosis is unclear, pilot studies using mice anticoagulated with the same coumadin compound used in these studies (as described for the experiment in Fig. 1) have shown attenuated tumor necrosis, based on reduction in tumor weight, by as much as 50% in anticoagulated compared with control mice. In this context, selective deposition of fibrin, a well-recognized stimu-
lant of the inflammatory response (30), in the tumor bed could be a factor promoting subsequent leukocytic infiltration. Further studies with a variety of anticoagulants and tumors will be required to gain further insight into this issue.

The studies presented in this report indicate that intravascular clot formation within the tumor vasculature is part of the early response of meth A sarcomas to TNF. The localization of clot formation to the tumor bed provides a potentially important model for examining mechanisms underlying intravascular thrombus formation.

Summary

Recent studies have indicated that TNF can promote activation of the coagulation mechanism by modulating coagulant properties of endothelial cells. In this report, we demonstrate that infusion of low concentrations of TNF (3 μg/animal) into mice bearing meth A fibrosarcomas leads to localized fibrin deposition with formation of occlusive intravascular thrombi in close association with the endothelial cell surface. Studies with 125I-fibrinogen showed tenfold enhanced accumulation of radioactivity in tumor within 2 h after TNF infusion. Western blots of tumor extracts subjected to SDS-PAGE and visualized with a fibrin-specific mAb indicated that fibrin forms in the tumor after the TNF infusion. Electron microscopic studies demonstrated fibrin strands, based on the characteristic 21-nm periodicity, which appeared to be adherent to the endothelial cell surface. Further ultrastructural studies indicated that fibrin formation, first evident within 30 min of the TNF infusion, led to occlusive thrombi limited to the tumor vascular bed (i.e., not in the normal mouse vasculature) within 2 h and was associated with an 80% reduction in tumor perfusion based on studies with Evans blue. In view of previous work concerning TNF induction of endothelial cell procoagulant activity, the hypothesis that tumor cell products prime the response of endothelium to this cytokine was tested. Supernatants of cultured meth A fibrosarcomas obtained serum-free conditions, which had no intrinsic procoagulant activity, considerably enhanced tissue factor induction in endothelium in response to submaximal concentrations of TNF. The factor(s) in the tumor-conditioned medium appeared to be distinct from IL-1, fibroblast growth factor, IFN-γ, TNF, endotoxin, TGF-α, and TGF-β. These studies delineate a novel model of localized clot formation in which thrombosis is initiated by a pathophysiological mediator, TNF, and provides an opportunity to examine mechanisms in the microenvironment directing clot formation to the tumor vascular bed.

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