

Regulation of Thrombomodulin by Tumor Necrosis Factor- α : Comparison of Transcriptional and Posttranscriptional Mechanisms

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The procoagulant properties of cultured vascular endothelial cells are enhanced in response to inflammatory cytokines such as tumor necrosis factor- α (TNF). A major component of this response is a reduction in expression of thrombomodulin, a cell surface cofactor for the activation of protein C. Regulation of thrombomodulin expression by TNF has been reported to occur through multiple mechanisms. To determine the relative roles of transcriptional and posttranscriptional regulation, the effect of TNF on the turnover of thrombomodulin protein and mRNA was examined in human and bovine endothelial cells. Quantitative nuclease S1 protection assays showed a 70% to 90% reduction in thrombomodulin mRNA within 4 hours of the addition of 1.0 nmol/L TNF to the culture medium. The decrease in thrombomodulin

mRNA resulted from inhibition of transcription, followed by rapid degradation of thrombomodulin transcripts ($t_{1/2} \leq 3$ hours). In pulse-chase incubations, thrombomodulin synthesis decreased parallel with mRNA, but the rate of degradation of radiolabeled thrombomodulin was not significantly altered by TNF. Human thrombomodulin was degraded with a $t_{1/2}$ of 8.2 ± 2.4 hours (SD) or 7.5 ± 1.3 hours (SD) in the absence or presence of TNF, respectively. We conclude that TNF acts primarily to inhibit thrombomodulin transcription. The subsequent decrease in activity results from the inherent instability of thrombomodulin mRNA and protein in these cells, and not from the regulation of thrombomodulin degradation.

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THROMBOMODULIN is an anticoagulant protein present on the surface of vascular endothelial cells. By modifying the substrate specificity of thrombin, thrombomodulin functions as a cofactor for the activation of protein C.^{1,2} Binding to thrombomodulin reduces the procoagulant properties of thrombin, and increases the rate of protein C activation at least 1,000-fold.³ After activation by the thrombin-thrombomodulin complex, activated protein C catalyzes the proteolysis of factors Va and VIIIa, thereby inhibiting coagulation.^{1,2} Recent studies suggest that bovine factor Xa can also bind to thrombomodulin and activate protein C,⁴ but this has not been observed with human factor Xa.⁵ The importance of thrombomodulin as a physiologic anticoagulant has been demonstrated by the potentiation of thrombin-induced thromboembolism in mice injected with anti-thrombomodulin antibodies.⁶

Thrombomodulin activity decreases when endothelial cells in culture are exposed to inflammatory mediators such as endotoxin,⁷ interleukin-1,⁸ or tumor necrosis factor- α (TNF).⁹⁻¹² Reduction in thrombomodulin and concomitant increases in the expression of the procoagulant protein tissue factor^{9,13} and plasminogen activator inhibitors^{14,15} result in the conversion of the endothelium from a thromboresistant to a thrombogenic surface. The procoagulant properties of stimulated endothelial cells may contribute to

the potential for vascular thrombosis in inflammatory disorders such as septic shock, disseminated intravascular coagulation, and malignancy.^{16,17}

Regulation of thrombomodulin by TNF may occur through multiple mechanisms. Decreased thrombomodulin activity in TNF-treated human umbilical vein endothelial cells (HUVEC) was reported to occur without changes in thrombomodulin mRNA levels, suggesting translational or posttranslational regulation.¹² In contrast, thrombomodulin gene transcription was rapidly inhibited when human or bovine endothelial cells were exposed to TNF, and was accompanied by decreases in thrombomodulin activity and mRNA level.¹⁰ It has also been suggested that the TNF-mediated reduction in thrombomodulin activity occurs through endocytosis and degradation of thrombomodulin in lysosomes.¹¹ These studies were all performed with primary cultures of endothelial cells, which have variable growth properties depending on cell source and culture conditions.¹⁸ In particular, thrombomodulin mRNA levels have been shown to vary several fold in HUVEC of different passage number.¹⁹ Thus, potential differences among endothelial cell preparations complicate the direct comparison of the multiple reported effects of TNF on thrombomodulin expression.

To clarify the relative roles of transcriptional and posttranscriptional regulation of thrombomodulin by TNF, we have examined the turnover of both thrombomodulin protein and thrombomodulin mRNA in the same preparations of human and bovine endothelial cells. The results suggest that endothelial cell thrombomodulin activity is controlled primarily by the level of thrombomodulin mRNA. Treatment with TNF reduces thrombomodulin transcription, but does not appear to alter thrombomodulin mRNA stability, translation, or protein degradation compared with unstimulated cells. The rate of decline of thrombomodulin activity after stimulation reflects the inherent instability of thrombomodulin mRNA and protein in these cells.

MATERIALS AND METHODS

Materials. Culture media, fetal bovine serum, and calf serum were obtained from GIBCO Laboratories (Grand Island, NY). Epidermal growth factor and endothelial cell growth supplement

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were obtained from Collaborative Research (Bedford, MA). Human fibronectin, heparin, bovine thyroglobulin, α -amanitin, actinomycin D, and nuclease S1 were purchased from Sigma Chemical Company (St Louis, MO). All radionuclides were from Amersham Corporation (Arlington Heights, IL). Recombinant human TNF (2×10^7 U/mg) was purchased from Genzyme Corporation (Boston, MA); succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate from Pierce Chemical Company (Rockford, IL); 5,6-dichloro-1- β -ribofuranosylbenzimidazole (DRB) from Calbiochem Corporation (San Diego, CA); and S-2366 from KabiVitrum (Stockholm, Sweden). Restriction enzymes *Mlu*I, *Eco*RI, *Sty*I, *Bst*EII, and *Bgl*II were from New England Biolabs (Beverly, MA). Calf intestinal alkaline phosphatase, proteinase K, and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), and T4 polynucleotide kinase from Pharmacia LKB Biotechnology (Piscataway, NJ).

Endothelial cells. HUVEC were purchased from the American Type Culture Collection, Rockville, MD (CRL 1730, used between passages 15 and 30), or Clonetics Corporation, San Diego, CA (used between passages 3 and 7). Cells were cultured in 45% 199 medium, 45% F-12 nutrient mixture, 10% fetal bovine serum, 90 μ g/mL heparin, 10 ng/mL epidermal growth factor, 30 μ g/mL endothelial cell growth supplement, and 100 μ g/mL gentamicin. JV015 fetal bovine aortic endothelial cells (BAEC) were obtained from Dr Sheila Knepper (Monsanto Co, St Louis, MO), used between passages 15 and 25, and cultured in Dulbecco's modified Eagle's medium, 5% calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

Metabolic labeling. Metabolic labeling with [³⁵S]cysteine was performed in cysteine-free labeling medium containing 10% dialyzed fetal bovine serum for HUVEC, and cysteine-free labeling medium without serum for BAEC. In pulse-chase experiments, cells were cultured to confluency in 35-mm wells ($\sim 0.5 \times 10^6$ cells/well), washed three times with labeling medium, and incubated for 15 hours in 1.0 mL labeling medium containing 100 μ Ci [³⁵S]cysteine. Cells were then washed twice with complete medium, and incubated for the indicated chase periods in 1.0 mL complete medium with or without 1.0 nmol/L TNF.

For measurement of thrombomodulin synthesis, cells cultured to confluency in 35-mm wells were pre-incubated for 0 to 24 hours in 1.0 mL complete medium containing 1.0 nmol/L TNF. Cells were then washed three times with labeling medium, and incubated for 2 hours in labeling medium containing [³⁵S]cysteine and 1.0 nmol/L TNF. Control cells were labeled for 2 hours without TNF.

Thrombomodulin immunoprecipitation. A peptide corresponding to the carboxyl terminal sequence of human thrombomodulin (H₂N-QHVRTERTPQRL-COOH) was synthesized and purified as previously described.²⁰ The synthetic peptide was coupled to bovine thyroglobulin using succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate as described by Barry et al.²¹ New Zealand White rabbits were immunized with the peptide-thyroglobulin conjugate according to the method of Vaitukaitus.²² [³⁵S]Cysteine-labeled cell lysates were immunoprecipitated with 5 μ L of rabbit polyclonal antipeptide antiserum as described,²⁰ except that an initial cycle of immunoprecipitation using pre-immune serum was included to reduce nonspecific background. Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with 10% polyacrylamide separating gels, followed by autoradiography and fluorography.²⁰

Thrombomodulin cofactor assay. Human thrombin was prepared as described.²⁰ Human protein C and human antithrombin III were gifts of Drs Joseph Miletich and Douglas Tollefsen (Washington University, St Louis, MO), respectively. Two-stage

thrombomodulin cofactor assays were performed as previously described.^{20,23} Briefly, lysates of $\sim 0.5 \times 10^6$ cells were incubated in assay buffer containing 0.6% Triton X-100 (J.T. Baker Chemical Co, Phillipsburg, NJ), 14 nmol/L thrombin, and 0.8 μ mol/L protein C for 30 minutes at 37°C, and activated protein C was determined by hydrolysis of the chromogenic substrate S-2366. Thrombomodulin-dependent activity was calculated from a standard curve generated with purified activated protein C.

Preparation of bovine thrombomodulin DNA probe. Total genomic DNA was prepared from JV015 cells by digestion with 100 μ g/mL proteinase K, 100 mmol/L NaCl, 250 mmol/L EDTA, and 0.5% SDS for 3 hours at 50°C, followed by phenol/chloroform extraction and ethanol precipitation. Polymerase chain reaction was performed as described²⁴ using *Taq* polymerase and synthetic oligonucleotide primers containing *Eco*RI restriction sites (5'-CCCAGGTGAATTCGGAGCGGGCACCC-3' and 5'-GGGTCTTGAATTCGACGCCAGCTAAG-3'). Amplified genomic bovine thrombomodulin DNA corresponding to nucleotides 783 through 1164 of the bovine cDNA sequence reported by Jackman et al²⁵ was digested with *Eco*RI and ligated into plasmid pUC18 with T4 DNA ligase. The sequence of subclone pBTM1 was confirmed by dideoxy sequencing.²⁶

Nuclease S1 protection analysis. Plasmid pBTM1 was linearized by digestion with *Sty*I. Plasmid pUC19TM12, containing the partial human thrombomodulin cDNA insert from λ HTM12,²⁷ was linearized by digestion with *Mlu*I. The full-length human γ -actin cDNA insert from plasmid pHF γ A-1 (provided by Dr L. Kedes, University of Southern California, Pasadena)²⁸ was subcloned into pBLUE-SCRIPT II (Stratagene, LaJolla, CA) and linearized by digestion with *Bgl*III. Plasmid pBA1,²⁹ containing a partial bovine actin cDNA (provided by Dr Jay Degen, University of Cincinnati, Cincinnati, OH) was linearized by digestion with *Bst*EII. The linearized plasmids were treated with calf intestinal alkaline phosphatase and end-labeled using [γ -³²P]adenosine triphosphate and T4 polynucleotide kinase. HUVEC or BAEC were incubated for the indicated times with 1.0 nmol/L TNF, and total cellular RNA was isolated by the method of Chomczynski and Sacchi.³⁰ Approximately 100,000 cpm of the thrombomodulin or actin probes were hybridized overnight at 55°C with 20 μ g or 5 μ g of RNA, respectively. All hybridizations were performed under conditions of probe excess. Hybridization conditions, nuclease S1 digestion, and analysis on denaturing PAGE were as described.³¹ Protected fragments of 242, 243, 260, and 325 nucleotides were detected for bovine thrombomodulin, human thrombomodulin, bovine actin, and human actin, respectively.

Runoff transcription assay. Nuclear runoff transcription was assayed in HUVEC as described previously¹² using purified plasmid DNA targets containing cDNA inserts from λ HTM15 (human thrombomodulin),²⁷ pHTF8 (human tissue factor),¹² or pHF γ A-1 (human γ -actin).²⁸ Vector plasmid was included as a control for nonspecific hybridization.

Densitometry. Autoradiograms from thrombomodulin immunoprecipitation and S1 nuclease experiments were analyzed with an UltraScan XL laser densitometer (Pharmacia LKB Biotechnology).

RESULTS

Effect of TNF on thrombomodulin mRNA. Addition of TNF to cultured endothelial cells produces an 80% reduction in thrombomodulin activity after 24 hours of incubation.⁹⁻¹² Results from Northern hybridization analyses have suggested that thrombomodulin mRNA levels may decrease¹⁰ or remain stable¹² after stimulation by TNF, implying that both transcriptional and posttranscriptional

mechanisms may be responsible for the decline in thrombomodulin activity. To more specifically evaluate thrombomodulin mRNA turnover in these cells, quantitative nuclease S1 protection assays were performed. Total cellular RNA was isolated from TNF-treated HUVEC or BAEC, hybridized to species-specific thrombomodulin or actin probes, and digested with nuclease S1. Protected fragments were detected by denaturing gel electrophoresis and autoradiography. Within 2 hours of the addition of 1.0 nmol/L TNF to the culture medium, both human (Fig 1A) and bovine (Fig 1B) thrombomodulin mRNA had decreased significantly, and levels remained low throughout the remainder of the 24-hour incubation. No changes were observed in actin mRNA levels. Quantitative densitometry of thrombomodulin mRNA, normalized to actin mRNA, showed similar rates of decline in BAEC and in early and late passage

HUVEC (Fig 1C), resulting in an 80% to 90% loss of thrombomodulin mRNA by 24 hours.

Concentrations of TNF used in previous studies of endothelial cell thrombomodulin expression have ranged between ~ 0.5 nmol/L¹⁰ and 2.0 nmol/L.¹¹ Thus, it was possible that the reported differences in the effects of TNF on thrombomodulin mRNA were concentration dependent. The relationship between thrombomodulin mRNA and activity was examined further by incubating HUVEC or BAEC for 12 hours with different concentrations of TNF. Thrombomodulin mRNA was quantitated by nuclease S1 protection, and cell lysates were assayed for the ability to promote protein C activation by thrombin (Fig 2). Equivalent reductions in thrombomodulin mRNA and activity were observed at each concentration of TNF, and the results agreed closely with previous dose-response data.^{9,11}

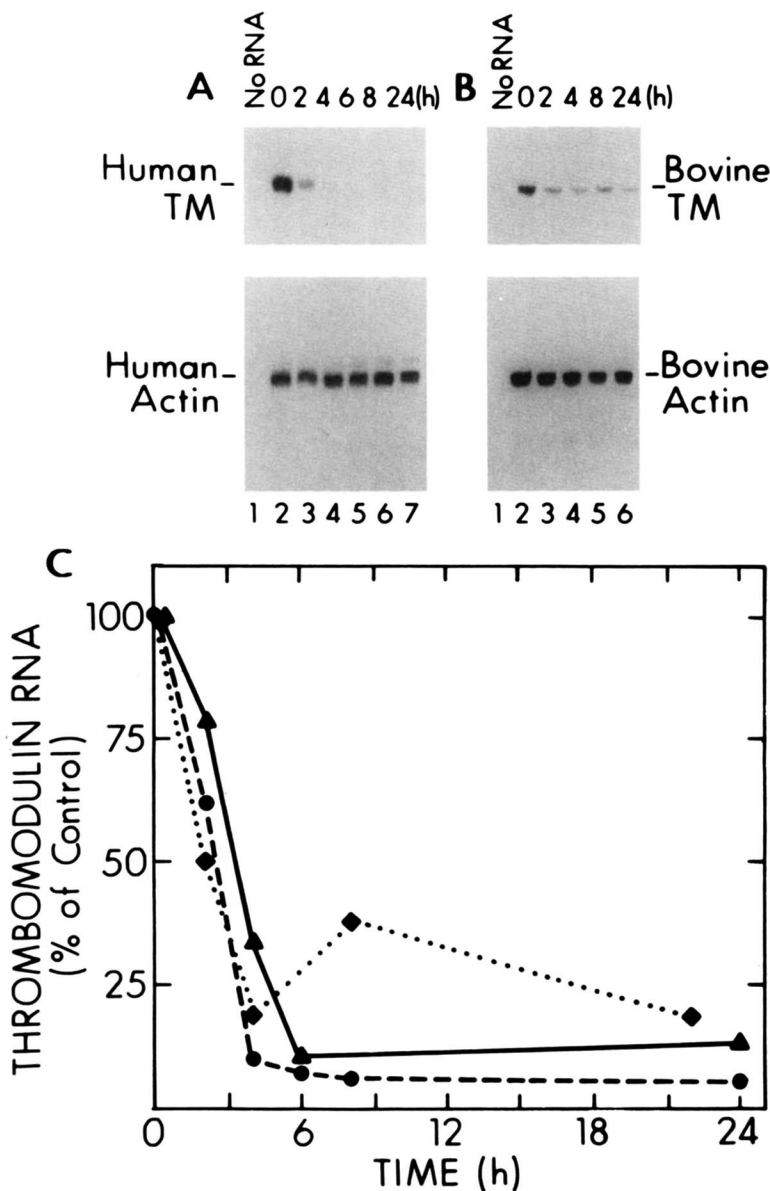


Fig 1. Nuclease S1 protection of thrombomodulin mRNA. At the indicated times after the addition of 1.0 nmol/L TNF, total cellular RNA was isolated from passage 18 HUVEC (A) or passage 26 BAEC (B), hybridized to excess amounts of end-labeled species-specific thrombomodulin or actin probes, digested with nuclease S1, and analyzed by denaturing gel electrophoresis and autoradiography. Bands of the expected fragment sizes for the individual probes are indicated. (C) Autoradiograms from three separate experiments were quantitated by laser densitometry. Thrombomodulin mRNA was normalized to actin mRNA and expressed as percent of thrombomodulin mRNA at time 0. (●), Passage 18 HUVEC; (▲), passage 7 HUVEC; (◆), passage 29 BAEC.

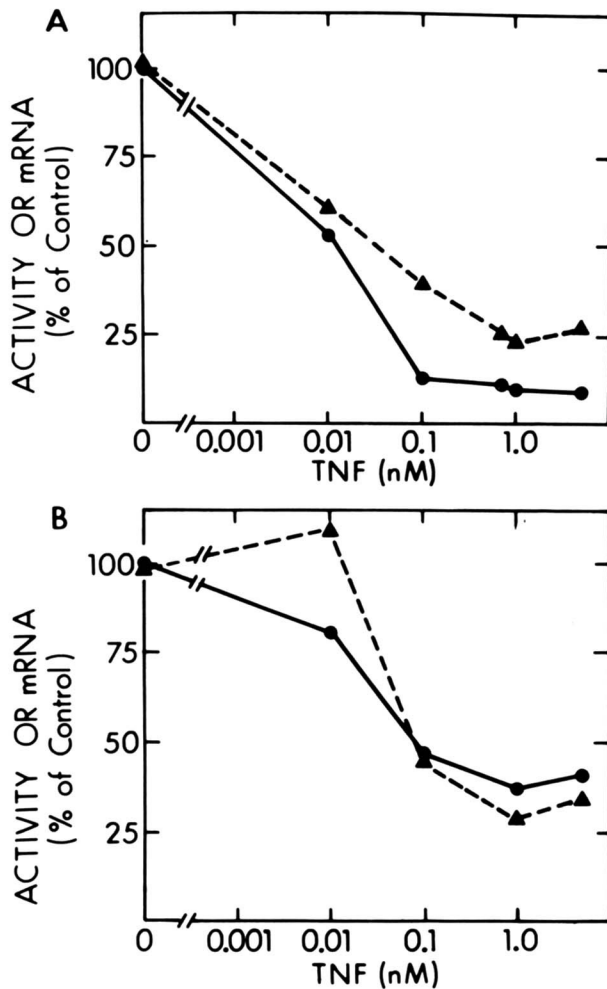


Fig 2. Dose response of thrombomodulin mRNA repression by TNF. Passage 5 HUVEC (A) or passage 23 BAEC (B) were incubated for 12 hours with the indicated concentrations of TNF. Thrombomodulin mRNA was quantitated by nuclease S1 protection, denaturing gel electrophoresis, autoradiography, and laser densitometry. Values were normalized to actin mRNA and expressed as percent of thrombomodulin mRNA in cells incubated without TNF. An aliquot of cells from each flask was assayed for total cellular thrombomodulin activity, expressed as percent of activity in cells incubated without TNF. (\blacktriangle), Activity; (\bullet), mRNA.

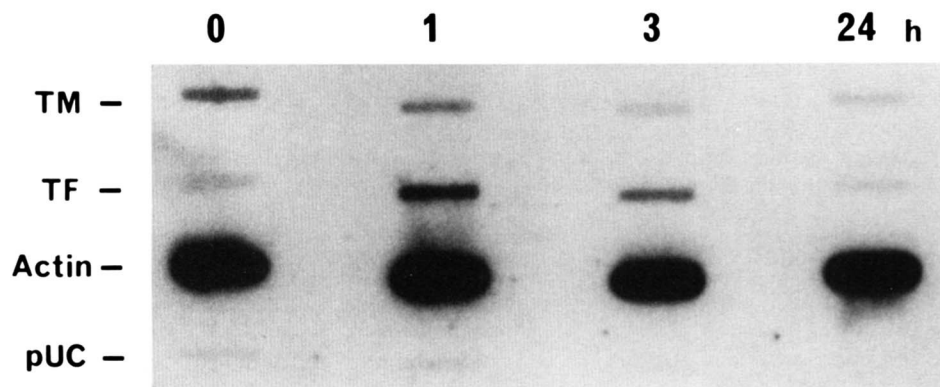
Therefore, under the conditions used here, the decrease in thrombomodulin activity produced by TNF was accompanied by a corresponding decrease in thrombomodulin mRNA.

Effect of TNF on thrombomodulin mRNA stability. Reduction in thrombomodulin mRNA after treatment with TNF could result from inhibition of transcription or from decreased mRNA stability. Thrombomodulin transcription was reported to be inhibited in nuclei from human and bovine endothelial cells treated with TNF.¹⁰ We were initially unable to duplicate these observations because of plasmid contamination in our earlier studies.^{12,32} When nuclear runoff assays were performed with purified plasmid targets, thrombomodulin transcription in HUVEC was reduced $\sim 80\%$ within 1 to 3 hours after addition of TNF (Fig 3), a finding consistent with the results of Conway and Rosenberg.¹⁰ As described previously,¹² tissue factor transcription was increased transiently and actin transcription was unchanged after addition of TNF (Fig 3).

The rapid decrease in thrombomodulin mRNA (Fig 1C) suggested that in addition to inhibiting transcription, TNF may also increase the rate of degradation of thrombomodulin transcripts. To determine if thrombomodulin mRNA was destabilized by TNF, total RNA was isolated from HUVEC after treatment with the transcriptional inhibitors DRB, actinomycin D, or α -amanitin. Analysis by nuclease S1 protection showed a $\sim 50\%$ decline in thrombomodulin mRNA within 2 hours of the addition of 100 $\mu\text{g}/\text{mL}$ DRB (Fig 4A) or 5 $\mu\text{g}/\text{mL}$ actinomycin D (Fig 4B), either in the absence or presence of TNF. When 2 $\mu\text{g}/\text{mL}$ α -amanitin was added to the culture medium in the absence of TNF, no reduction in thrombomodulin mRNA was observed (Fig 4C), possibly because of impaired entry of α -amanitin into intact cells.³³ In the presence of both α -amanitin and TNF, thrombomodulin mRNA was degraded at a rate comparable to that observed with the other inhibitors ($t_{1/2} \leq 3$ hours). Actin mRNA degradation was negligible during 8-hour incubations with all inhibitors. These results demonstrate that thrombomodulin mRNA is unstable in both untreated and TNF-stimulated HUVEC. The rapid decrease in thrombomodulin mRNA after inhibition of transcription by TNF reflects this instability.

Effect of TNF on thrombomodulin degradation. In addition to its effect on thrombomodulin mRNA, TNF has been

Fig 3. Effect of TNF on runoff transcription. After the indicated times of incubation with 1.0 nmol/L TNF, nuclei were isolated from HUVEC at passage 3 and runoff transcription assays were performed with guanosine 5'-[α -³²P] triphosphate. Nitrocellulose filters containing human thrombomodulin (TM), human tissue factor (TF), human γ -actin, and control plasmid targets were hybridized with transcript RNA and analyzed by autoradiography.



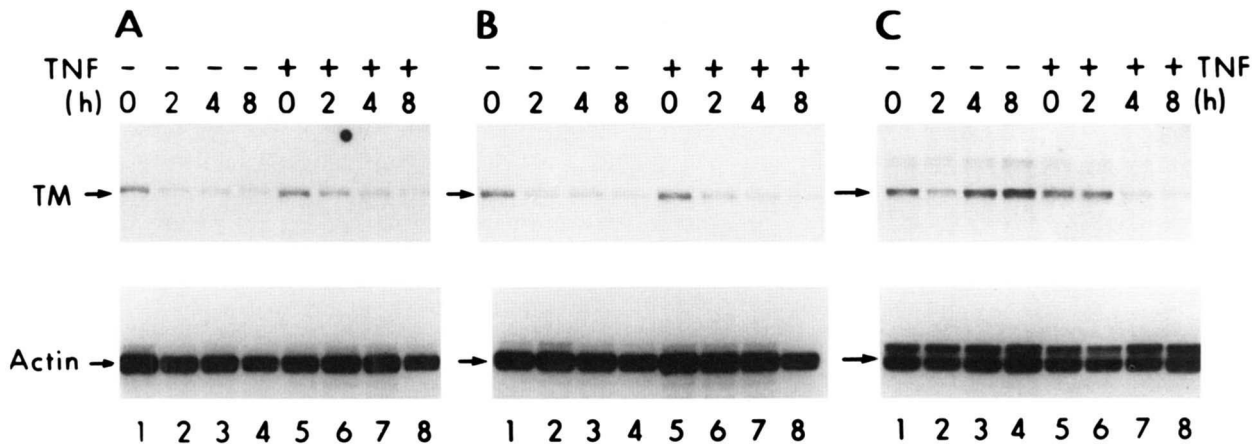


Fig 4. Time course of thrombomodulin mRNA degradation. Passage 5 HUVEC were incubated for the indicated times, either in the absence (lanes 1 through 4) or presence (lanes 5 through 8) of 1.0 nmol/L TNF, with the transcriptional inhibitors DRB (A), actinomycin D (B), or α -amanitin (C). Total cellular RNA was isolated and analyzed for thrombomodulin or actin mRNA by nuclease S1 protection, denaturing gel electrophoresis, and autoradiography.

proposed to stimulate the degradation of thrombomodulin in lysosomes.¹¹ To determine whether the rate of thrombomodulin degradation was altered by TNF, HUVEC were labeled for 15 hours with [³⁵S]cysteine, washed, and then chased for up to 24 hours in the presence or absence of 1.0 nmol/L TNF. Following the chase incubation, radiolabeled thrombomodulin was immunoprecipitated from cell lysates and analyzed by SDS-PAGE. Immunoprecipitations were performed using a rabbit polyclonal antiserum raised against a synthetic peptide identical in sequence to the carboxyl terminal 13 amino acids of human thrombomodulin. This antiserum specifically immunoprecipitated a 90,000-dalton band corresponding to mature human thrombomodulin (Fig 5A). A band of the same mobility was obtained when immunoprecipitations were performed with a monoclonal antibody to human thrombomodulin,²⁷ but not with rabbit or mouse nonimmune sera (data not shown).

The time course of thrombomodulin degradation was identical in the absence (lanes 1 through 4) or presence (lanes 5 through 8) of TNF. No immunoprecipitable thrombomodulin was detected in the culture medium at any time (data not shown). Autoradiograms from three separate pulse-chase experiments using both early and late passage HUVEC were quantitated by laser densitometry. The half-life of radiolabeled thrombomodulin was 8.2 ± 2.4 hours (SD) without TNF (Fig 5B), compared to 7.5 ± 1.3 hours (SD) with TNF (Fig 5C). Similarly, TNF had no effect on the rate of degradation of bovine thrombomodulin from BAEC, which was also immunoprecipitated by this antiserum (data not shown).

Effect of TNF on thrombomodulin synthesis. Determination of the rate of thrombomodulin synthesis required the use of a short labeling time relative to the half-life of thrombomodulin. HUVEC were continuously labeled with [³⁵S]cysteine for various times and radiolabeled thrombomodulin was recovered by immunoprecipitation (Fig 6A). With labeling times less than 2 hours, a small amount of incompletely glycosylated thrombomodulin was observed (lanes 1 and 2). As the labeling time was lengthened, the

recovery of radiolabeled thrombomodulin increased, and mature thrombomodulin became the predominant band (lanes 3 through 6). Based on this result, a labeling time of 2 hours was used to evaluate the effect of TNF on the rate of thrombomodulin synthesis. HUVEC or BAEC were incubated for up to 26 hours in medium containing 1.0 nmol/L TNF. [³⁵S]Cysteine was added for only the final 2 hours of the incubation, so that radiolabeled thrombomodulin reflected newly synthesized protein. Synthesis of both partially and fully glycosylated forms of thrombomodulin decreased by ~80% after 4 hours of incubation with TNF, and remained reduced throughout the remainder of the incubation (Fig 6B and 6C, lanes 3 through 6). The high molecular weight bands migrating near the top of the gel (Fig 6) were variably observed with both immune and nonimmune sera, and therefore do not represent thrombomodulin.

A comparison of the time course of thrombomodulin synthesis and activity after addition of TNF to BAEC and to early and late passage HUVEC is shown in Fig 7. The rates of thrombomodulin synthesis (Fig 7A) were determined by quantitative densitometry of autoradiograms from three experiments similar to those shown in Fig 6. Thrombomodulin activity (Fig 7B) was determined by measuring the ability of cell lysates to promote protein C activation by thrombin. After a lag period of 2 to 3 hours, thrombomodulin synthesis was rapidly inhibited, and thrombomodulin activity decreased at a rate consistent with the experimentally determined half-life of approximately 8 hours. Therefore, inhibition of thrombomodulin synthesis completely accounted for the observed reduction in thrombomodulin activity in both human and bovine endothelial cells. Furthermore, thrombomodulin synthesis declined with a time course identical to thrombomodulin mRNA (Fig 1C). This suggests that endothelial cell thrombomodulin synthesis is determined primarily by the level of mRNA, rather than by regulation of translation. We conclude that the reduction in thrombomodulin activity in TNF-treated endothelial cells can be completely accounted for by inhibition of transcrip-

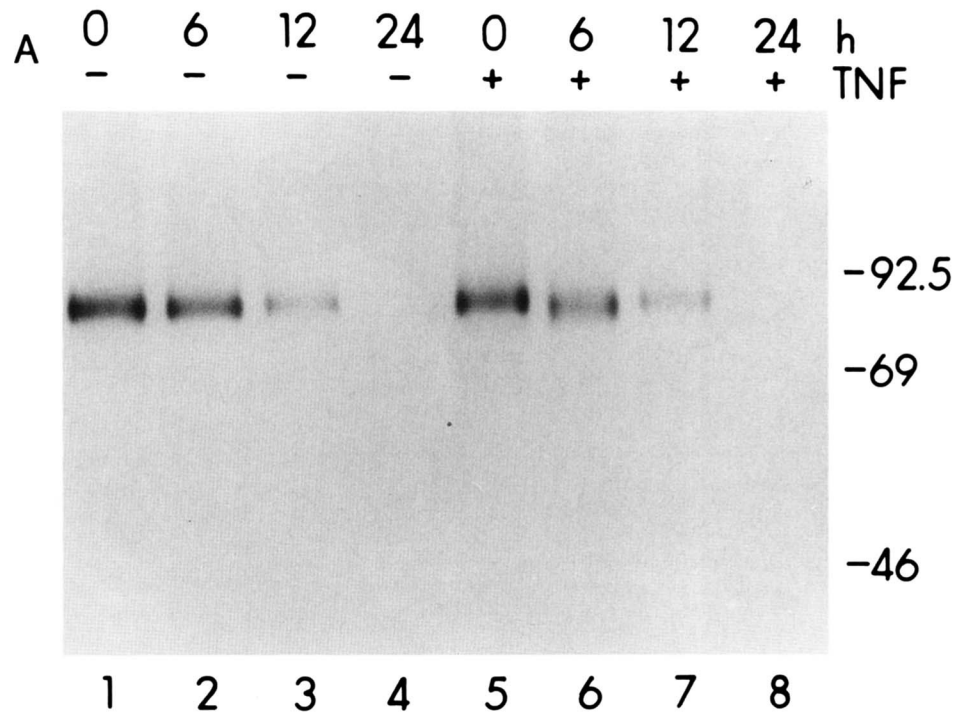


Fig 5. Determination of the rate of thrombomodulin degradation by pulse-chase labeling. (A) Passage 28 HUVEC were labeled for 15 hours with [³⁵S]cysteine, washed, and incubated for the indicated times in the absence or presence of 1.0 nmol/L TNF. Thrombomodulin was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and autoradiography. The mass in kilodaltons of protein standards is indicated on the right. (B and C) Quantitative densitometry of the thrombomodulin bands from autoradiograms of three experiments, expressed as percent of thrombomodulin at time 0. (○,●), Passage 24 HUVEC; (△,▲), passage 28 HUVEC; (◇,◆), passage 4 HUVEC. (B) Open symbols, no TNF; (C) closed symbols, 1.0 nmol/L TNF.

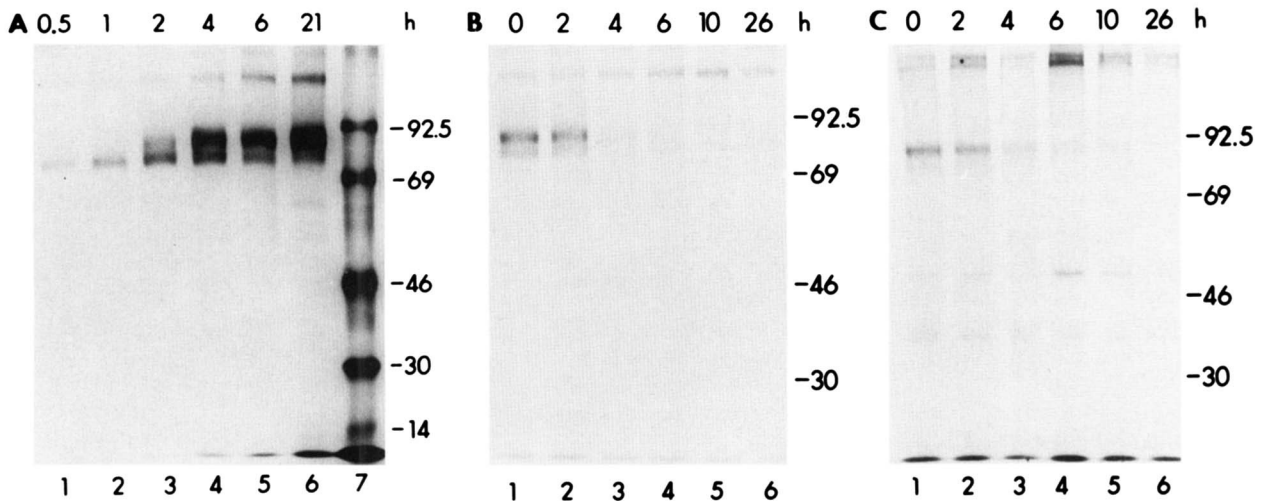
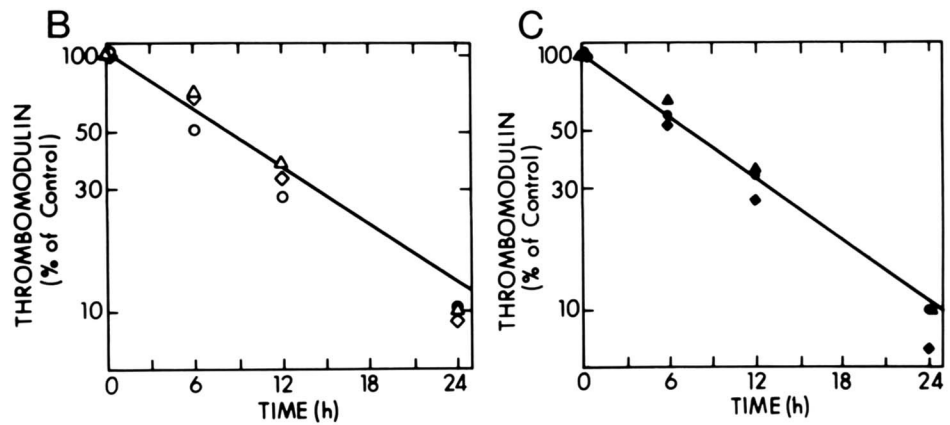


Fig 6. Effect of TNF on thrombomodulin synthesis. (A) Passage 23 HUVEC were incubated with [³⁵S]cysteine in the absence of TNF for the indicated times (lanes 1 through 6). Thrombomodulin was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and autoradiography. Lane 7, protein size standards. Passage 26 HUVEC (B) or passage 19 BAEC (C) were incubated for the indicated times with 1.0 nmol/L TNF. [³⁵S]Cysteine was added for only the final 2 hours of the incubation. Thrombomodulin was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Control cells (lane 1) were labeled for 2 hours without TNF. The mass of the protein standards is indicated in kilodaltons in each panel.

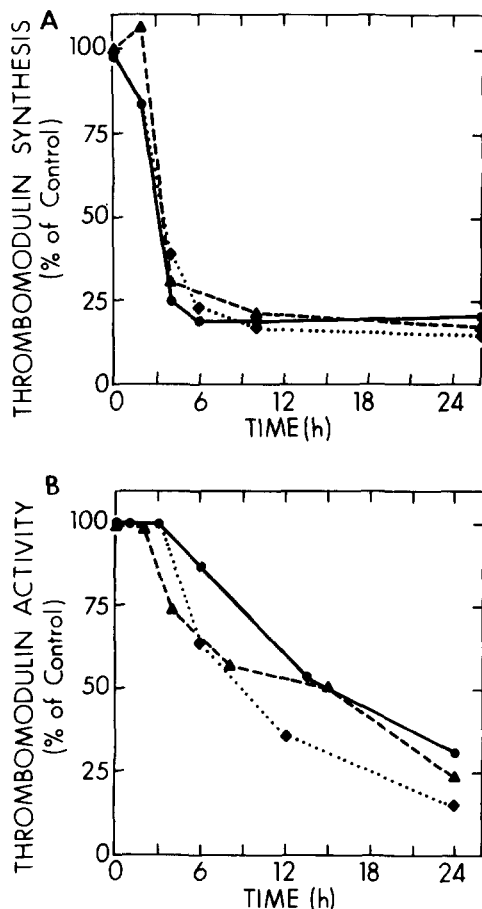


Fig 7. Comparison of the time course of thrombomodulin synthesis and activity. (A) Thrombomodulin synthesis, at the indicated times after the addition of 1.0 nmol/L TNF, was determined by quantitative densitometry of autoradiograms obtained as described for Fig 6. Values are expressed as percent of synthesis at 0 hours. (●), Passage 26 HUVEC; (▲), passage 4 HUVEC; (◆), passage 19 BAEC. (B) Thrombomodulin activity was determined at the indicated times after the addition of 1.0 nmol/L TNF and is expressed as percent of activity at 0 hours. (●) Passage 30 HUVEC; (▲), passage 6 HUVEC; (◆), passage 23 BAEC.

tion. The time-dependent loss of activity reflects inherent rates of degradation of thrombomodulin mRNA and protein, which are not altered by TNF.

DISCUSSION

Thrombomodulin is expressed on the luminal surface of resting endothelium, serving as a physiologic anticoagulant. Stimulation of endothelial cells with inflammatory cytokines, including TNF and interleukin-1, results in a dose- and time-dependent decrease in thrombomodulin expression.^{8,9} Thrombomodulin activity may also be reduced when endothelial cells are perturbed by growth under hypoxic conditions³⁴ or exposed to elevated levels of homocysteine, which has been associated with a congenital thrombotic disorder.³⁵

Previous reports have suggested that regulation of endothelial cell thrombomodulin activity by TNF occurs through

both transcriptional¹⁰ and posttranscriptional^{11,12} mechanisms. Studies of this type require the use of primary cultures of endothelial cells, which have limited viability, and are subject to variation between lineage, passage number, and culture media, which often contain supplemental growth factors. Comparison of the effects of TNF on thrombomodulin expression in different cultures is difficult, especially because thrombomodulin mRNA levels have been observed to vary considerably in cultured human endothelial cells.¹⁹ To minimize potential variability among cultures, we have examined the turnover of both thrombomodulin protein and thrombomodulin mRNA in the same preparations of TNF-treated HUVEC and BAEC.

Quantitative nuclease S1 protection showed that thrombomodulin mRNA levels decreased after addition of TNF to human or bovine cells. As reported previously,¹⁰ the decrease in thrombomodulin mRNA appeared to result from inhibition of transcription followed by rapid degradation of thrombomodulin transcripts. The effect of TNF on thrombomodulin mRNA stability was investigated using DRB, a specific inhibitor of transcription initiation that rapidly enters cells,³⁶ as well as the transcriptional inhibitors actinomycin D and α -amanitin. With DRB or actinomycin D, the rate of thrombomodulin mRNA degradation was comparable in the absence or presence of TNF ($t_{1/2} \leq 3$ hours). This suggests that thrombomodulin mRNA is inherently unstable in both untreated and TNF-stimulated endothelial cells. In contrast to Conway and Rosenberg,¹⁰ we did not observe significant thrombomodulin degradation after the addition of 2 μ g/mL α -amanitin to the culture medium in the absence of TNF (Fig 7), despite complete inhibition of thrombomodulin transcription by the same concentration of α -amanitin in nuclear runoff assays (data not shown). This may be due to the failure of α -amanitin to enter intact cells in adequate concentration to inhibit transcription by DNA polymerase II.³³ Alternatively, α -amanitin may have secondary effects that impair thrombomodulin mRNA degradation, as has been reported with actinomycin D.^{37,38}

The 3' untranslated regions of human and bovine thrombomodulin mRNA contain AU-rich sequences that have been implicated as mediators of mRNA instability in several cytokines and protooncogenes.³⁹ The role of these or other potential regulatory elements in thrombomodulin mRNA degradation is unclear, but could be evaluated further by testing their ability to confer instability to chimeric transcripts.^{38,39} Such AU-rich 3' noncoding sequences have also been shown to inhibit translation of some mRNAs.^{40,41} Under the conditions used here, the rate and magnitude of reduction in thrombomodulin synthesis (Fig 7A) correlated closely with decreased levels of thrombomodulin mRNA (Fig 1C). Therefore, inhibition of translation was not required to explain the decline in thrombomodulin synthesis in these cells. However, our previous observation of decreased thrombomodulin activity without decreased thrombomodulin mRNA in some preparations of TNF-treated HUVEC¹² suggests that translational regulation of

thrombomodulin expression may occur under some conditions.

In addition to its effect on thrombomodulin synthesis, TNF has been proposed to enhance thrombomodulin degradation.¹¹ Our results suggest that accelerated thrombomodulin degradation does not contribute significantly to the decrease in thrombomodulin activity in TNF-treated cells. [³⁵S]cysteine-labeled thrombomodulin was degraded with a half-life of 8.2 ± 2.4 hours (SD) in untreated cells, compared with 7.5 ± 1.3 hours (SD) after TNF stimulation. Human thrombomodulin appeared to be somewhat less stable than mouse thrombomodulin, which was degraded with half-lives of 19.8 ± 3.9 hours and 10.9 ± 1.1 hours in unstimulated and phorbol ester-treated mouse hemangioma cells, respectively.³¹

The rate of thrombomodulin degradation was consistent with the observed decrease in total cellular thrombomodulin activity after addition of TNF (Fig 7B). Although TNF acted primarily to reduce thrombomodulin synthesis, the subsequent loss of activity was dependent on continued thrombomodulin degradation, presumably through internalization of cell surface thrombomodulin and degradation in lysosomes. This may provide an explanation for the blunted response to TNF observed when the lysosomal degradation pathway was blocked by treatment of BAEC with

chloroquine.¹¹ It is possible that in the presence of chloroquine, thrombomodulin that enters the cell by endocytosis can be recycled to the plasma membrane. Alternatively, chloroquine may block the constitutive endocytosis of thrombomodulin.

TNF has been shown to stimulate the transcription of several genes in endothelial cells, including tissue factor,¹² leukocyte adhesion molecules,⁴² and AP-1/*jun*, which may be responsible for the activation of additional genes.⁴³ Phorbol myristate acetate, which also activates AP-1/*jun* through stimulation of protein kinase C,⁴⁴ fails to reduce thrombomodulin activity in HUVEC,¹² suggesting that alternative pathways may be responsible for the inhibition of thrombomodulin transcription by TNF. Human thrombomodulin is encoded by a single gene, which is free of introns.^{45,46} The putative promoter region contains four potential recognition sequences for the transcription factor Sp1, but no consensus AP-1 binding sites. Recent data suggest that thrombomodulin transcription can be enhanced by analogues of cyclic adenosine monophosphate in human megakaryoblastic cell lines,⁴⁷ and may be activated in developing nervous tissue.⁴⁸ Analysis of the thrombomodulin promoter region using reporter constructs may provide additional insight into the positive and negative regulation of thrombomodulin expression in these multiple cell types.

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