ENHANCED RESPONSIVENESS OF ENDOTHELIUM IN THE GROWING/MOTILE STATE TO TUMOR NECROSIS FACTOR/CACHECTIN

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Generalized vascular collapse can be induced by the infusion of high concentrations of tumor necrosis factor/cachectin (TNF) (1); at lower concentrations, lesions may be localized to particular parts of the vascular tree. Exposure of tumor-bearing mice to these lower concentrations of TNF, for example, results in intravascular clot formation confined only to the tumor vascular bed (2). This observation raises questions about mechanisms that direct the action of TNF within the vasculature.

Endothelium is a target of cytokines, such as TNF, which are central mediators of the host response. Quiescent endothelium maintains vascular fluidity and constitutes a barrier to the escape of solutes and cellular elements of the blood from the intravascular compartment. Exposure to TNF, however, activates specific endothelial cell-dependent mechanisms in vitro and, presumably, in vivo contributes to the inflammatory response: these include promotion of leukocyte adhesion and coagulation, and an increase in monolayer permeability (1, 3–5).

Although in vitro experiments would suggest that the endothelial response to TNF is uniform, it is evident that a characteristic feature of the host response is its localization to particular sites of the vascular tree. Induction of endothelial growth/motility occurs in regeneration after damage to the endothelial monolayer and in angiogenesis, whether reparative, or in the neovascularization of a tumor. We have found that such proliferating and/or motile endothelial cells would constitute a selective target for TNF. The studies reported here demonstrate that compared with quiescent cultures, endothelial cells in the growth and/or motile state have enhanced responsiveness to and increased affinity for TNF. In parallel, crosslinking studies showed two additional bands on autoradiograms from SDS-PAGE of cell-bound 125I-TNF. These data suggest a model by which an injury or neoplastic focus, resulting in induction of endothelial growth or motility, renders these cells more sensitive to perturbation by TNF, and offers insight into a possible mechanism by which TNF effects are targeted within the vasculature.

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Materials and Methods

Reagents. Recombinant human TNF (~10⁸ U/mg) was generously provided by Dr. P. Lomedico of Hoffmann-LaRoche (Nutley, NJ), and this preparation was homogeneous on SDS-PAGE and distinct from lymphotxin. TNF was radiiodinated by the lactoperoxidase method (6) by mixing TNF (100 μg) with lactoperoxidase-glucose oxidase beads (0.05 ml; Bio-Rad Laboratories, Richmond, CA), glucose (0.025 ml of a 2% solution), and phosphate buffer (0.05 ml; 0.2 M, pH 7.2) for 1.5 min at 4°C. The reaction was stopped by removal of the beads by centrifugation, and free iodine was separated by gel filtration on a Sephadex G-25 column pre-equilibrated with Tris-buffered saline (20 mM Tris, pH 7.4, 0.1 M NaCl) followed by exhaustive dialysis in the same buffer. The specific radioactivity of the tracer was 4,800-6,000 cpm/mg over eight iodinations, and autoradiograms prepared from SDS-PAGE (7) (7.5%; see Fig. 7, lane F) showed a single major band on reduced gels corresponding to an Mₐ ~17,000. Pilot experiments indicated that TNF radiolabeled by this method bound to endothelium in a manner comparable to TNF radiiodinated using 1,3,4,6-tetraclororo-3α, 6α-diphenyl glycoluril (8), which we used in a prior study (9). Monospecific polyclonal antibody to TNF was generously provided by Dr. D. Blohm (Badische Anilin-und Soda-Fabrik, Ludwigshafen, FRG).

Antibody to bovine thrombomodulin was prepared from purified thrombomodulin (10) in rabbits by standard procedures (11). Murine mAb to bovine tissue factor was prepared as described (12). Vinblastine and colchicine were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine protein C, thrombin, and antithrombin III were purified by previously described methods (13). Tritiated inulin (271 mCi/g) was purchased from New England Nuclear (Boston, MA).

Cell Culture. Bovine aortic endothelial cells were isolated from calf aortas as described (14) and were grown in MEM (Flow Laboratories, Rockville, MD), supplemented with penicillin-streptomycin (50 U/ml to 50 μg/ml), glutamine (1%), and FCS (15%; HyClone Laboratories, Logan, UT). Cells were grown in a humidified, 5% CO₂ atmosphere and were transferred either by trypsinization or by exposing the cells to EDTA (1 mM). For experiments, cells (passages 2-10) from different aortas were grown to the indicated density in 35-mm dishes (for tissue factor assays), 24-well (for TNF radioligand binding studies and thrombomodulin assays), or Transwell plates (6.5-mm polycarbonate membranes, with a pore size of 0.4 μm, mounted on polystyrene inserts; Costar, Cambridge, MA). Cell densities were determined from duplicate wells electronically using a Coulter Counter (model ZM; Coulter Electronics, Hialeah, FL). For morphologic studies, cells were grown on coverslips inserted in 35-mm dishes. Cultures were characterized as endothelial by the formation of cobblestone-like monolayers, contact inhibition at confluence, and the presence of von Willebrand factor, protein S, and thrombomodulin (14-17).

Wounding of endothelial monolayers was carried out using a 2-mm cork borer (18). Labeling index was determined using a kit from Amersham Corp. (Arlington Heights, IL). According to this procedure cultures are allowed to incorporate 5-bromouridine, which is then detected using a specific antibody and the peroxidase method for visualization of labeled cells.

TNF Radioligand Binding Studies. Binding studies were carried out by washing monolayers three times over 15 min with MEM containing HEPES (10 mM, pH 7.4) and BSA (1 mg/ml). Cooling cultures to 4°C, and then adding the same buffer such that monolayers were completely covered with fluid. Where indicated, cultures were preincubated with either colchicine or vinblastine before the binding study was performed. Irradiation was carried out using a Siemens x-ray machine. ¹²⁵Ι-TNF was then added either alone (total binding) or in the presence of excess unlabeled TNF (a 100-fold molar excess; nonspecific binding). After 2 h at 4°C, cultures were washed rapidly 10 times with the same buffer used for binding and then solubilized with SDS (2%). Specific binding, total minus nonspecific binding, was then fit to the equilibrium binding equation \( B = nK/(1 + K) \) (19), where \( B \) is the amount of specifically bound ligand, \( n \) is the number of binding sites per cell, \( a \) is the free concentration of radioligand, and \( K \) is the association constant. A nonlinear least-squares program was used to obtain the best fit curve, to solve for \( n \) and \( K \), and to determine the standard error. These methods have been used by us previously to perform equilibrium binding studies of ¹²⁵I-TNF-endothelial cell interaction (9).
Crosslinking Studies. Crosslinking studies were carried out using disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) using the method described by Kull et al. (20). In brief, a radioligand binding assay was performed as described above and monolayers were washed 10 times with HBSS and allowed to incubate for 20 min in the presence of the crosslinking agent (0.5 mM) at room temperature. Cultures were then washed five times with ice cold HBSS containing PMSF (1 mM), scraped with a rubber policeman, and pelleted by centrifugation. The pellet was solubilized using SDS (2%) containing PMSF (1 mM), boiled for 10 min, recentrifuged, and the supernatant was subjected to SDS-PAGE (7.5%) (7). Controls were carried out in which TNF (0.5 nM) was incubated for 20 min at room temperature with disuccinimidyl suberate at the same concentration as above, but in the absence of cells. Samples of this reaction mixture were also obtained for SDS-PAGE.

Thrombomodulin Assays. Thrombomodulin assays were performed by a previously described method (21). In brief, cultures were washed four times in balanced salt solution and then incubated for 45 min at 37°C in 10 mM Hepes, pH 7.45, 137 mM NaCl, 11mM Glucose, 4 mM KCl, 2 mM CaCl2, and 2 mg/ml BSA containing protein C (100 µg/ml) and thrombin (0.1 U/ml). Formation of activated protein C was terminated by the addition of antithrombin III (100 µg/ml)/heparin (1 U/ml) and the amount of enzyme formed was determined using a chromogenic assay, hydrolysis of the substrate Lys-Pro-Arg-p-nitroanilide (Spectrozyme, American Diagnostica, New York, NY). Enzyme concentration was determined by comparison with a standard curve made in the presence of known amounts of activated protein C.

Tissue Factor Activity. Tissue factor activity of endothelial cell monolayers was determined using a coagulant assay after detaching cells from the growth surface by scraping as described previously (22). In brief, after washing monolayers to remove the incubation mixture, cells were scraped with a rubber policeman and suspended in barbital-buffered saline. Cells were washed twice, by centrifugation, and resuspended in the same buffer, and finally suspended in 0.1 ml of barbital-buffered saline. A two-stage coagulant assay was then carried out as described previously (22). Incubation of cells with the mAb to bovine tissue factor (12) blocked endothelial procoagulant activity induced in response to TNF, indicating that tissue factor was responsible for the procoagulant activity. Quantification of induced tissue factor was accomplished by comparing clotting times with a standard curve generated with known amounts of purified tissue factor (23).

Barrier Function. Barrier function of confluent or postconfluent monolayers in Transwell inserts was studied by washing cultures with Earle's balanced salt solution and then adding MEM containing fatty acid-free BSA (1%; Sigma Chemical Co.) to each compartment (avoiding an oncotic gradient; this method has been described in detail previously (24)). Final volumes present in the inner and outer (corresponding upper and lower, respectively) chambers were adjusted to yield no hydrostatic gradient across the monolayer. Radiolabeled inulin was then added in trace concentrations (3 µg/ml) in the presence or absence of TNF. The mixture was then incubated at 37°C in a 5% CO2 atmosphere with continuous agitation and samples (0.005 ml) were withdrawn at the indicated times. Sampling of wells changed volume in chambers during the experiment by <5%. Transport of tracers from the inner to outer chamber, i.e., across the endothelial monolayer, was determined by dividing radioactivity in the outer well by radioactivity in the inner well (measured from duplicate 0.005 ml samples from each well). Data were normalized by dividing transport of tracer observed across TNF-treated monolayers by that observed in the matched set of untreated monolayers. Wells were also sampled within 5 min of tracer addition to establish a background level in order to exclude "damaged" monolayers from the experiment.

Microscopy. Cell monolayers grown on coverslips were fixed in 3.5% paraformaldehyde in PBS, pH 7.2, containing 0.1% NP-40 for 5-10 min and then washed in PBS. For visualization of Factin, coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Junction City, OR) for 30-45 min, washed in PBS, and mounted in Gelvatol containing 1 mg/ml p-phenylenediamine. Mounted coverslips were examined in a Leitz Dialux 20 microscope with a 2.4 Plöempak filter block and water immersion fluorescent objectives, and recorded on Kodak Tri-X film. Immunofluorescence for TNF, thrombomodulin, and tissue factor was carried out by the procedures previously described to visualize endothelial cell protein S (16). Cell surface bound TNF was also visualized by scanning electron microscopy. TNF-colloidal gold conjugates were prepared as described (9) and bound to the endothelial
cell surface during a 2-h incubation at 4°C. Monolayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, postfixed in 2% osmium tetroxide, dehydrated in ethanol, dried from Pel-Dry (Ted Pella Inc., Redding, CA); and viewed in a Jeol T300 scanning electron microscope.

Results

TNF-induced Modulation of Endothelial Cell Function: Comparison of Subconfluent, Confluent, and Postconfluent Cultures. TNF can modulate endothelial cell surface coagulant properties by several mechanisms, including suppression of the anticoagulant cofactor thrombomodulin and induction of the procoagulant cofactor tissue factor, which potentially results in promotion of coagulation on the vessel surface (1, 3-5). At a low concentration of TNF (0.1 nM), thrombomodulin was suppressed in subconfluent cultures (2 d after plating; labeling index 30%; 3.6 x 10⁴ cells/cm²) with a decrease in cell surface activity of ~65% after 8 h (Fig. 1, lower left inset). In contrast, cultures that had just achieved confluence (4 d after plating; labeling index 2-5%; 4.8 x 10⁴ cells/cm²) were less responsive, with a decline in thrombomodulin activity of only ~25%; and late postconfluent cultures (14 d after plating; labeling index <1%; 2 x 10⁵ cells/cm²) showed little if any response. These results led us to examine the response of endothelium to a higher concentration of TNF (Fig. 1, right upper inset).

At a concentration of 10 nM, TNF induced similar suppression of thrombomodulin in subconfluent, just confluent, and postconfluent cultured endothelium. In experiments carried out with a wide range of TNF concentrations (Fig. 1), half-maximal suppression of endothelial cell surface thrombomodulin activity occurred at TNF concentrations of ~0.1, 1.0, and 2.0 nM, respectively, in subconfluent, confluent,
and late postconfluent cultures. Suppression of thrombomodulin activity was paralleled by a fall in total cell-associated thrombomodulin antigen (data not shown). Comparing these results, the effectiveness of TNF for modulating endothelial cell thrombomodulin appears to be ~20 times greater on growing than postconfluent, quiescent cultures.

Several mechanisms underly the effect of TNF on thrombomodulin, including accelerated degradation, shedding of cell surface thrombomodulin, and suppression of synthesis (25–27). This led us to examine whether TNF-induced modulation of endothelial cell tissue factor, which involves de novo synthesis (21, 28), would also vary with the growth state of endothelial cell cultures (Fig. 2). Subconfluent cultures exposed to TNF showed half-maximal induction of tissue factor activity at a concentration of ~0.2 nM; in contrast, confluent and late postconfluent cultures showed a similar effect at 1–2.5 nM. Thus, growth state appears to be a determinant of endothelial cell sensitivity to TNF, both in terms of the induction of tissue factor and suppression of thrombomodulin.

Maintenance of a barrier against passage of solutes from the intravascular space to the interstitium is an important homeostatic property of the endothelial cell monolayer. A general characteristic ascribed to newly formed blood vessels is increased “leakiness,” indicating that barrier function has not attained optimal levels. This observation, taken together with previous work showing that TNF can perturb endothelial cell barrier function to permit the passage of macromolecules and lower molecular weight solutes (24, 29–30), led us to compare the effect of TNF on confluent and late postconfluent cultures (Fig. 3). When confluent cultures (monolayers that had just achieved barrier function) were exposed to TNF (0.5 nM), an increase in the passage of [3H]inulin could be observed within 1 h, as compared with untreated confluent cultures, and was increased twofold by 2–3 h. In contrast, late postconfluent cultures showed a slower response: increase in permeability, as compared with untreated postconfluent cultures, was not evident until 3–4 h after exposure to TNF. Furthermore, the extent of increased passage of tracer across postconfluent monolayers was only 50% of that observed in confluent cultures.

Coincident with TNF-induced perturbation of monolayer barrier function, there was an alteration in cell shape/cytoskeletal organization. Subconfluent endothelial cells are polar to polygonal, with prominent lamellae, sublamellar microfilament

![Figure 2](image-url)
arcs, and oriented stress fiber arrays (Fig. 4 A). At confluence, cultures form a contiguous monolayer with actin-based cytoskeletons characterized by parallel central stress fiber arrays and prominent circumferential stress fiber bundles (Fig. 4 C). Postconfluent endothelial cells form tightly apposed monolayers with dense marginal actin bands and few central stress fibers (Fig. 4 E). After TNF treatment of endothelial cultures (Fig. 4, B, D, and F), circumferential stress fibers were lost, central stress fibers become fragmented, and actin aggregates appeared in the peripheral cytoplasm. These changes were most marked in the subconfluent and just confluent cells (Fig. 4, B and D), consistent with the hypothesis that these cells have increased sensitivity to TNF compared with postconfluent cultures (Fig. 4 F).

Taken together, these data showed that the response of endothelium to TNF was dependent on the growth/motile state of the cultures: subconfluent cultures appeared to be more sensitive to the effects of TNF than postconfluent cultures, possibly due to a difference in the apparent affinity of the endothelial cell for TNF in growing/motile and quiescent cultures. To assess the basis for this differential responsiveness of endothelium to TNF, expression of endothelial cell surface TNF binding sites/putative receptors was examined.
Binding of TNF to Endothelium: Induction of High Affinity Cell Surface Binding Sites. Radioiodinated binding studies using $^{125}$I-TNF were performed under equilibrium conditions using subconfluent, confluent, and early and late postconfluent monolayers, as described in the legend to Fig. 1 (Fig. 5). 2 d after subculture, the apparent $K_d$ of TNF-cell surface binding to subconfluent endothelium was $\sim0.12$ nM. As cells remained in culture for longer periods, the affinity of TNF-endothelial cell interaction appeared to decrease: at 4 (confluent), 7 (early postconfluent), and 14 d (late postconfluent) after plating the apparent $K_d$s were $\sim0.27$, $\sim0.85$, and $\sim1.87$ nM, respectively. When a series of six representative TNF binding experiments with cells derived from different calf aortas and different passages were compared, the affinity of TNF-endothelial cell binding appeared to decrease from 6- to 16-fold from the subconfluent to the late postconfluent state. There was also a change in receptor number as the cells grew to confluence consisting of less than a twofold decrease in binding sites (Fig. 5).

One important consideration was whether these changes in TNF binding to endothelium reflected better access of ligand to the receptor in subconfluent cultures as compared with tightly packed late postconfluent cultures. To address this issue, radioligand binding studies were carried out in suspension using endothelial cells released from the culture surface by exposure to EDTA-containing buffer. Under these conditions, cells derived from subconfluent cultures still displayed higher affinity binding sites than cells derived from late postconfluent cultures (apparent $K_d$s were $\sim0.2$ and $\sim1.5$ nM, respectively), the total number of TNF molecules bound to suspended cells followed the same pattern observed with adherent cells. The decreased affinity of confluent/postconfluent cultures for $^{125}$I-TNF was not due to endothelial cell-dependent processing of the tracer. When the supernatant of $^{125}$I-TNF binding mixtures previously incubated with late postconfluent cultures was subsequently exposed to subconfluent cultures, high affinity binding comparable to that observed with tracer not previously exposed to endothelium was observed (data not shown).

![Figure 5. Binding of $^{125}$I-TNF to endothelium: comparison of subconfluent (.), confluent (■), early (●), and late postconfluent cultures (▲). (A) Endothelial monolayers were incubated for 2 h at 4°C with the indicated concentrations of $^{125}$I-TNF alone (total binding) or $^{125}$I-TNF in the presence of a 100-fold molar excess of unlabeled TNF (nonspecific binding). The binding assay was carried out as described in the text. Specific binding (total minus nonspecific binding) is plotted versus free $^{125}$I-TNF. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. Cultures were classified based on confluency as described in the legend to Fig. 1. (B) Scatchard analysis of the data shown in A. Parameters of binding, $K_d$ and the number of molecules bound per cell, follow: preconfluent (2 d after plating; 0.12 ± 0.02 nM; $5.6 \pm 0.2 \times 10^3$ molecules/cell), confluent (4 d after plating; 0.27 ± 0.02 nM, $5.4 \pm 0.2 \times 10^3$ molecules/cell), early postconfluent (7 d after plating, 0.85 ± 0.02 nM, $4.3 \pm 0.1 \times 10^3$ molecules/cell), and late postconfluent (14 d after plating, 1.87 ± 0.03 nM, $2.7 \pm 0.4 \times 10^3$ molecules/cell).]
Conversely, when $^{125}$I-TNF that had been exposed to subconfluent cultures was then incubated with late postconfluent monolayers, binding was the same as that observed with fresh tracer. These data indicate that the change in affinity of TNF-endothelial cell interaction as cells move from the sub- to postconfluent state is not due to modification of the ligand, but rather occurs due to a change at the level of the cellular receptor.

To assess TNF-endothelial cell interaction at the morphological level, cell-bound TNF was visualized in subconfluent and late postconfluent cultures (Fig. 6). For immunofluorescence, endothelium was incubated with a low concentration of TNF (to promote occupancy of high affinity TNF binding sites) for 1 h at 4°C and then after 5 min at room temperature (during which time culstering/patching presumably occurred, facilitating visualization of the ligand) fixation was carried out. Indirect immunofluorescence staining of cultures using anti-iodinated TNF IgG demonstrated greater staining of subconfluent than late postconfluent cultures (Fig. 6, A-B). In view of recent data indicating that cytokines can modulate membrane fluidity (31), we also carried out experiments in which TNF-colloidal gold conjugates were bound to the cell surface and visualized by electron microscopy (in this case, visualization of the ligand is independent of receptor redistribution). At low concentrations of TNF, TNF-gold particles were consistently observed only on subconfluent endothelial cultures (Fig. 6, C-D). These results support the data from the radioligand binding studies and suggest that there is a higher affinity of TNF for its cell surface binding sites on growing endothelium.

To gain insight into molecular mechanisms responsible for formation of the high affinity cell surface binding site, crosslinking studies were carried out (Fig. 7). Late postconfluent monolayers (lane A) demonstrated two bands, corresponding to $M_r$ ~90,000 and ~108,000, which were similar to those observed in other crosslinking studies (1, 20, 32–34). Subconfluent cultures consistently showed two additional bands corresponding to $M_r$ ~66,000 and ~84,000 (lane B). Addition of excess unlabeled TNF blocked in parallel binding of $^{125}$I-TNF to the cell surface and the appearance of these four bands on autoradiograms (lanes C-D). Since TNF has been reported to form multimers (35–36), it was important to compare these results with studies performed in the absence of cells: none of the four bands (corresponding to $M_r$ of ~66,000, ~84,000, ~90,000, and ~108,000) were observed when radioiodinated TNF was incubated with the crosslinking agent in the absence of cells (lane E), although bands corresponding to $M_r$ ~35,000 and ~52,000, representing TNF dimer and trimer, were seen (in lanes A–E, TNF monomer, $M_r$ ~17,500, was allowed to run off the gel). The initial tracer, $^{125}$I-TNF, in the absence of crosslinker, demonstrated only a single band with $M_r$ ~17,500 (lane F). These results are consistent with the hypothesis that subconfluent cells express new polypeptides on the cell surface, with which TNF becomes associated.

**Role of the Growth State and Cytoskeletal Organization/Cell Shape in Expression of High Affinity TNF Binding Sites.** Since growing endothelial cells are in a radially extended/motile configuration (37–38), it was important to distinguish between the contribution made by alterations in cell shape/cytoskeletal configuration, and that related to the proliferative state of the cell (i.e., cell cycle, entry into S phase), on the expression of high affinity TNF binding sites (39). To address this question, cultures were irradiated to block proliferation and TNF-endothelial cell interaction was
FIGURE 6. Visualization of TNF bound to subconfluent (A and C) and late postconfluent (B and D) endothelial monolayers by immunofluorescence and electron microscopy. (A-B) Immunofluorescence. Endothelial cultures (subconfluent [A] and postconfluent [B]) were incubated with TNF (0.3 nM) for 1 h at 4°C, washed, and then after 5 min at room temperature, fixation was carried out. TNF was visualized by adding rabbit anti-TNF antibody and FITC-conjugated anti-rabbit IgG. Cultures were characterized based on confluency as described in the legend to Fig. 1. Details of methods are described in the text. Magnification: x650. (C-D) Scanning electron microscopy. Endothelial cells (subconfluent [C] and postconfluent [D]) were incubated with TNF (0.1 nM) conjugated to colloidal gold particles for 1 h at 4°C as described in the text. Demar-
FIGURE 7. Crosslinking of $^{125}$I-TNF to endothelium: comparison of subconfluent and late postconfluent cultures. Endothelial monolayers were classified based on confluency as described in the legend to Fig. 1. Cultures were incubated with $^{125}$I-TNF (0.7 nM) alone or in the presence of a 100-fold molar excess of unlabeled TNF for 2 h at 4°C. Cells were then washed, crosslinked, solubilized, and subjected to SDS-PAGE followed by autoradiography as described in the text. (A) Late postconfluent endothelial culture incubated with $^{125}$I-TNF alone; (B) Subconfluent endothelial culture incubated with $^{125}$I-TNF alone; (C) same as A, but the binding study was carried out in the presence of excess unlabeled TNF; (D) same as B, but the binding study was carried out in the presence of excess unlabeled TNF; (E) $^{125}$I-TNF (0.7 nM) incubated with crosslinker under the same conditions as described above, but in the absence of cells; (F) $^{125}$I-TNF after radiolabeling (no crosslinking or exposure to cells). In A–E, the monomeric form of TNF ($M_r \sim 17,500$) was allowed to run off the gel. Arrows on the left point to bands seen in lane A corresponding to $M_r \sim 90,000$ and $\sim 108,000$, and in lane B corresponding to $M_r \sim 66,000$, $\sim 84,000$, $\sim 90,000$, and $\sim 108,000$. Approximate molecular weights were determined by comparing mobilities of the above bands with that of standard proteins run simultaneously: myosin heavy chain ($M_r 200,000$), phosphorylase B ($M_r 97,400$), BSA ($M_r 68,000$), ovalbumin ($M_r 43,000$), and $\alpha$-chymotrypsin ($M_r 25,700$).

Subconfluent cultures irradiated with 1,100 rad and then maintained in culture for up to 12 d had a labeling index of $\sim 0\%$ (Fig. 8). No increase in cell number was observed until after 12–14 d, consistent with the previously reported effect of irradiation on cultured endothelium (40). Expression of high affinity TNF binding sites was maintained for the next 7 d (at 4 d, $K_d \sim 0.08$ nM; at 7 d, $K_d \sim 0.14$ nM) with only a small decrease in affinity 14 d later ($K_d \sim 0.37$ nM). Furthermore, these cells displayed an effective TNF response, assessed in terms of thrombomodulin suppression (data not shown).

Conversely, when late postconfluent cultures expressing lower affinity TNF binding sites ($K_d \sim 2.1$ nM) were irradiated and then replated at subconfluent density, with radial extension and motility, high affinity receptors for TNF were induced (Fig. 9). For these experiments, cultures were irradiated, allowed to recover for 2 d, then subcultured at equal density and maintained for 3 d more before the radioligand binding study. Such cultures, irradiated with 1,100 rad (labeling index <0.1%), had binding sites for $^{125}$I-TNF with $K_d \sim 0.08$ nM, compared with $K_d \sim 0.16$ nM for cultures irradiated with 880 rad (labeling index 0.1–0.5%) and $K_d \sim 0.25$ nM for 660 rad (labeling index $\sim 0.5\%$); in nonirradiated controls the $K_d$ was $\sim 0.2$ nM. Cultures with the lowest cell density, in which the cells were maximally extended (those irradiated with 1,100 rad in which cell division was most completely suppressed), demonstrated the highest affinity TNF binding sites. Whereas, cultures irradiated with 660 rad, which were still capable of cell division after subculturing and had achieved a higher cell density when the $^{125}$I-TNF binding assay was performed 3

Cated areas in C–D are shown at higher resolution in the insets on the right. Cell surface–bound TNF–colloidal gold particles (arrow heads) are demonstrated in the inset to C. Addition of a 100-fold molar excess of TNF not bound to gold particles blocked binding of TNF–gold particles to endothelium by $\sim 90\%$. $\times 2,000$; inset, $\times 15,000$. 
1.4 0 101 2100 3000 4001 SW 610 subconfluent controls (* day 2 after plating, 3.6 x 10^4 cells/cm^2, labeling index 30%) and irradiated cultures (day 4, 7, day 14, ▲). The labeling index and cell number was the same in irradiated cells on days 4 and 7 (0% labeling index; 3.2-3.4 x 10^4 cells/cm^2). After the 10-12th day, cell growth began (the results on day 14 [▲] are shown when there were 4.2 cells/cm^2). Specific binding is plotted versus free ^125I-TNF. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. (B) Scatchard analysis of the data shown in A. Parameters of binding, Kd, and the number of molecules bound per cell, follow: nonirradiated control (0.13 ± 0.02 nM, 5.6 ± 0.2 x 10^3 molecules/cell), irradiated cultures (day 4, 0.09 ± 0.01 nM, 5.9 ± 0.1 x 10^3 molecules/cell; day 7, 0.14 ± 0.05 nM; 5.4 ± 0.9 x 10^3 molecules/cell; day 14, 0.37 ± 0.04 nM; 4.7 ± 0.2 x 10^3 molecules/cell).

d later, showed a slightly lower affinity for TNF. Parallel functional studies showed that thrombomodulin was as effectively suppressed by TNF in irradiated cultures as in the subconfluent cultures (Fig. 1) where there was expression of high affinity TNF sites (data not shown). These data indicated that expression of high affinity TNF binding sites did not require cellular proliferation, but appeared to be correlated more closely with changed cell shape and assumption of the motile or radially extended configuration.

Perturbation of the microtubule network in postconfluent endothelial cultures by agents such as vinblastine or colchicine leads to exaggerated lamellar activity, dis-

Figure 8. Effect of radiation-induced arrest of endothelial cell proliferation on the expression of TNF binding sites by subconfluent cultures. (A) Subconfluent cultures were irradiated 2d after plating with 1,100 rad, maintained in culture and then radioligand binding studies with ^125I-TNF were carried out when indicated: nonirradiated subconfluent controls (○) (day 2 after plating, 3.6 x 10^4 cells/cm^2, labeling index 30%) and irradiated cultures (day 4, ▲; day 7, ●; day 14, ▼). The labeling index and cell number was the same in irradiated cells on days 4 and 7 (0% labeling index; 3.2-3.4 x 10^4 cells/cm^2). After the 10-12th day, cell growth began (the results on day 14 [▲] are shown when there were 4.2 cells/cm^2). Specific binding is plotted versus free ^125I-TNF. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. (B) Scatchard analysis of the data shown in A. Parameters of binding, Kd, and the number of molecules bound per cell, follow: nonirradiated control (0.13 ± 0.02 nM, 5.6 ± 0.2 x 10^3 molecules/cell), irradiated cultures (day 4, 0.09 ± 0.01 nM, 5.9 ± 0.1 x 10^3 molecules/cell; day 7, 0.14 ± 0.05 nM; 5.4 ± 0.9 x 10^3 molecules/cell; day 14, 0.37 ± 0.04 nM; 4.7 ± 0.2 x 10^3 molecules/cell).

Figure 9. Effect of radiation-induced arrest of endothelial cell proliferation on the expression of TNF binding sites by postconfluent cultures. (A) Late postconfluent cultures (see Fig. 1, legend) were irradiated with either 660 (▲), 880 (●), or 1,100 (▼) rad, maintained in culture for 2 d, and then subcultured to a subconfluent density (3.5 x 10^4 cells/cm^2). 3 d later a TNF radioligand binding study was carried out. Specific binding is plotted versus free ^125I-TNF (○). Binding data from late postconfluent cells tested before irradiation. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. The number of cells per well at the time of the binding assay follows: irradiated with 660 rad (4.4 x 10^4 cells/cm^2), irradiated with 880 rad (3.8 x 10^4 cells/cm^2), and 1,100 rad (3.4 x 10^4 cells/cm^2). (B) Scatchard analysis of the data shown in A. Parameters of binding, Kd, and the number of molecules bound per cell, follow: irradiated with 660 rad (0.25 ± 0.01 nM, 5.0 ± 0.1 x 10^3 molecules/cell), 880 rad (0.16 ± 0.02 nM, 5.7 ± 0.4 x 10^3 molecules/cell), and 1,100 rad (0.08 ± 0.01 nM, 6.5 ± 0.1 x 10^3 molecules/cell). Cultures that were tested for binding before irradiation demonstrated Kd = 2.14 ± 0.02 nM and 2.9 ± 0.1 x 10^3 molecules/cell.
ruption of cell–cell contacts, and radial extension (our unpublished observation) followed by induction of DNA synthesis (18). This led us to examine the effects of these agents on the induction of high affinity TNF binding sites (Fig. 10). Dismantling of microtubules by incubation of late postconfluent cultures ($K_d$ for TNF of $\sim 1.43$ nM) with vinblastine for 8 h, followed by washing and a 22-h exposure to fresh medium, resulted in the induction of high affinity TNF binding sites ($K_d \sim 0.14$ nM); similarly, exposure of cultures to colchicine also increased the affinity of TNF for late postconfluent monolayers ($K_d \sim 0.19$ nM). At the time the TNF binding assay was performed, 22 h after addition of fresh medium, monolayers displayed alteration in cell shape and the actin-based cytoskeleton (i.e., loss of tight cell–cell apposition, loss of marginal actin bands, and induction of axial stress fiber arrays), but no increase in labeling index. At longer times (>36 h), a considerable increase in the labeling index (≈ 30%) was observed. These data lend support to the hypothesis that cytoskeletal organization/cell shape may play an important role in determining the expression of high affinity TNF binding sites on endothelium, even before the initiation of cell division and/or DNA synthesis.

Wounding of an Endothelial Cell Monolayer: Effect on a TNF–Endothelial Cell Interaction. These results suggested a mechanism for localizing TNF action within endothelial cultures: local stimulation of endothelial cell growth/motility, in response to injury, could enhance expression of high affinity TNF receptors and in parallel magnify the TNF response. To test this, a late postconfluent monolayer was wounded by physically removing a section of the culture, allowed to recover for 36 h, and then exposed to a low concentration of TNF (0.3 nM) (Fig. 11, A–F). Cells that had migrated into the wound (corresponding to zone 1) (38, 41–42) or those near the wound edge (corresponding to zones 2 and 3) showed enhanced immunofluorescence staining for TNF (Fig. 11 A), compared with cells further back from the wound (corresponding to zone 4) (Fig. 11 B). In addition to increased TNF binding, the cells in zones 1–3 had perturbed coagulant properties: there was enhanced immunofluorescence staining for tissue factor after exposure to TNF in cells close to the wound (zones 1–3, Fig. 11 C), compared with cells further away from the wound.

![Figure 10](image-url). Effect of vinblastine (◊) and colchicine (●) on the binding of $^{125}$I-TNF to late postconfluent endothelial monolayers. (A) Late postconfluent endothelial monolayers were incubated 8 h at 37°C with vinblastine (0.1 μg/ml) or colchicine (0.1 μg/ml), washed, and incubated in normal growth medium for an additional 22 h. Then, a $^{125}$I-TNF binding assay was carried out as described. Control late postconfluent monolayers (●) were treated identically except that no drug was added. Specific binding is plotted versus free $^{125}$I-TNF. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. (B) Scatchard analysis of the data shown in A. Parameters of binding, $K_d$, and the number of molecules bound per cell, follow: vinblastine ($0.14 \pm 0.01$ nM; $4.2 \pm 0.1 \times 10^3$ molecules/cell), colchicine ($0.19 \pm 0.03$ nM; $3.9 \pm 0.01 \times 10^3$ molecules/cell), and untreated control ($1.43 \pm 0.1$ nM; $2.9 \pm 0.2 \times 10^3$ molecules/cell).
Figure 11. Wounding of a late postconfluent endothelial cell monolayer: effect on TNF association and responsiveness. Quiescent, postconfluent endothelial monolayers were wounded as described in the text. Then, they were exposed to either medium supplemented with 0.3 nM TNF (A-F) or fresh medium alone (G and H). Immunofluorescence studies for cell-associated TNF, tissue factor, and thrombomodulin were carried out. (A and B) TNF binding: cultures were incubated with TNF at 4°C for 1 h followed by washing, 5 min at room temperature, fixation, and immunofluorescence staining for TNF of cells at the wound edge (A) or quiescent cells further away from the wound edge (B); (C and E) tissue factor expression after TNF: cultures were incubated with TNF at 37°C for 8 h followed by immunofluorescence staining for tissue factor of cells at the wound edge (C) or quiescent cells further away from the wound edge (E); (D and F) thrombomodulin expression after TNF: cultures were incubated
edge (zone 4; Fig. 11E) or cells close to the wound in control cultures (no TNF added; Fig. 11G). The latter control was important, since growing cells have been shown to have more tissue factor than quiescent cultures (12), although this is clearly less than that observed in the presence of TNF. Suppression of thrombomodulin was also accentuated after exposure to TNF in cells at or near the wound (Fig. 11D), compared with cells further away from the wound (Fig. 11F) or cells close to the wound in control cultures (no TNF; Fig. 11H). Endothelial cells that showed enhanced TNF binding and modulation of coagulant properties had also assumed the motile configuration with concomitant rearrangement of the actin-based cytoskeleton (as described in Fig. 4A). These observations emphasize the strong association between induction of high affinity TNF binding sites and the alteration in cell shape/cytoskeleton.

Discussion

Endothelial cells respond to TNF by modulating coagulant function, barrier properties, and a range of other activities that allow them to participate actively in the host response (1-3, 5). These studies demonstrate that the endothelial cell response to TNF can be regulated by alterations in cytoskeletal organization/cell shape and is correlated with induction of the motile configuration. Cultured endothelium in the growth state, which display an extended, motile form, express high affinity endothelial cell surface TNF binding sites whose occupancy correlates closely with modulation of endothelial cell coagulant properties. Although TNF could induce similar modulation of cellular properties in quiescent, postconfluent cultures in contact inhibition, about 15-fold higher concentrations of cytokine were required, consistent with the observed decrease in affinity of TNF for such postconfluent endothelium. A possible mechanism underlying this alteration in TNF-endothelial cell interaction was indicated by the results of crosslinking studies: both postconfluent and subconfluent cultures demonstrated bands, presumably due to ligand-receptor complex, corresponding to $M_r$ of ~90,000 and ~108,000. Bands of similar molecular weights have been observed when TNF has been crosslinked to other cellular surfaces (1, 20, 32-34). Only subconfluent cultures, however, demonstrated bands corresponding to $M_r$ ~66,000 and ~84,000. Although the functional significance of these new bands, presumably indicating novel cell surface proteins to which TNF becomes crosslinked, is not yet clear, by analogy with the IL-2 receptor, they may modulate the affinity of the receptor for the ligand (43).

Regulation of cell surface TNF binding sites by environmental stimuli such as IFN-$\gamma$, which increases the number of sites, and pharmacologic agents such as PMA, which decreases the number of receptors and possibly efficacy of TNF cellular interaction, has been reported (1, 44-45). We have also observed modulation of the number

with medium supplemented with TNF at 37°C for 8 h followed by immunofluorescence staining for thrombomodulin of cells at the wound edge (D) or cells further away from the wound edge (F). (G and H) Control without TNF: Cultures were incubated with fresh medium at 37°C for 8 h followed by immunofluorescence staining for tissue factor (G) or thrombomodulin (H) of cells at the wound edge. x650.
of endothelial cell surface TNF binding sites with these agents (data not shown), but they induced no change in the affinity of TNF for its receptor similar to that found with growing/motile cells (Fig. 5). The number of binding sites for TNF also changed as subconfluent cultures achieved confluence (Fig. 5), consisting of about a twofold decrease. This may be due to alteration in the relationship between the apparent number of binding sites and cell area, shape, and cell–cell contact. Alternatively, there may be a true difference in the number of cell surface sites, as suggested by the fact that when binding studies were carried out using suspended cells there were still about twofold more sites on preparations derived from sub- versus postconfluent cultures.

Especially in endothelium, changes in cell shape are usually tightly coupled to modulation of the cytoskeleton and, in turn, to the growth state of the cell (37–39). Growing, subconfluent endothelial cultures, for example, display a characteristic motile configuration (Fig. 4) (38, 41) and express high affinity TNF sites. However, irradiated cultures, which had lost their proliferative capacity but are capable of extensive or motile activity, retained the ability to express high affinity TNF sites. Similarly, exposure of cultures to microtubule poisons, which resulted in changes in cell shape and cytoskeleton (before entry into the proliferative state) (18), led to induction of high affinity TNF binding sites. These results suggest the importance of cytoskeletal organization/cell shape in regulating expression of high affinity TNF binding sites. Alternatively, other mechanisms could be involved. For example, cell–cell contacts resulting in growth inhibition (this could be due to expression of certain membrane proteins) (46) or formation of gap junctions (47) may make an important contribution to determining the TNF sensitivity of endothelium.

These data lead us to propose a model for examining TNF vascular interactions: local stimulation of endothelial cell growth and/or motility should result in expression of high affinity TNF sites and targeting of the effects of this cytokine to a particular locus in the vasculature. Results of the in vitro wounding study, in which enhanced TNF binding and responsiveness was associated with the proliferating/motile cells, support this concept. Expression of high affinity TNF binding sites is a potential parameter of activated endothelium providing insight into one mechanism by which cytokine action can be localized within the vascular tree. We are testing this hypothesis using angiogenesis models in vivo.

Summary

Some in vivo observations have suggested that growing or perturbed endothelium, such as that which occurs during angiogenesis, is more sensitive to the action of cytokines (TNF/cachectin, TNF, or IL-1) than normal quiescent endothelial cells. This led us to examine the responsiveness of endothelium to TNF as a function of the growth/motile state of the cell. TNF-induced modulation of endothelial cell surface coagulant function was half-maximal at a concentration of ∼0.1 nM in subconfluent cultures, whereas 1–2 nM was required for the same effect in postconfluent cultures. Perturbation of endothelial cell shape/cytoskeleton was similarly more sensitive to TNF in subconfluent cultures. Consistent with these results, radioligand binding studies demonstrated high affinity TNF binding sites, $K_d$ ∼0.1 nM on subconfluent cultures, whereas only lower affinity sites ($K_d$ ∼1.8 nM) were detected on postconfluent cultures. The mechanisms underlying this change in the
affinity of endothelium for TNF were studied in four settings. Crosslinking experiments with $^{125}$I-TNF and endothelium showed additional bands corresponding to $M_r$ ~66,000 and ~84,000 with subconfluent cultures that were not observed with postconfluent cultures. Experiments with X-irradiated endothelium, whose growth but not motility was blocked, indicated that proliferation was not required for induction of high affinity TNF sites. Postconfluent endothelium, triggered to enter the proliferative cycle by microbutuble poisons, expressed high affinity TNF binding sites together with changes in cell shape/cytoskeleton well before their entry into S phase. Using wounded postconfluent monolayers, cells that migrated into the wound and those close to the wound edge displayed enhanced TNF binding and modulation of coagulant properties. These results suggest a model for targeting TNF action within the vasculature; regulation of high affinity endothelial cell binding sites can direct TNF to activated cells in particular parts of the vascular tree.

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