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Tumor Necrosis Factor Suppresses Transcription of the Thrombomodulin Gene in Endothelial Cells

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Tumor necrosis factor (TNF) dramatically alters the levels of various surface components of the blood vessel wall, such as blood coagulation enzyme receptors, leukocyte-adhesive receptors, and class 1 major histocompatibility complex antigens, which may have relevance to its effects in septic shock, angiogenesis, and tumor growth. However, the precise mechanism by which the cytokine is able to accomplish this remodeling of the endothelial cell surface has not been defined. We have demonstrated that exposure of bovine and human endothelial cells to TNF leads to suppression of the functional cell surface thrombin receptor, thrombomodulin (TM), and TM mRNA of virtually identical magnitude. The cytokine has no significant effect on the stability of TM mRNA or endothelial receptor turnover. Nuclear run-on studies reveal that the treatment of endothelial cells with TNF for short periods reduces TM gene transcription to as little as 3% of control values and that this inhibition does not require new protein synthesis.

Thrombomodulin (TM) is a specific endothelial cell receptor which forms a 1:1 complex with thrombin (7). This interaction product is capable of rapidly converting protein C to activated protein C, which proteolytically destroys the activated cofactors of the coagulation mechanism and thereby suppresses the amount of thrombin generated. The structure of TM was completely unknown until the cloning and sequencing of a cDNA which codes for the bovine and human endothelial cell receptors (12, 13, 27, 31). These studies revealed that TM is structurally similar to coated-pit receptors and is organized into domains which resemble those of the low-density lipoprotein receptor (10). The nucleotide sequence of the human TM gene has also been reported from our laboratory and is noteworthy because of the complete absence of introns (12).

Tumor necrosis factor (TNF) is a polypeptide cytokine which is produced by activated monocytes or macrophages and was originally described as a mediator of hemorrhagic necrosis of certain murine tumors (3, 15, 21, 23). Nawroth and Stern (19) and Bevilacqua et al. (1) have demonstrated that this cytokine enhances the expression of the endothelial cell product, tissue factor, which leads to activation of the coagulation mechanism. Nawroth and Stern (19) have also noted that TNF induces a dramatic dose-dependent reduction in functional cell surface TM, which removes a potent brake on the generation of hemostatic-system enzymes. Schleef et al. (24) have reported that this cytokine augments the synthesis of plasminogen activator inhibitor type 1 and suppresses the production of tissue-type plasminogen activator, which favors the formation of fibrin thrombi. These multiple actions of TNF on endothelial cells may have relevance to its effects in septic shock, angiogenesis, and tumor growth (9, 14, 28). However, the precise mechanism by which the monokine is able to alter the levels of endothelial cell receptors has not been defined at the molecular level.

We have investigated the effects of recombinant TNF alpha on bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) with respect to the synthesis of functional cell surface TM, the levels and stability of TM mRNA, and the in vitro transcription of the TM gene. These studies allowed us to conclude that the monokine rapidly and profoundly inhibits transcription of the TM gene without requiring de novo protein synthesis.

We initially examined the effects of TNF (400 U/ml, 10 nM) on the level of functional cell surface TM with two clones of BAEC and early-passage HUVEC. Previous investigations conducted in our laboratory by using morphological criteria, immunofluorescence staining for von Willebrand factor, and binding of di-iodo-acyl low-density lipoprotein have confirmed the authenticity of these endothelial cells (17). The functional levels of TM were quantitated by measuring the time-dependent conversion of protein C to activated protein C in the presence of exogenously added thrombin. The extent of activated protein C generation is dependent on the presence of functional cell surface TM, since more than 80% of this conversion could be inhibited by the addition of an affinity-fractionated, rabbit anti-bovine TM antibody population to either BAEC clone (data not shown). As previously reported by Nawroth and Stern (19), TNF suppressed functional cell surface TM of BAEC and HUVEC within 4 h to 43.6% ± 16.8% (n = 5) and 45.0% ± 3.4% (n = 5) of control values, respectively. Throughout our studies, we noted that extended incubation of BAEC and HUVEC with the monokine for up to 24 h caused no significant change in cell number, morphology, or cell viability, as determined by trypan blue exclusion. On this basis, one could exclude any inhibitory effect of TNF on DNA synthesis.

We therefore investigated whether the monokine could inhibit the expression of TM by enhancing endogenous degradation or internalization of the endothelial cell receptor, as suggested previously [N. L. Esmon, K. L. Moore, and C. T. Esmon, Blood **70**(Suppl. 1):401a, 1987]. This possibility was examined by ascertaining the effects of TNF on the stability of functional cell surface TM while suppressing de novo synthesis of the endothelial cell receptor. The monokine was added to cloned BAEC or HUVEC for

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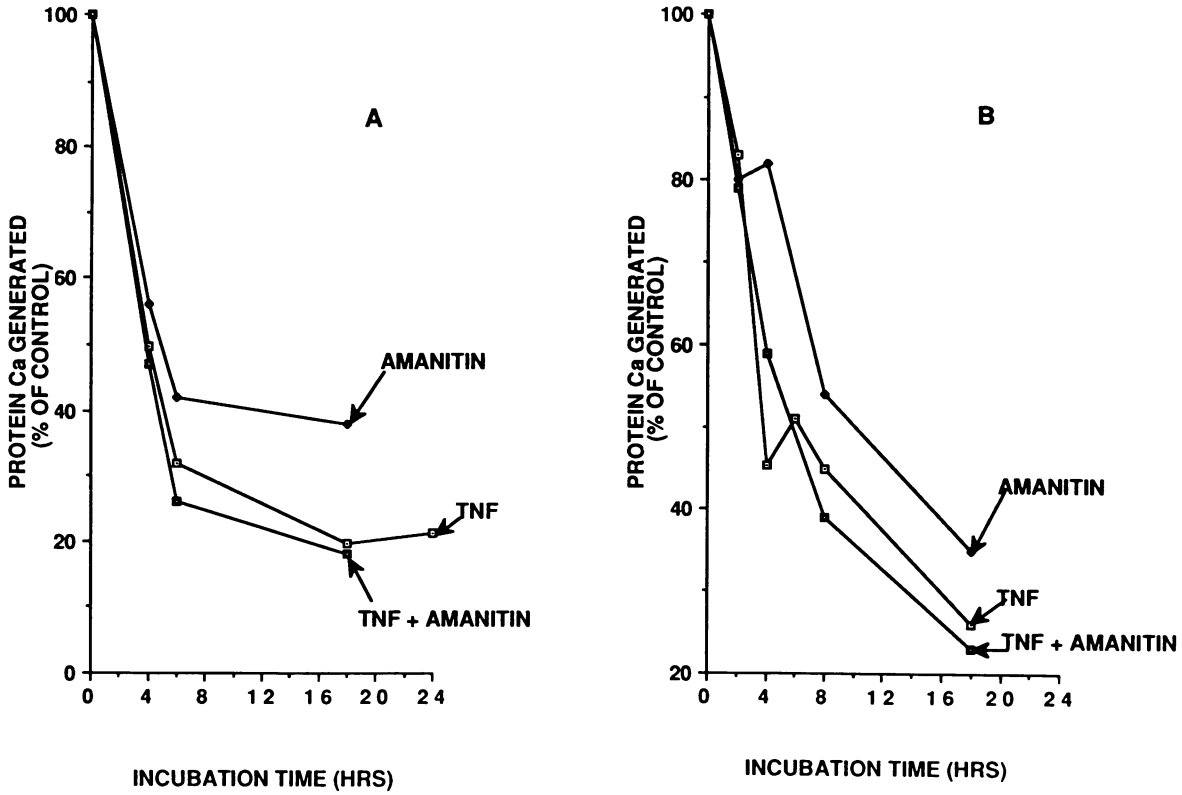

FIG. 1. Effect of TNF and alpha amanitin on cell surface TM function. BAEC (A) and HUVEC (B) were grown in 2-cm^2 tissue culture wells until 2 to 3 days postconfluence and exposed for various times to TNF (400 U/ml), TNF with α-amanitin at 2 μg/ml, or α-amanitin alone (2 μg/ml). The recombinant *Escherichia coli*-derived TNF alpha (Asahi Chemical Industry America, New York, N.Y.) exhibited a specific activity of 2.2×10^6 U/mg. Monolayers were washed and then incubated for 90 min with 200 μl of thrombin and protein C at final concentrations of 2.1 and 1,333 nM, respectively. The reaction was quenched by the addition of hirudin and antithrombin III at final concentrations of 3 U/ml and 600 nM, respectively. The TM-dependent conversion of protein C to activated protein C was measured by adding the reaction mixture to 500 μl of 0.4 mM amidolytic substrate S2238 (Helena Laboratories, Beaumont, Tex.) and quantitating the rate of change of A_{405}. The results provided represent the means of at least three individual experiments performed in duplicate and normalized to the initial level of functional cell surface TM.

various periods in the presence of the specific RNA polymerase II inhibitor, α-amanitin. The transcription of specific RNA polymerase II-dependent RNA, including TM mRNA, is dramatically inhibited at an α-amanitin concentration of 2 μg/ml (29, 30; see below). Figure 1 depicts the cytokine-induced, time-dependent reduction of cell surface TM of a BAEC clone and HUVEC, respectively. This inhibitory phenomenon is not secondary to contamination by endotoxin, since heat inactivation of TNF eliminates the decrease in endothelial-cell receptor activity. Figure 1 also shows the results of experiments in which the half-life of functional cell surface TM on BAEC and HUVEC was determined by exposing cells to α-amanitin in the presence or absence of TNF. The difference in endothelial-cell receptor activity observed at 2 to 18 h of incubation with or without added TNF was not statistically significant ($P < 0.05$).

These data indicate that TNF does not significantly enhance the destruction of cell surface TM or cause the endothelial-cell receptor to be more rapidly internalized. However, it is interesting to note the rapid time-dependent reduction of functional cell surface TM in the presence of α-amanitin. This observation is consistent with the hypothesis that TM is constitutively internalized without formation of a ligand-receptor complex. The low-density-lipoprotein receptor is structurally homologous to TM and can also enter cells via coated pits in the absence of ligand (10). Detailed studies of the internalization of labeled cell surface TM in the presence and absence of TNF will be required to confirm this aspect of endothelial-cell receptor function.

We next determined the effect of the monokine on the steady-state concentrations of BAEC and HUVEC TM mRNA and compared the results with those obtained by measuring the functional cell surface endothelial-cell receptor. After various times of incubation with TNF, BAEC or HUVEC monolayers were washed with phosphate-buffered saline, total RNA was isolated, and (RNA blot) analyses were conducted (4, 16). Figure 2A shows the autoradiographs of Northern gels of TNF-treated BAEC hybridized with the ^{32}P-labeled cDNA probe for the endothelial-cell receptor (8). The intensity of the TM mRNA band rapidly decreased as a function of time of incubation with monokine, whereas the size of the TM mRNA remained unchanged at 3.8 kilobases. We excluded the possibility that TNF was causing a nonspecific reduction in total cellular mRNA by demonstrating that the cytokine had no suppressive effect on the concentrations of tubulin mRNA (2) (Fig. 2A) or the levels of glyceraldehyde-3-phosphate-dehydrogenase mRNA (6) and angiotensinogen mRNA (20) (data not shown). The various bands of the autoradiographs were quantitated by scanning-laser densitometry (Fig. 2B). The data show that TNF suppressed the levels of BAEC TM mRNA to 44% ± 8% ($n = 3$) of control values after 4 h of incubation. Similar

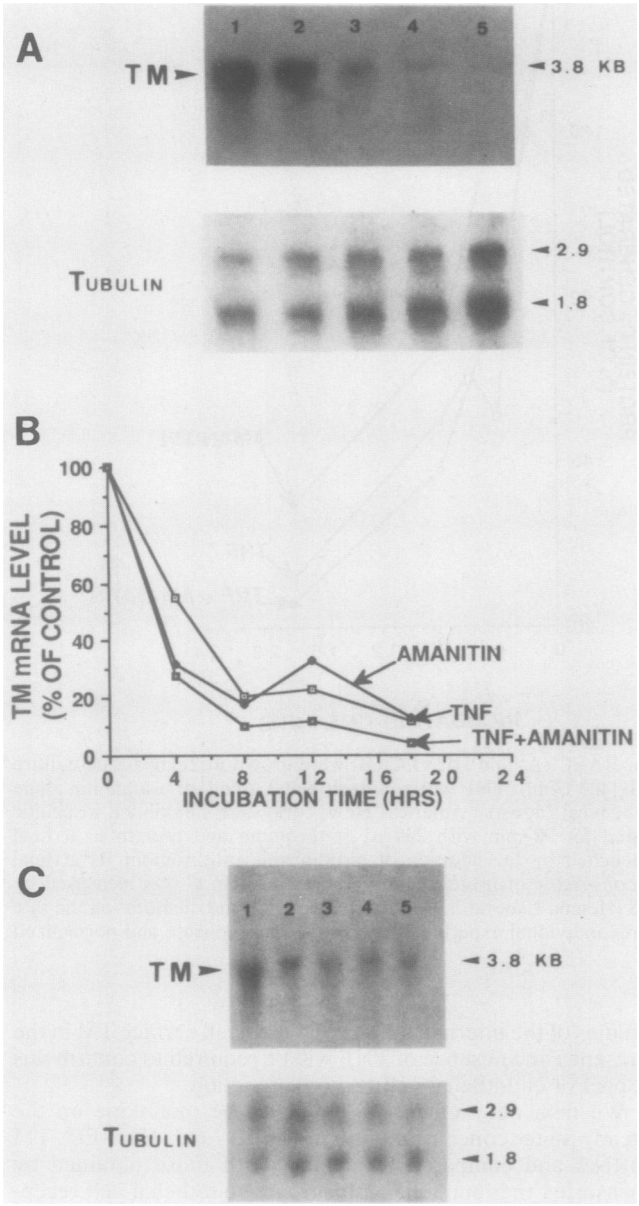

FIG. 2. Effect of TNF on the expression and stability of endothelial-cell TM mRNA. (A) BAEC were exposed to buffer control (lane 1) or TNF (400 U/ml) for 4, 8, 12, and 18 h (lanes 2, 3, 4, and 5, respectively). Total RNA was isolated from the cell pellets by the cesium chloride method (4, 16), and 10 μg of total RNA per lane was electrophoresed on a 1% agarose–formaldehyde gel. The bands were transferred to a GeneScreen filter (Du Pont, NEN Research Products, Boston, Mass.) by capillary blotting, and the filter was hybridized with random primer ^{32}P-labeled DNA probes (8) for TM (12, 13) and rat brain β-tubulin (2). Transcription of the β-tubulin gene is known to produce two separate mRNAs. (B) BAEC were exposed to TNF (400 U/ml), TNF with α-amanitin (2 μg/ml), or α-amanitin alone (2 μg/ml). Total RNA was isolated, and Northern analyses were done as described for panel A. The band intensity of autoradiographs was quantitated by scanning densitometry with results normalized to buffer control values. (C) HUVEC were exposed to buffer control (lane 1) or TNF (400 U/ml) (lanes 2, 3, 4, and 5), total RNA was isolated, and Northern analysis was conducted as outlined for panel A. KB, Kilobases.

experiments were conducted with HUVEC (Fig. 2C), and quantitation of the bands demonstrated that the levels of TM mRNA were reduced to 30.3% ± 18% (n = 3) of control values after 4 h of incubation with TNF (data not shown). The kinetics of the time-dependent suppression of BAEC or HUVEC TM mRNA produced by TNF are virtually identical to those of the time-dependent loss of functional cell surface endothelial-cell receptor produced by the monokine (Fig. 1A and 2B). The quantitatively similar effects of TNF on the levels of specific mRNA and functional cell surface TM allowed us to exclude the possibility that the cytokine caused a significant translational defect which led to inhibition of endothelial-cell receptor expression.

Given the results described above, we ascertained whether TNF altered the stability of TM mRNA by evaluating the effect of the monokine on the α-amanitin-induced, time-dependent reduction of the endothelial-cell message. Endothelial-cell monolayers were incubated with or without TNF in the presence of α-amanitin. Total RNA was extracted at various times and the levels of TM mRNA and tubulin mRNA were evaluated by Northern analyses. Laser densitometric quantitation of the filters revealed similar half-lives of approximately 3.5 to 4 h for TM mRNA from both BAEC (Fig. 2B) and HUVEC (data not shown) in the presence and absence of the monokine, utilizing concentrations of tubulin mRNA for normalization.

The lack of any substantial effect of TNF on the stability of TM mRNA indicates that the dramatic suppressive action of the cytokine on the expression of this endothelial-cell receptor must be attributed to a transcriptional mechanism. To support this hypothesis, we carried out nuclear run-on experiments to measure in vitro transcription rates in both TNF-treated and control endothelial cells (11, 18). The data show that basal levels of transcription of the TM gene and the tubulin gene in control BAEC and HUVEC were readily detectable (Fig. 3). When the endothelial cells were exposed to TNF, no significant changes in tubulin gene transcription were observed over 18 h. However, in two experiments, the addition of monokine suppressed BAEC TM gene transcription to 3 and 9% of control values and HUVEC TM gene transcription to 22 and 9% of control values within 2 h. The results of one of the studies are provided in Fig. 3. The nuclear run-on experiments were also conducted at earlier times, which revealed that incubation of HUVEC with TNF for 5 to 20 min produced no change in TM gene transcription, but addition of the monokine to HUVEC for 40 and 60 min suppressed TM gene transcription to 77 and 31% of control values, respectively (data not shown).

Our earlier observation that time-dependent changes in the levels of BAEC or HUVEC TM mRNA produced by α-amanitin were identical to those caused by a combination of TNF and α-amanitin suggests that the monokine is able to almost immediately suppress transcription of the endothelial-cell receptor gene. This conclusion is somewhat at variance with the nuclear run-on experiments cited above, which indicate that TNF requires about 40 to 60 min to exert its inhibitory effect on TM gene transcription. The above-mentioned minor discrepancy is probably attributable to the inherent experimental errors of Northern analyses as well as to minor biologic variations in BAEC and HUVEC which make it difficult to detect the small time delay in the action of the monokine. The hypotheses that TNF binds to specific endothelial-cell receptors and that the internalized monokine-receptor complex functions directly or indirectly to regulate TM gene transcription are consistent with a small time delay in the action of TNF.

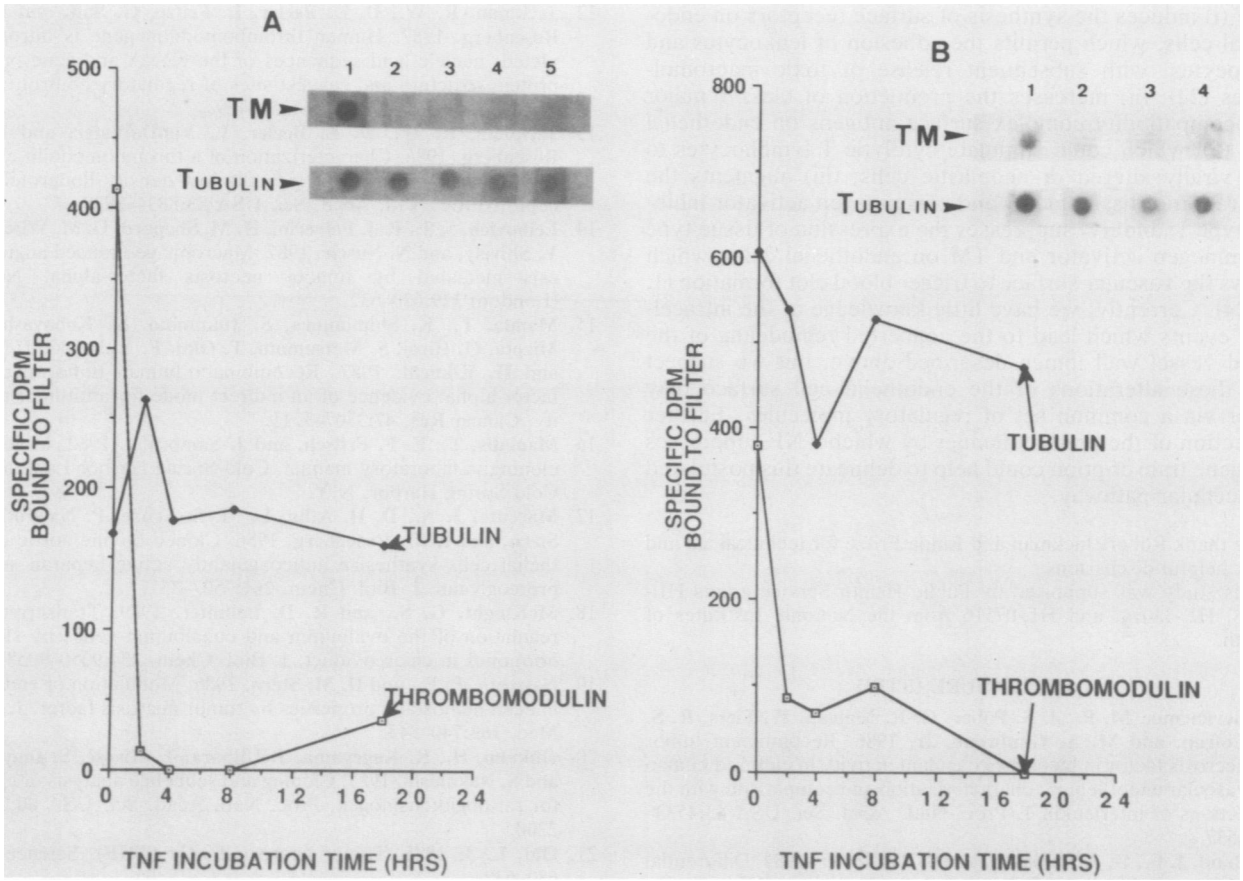

FIG. 3. Effect of TNF on TM gene transcription. BAEC (A) or HUVEC (B) were exposed to buffer control or TNF (400 U/ml), and nuclei were isolated from control cells (lane 1) and from cells exposed to cytokine for 2, 4, 8, and 18 h (lanes 2, 3, 4, and 5, respectively). Transcriptional activity was determined by incubating 30×10^6 nuclei for 20 min at 28°C with 400 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) in 5 mM Tris hydrochloride (pH 8.0)–2.5 mM $MgCl_2$–0.15 M KCl with 5 mM dithiothreitol–1 mM (each) ATP, CTP, and GTP. Samples were treated for 7.5 min with 200 U of DNase 1 and were extracted to obtain RNA. Equal numbers of counts were hybridized at 60°C for 48 to 72 h with 5 μg of denatured plasmid cDNA immobilized on nitrocellulose filters. These filters were washed in the presence of RNase A (10 μg/ml) and RNase T_1 (10 μg/ml). Hybridization efficiency varied from 20 to 30% as determined by a parallel reaction with a ^{32}P-labeled riboprobe. The autoradiographs show hybridization of ^{32}P-labeled transcripts to the TM and rat brain β-tubulin DNA probes. Nascent TM and β-tubulin ^{32}P-labeled RNA transcripts were quantitated by determining the specific number of disintegrations per minute bound to an excess of denatured TM and β-tubulin cDNA, respectively. Individual filters were counted by using the Betagen 603 Blot Analyzer (Betagen Corp., Waltham, Mass.) (26).

The delayed effect of TNF on the transcription of the TM gene prompted us to determine whether protein synthesis was required to observe inhibition of endothelial-cell receptor gene expression. HUVEC were incubated with or without monokine (400 U/ml), in the presence or absence of cycloheximide (10 μg/ml) or buffer control. At this concentration of protein synthesis inhibitor, [^{3}H]leucine incorporation is suppressed by >99%. Following exposure of HUVEC to the above-mentioned agents for 6 h, total RNA was extracted and the levels of TM mRNA and tubulin mRNA were determined by Northern analysis. Densitometric examination of the autoradiographs showed that TNF produced a dramatic decrease in the levels of TM mRNA normalized to tubulin mRNA compared with the control values. Mean levels from three experiments were 32.8% ± 3.5% for TNF alone and 32.3% ± 9.5% for TNF with cycloheximide. Exposure of the endothelial cells to both monokine and cycloheximide resulted in an identical reduction of TM mRNA levels. These data indicate that de novo protein synthesis is not required for TNF to suppress transcription of the TM gene. However, it is interesting to note that the addition of cycloheximide alone for 6 h caused an increase in the levels of TM mRNA, i.e., 252% ± 12%, compared with control values. Certain proto-oncogenes, hemopoietic growth factors, and cytokine mRNAs exhibit AUUUA repeats within their 3′ untranslated regions, which presumably give these species a relatively brief survival because of the function of a metabolically unstable RNase (25). The 3′ untranslated tail of the TM mRNA possesses two AUUUA repeats, and inhibitors of protein synthesis significantly increase the levels of this message. The observations described above suggest that this region of TM mRNA may also function as a target for the degradative pathway outlined above and could play an important regulatory role under certain conditions.

In summary, we have determined that the predominant suppressive effect of TNF on TM expression of endothelial cells is at the level of transcription and that this suppression occurs independent of de novo protein synthesis. The endothelial cell represents one of the major cellular targets of TNF and appears to be intimately involved in many biologic phenomena. Previous investigations have demonstrated that

TNF (i) induces the synthesis of surface receptors on endothelial cells, which permits the adhesion of leukocytes and monocytes, with subsequent release of toxic macromolecules (22); (ii) increases the production of class 1 major histocompatibility complex surface antigens on endothelial cells (5), which could stimulate cytolytic T lymphocytes to lyse virally altered or neoplastic cells; (iii) augments the generation of tissue factor and plasminogen activator inhibitor type 1; and (iv) suppresses the expression of tissue type plasminogen activator and TM on endothelial cells, which allows the vascular surface to trigger blood clot formation (1, 19, 24). Currently, we have little knowledge of the intracellular events which lead to the concerted remodeling of the blood vessel wall lumen described above, but we suspect that these alterations of the endothelial-cell surface may occur via a common set of regulatory molecules. Further dissection of the precise manner by which TNF suppresses TM gene transcription could help to delineate this postulated intracellular pathway.

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