

# TUMOR NECROSIS FACTOR/CACHECTIN INTERACTS WITH ENDOTHELIAL CELL RECEPTORS TO INDUCE RELEASE OF INTERLEUKIN 1

BY P. P. NAWROTH,\* I. BANK,‡ D. HANDLEY,§ J. CASSIMERIS,‡ L. CHESSE,‡  
AND D. STERN\*

*From the \*Thrombosis Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73014; and the Departments of ‡Medicine and §Physiology, Columbia University College of Physicians and Surgeons, New York, New York 10032*

Tumor Necrosis Factor/cachectin (TNF)<sup>1</sup> has been implicated as a mediator of the host response in sepsis and neoplasia (1-2). The close relationship between the septic state and coagulation abnormalities (e.g., disseminated intravascular coagulation), and the hemorrhagic nature of the lesions in the tumor bed induced by TNF (2) suggest that defects in the hemostatic system may result from the action of TNF. In this context, recent studies have indicated that TNF can modulate hemostatic properties of the cells forming the luminal vascular surface, the endothelium (3). Through the concomitant induction of endothelial cell procoagulant activity and suppression of anticoagulant mechanisms, TNF renders the vessel wall a potentially effective surface for thrombotic phenomena and the ensuing coagulopathies. These data suggest the hypothesis that endothelium is a target tissue for TNF.

To serve as a target tissue for an inflammatory mediator such as TNF, endothelium would be expected to provide both binding sites and an amplification mechanism through which the TNF-stimulated cell could augment the inflammatory response. This would be analogous to the chain of events set into motion by IL-1 acting on the lymphocyte to promote IL-2 generation, and thus lymphocyte proliferation potentiating the immune response (4). In this study cultured human umbilical vein endothelial cells are shown to possess saturable and high affinity binding sites for TNF. Furthermore, specific interaction of TNF with endothelium leads to the synthesis and release of IL-1. These data indicate that TNF can initiate a cascade of inflammatory events on the vessel surface both directly and through the stimulation of endothelial cell IL-1 generation.

## Materials and Methods

*Cell Culture.* Endothelial cells derived from human umbilical cord veins were prepared by the method of Jaffe et al. (5 and 6), as modified by Thornton et al. (7). Endothelial cells were harvested from umbilical veins, cells were placed in tissue culture flasks

Address correspondence to Drs. Peter P. Nawroth and David M. Stern, Thrombosis Research, Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104.

<sup>1</sup> *Abbreviation used in this paper:* TNF, tumor necrosis factor/cachectin.

pretreated with gelatin (0.25%; gelatin solutions contained 1  $\mu\text{g}/\text{ml}$  polymyxin B) and grown in medium 199 (Gibco Laboratories, Grand Island, NY) containing heparin (90  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co., St. Louis, MO), endothelial cell growth factor (20  $\mu\text{g}/\text{ml}$ ; Meloy Laboratories, Springfield, VA), human serum (20%; prepared from normal human volunteers), and penicillin-streptomycin (50 U/ml–50  $\mu\text{g}/\text{ml}$ ; Gibco Laboratories). After confluence was achieved ( $0.7\text{--}1.1 \times 10^5$  cells/cm<sup>2</sup>), cells were subcultured using trypsin-EDTA (0.25–0.05%; Gibco Laboratories). Cultures were characterized as endothelial cells based on morphologic criteria (5) and by positive immunofluorescence staining with a specific anti-Factor VIII/von Willebrand factor antibody (generously provided by Drs. Chopek and Davie, University of Washington, Seattle, WA) (8). By passage 1, endothelial cell cultures contained no monocyte/macrophage cells, as judged by morphologic criteria and by cytofluorometry using OKM2, an mAb reactive with the majority of human monocytes/macrophages (9). Absence of T lymphocytes from cultures was also assessed cytofluorometrically using the monoclonal antibody OKT3 (10). All experiments were carried out 24 h after endothelial cells achieved confluence in 9.6-cm<sup>2</sup> dishes except for the rebinding studies (Fig. 3) which employed 75 cm<sup>2</sup> tissue culture flasks.

*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 employed in these studies, migrated as a single band with an apparent molecular weight of 17,500 under both reducing and nonreducing conditions on SDS-PAGE (11). This material was distinct from lymphotoxin, had no activity in an IL-1 thymocyte costimulation assay, and had a specific activity of  $\sim 10^8$  U/mg using a TNF cytolytic assay (12). The amino acid composition and N-terminal sequence corresponded to that previously reported (13; 14; and Kirchner et al., manuscript in preparation). Heat-treatment of TNF was carried out at 70°C for 1 h and resulted in loss of cytolytic activity (15).

Radiolabeling of TNF was accomplished using 1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenyl glycoluril (Iodogen; Pierce Chemical Co. Rockville, MD) (16). Free iodine was separated from TNF by gel filtration using a column (20 cm) of Sephadex G-25. The specific radioactivity of <sup>125</sup>I-TNF was 25–40,000 cpm/ng over four radioiodinations. Radioiodinated TNF migrated as a single band on reduced (Fig. 2) or nonreduced SDS-PAGE and comigrated with the unlabeled material. After the radiolabelling procedure, TNF retained 70–90% of its original activity as determined by a cytotoxic assay on L929 cells (12).

Monospecific rabbit antiserum to human IL-1 was provided by BASF. The IgG fraction of rabbit antiserum to human IL-1 was purchased from Genzyme (Boston, MA). Control serum was obtained from rabbits not specifically immunized with any protein. The IgG fractions of antisera were prepared using staphylococcal protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) by the method of Goding (17). IgGs were immobilized on affigel 10 ( $\sim 2$  mg of protein per ml of resin) following the manufacturer's protocol. Columns were then equilibrated and run in serum-free medium (minimal essential medium containing penicillin-streptomycin [50 U/ml–50  $\mu\text{g}/\text{ml}$ ], 1  $\mu\text{g}/\text{ml}$  polymyxin B, 10 mM Hepes [pH 7.4], 20  $\mu\text{g}/\text{ml}$  transferrin, and 10  $\mu\text{g}/\text{ml}$  insulin and 5 mg/ml BSA).

Electrophoresis was carried out by the method of Laemmli (11) employing 12.5% gels. For autoradiography, gels were dried and exposed to X-Omat (XAR5) film (Eastman Kodak Co., Rochester, NY) in the presence of a Cronex intensifying screen (Dupont Co., Wilmington, DE). Standard proteins, including lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), OVA (45,000), BSA (66,200) and phosphor-ylase B (92,500) (Bio-Rad Laboratories, Cambridge, MA.) were run simultaneously.

IL-1 activity was measured in a costimulation assay using murine thymocytes as described previously (18). Thymocytes ( $1.5 \times 10^6$ /well) from 4–8-wk-old C3H/HEJ mice (The Jackson Laboratory, Bar Harbor, ME) were cultured for 72 h in Iscove's modified Dulbecco's medium (Gibco Laboratories) containing FCS (10%), glutamine (1%),  $\beta$ -ME (50  $\mu\text{M}$ ), and PHA 1%. Endothelial cell supernatants (50  $\mu\text{l}$ ) were added to wells at the start of a 72 h assay, and [<sup>3</sup>H]thymidine (1  $\mu\text{Ci}/\text{well}$ ; New England Nuclear, Boston, MA) was added 18 h before harvesting. Before assaying supernatants of endothelial cells

containing added cycloheximide, samples were extensively dialyzed vs. saline (dialysis tubing had a molecular weight cutoff of 3,500). Control samples containing cycloheximide and medium not exposed to cells were also dialyzed and assayed. Under these conditions cycloheximide had no effect on thymocyte proliferation in response to added IL-1 (Genzyme). In addition to studying culture supernatants, a lysate of endothelial cells was also assayed. The lysate was prepared by three freeze-thaw cycles ( $-80$  to  $37^{\circ}\text{C}$ ) and cellular debris was removed by centrifugation ( $10,000\text{ g}$  for 20 min). Samples were assayed in triplicate. TNF, up to 100 nM, did not induce thymocyte proliferation above background in this assay.

*TNF Binding Assays.* Binding studies were carried out at  $4^{\circ}\text{C}$  after washing endothelial cells twice with calcium- and magnesium-free HBSS. 1 ml serum-free medium (Medium 199 containing penicillin-streptomycin [ $50\text{ U/ml}$ - $50\text{ }\mu\text{g/ml}$ ], 10 mM Hepes [pH 7.4], 20  $\mu\text{g/ml}$  transferrin, 10  $\mu\text{g/ml}$  insulin, 1  $\mu\text{g/ml}$  polymyxin B, 5 mg/ml human serum albumin) containing radiolabeled TNF alone or radiolabeled TNF in the presence of excess unlabeled TNF was then added for the indicated times. The concentration of unlabeled TNF used to displace specific binding in these studies was a 300-fold molar excess compared with  $^{125}\text{I}$ -TNF. Addition of more unlabeled material had no effect on specific binding. Binding was terminated by five rapid washes (1 ml/wash) with serum-free medium ( $4^{\circ}\text{C}$ ). Cells were solubilized with tris (50 mM; pH 7.4)-saline (0.1 M) containing NP-40 (1%). No binding was observed in wells without cells. Data from binding experiments were fit to the equilibrium binding equation:  $[B = nK \div (1 + KA)]$  (19), assuming a one-site model, where B is the amount bound, n is the number of sites per cell, A is the free concentration of radioligand, and K is the association constant. A nonlinear, least-squares program (SAS Institute, Cary, NC) was used to obtain the best fit curve, to solve for n and K, and to determine the standard error. A plot of residuals vs. free radioligand concentration for the binding data shown in Fig. 4 indicated no systematic error was involved in fitting the binding to the model used (data not shown).

Dissociation studies were carried out using the method of infinite dilution (Fig. 1B) as described by Lollar et al. (20); after cell monolayers were incubated with  $^{125}\text{I}$ -TNF and washed free of unbound protein, fresh, serum-free medium was added (1 ml). At the indicated times, incubation buffer was aspirated, the dishes were washed twice (1 ml/wash) with the same buffer, and solubilized as described above. Dissociation of  $^{125}\text{I}$ -TNF was also studied in the presence of excess unlabeled TNF and by incubating monolayers with glycine (50 mM; pH 3.0)-saline (0.1 M)-EDTA (5 mM) (21) for 2 min at  $4^{\circ}\text{C}$ . The latter treatment did not result in loss of endothelial cell viability or detachment from the culture dish (see Results).

The ability of  $^{125}\text{I}$ -TNF to rebind to endothelial cell monolayers after elution from cells was tested as follows (Fig. 3):  $^{125}\text{I}$ -TNF (300 pM) was incubated with endothelial cell monolayers in  $75\text{-cm}^2$  tissue culture flasks (five flasks in total) for 2 h at  $4^{\circ}\text{C}$ . After washing to remove unbound material, cell-bound  $^{125}\text{I}$ -TNF was eluted with glycine (50 mM; pH 3.0)-saline (0.1 M; 3 ml/flask) as described above. Eluted  $^{125}\text{I}$ -TNF was immediately restored to pH 7.4 by the addition of 2 M tris (pH 8.0), dialyzed vs. protein-free, serum-free medium ( $4^{\circ}\text{C}$ ), concentrated by exposure to Sephadex G-10 (Pharmacia Fine Chemicals) while still within the dialysis bag, and further dialyzed vs. the same medium. Finally, eluted  $^{125}\text{I}$ -TNF was diluted with serum-free medium to a final  $^{125}\text{I}$ -TNF concentration of 45 pM. The endothelial cell binding of  $^{125}\text{I}$ -TNF eluted from the cell surface was then compared with the binding of  $^{125}\text{I}$ -TNF not previously exposed to cells (Fig. 3).

*Colloidal Gold Labeling and Electron Microscopy.* Suspensions of colloidal gold were prepared (22) with an average diameter of  $15.6 \pm 1.1\text{ nm}$  (mean  $\pm$  S.D.) and at a concentration of  $1.9 \times 10^{12}$  particles/ml (23). Optimal adsorption of TNF to colloidal gold occurred when the pH of the reaction mixture was adjusted to the pI of TNF (13). The completeness of TNF adsorption to the gold particles was determined using the serial dilution-electrolyte flocculation test (24–25). After labeling, TNF-gold suspensions were adjusted to isotonicity with sucrose and albumin. Colloidal gold was also labeled with albumin to assess nonspecific binding in the cell labeling studies. Experiments were then carried out as described for radioligands. Nonspecific binding of TNF-gold probes to the

cell surface was determined in the presence of one hundredfold molar excess of free TNF. Nonspecific colloid binding was evaluated using human serum albumin, colloidal gold conjugates. The surface density of TNF-gold probes on endothelial cells was determined from thin sections of each culture plate (26). A total of 25 electron micrographs was photographed from thin sections of each sample.

For transmission electron microscopy, monolayers were fixed in a glutaraldehyde (1%) and acrolein (0.5%) in sodium cacodylate (0.1 M, pH 7.35, 475 mosmol) for 30 min. Cells were postfixed for one hour in osmium tetroxide (1%), stained with uranyl acetate (2%), dehydrated in an ascending ethanol series, and embedded in Spurr's resin (27). Thin sections (50 nm) were contrasted with lead citrate and viewed in a EM-95 electron microscope (Carl Zeiss, Inc., Thornwood, NY).

*Treatment of Endothelial Cells with Unlabeled TNF.* Confluent endothelial cell monolayers (9.6-cm<sup>2</sup> dishes) were washed twice with calcium- and magnesium-free HBSS, then serum-free medium (1 ml) was added in the presence of other proteins as indicated, and cultures were incubated at 37°C. In experiments using cycloheximide (1 µg/ml; Sigma Chemical Co.), this inhibitor was added simultaneously with the TNF. Throughout these experiments, endothelial cell viability was >95%, as assessed by trypan blue exclusion and morphologic criteria.

## Results

Previous studies have shown that incubation of confluent, cultured endothelial cells with TNF is not lethal for the cells and leads to the modulation of cell surface coagulant properties (3). These data suggested that endothelium would possess specific interaction sites for TNF. When cultured human umbilical vein endothelial cells were incubated with recombinant human <sup>125</sup>I-TNF, time-dependent binding was observed (Fig. 1A). At 4°C, binding reached an apparent maximum by 80 min even at the lowest concentrations of <sup>125</sup>I-TNF used (30 pM). The second order rate constant for association, calculated from the data in Fig. 1A was  $\sim 4.1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ . The concentration of binding sites used in this calculation was taken from the data in Fig. 4. At 37°C, cellular association was more rapid, but surface binding was followed by cellular processing. This was indicated by the incomplete elution of cell-associated <sup>125</sup>I-TNF during a brief exposure to trypsin (2 mg/ml for 5 min at 21°C), when binding was carried out at 37°C, compared with complete elution when binding was carried out at 4°C. These results, which suggest that cell-associated <sup>125</sup>I-TNF is not confined to the cell surface at the higher temperature, are consistent with endocytosis of the ligand. Endocytosis of surface bound TNF has been reported by Baglioni et al. (21) during studies of the interaction of TNF with HeLa cells. Thus, in order to define equilibrium-binding parameters in the present study, experiments were carried out at 4°C.

Dissociation studies (Fig. 1B) indicated that interaction of <sup>125</sup>I-TNF with endothelium was reversible with a first-order dissociation constant of  $5.1 \times 10^{-3} \text{ min}^{-1}$ . A similar dissociation rate was observed whether elution of cell-bound TNF was studied by the method of infinite dilution or in the presence of excess unlabeled TNF. Although several hours were required for complete dissociation, TNF was on the cell surface, since a 2 min exposure at 4°C to glycine (50 mM, pH 3.0)-saline (0.1 M)-EDTA (5 mM) buffer (21) resulted in rapid elution of cell-associated <sup>125</sup>I-TNF (Fig. 1B), but did not disrupt the intact monolayer. Elution of cell-bound TNF was also complete in the presence of NP-40 (1%). SDS-PAGE of the detergent eluates indicated that the initial tracer and cell-

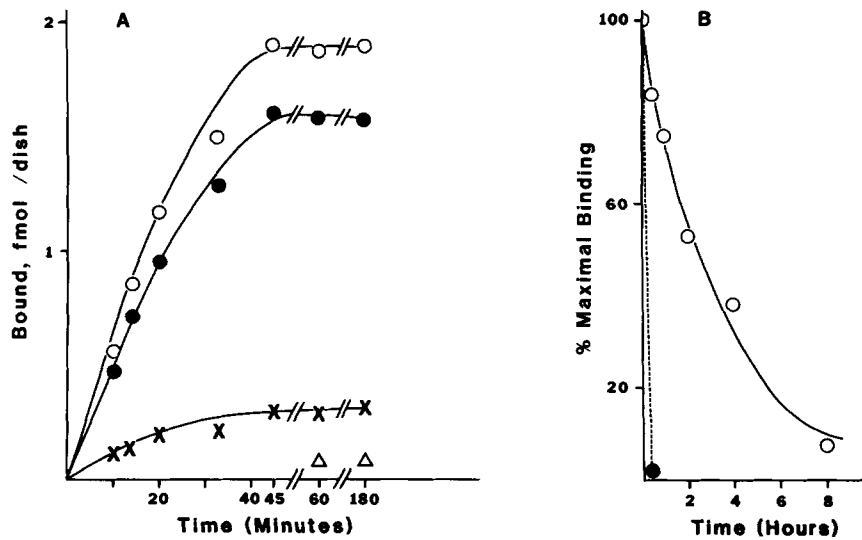


FIGURE 1. Time course and reversibility of <sup>125</sup>I-TNF binding to endothelial cells. (A) Time Course: Endothelial cell monolayers (passage 2) were incubated for the indicated times with <sup>125</sup>I-TNF (150 pM) alone or <sup>125</sup>I-TNF in the presence of excess unlabeled TNF (45 nM). Total binding (O), binding observed with <sup>125</sup>I-TNF alone; nonspecific binding (x), binding observed with <sup>125</sup>I-TNF in the presence of unlabeled TNF; and specific binding (●), the difference between total and nonspecific binding. The mean of duplicates is shown. (B) Reversibility: Endothelial cell monolayers (passage 1) were incubated with <sup>125</sup>I-TNF (180 pM) for 2 h at 4°C and then washed four times as described in Materials and Methods. At this point (time 0), fresh, serum-free medium (1.0 ml) (O), or glycine (50 mM; pH 3.0)-saline (0.1 M)-EDTA (5 mM) buffer (1 ml) (●) was added. The dishes were then incubated at 4°C for the indicated times, washed, and solubilized. Nonspecific binding was determined from a set of dishes in which <sup>125</sup>I-TNF (180 pM) and unlabeled TNF (50 nM) were added simultaneously. Maximal specific binding (time 0) was 1.6 fmol/well. The mean and SEM of the percent maximal specific binding is plotted vs. time.

bound material migrated identically with  $M_r \sim 17,500$  (data not shown). Although the molecular weight of cell-bound <sup>125</sup>I-TNF on SDS-PAGE appeared to be unchanged, since only a small portion (1–11%) of the total added <sup>125</sup>I-TNF became cell-associated it was important to determine whether cell-bound <sup>125</sup>I-TNF was representative of the total pool of radiolabelled material, i.e. not a subpopulation of damaged molecules. To test this cell-bound <sup>125</sup>I-TNF was eluted from monolayers, dialyzed, and reapplied to fresh cultures. Simultaneously, fresh <sup>125</sup>I-TNF was diluted to the same concentration and incubated with other endothelial cell monolayers. In each case a comparable amount of <sup>125</sup>I-TNF bound to the endothelium. Thus, cell-bound <sup>125</sup>I-TNF is representative of the total pool of <sup>125</sup>I-TNF.

Employing these conditions for equilibrium binding, the association of <sup>125</sup>I-TNF with endothelium was observed to be saturable (Fig. 2). Binding was half-maximal at  $105 \pm 40$  pM and, at saturation there were  $1.5 \pm 0.5 \times 10^3$  sites/cell. Experiments in which <sup>125</sup>I-TNF was diluted with unlabeled TNF showed comparable binding parameters, indicating that unlabeled and labeled TNF interacted with endothelium similarly. In contrast, heat-treated TNF, which lacks biologic activity (3, 15), does not bind to endothelial cell monolayers (Fig. 2).

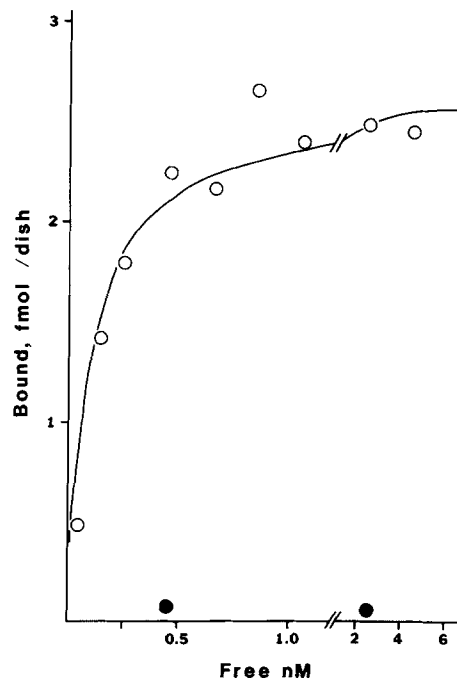


FIGURE 2. Saturability of  $^{125}\text{I}$ -TNF binding to endothelial cell monolayers (passage 1). Endothelial cell monolayers were incubated for 2 h at  $4^\circ\text{C}$  with the indicated concentrations of  $^{125}\text{I}$ -TNF alone (total binding or  $^{125}\text{I}$ -TNF in the presence of a 300-fold molar excess of unlabeled TNF. The binding assay was carried out as described in Materials and Methods. Specific binding (total minus nonspecific binding) is plotted vs. free  $^{125}\text{I}$ -TNF (O). Data were analyzed by the nonlinear least squares program and the curve (-) indicates the best fit line. Nonspecific binding accounted for 15–23% of the total binding. Where indicated, heat-treated  $^{125}\text{I}$ -TNF and unlabeled TNF were used (●).

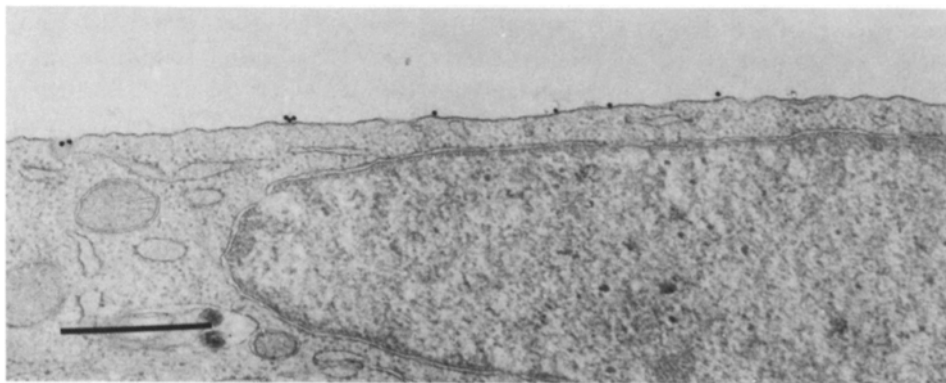


FIGURE 3. Binding of TNF-colloidal gold particles to endothelial cell monolayers. Endothelial cell monolayers (passage 2) were incubated for 2 h at  $4^\circ\text{C}$  with TNF-gold probes (corresponding to a TNF concentration of 177 pM and  $1.9 \times 10^{12}$  probes/ml). After washing ( $4^\circ\text{C}$ ) to remove unbound material, samples were immediately processed for electron microscopy (see in Materials and Methods). TNF-gold particles are randomly distributed at the outer membrane surface. Bar,  $0.1 \mu\text{m}$ .

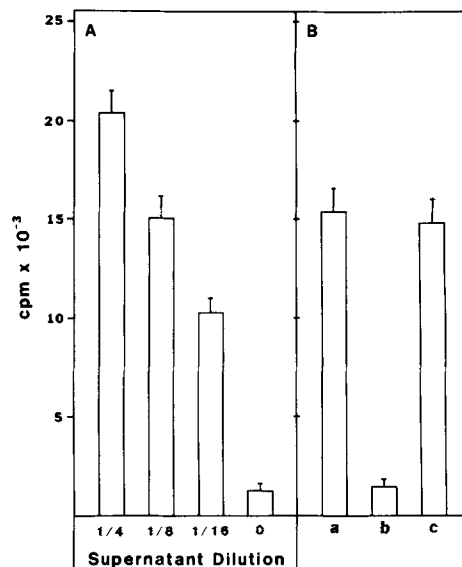


FIGURE 4. Elaboration of IL-1 activity by TNF-treated endothelial cells. (A) Endothelial cell monolayers (passage 4) were incubated in serum-free medium in the presence or absence of TNF (250 pM) for 18 h at 37°C. Samples from TNF-treated cells were diluted in serum-free medium, as indicated, and assayed for IL-1 activity in the thymocyte proliferation assay. Samples from untreated cells (○) were assayed at a 1:2 dilution; identical results were observed at 1:1 and 1:4 dilutions. Sample dilution is plotted vs. [<sup>3</sup>H]thymidine incorporation. (B) Effect of anti-IL-1 affigel 10 on the IL-1 activity of TNF-treated endothelial cell supernatants. Endothelial cell monolayers (passage 3) were incubated in serum-free medium for 17 h at 37°C. Aliquots of cultured supernatants (0.3 ml) were then chromatographed on an anti-IL-1 column (bed volume, 0.5 ml) or a column made by coupling nonimmune rabbit IgG to the resin (bed volume, 0.5 ml). The initial culture supernatant (*a*), the pass-through fraction from the anti-IL-1 column (*b*), and the pass through fraction from the control IgG column (*c*) were assayed in the thymocyte proliferation assay. The mean ± 1 SD of triplicate determinations is shown in both A and B.

To complement the radioligand binding studies and establish that bound TNF was associated with the endothelial cell surface, morphologic studies using gold-labeled TNF were carried out (Fig. 3). Gold-labeled TNF bound to endothelium over a similar range of concentrations and incubation times as that of radiolabeled TNF. The presence of a three hundredfold molar excess of TNF not bound to gold blocked endothelial cell binding of TNF colloidal gold conjugates by >85%. At 4°C, TNF-gold particles were distributed in a random manner, but closely opposed to the luminal cell surface. The binding of BSA-gold conjugates was <5% of that observed with TNF-gold conjugates. These data indicate that TNF does interact with sites on the endothelial cell surface.

To more fully understand the pathophysiologic implications of the interaction of endothelium with TNF, generation of IL-1 (28) by TNF-treated endothelial cell monolayers was studied. When TNF was incubated with confluent human endothelial cells, release of IL-1 activity occurred (Fig. 4A). Using the thymocyte costimulation assay (18), IL-1 activity was clearly detectable in serially diluted supernatants of TNF-treated endothelial cells. Adsorption of IL-1 activity in culture supernatants by an antibody to IL-1 coupled to a solid-phase support

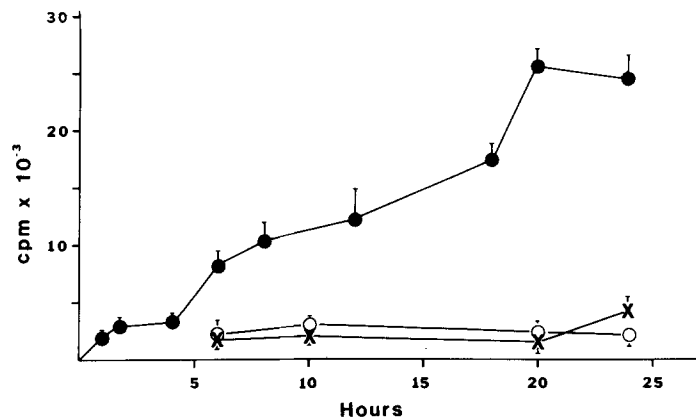


FIGURE 5. Time course of TNF-induced elaboration of IL-1 activity by endothelial cells. Endothelial cell monolayers (passage 2) were incubated in the presence of either TNF (200 pM) (●), or heat-treated TNF (220 pM) (x), or in serum-free medium alone (○) for the indicated time at 37°C. The supernatants were then assayed for IL-1 activity in the thymocyte proliferation assay as described. The mean  $\pm$  1 SD of triplicate determinations is shown.

(Fig. 4B) suggests that IL-1-like molecules were responsible for the observed proliferative response. A control column with nonimmune IgG coupled to the resin had no effect on IL-1 activity in culture supernatants.

Since lipopolysaccharide has been shown to cause release of endothelial cell IL-1 (28), it was important to determine whether TNF in the preparation was responsible for the elaboration of IL-1 activity. In this context, following chromatography of a stock TNF solution (5 nM) over an affigel 10 column (0.6  $\times$  20 cm) with immobilized rabbit antiserum to TNF, pass-through fractions were ineffective in the induction of endothelial cell IL-1. A control column with nonimmune IgG coupled to the resin did not diminish the potency of the TNF preparation for the induction of endothelial cell IL-1. Furthermore, heat treatment of the TNF preparation destroyed its efficacy for IL-1 generation (Figs. 5, 6), although there was no effect on IL-1 release initiated by exogenous lipopolysaccharide (data not shown). Thus, TNF was responsible for the observed IL-1 generation in these experiments.

Induction of IL-1 by TNF occurred in a time-dependent manner (Fig. 5). Increased IL-1 activity was detectable in culture supernatants by 6 h and reached a maximum by 24 h. Addition of cycloheximide (1  $\mu$ g/ml) blocked elaboration of IL-1 activity (Fig. 7), suggesting that de novo protein synthesis was required for endothelium to generate IL-1. Further data to support this view are provided by experiments indicating that endothelial cell lysates did not contain significant amounts of IL-1 before TNF-treatment (data not shown and reference 28). These results are similar to lipopolysaccharide-induced endothelial cell IL-1 generation, where protein synthesis was also required (28).

Generation of IL-1 by endothelium was also dependent on the concentration of TNF incubated with the cultures (Fig. 6). IL-1 generation was half-maximal at a TNF concentration of 40–80 pM and reached a maximum by 300 pM. The general correspondence between parameters of TNF induction of endothelial cell IL-1 and <sup>125</sup>I-TNF binding to the monolayers suggests that it is the cell-



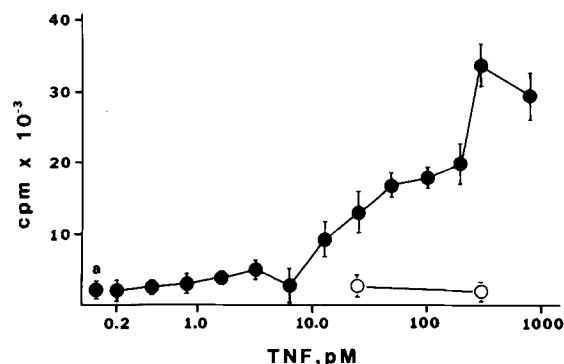


FIGURE 6. The effect of TNF-concentration on the generation of IL-1 by endothelial cells. Endothelial cell monolayers (passage 2) were incubated for 18 h at 37°C in serum-free medium in the presence of either the indicated concentration of TNF (●) or heat-treated TNF (○). Samples were then assayed in the thymocyte proliferation assay. The direct effect of TNF (0.8 nM) added to the thymocyte assay in the absence of endothelial cell supernatant is shown (a). The mean  $\pm$  1 SD of triplicated determinations is shown.

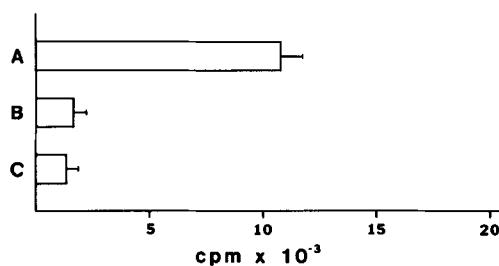


FIGURE 7. Effect of cycloheximide on TNF-induced IL-1 elaboration. Endothelial cells (passage 1) were incubated either with TNF (25 pM) (A), TNF (25 pM) and cycloheximide (1 µg/ml) (B), or serum-free medium alone (C). This concentration of cycloheximide did not reduce cell viability. Culture supernatants were dialyzed extensively vs. saline, and assayed for IL-1 as described in Materials and Methods. 1 µg/ml cycloheximide, after dialysis, had no effect on the thymocyte proliferation assay. The mean  $\pm$  1 SD. is shown.

bound TNF that stimulates IL-1 generation. This hypothesis is supported by the observation that heat-treated TNF neither binds to endothelium nor induces IL-1 generation even at levels as high as 300 pM (Fig. 6).

### Discussion

The results reported here indicate that cultured endothelium provides a target for the monokine TNF. In contrast to its lethal effect on certain transformed tumor cell lines (2), TNF is not toxic to confluent cultured human endothelium (3). Rather, it binds to the cell surface in a saturable, high affinity manner and promotes IL-1 generation. Consistent with these results, the febrile response observed in patients and mice infused with TNF may involve TNF-induced IL-1 release (2, 29). This suggests that the pathophysiology of disorders in which TNF plays an integral role involves not only direct action of TNF on cellular targets, but also an amplification mechanism involving IL-1 and its cellular targets. For example, within 2 h of exposure to TNF, before the release of

detectable amounts of IL-1, endothelial cells undergo a modulation of their coagulant properties allowing them to promote activation of the hemostatic mechanism (3). Subsequently, IL-1 is generated, both in response to TNF and thrombin formed by the activated coagulation system (28), and can interact with other vascular beds, resulting in the induction of procoagulant activity in their endothelium. This series of events could well underlie the diffuse coagulopathy associated with the septic state or the prothrombotic diathesis observed in patients with malignancies.

An alternative possibility suggested by the data presented here is that IL-1 mediates certain changes in endothelial cell physiology initiated by TNF. In support of this hypothesis, both TNF and IL-1 result in the induction of endothelial cell tissue factor (3, 30, 31). To test this, endothelial cells were incubated with TNF (50 pM) in the presence of anti-IL-1 IgG at a concentration that inhibits thymocyte proliferation (18) in response to 50 U/ml of IL-1. The latter concentration of IL-1 is considerably more than that detected in endothelial cell supernatants after treatment of monolayers with TNF. The antibody to IL-1 had no effect on tissue factor induction compared with nonimmune IgG or medium alone. In contrast, this antibody blocked tissue factor induction in response to purified exogenous IL-1 (10 U/ml). In this context, when endothelial cells were incubated simultaneously with TNF and IL-1 more tissue factor was observed than with either agent alone. These results suggest that TNF and IL-1 may act independently to bring about the induction of endothelial cell tissue factor. However, these results must be interpreted with caution since the possibility remains that IL-1 produced in response to TNF acts at a location inaccessible to the IL-1 antibody.

The close relationship between the concentrations of TNF that modulate endothelial cell hemostatic properties (half-maximal at 40–70 pM) observed in a previous study (3), and levels that induce endothelial cell IL-1 and result in occupancy of TNF cell surface binding sites suggests that these sites may be involved in mediating the effect of TNF on the vessel surface. Recent data of Rubin et al. (32), indicating that mouse L(M) cells sensitive to TNF bound the monokine specifically, but resistant L(M) cells did not, support the concept of a relationship between the presence of TNF binding sites and a cellular response to TNF. The affinity of  $^{125}\text{I}$ -TNF for the endothelial cell surface ( $105 \pm 40$  pM) is similar to the affinity of radiolabeled TNF for other cellular surfaces with specific binding sites (100–300 pM) (21, 32–33). The number of binding sites on the endothelial cell surface ( $\sim 1,500$  sites/cell) is somewhat less than that reported in two studies (21, 33). In contrast, Baglioni et al. (21) and Beutler et al. (33) found  $\sim 6,000$  and  $\sim 10,000$  TNF-binding sites/cell, respectively, on cell populations sensitive to TNF. Furthermore Baglioni et al. (21) noted decreased sensitivity of cells to TNF with fewer TNF-binding sites. This suggests the hypothesis that TNF could become toxic to endothelium or more effective in IL-1 induction if the number of endothelial cell TNF sites was increased in pathologic states. For example, the *in vivo* tumoricidal effect of TNF could be related to concentration of TNF in the tumor vascular bed by endothelium with increased numbers of TNF receptors.

The demonstration of TNF binding sites on endothelium coupled with TNF-

induced IL-1 generation and modulation of endothelial cell hemostatic properties contributes to an emerging picture in which the vessel surface could play a central role in the pathophysiology of TNF-mediated disorders. The interaction of TNF with endothelium may thus contribute to the pathogenesis of the diffuse coagulopathy associated with sepsis and the apparent "collapse of vasculature around tumors" noted by investigators who examined destruction of tumors in vivo by endotoxin 30 years ago (34–36).

### Summary

Tumor necrosis factor/cachectin (TNF) has been implicated as a mediator of the host response in sepsis and neoplasia. Recent work has shown that TNF can modulate endothelial cell hemostatic properties, suggesting that endothelium is a target tissue for TNF. This led us to examine whether endothelial cells have specific binding sites for TNF and augment the biological response to TNF by elaborating the inflammatory mediator, IL-1. Incubation of <sup>125</sup>I-recombinant human TNF with confluent, cultured human umbilical vein endothelial cells resulted in time-dependent, reversible, and saturable binding. Binding was half-maximal at a TNF concentration of  $105 \pm 40$  pM, and at saturation 1,500 molecules were bound per cell. Heat-treated TNF, which is biologically inactive, did not bind to endothelium. In addition to surface binding, TNF induced the elaboration of IL-1 activity by endothelial cells in a time-dependent manner. Generation of IL-1 activity required protein synthesis and was half-maximal at a TNF concentration of  $50 \pm 20$  pM. IL-1 activity from TNF-treated endothelium could be adsorbed by an immobilized antibody to IL-1. Heat-treated TNF was ineffective in eliciting endothelial cell IL-1. These data indicate that TNF can bind specifically to endothelium and initiate a cascade of inflammatory and coagulant events on the vessel surface potentially central to the host response to neoplasia and sepsis.

We gratefully acknowledge the technical assistance of V. Guinn throughout these studies. D. Stern completed this work during the tenure of a Clinician Scientist Award from the American Heart Association, with funds contributed in part by the Oklahoma affiliate. This work was supported by a Young Investigator Award from the Oklahoma Affiliate of the American Heart Association (D. Stern) and National Institutes of Health grants HL-34625 (D. Stern) and AI 14969 (L. Chess).

*Received for publication 27 January 1986 and in revised form 3 March 1986.*

### References

1. Beutler, B., I. W. Milsark, and A. C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effects of endotoxin. *Science (Wash. DC)*. 229:869.
2. Old, L. J. 1985. Tumor necrosis factor. *Science (Wash. DC)*. 230:630.
3. Nawroth, P., and D. Stern. 1986. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* 164:740.
4. Dinarello, C. A. 1984. Interleukin-1. *Rev. Infect. Dis.* 6:51.
5. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745.

6. Jaffe, E. A. 1984. Culture and identification of large vessel endothelial cells. *In* Biology of Endothelial Cells. E. A. Jaffe, editor. Martinus Nijhoff, Boston. 1-13.
7. Thornton, S. C., S. N. Mueller, and E. M. Levine. 1983. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. *Science (Wash. DC)*. 222:623.
8. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* 52:2757.
9. Breard, J., E. L. Reinherz, P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with human peripheral blood monocytes. *J. Immunol.* 124:1943.
10. Reinherz, E. L., and S. F. Schlossman. 1980. The differentiation and function of human T lymphocytes. *Cell.* 19:821.
11. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature (Lond.)*. 227:680.
12. Ruff, M. R., and G. E. Gifford. 1980. Purification and physicochemical characterization of rabbit tumor necrosis factor. *J. Immunol.* 125:1671.
13. Pennica, D., G. Nedwin, J. Hayflick, P. Seeburg, R. Derynck, M. Palladino, W. Kohr, B. Aggarwal, and D. Goeddel. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.)*. 312:724.
14. Wang, A. M., A. Creasey, M. Ladner, L. Lin, J. Strickler, J. Van Arsdel, R. Yamamoto, and D. Mark. 1985. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science (Wash. DC)*. 228:149.
15. Williamson, B., E. Carswell, B. Rubin, J. Prendergast, and L. Old. 1983. Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. *Proc. Natl. Acad. Sci. USA.* 80:5397.
16. Salacinski, P., C. McLean, J. Sykes, V. Clement-Jones, and P. Lowry. 1981. Iodination of proteins, glycoproteins, and peptides using solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenylglycoluril. *Anal. Biochem.* 117:136-146.
17. Goding, J. 1976. Conjugation of antibodies with fluorochromes: modifications to standard methods. *J. Immunol. Methods.* 13:215.
18. Meltzer, M. A., and J. J. Oppenheim. 1977. Bidirectional amplification of macrophage-lymphocyte interaction: enhanced lymphocyte activation factor production by activated adherent mouse peritoneal cells. *J. Immunol.* 118:77.
19. Klotz, I., and D. Hunston. 1971. Properties of graphical representations of multiple classes of binding sites. *Biochemistry.* 10:3065-3069.
20. Lollar, P., J. Hoak, and W. Owen. 1980. Binding of thrombin to cultured human endothelial cells. *J. Biol. Chem.* 255:10279.
21. Baglioni, C., S. McCandless, J. Tauernier, and W. Fiers. 1985. Binding of human tumor necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. *J. Biol. Chem.* 260:13395.
22. Frens, G. 1973. Controlled nucleation for the regulation of particle size in monodisperse gold suspensions. *Nature (Lond.)*. 241:22.
23. Ackerman, G., J. Yang, and K. Wolken. 1983. Differential surface labeling and internalization of glucagon by peripheral leukocytes. *J. Histochem. Cytochem.* 31:433.
24. Horrisberger, M., and M. VonLanthen. 1980. Ultrastructural localization of soybean agglutinin on thin sections of glycine max (soybean) Var. Altona by the gold method. *Histochemistry.* 65:181.
25. Handley, D., and L. Witte. 1984. Platelet derived growth factor labeled with colloidal gold for use as a mitogenic receptor probe. *Eur. J. Cell. Biol.* 34:281.
26. Handley, D. A., and S. Chien. 1983. Colloidal gold: A pluripotent receptor probe. *Proc. Soc. Exp. Biol. Med.* 174:1.

27. Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31.
28. Stern, D. M., I. Bank, P. P. Nawroth, J. Cassimeris, W. Kisiel, J. W. Fenton, C. Dinarello, L. Chess, and E. A. Jaffe. 1985. Self-regulation of procoagulant events on the endothelial cell surface. *J. Exp. Med.* 162:1223.
29. Beutler, B., Milsark, J. W., Krochin, N., and Cerami, A. 1985. Cachectin (Tumor Necrosis Factor, TNF): The monokine responsible for the lethal effect of endotoxin. *Blood.* 66(Suppl.)83a.
30. Bevilacqua, M., J. S. Pober, G. R. Majeau, R. S. Cotran, and M. A. Gimbrone, Jr. 1984. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J. Exp. Med.* 160:618.
31. Nawroth, P. P., D. Handley, C. T. Esmon, and D. M. Stern. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA.* In press.
32. Rubin, B. Y., S. L. Anderson, S. A. Sullivan, B. D. Williamson, E. A. Carswell, and L. J. Old. 1985. High affinity binding of <sup>125</sup>I-labeled human tumor necrosis factor (LuKII) to specific cell surface receptors. *J. Exp. Med.* 162:1099.
33. Beutler, B. J., J. Mahoney, N. Le Trang, P. Pekala, and A. Cerami. 1985. Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. *J. Exp. Med.* 161:984.
34. Algive, G. H., and F. Y. Legullais. 1951. Vascular reactions of normal and malignant tissues *in vivo*. IV. The effect of peripheral hypotension on transplanted tumors. *J. Natl. Cancer Inst.* 12:399-422.
35. Algive, G. H., F. Y. Legullais, and B. F. Anderson. 1952. Vascular reactions of normal and malignant tissues *in vivo*. V. The role of hypotension in the action of a bacterial polysaccharide on tumors. *J. Natl. Cancer Inst. Monogr.* 13:1279-1295.
36. Younger, J. S., and G. H. Algive. 1949. The effect of vascular occlusion on transplanted tumors. *J. Natl. Cancer Inst.* 10:565-579.