Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated via activation of nuclear factor-kappa B

Short title: Thrombomodulin and NF-κB

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Abstract

Inflammation and thrombosis are increasingly recognized as inter-related biological processes. Endothelial cell expression of thrombomodulin (TM), a key component of the anticoagulant protein C pathway, is potently inhibited by inflammatory cytokines. Because the mechanism underlying this effect is largely unknown, we investigated a potential role for the inflammatory transcription factor nuclear factor kappa-B (NF-κB). Blocking NF-κB activation effectively prevented cytokine-induced down-regulation of TM, both in vitro and in a mouse model of TNF-α-mediated lung injury. Though the TM promoter lacks a classic NF-κB consensus site, it does contain tandem Ets transcription factor binding sites previously shown to be important for both constitutive TM gene expression and cytokine-induced repression. Using electrophoretic mobility shift assay and chromatin immunoprecipitation, we found that multiple Ets species bind to the TNF-α response element within the TM promoter. Though cytokine exposure did not alter Ets factor binding, it did reduce binding of p300, a co-activator required by Ets for full transcriptional activity. Over-expression of p300 also prevented TM repression by cytokines. We conclude that NF-κB is a critical mediator of TM repression by cytokines. Further evidence suggests a mechanism involving competition by NF-κB for limited pools of the transcriptional co-activator p300 necessary for TM gene expression.
Introduction

Systemic inflammatory conditions, such as bacterial sepsis and vasculitis, are frequently complicated by the development of pathologic thrombosis. Inflammatory processes can shift the hemostatic balance toward thrombus formation not only by stimulating tissue factor-dependent coagulation but also by inhibiting anticoagulant and fibrinolytic pathways. Thrombomodulin (TM), a 100 kD transmembrane protein expressed in abundance by vascular endothelial cells, is a critical component of the anticoagulant protein C pathway. TM binds thrombin and alters its active site specificity to facilitate proteolytic activation of circulating protein C. In concert with its cofactor protein S, activated protein C (APC) enzymatically degrades factors Va and VIIIa of the clotting cascade thereby suppressing further thrombin generation. Growing clinical evidence suggests that dysfunction of the TM-APC pathway caused by inflammation compromises vascular thromboresistance. For example, TM expression is markedly reduced in skin-biopsy specimens taken from patients with severe bacterial sepsis complicated by microvascular thrombosis and associated with abnormally low circulating levels of APC. Impaired TM activity provides the rationale for administering recombinant APC to patients with severe sepsis, a therapy that has recently been shown to reduces the risk of death in such patients by nearly 20%.

In vitro studies have demonstrated that endothelial TM expression is potently inhibited by inflammatory mediators such as bacterial endotoxin and several inflammatory cytokines. Of these mediators, the effects of tumor necrosis factor-α (TNF-α) are the...
most widely studied. Exposure of endothelial cells to TNF-α causes an abrupt down-regulation of TM gene expression that does not require new protein synthesis or involve changes in message stability. While it is not known how this occurs, promoter studies provide clues to potential mechanisms. Two groups have independently identified a discrete region in the TM promoter immediately up-stream of the TATA box (-76 to –29 relative to the transcriptional start site) that is important for both constitutive gene expression and repression in response to TNF-α stimulation. Within this purported TNF-α response element are three direct repeats of the binding sequence (GGAA) for the Ets family of transcription factors in reverse orientation. The physiologic importance of these sites in regulating TM gene expression was confirmed by promoter-reporter studies demonstrating that mutation of these sequences significantly reduced basal promoter activity and prevented TNF-α-induced promoter repression. The mechanism by which TNF-α could inhibit TM gene expression via the modulation of Ets transcriptional activity remains unknown.

Many of the transcriptional effects of inflammatory cytokines, including TNF-α, are mediated via activation of the transcription factor nuclear factor kappa-β (NF-κB; reviewed in reference 14). The predominant form of NF-κB is a heterodimer composed of two subunits, p65 (RelA) and p50, though several other hetero- and homodimers have been described. In quiescent cells, NF-κB is complexed in the cytoplasm to the inhibitory protein IκBα. Stimulation by cytokines or endotoxin induces the phosphorylation and subsequent degradation of IκBα, allowing the p65/p50 heterodimer to translocate to the nucleus where it acts as a transcriptional activator via binding to
specific consensus sequence in the promoter region of target genes. In addition to
inducing the expression of genes that control the inflammatory response, NF-κB is also
increasingly recognized as an important modulator of hemostasis. NF-κB activation is
required for cytokine-induced expression of tissue factor expression in endothelial cells
and monocytes as well as contributes to the up-regulation of plasminogen activator
inhibitor-1 (PAI-1) by TNF-α and urokinase by phorbol esters.\textsuperscript{16-20}

We hypothesize that inflammation modulates the expression of both procoagulant and
anticoagulant molecules via common signaling pathways. Given its prominence in
mediating tissue factor expression, the role of NF-κB in mediating cytokine-induced
inhibition of TM was investigated, both in vitro and in a mouse model of acute lung
injury. Our findings reveal that activation of NF-κB is a critical requirement for this effect.
Because the TM promoter lacks a consensus NF-κB binding sequence, we further
explored mechanisms by which NF-κB could alter TM expression via direct binding to
non-canonical promoter sequences or indirectly through modulation of the
transcriptional factors required for TM expression.
Materials and Methods

Construction of Adenovirus Vectors. The plasmid, pCMV-IκBα-S32/36A, containing the full-length cDNA sequence of an IκBα mutant with an amino-terminus FLAG peptide tag (DYKDDDK), was a generous gift of Dr. Albert S. Baldwin (University of North Carolina, Chapel Hill). This IκBα super-repressor (IκBsr) contains alanine substitutions at Ser32 and Ser36 that prevent its phosphorylation and subsequent degradation, thereby enabling it to act as a constitutive repressor of NF-κB.21 The FLAG-IκBsr sequence was ligated into the multi-cloning site of pAdloxRSV, a shuttle plasmid containing the RSV promoter and SV-40 virus polyadenylation signal. The first-generation recombinant adenovirus, AdIκBsr, was generated by cotransfection of pAdFLAG-IκBsr and purified ψ5 adenovirus DNA into CRE8 cells as previously described.22 CRE8 cells, ψ5 adenovirus, and pAdlox, the parent plasmid of AdLoxRSV, were generous gifts by Dr. Stephen Hardy (Cell Genesys, Inc). AdNull, a control virus expressing no transgene was generated in similar fashion. Recombinant adenovirus vectors were propagated in 293 cells and purified by double cesium chloride centrifugation. Viral stocks were plaque-titered on 293 cells and replication incompetence verified using A549 cells.

The plasmid, pVR1012p300, containing the full-length cDNA sequence of the human p300 gene was a generous gift of Dr. Gary J. Nabel (National Institutes of Health, Bethesda, MD). To generate an adenovirus vector that could package the entire p300 coding sequence (~7.5 kbp), a modified ψ5 virus (ψ5-FB) containing an added deletion of the fiber gene deleted was constructed. To create this virus, CRE8 and 911 cell lines were first stably transfected with pDV67, a plasmid expressing the type 5 adenovirus
fiber gene.\textsuperscript{23,24} The ψ5-FB virus was generated by co-transfecting the left end of the ψ5 genome (containing a \textit{loxP} site in the E1 region) and the right end of the fiber-less virus, Ad5.GFPΔF, into 911-FB cells.\textsuperscript{25} Resulting recombinant viral clones were screened for their inability to form plaques on non-fiber expressing cells and for lack of GFP expression. The p300 coding sequence was ligated into the multi-cloning site of pAdloxRSV to generate pAdp300. Recombinant virus was generated by co-transfecting pAdp300 and purified ψ5-FB DNA into CRE8-FB. The resulting recombinant adenovirus was propagated and titered on 911-FB cells as above.

\textit{Cell Culture, Viral Transduction and Cytokine Stimulation.} Human umbilical vein endothelial cells (HUVEC) from a single donor were maintained in EGM-2 media (BioWhittaker, Walkersville, MD) under 5% CO\textsubscript{2} at 37°C. Cells of passage 2-6 were used for all experiments. To inhibit NF-κB activation prior to cytokine stimulation, parthenolide (Sigma; St. Louis, MO) was added to the medium at the indicated concentrations for 1 hour or cells were transduced with the indicated adenoviral construct. Adenoviral transduction was performed by incubating confluent HUVEC with EGM-2 medium containing vector at the indicated dose for 90 minutes, followed by addition of fresh EGM-2 medium to dilute the viral suspension by 1:4. After 24 hours, the cells were rinsed and refreshed with medium and incubated for an additional 48 hours prior to cytokine stimulation to allow adequate transgene expression. For cytokine stimulation, the medium was replaced with EGM-2 containing either vehicle or 80-100 ng/ml human recombinant TNF-α (BD Biosciences, Palo Alto, CA), 10 ng/ml human recombinant IL-1β (Sigma), or 10 µg/ml LPS (E. coli serotype O26:B6; Sigma) for the
Quantification of NF-κB Activation. An ELISA-based method (Trans-AM NF-κB; Active Motif, Carlsbad, CA) was used because of its increased sensitivity to detect and ability to quantify NF-κB activation in small amounts of tissue compared to the more traditional EMSA. Cells were washed in saline, lysed in 20 mM HEPES, pH 7.5, 350 mM NaCl, 20% glycerol, 1% Triton X-100, 1 mM MgCl2 and 0.5 mM EDTA containing a protease inhibitor cocktail (P 8340; Sigma) for 10 minutes on ice then cleared by centrifugation. Lysates were then placed in 96-well plates coated with an oligonucleotide containing the NF-κB consensus sequence and the presence of active NF-κB detected using antibodies specific for p50 subunits that are not complexed to IκB and thus able to bind the consensus sequence. An HRP conjugated secondary antibody is used to quantify NF-κB binding by conversion of an applied chromogenic substrate.

Western Blot Analysis. Cells were rinsed twice with ice-cold phosphate-buffered saline and incubated for 30 minutes at 4°C in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, and 100 µg/ml phenylmethanesulfonyl fluoride. Cell lysates were clarified by centrifugation and total protein concentrations determined using BCA protein assay kit (Pierce, Rockford, IL). 2 µg of each sample was electrophoresed through a 4-20% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred overnight to an Immobilon-P PVDF membrane (Millipore, Bedford, MA) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer’s instructions. After blocking for 30 minutes with 3% nonfat milk in Tris-buffered saline, pH 7.6 (TBS),
0.1% Tween 20, blots were incubated simultaneously with 1:500-1500 dilutions of an anti-human TM monoclonal antibody (#2375; American Diagnostica, Stamford, CT) and a 1:10,000 dilution of an anti-actin monoclonal antibody (AC-40; Sigma) in TBS-T containing 1% nonfat milk for one hour, followed by incubation with peroxidase-labeled sheep anti-mouse IgG polyclonal secondary antibody (NA 931; Amersham, Little Chalfont, UK) diluted 1:10,000. Detection was performed by autoradiography with enhanced chemiluminescence (ECL-Plus, Amersham), and densitometric quantification using UN-SCAN-IT software (Silk Scientific, Orem, UT).

Real-Time PCR. Cells were washed twice with ice-cold PBS then incubated for 3 minutes at room temperature in 1 ml/well of TRIZOL reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted per manufacturer’s instructions. Each sample was subsequently treated with RNase-free DNase I (Roche, Indianapolis, IN) to remove traces of genomic DNA. Real-time quantitative PCR was performed using a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Duplicates of each sample were subjected to reverse transcription (48°C for 30 minutes) and standard multiplex real-time PCR (95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds) using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) and primers and probes designed using Primer Express software (Applied Biosystems) specific for either human TM (GenBank #M74564; forward: 5’-CCCAACACCCAGGCTAGCT-3’; reverse: 5’-CGTCGATGTCCGTGCAGAT-3’; 6FAM-labeled probe: 5’-TGCCCTGAAGGTAC-3’).
ATCCTGGACG-3’) or mouse TM (GenBank #X14432; forward: 5’-GAAACTTCCCTGGCTCC TATGA-3’; reverse: 5’-GTCTTTGCTAATCTGACCAGC AAG-3’; 6FAM-labeled probe: 5’-TGTATC TGCGGGCCTGACACAGCC-3’). The mean threshold cycle (C_T) values for each sample was compared with those of standard curves derived from serial dilutions of a reference sample. The coefficient of variance between duplicates was routinely less than 20%. For each sample, TM gene expression was normalized to 18S ribosomal RNA (TaqMan Ribosomal RNA Reagents with VIC-labeled probe; Applied Biosystems).

**Acute Lung Injury Model.** All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Male C3H/HeN mice weighing 22–29 g were anesthetized with thiopentobarbital (85–95 µg/kg i.p.) and ketamine (3 µg/kg i.p.) and placed in a supine upright position. Using sterile technique, the trachea was approached via a midline neck incision and isolated by blunt dissection. Using a 27-gauge needle attached to a microsyringe, 60 µl of phosphate-buffered saline (PBS) vehicle or 10^7 plaque-forming units (pfu) of AdNull or AdIκBsr in PBS was instilled into the trachea. Immediately before instillation, forced expiration was achieved by compression of the thorax. After viral installation, 60 µl of air was instilled to produce a deep inspiration that facilitated adenoviral dispersion to distal air spaces. The neck incision was closed, and the animals recovered. 4 days after adenoviral transduction, 60 µl of PBS alone or containing 20 µg human recombinant TNF-α (Research Diagnostics, Flanders, NJ) was instilled into the trachea in similar fashion. Sixteen hours later, animals were sacrificed by lethal administration of thiopentobarbital and
ketamine. Fresh tissue from both lungs was processed for assayed for Western blot analysis, real-time PCR and NF-κB activation as previously described.

*Electrophoretic Mobility Shift Assay (EMSA).* Nuclear extracts were prepared as previously described. Double-stranded oligonucleotide probes containing either the TNF-α response element within the TM promoter (5'-ACCAGGCCTTTGCCTCCTCGAGCAG-3'), the potential non-canonical NF-κB binding site (5'-ATGTCAGAGGCTGCCTCGCAG-3') or the consensus NF-κB binding site from the murine immunoglobulin κ-light chain enhancer (5'-AGTTGAGGGACTTTCCCAGGCA-3') were 5'-end-labeled with α-32P-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), column purified (QIAquick; QIAGEN, Valencia, CA) and diluted to 10^5 cpm/µl. 5 µg of nuclear extracts and 10^5 cpm end-labeled probe were incubated at room temperature for 20 minutes in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiotreitol, 5% glycerol, 1 mg/ml bovine serum albumin and 1 µg poly(dI-dC) (Amersham). Reaction samples were electrophoresed through a 6% polyacrylamide gel in 0.5X Tris-boric acid-EDTA (TBE; Invitrogen). The gel was vacuum dried onto filter paper prior to autoradiography. Competition assays were performed by pre-incubating nuclear extracts with unlabeled oligonucleotides as indicated, or an oligonucleotide containing the Ets binding site sequence found in the stromelysin promoter (5'-AATTCGTCAGTTAAGCAGGAATGACTAACG-3'). Super-shift analyses were performed by pre-incubating nuclear extracts with antibodies (2 µg per reaction) purchased from Santa Cruz Biotechnology, Santa Cruz, CA against p50 (H-119, sc-7178X), p52 (K-27, sc-298X), and p65 (C-20, sc-372X).
Chromatin Immunoprecipitation (ChIP). ChIP was performed using a commercially available assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY). Prior to harvest, DNA and associated proteins were cross-linked by the addition of 37% formaldehyde to culture medium (1% final concentration). After a 10-minute incubation at 37°C, cells were washed in ice-cold PBS, harvested in 1.4 ml PBS per well, and lysed in the presence of a protease inhibitor cocktail (P 8340; Sigma) according to the manufacturer’s instructions. DNA was sheared to lengths of <1,000 base pairs by sonication at 30% power for two cycles of five seconds each at 4°C. Samples were immunoprecipitated using specific antibodies, the cross-linking reversed, and DNA purified according to instructions. Quantitative RT-PCR using primers and probe specific to the gene segment assessed enrichment of the gene segment of interest by protein-specific immunoprecipitation. Values were normalized as the percent of input DNA. Samples processed in the absence of antibody, as well as primers and probe for a distant gene segment that would not be expected to bind the proteins of interest, were used as negative controls. Antibodies against the following proteins were purchased from Santa Cruz Biotechnology, Inc: Ets-1 (C-20, sc-350X), Ets-2 (C-20, sc-351X), Erg (C-20, sc-353X), Tel (N-19, sc-8546X), Nerf (V-19, sc-6829X), p50 (H-119, sc-7178X), p52 (K-27, sc-298X), and p65 (C-20, sc-372X). A polyclonal anti-p300 antibody was affinity purified from rabbit serum as previously described. Primers and probes were specific for the TNF-α response element within the human TM promoter (forward: 5'-TCCGAGTATGCAGCTACAG-3'; reverse: 5'-GGCCAGGGCTCGAGTTTATAA-3'; 6FAM-labeled probe: 5'-CAGGCAC TTCCTTCTTTTCCGAAC-3'), and for a
segment ~18,000 base pairs upstream of the TM promoter (forward: 5’-AGGAATGAAACTGAAGTCTGTCTGACT-3’; reverse: 5’-AGACTGATGCCCACCACTATCC-3’; 6FAM-labeled probe: 5’-CTCAGAATCATGCTCTTATTCCAAACTTGTCTCCA-3’).

Protein C Activation. To measure in situ APC-generating capacity, HUVEC plated in 96-well plates and subjected to the described conditions were first washed with Hank’s buffered salt solution (HBSS; Life Technologies) then incubated with 25 µg/mL human protein C (American Diagnostica), 1 NIH U/mL human α-thrombin (Sigma), 2.5 mM CaCl₂ and 1mg/mL bovine serum albumin (Sigma) in HBSS at 37°C. After 1 hour the thrombin was neutralized by addition of 50 µL of 50 mg/mL lepirudin (Hoechst Marion Roussel, Kansas City, MO) 100 µL aliquots were removed and incubated with 50 µL of a 3 mM solution of the chromogenic substrate S-2366 (Chromogenix, Milano, Italy) at room temperature. The rate of conversion of the substrate by APC was determined spectrophotometrically using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis. All data are presented as mean ±SEM. Where indicated, comparisons between two groups were by two-tailed t test. P values <0.05 was considered statistically significant.
Results

Inhibition of TM expression by inflammatory mediators is dependent on NF-κB activation. To determine a role for NF-κB in mediating cytokine-induced down-regulation of TM, its activation was inhibited both pharmacologically, using the soluble inhibitor parthenolide29,30, and by molecular means, via transduction with an adenovirus vector (AdIkBsr) expressing the constitutively active IkB-S32/36A mutant.21 Both the pretreatment of HUVEC with parthenolide and transduction with AdIkBsr effectively blocked the activation of NF-κB (Figure 1A) and prevented the down-regulation of TM gene and protein expression (Figures 1B-C) in response to TNF-α stimulation. To ascertain if these results are generalizable to the inhibition of TM by other inflammatory mediators, HUVEC pretreated with parthenolide or transduced with AdIkBsr were also stimulated with IL-1β and bacterial endotoxin (Figure 2). Blocking NF-κB activation also prevented TM gene down-regulation in response to both inflammatory mediators. Interestingly, infection with the adenovirus control vector itself blunted the down-regulation of TM by endotoxin. This effect was consistently observed over a wide range of endotoxin dosages (data not shown).

To determine if NF-κB mediates TNF-α-induced TM down-regulation in vivo, we employed a mouse model of cytokine-mediated acute lung injury.31 Intra-tracheal instillation of 20 μg of TNF-α resulted in a 5–fold increase in NF-κB activation and a near 90% inhibition of pulmonary TM gene expression within 16 hours of exposure (Figures 3). In contrast to mouse lungs transduced 4 days previously with the AdNull control virus, lungs transduced with AdIkBsr had blunted NF-κB activation and no
significant down-regulation of TM gene expression following TNF-α challenge. These data confirm the physiologic relevance of NF-κB in regulating in vivo TM expression.

*Binding of NF-κB to the TM promoter.* While NF-κB traditionally mediates transcriptional activation, there is a small but growing list of examples where NF-κB can function as a transcriptional repressor.³²-³⁴ To dissect the mechanism by which NF-κB mediates cytokine-induced transcriptional repression of TM, the ability of NF-κB to bind to the TM promoter was first investigated. Sequence analysis of the 5’ untranslated region did not reveal the presence of a classic NF-κB consensus site (GGGRNNYYCC) within the TM promoter (-1539 to +28, relative to the transcriptional start site).¹²,³⁵ However, a region immediately downstream of the transcriptional start site (+9 to +17) does contain a sequence (AGGCTGCCT) identical to a non-canonical NF-κB binding site in the osteocalcin promoter that mediates transcriptional repression via binding of p50 homodimers.³² To evaluate whether this site is functional, EMSA was performed on HUVEC nuclear extracts ± TNF-α stimulation for 1 hour, using a radiolabeled oligonucleotide probe containing the TM promoter sequence spanning this motif (Figure 4A). While a dominant protein-DNA complex was identified, the lack of competition from a cold oligonucleotide suggests only a non-specific interaction. In contrast, incubation of the same nuclear extracts with a probe containing a classic consensus NF-κB sequence revealed inducible DNA-protein complexes that were competed by a cold oligonucleotide probe. To confirm these results and to exclude the presence of cryptic NF-κB binding sites elsewhere in the TM promoter, chromatin immunoprecipitation was performed using antibodies to several of the NF-κB subunits known to bind to non-
canonical sites (Figure 4B). There was no identifiable binding of the p50, p52 or p65 subunits above background to the TM promoter. As a positive control, exposure to TNF-α did result in an expected rise in p65 subunit binding to the tissue factor promoter. These data argue against direct binding of NF-κB to the TM promoter and suggest an indirect mechanism by which its activation mediates cytokine-induced repression of TM.

**Binding of Ets transcription factors to the TM promoter.** The TM promoter contains three direct repeats of the Ets consensus binding sequence within a discrete region immediately up-stream of the TATA box (-76 to -56) that have previously been shown to mediate constitutive TM expression as well as responsiveness to TNF-α.\(^\text{13}\) We hypothesize that NF-κB might repress TM expression by interfering with Ets binding to the TM promoter or by inhibiting its transcriptional activity. To determine if TNF-α stimulation modulates Ets binding, EMSA was performed on nuclear extracts from HUVEC ± TNF-α stimulation, using a radiolabeled oligonucleotide probe containing the TM promoter sequence spanning the Ets binding sites (Figure 5A). A dominant protein-DNA complex was identified that exhibited competition from a cold oligonucleotide containing Ets consensus sequences from the stromelysin promoter but not from a nonspecific oligonucleotide, confirming binding of an Ets factor to the TM promoter. However, neither the intensity of the band nor its electrophoretic mobility changed with TNF-α treatment, suggesting that NF-kB activation does not grossly interfere with Ets binding to the TM promoter.

While several of the highly conserved Ets transcription factors are expressed in
endothelial cells, it is not known which of these mediate constitutive TM expression. It is plausible that NF-κB activation might cause displacement of an Ets protein with transcriptional stimulatory activity by one with repressor activity. To investigate this possibility, chromatin immunoprecipitation was performed on extracts from HUVEC ± TNF-α stimulation for 16h, using antibodies to Ets proteins known to be expressed in endothelial cells (Figure 5B). While Ets-1, Ets-2 and Erg were found to associate with the TM promoter at baseline, their binding was not altered by TNF-α stimulation. The binding of Tel, an Ets factor with repressor activity, was also not enhanced by TNF-α. These data confirm that NF-κB activation does not inhibit transcription by grossly altering Ets binding to the TM promoter.

**Modulation of TM expression by p300.** Full transcriptional activity of both the Ets family of proteins and NF-κB requires physical interaction with the closely-related transcriptional co-activators, p300 and cAMP response element binding protein [CREB]-binding protein (CBP). Recent evidence indicates that NF-κB can inhibit the activities of certain transcription factors by competing for the stable, but limited, quantities of p300/CBP within the nucleus. To investigate whether NF-κB inhibits TM expression by this mechanism, we first determined if stimulation with TNF-α alters the association of p300/CBP with the TM promoter. Chromatin immunoprecipitation was performed on extracts from HUVEC ± TNF-α stimulation for 1h, using an antibody against p300. The baseline association of p300 with the TM promoter was reduced by ~50% upon stimulation with TNF-α for 1 hour, an effect that was reversed by transduction with AdIkBsr (Figure 6). As a positive control, exposure to TNF-α resulted
in the expected marked rise in p300 association with the tissue factor promoter.

We then determined if over-expression of p300 might prevent cytokine-induced TM down-regulation by relieving the competition for limited pools of p300. To accomplish this, a high-capacity adenovirus vector, Adp300, was constructed expressing the full-length human p300 gene. Transduction of HUVEC with Adp300 effectively prevented the down-regulation of TM gene expression in response to TNF-α stimulation for 16h, compared to untransduced or AdNull-transduced HUVEC (Figure 7A). Furthermore, both the over-expression of p300 and IκB-S32/36A restored the capacity of HUVEC stimulated with TNF-α to activate protein C, an indicator of TM functional activity (Figure 7B). Taken together, these data are consistent with a model in which cytokine-induced NF-κB activation represses TM expression and function indirectly via competition for limited quantities of p300/CBP.
Discussion

The major findings of the present study are: 1) The inhibition of TM in response to inflammatory cytokines and endotoxin is mediated via activation of NF-κB. 2) NF-κB does not directly associate with the TM promoter, either through a classic consensus sequence or through a non-canonical binding site. 3) Multiple Ets transcription factors bind to the TM promoter, though binding is not altered by cytokine stimulation. 4) Cytokine-induced NF-κB activation appears to indirectly inhibit TM expression via competition for limited pools of the transcriptional co-activator p300/CBP.

While it has been known for some time that TNF-α and other inflammatory mediators potently down-regulate TM expression, the mechanism underlying this effect have remained obscure. Recognition that NF-κB activation mediates this effect fills in several gaps in the molecular understanding of TM regulation. The activation of NF-κB is a rapid post-translational process, thereby providing an explanation for why TNF-α is able to inhibit TM gene expression without new protein synthesis. It also explains the observation that heat shock stress is able to block the inhibitory effects of TNF-α on TM expression in endothelial cells. Induction of the heat shock response is known to inhibit NF-κB activation in response to TNF-α stimulation both by increasing the expression of IκB and by preventing its phosphorylation and subsequent degradation.

Realizing that NF-κB appears to exert its effect on TM expression via competition for p300/CBP also places into context the findings of von der Ahe, who used promoter
analysis to convincingly demonstrate that an Ets-like transcription factor is responsible for both constitutive TM expression as well as TNF-α- responsiveness. As part of that study, footprint analysis revealed protein binding to the tandem Ets consensus sequences in the proximal TM promoter that did not change with TNF-α stimulation. Based on this and the observation that over-expression of c-Ets-1 prevented the down-regulation of TM by TNF-α, the authors speculated that an Ets cofactor was the ultimate target for TNF-α. Our data demonstrate that several different Ets species bind the TM promoter and confirm that binding is not altered by TNF-α stimulation. Furthermore, we identify p300/CBP as the required cofactor for Ets transcriptional activity that is the molecular target of TNF-α stimulation.

While NF-κB predominantly acts as a transcriptional activator, there is a small but growing list of examples where it can act as a repressor. One mechanism by which this occurs is by transcriptional interference caused by the binding of atypical NF-κB species, usually homodimers of p50 and p52 subunits which lack transcriptional activation domains, to non-canonical promoter binding sites. This has been described for TNF-α-induced inhibition of the human osteocalcin and α2(I) collagen genes and as well as IL-1β-mediated inhibition of CYP2C11, a rat cytochrome P450 gene.32,33,43 While the TM promoter does contains a sequence similar to the non-canonical NF-κB binding site in the human osteocalcin promoter, both EMSA and chromatin immunoprecipitation analyses failed to indicate evidence of functionality.

A second mechanism by which NF-κB can inhibit gene expression is by competing for
the cellular machinery used by other transcription factors. The transcriptional co-activators p300 and CBP are nearly identical proteins with histone acetyltransferase activity that modulate the activities of many different transcription factors, including NF-κB and members of the Ets family (reviewed in references 44,45). p300/CBP is present in limited but stable quantities within the nucleus. There are several examples where NF-κB can mediate signal-induced transcriptional repression via competition for p300/CBP. This has been described for NF-κB-mediated transcriptional suppression of p53 and the human estrogen receptor as well as TNF-α-induced down-regulation of epithelial cell adhesion molecule (EpCAM) expression. Two pieces of evidence from the present study support a similar mechanism for cytokine-induced down-regulation of TM. The first is the decrease in p300 association with the TM promoter after TNF-α stimulation that is prevented by blockade of NF-κB activation. The second is the abrogation of TNF-α-induced TM inhibition by over-expression of p300.

Competition for p300/CBP may also provide an explanation for the observations that all-trans-retinoic acid (RA) and cAMP analogues, agents known to stimulate basal TM gene expression, prevent the down-regulation of TM in response to TNF-α. Extensive promoter analysis has revealed that RA induction of TM is mediated by the binding of Sp1 to two Sp sites (-145 to –121) in addition to the binding of the RAR/RXR heterodimer to a classic DR4 RA receptor binding site (-1531 to –1516). There is strong evidence that Ets and Sp1 transcription factors physically interact with each other and p300/CBP in a multi-protein complex to enhance transcriptional activity. Both RA and cAMP analogues increase phosphorylation of Sp1 that increases its
transcriptional activity. It is possible that RA or cAMP-induced phosphorylation of Sp1 increases the affinity of p300/CBP for the Sp1-Ets nucleosome, thereby competing it away from NF-κB. Mackman’s group has described a similar mechanism by which elevations in intracellular cAMP prevent NF-κB-mediated up-regulation of tissue factor. In this case, cAMP results in protein kinase A-mediated phosphorylation of CREB that increases its affinity for p300/CBP which effectively competes it away from NF-κB. While the mechanism by which cAMP increases TM transcription remains unknown, its effects potentiate those of RA. The above data provide a theoretical framework for future studies aimed at elucidating the mechanism by which RA and cAMP prevent cytokine-induced inhibition of TM.

The finding that NF-κB can regulate TM expression provides evidence for a common molecular pathway by which inflammation affects hemostasis by modulating the expression of both procoagulant and anticoagulant molecules. It is well established that the induction of tissue factor expression in endothelial cells by cytokines is dependent on binding of c-Rel-p65 heterodimers to an NF-κB consensus site within the tissue factor promoter. c-Rel-p65 heterodimers are also known to mediate the induction of urokinase gene expression by phorbol esters in some cancer cell lines. Recently, inhibition of NF-κB by emodin has been shown to partially prevent the induction of PAI-1 by TNF-α in HUVEC. Further studies will likely identify other hemostatic molecules that are regulated by NF-κB. It is also intriguing to speculate that local differences in the NF-κB activation cascade may contribute to the differential susceptibility of certain vascular beds to thrombus formation during inflammation.
In summary, our results identify the activation of NF-κB as a critical mediator of TM inhibition by inflammatory mediators, both in vitro and in a mouse model of acute lung injury. This finding establishes a common pathway by which inflammation modulates both procoagulant and anticoagulant pathways to produce pathologic thrombosis. We further show evidence suggesting that NF-κB inhibits TM expression indirectly via competition for the co-activator p300/CBP. Elucidation of this regulatory pathway provides new opportunities to develop novel therapeutic strategies to prevent the thrombotic sequelae of inflammatory diseases.
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References


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Figure Legends

Figure 1. Effect of blocking NF-κB activation on TNF-α-induced TM repression. HUVEC were pretreated with parthenolide at the indicated doses or transduced with either AdNull or AdIκBsr (MOI =100pfu/cell) prior to stimulation ±100 ng/mL human TNF-α. A. NF-κB activity measured in nuclear extracts by ELISA 1 hour after TNF-α stimulation. B. TM gene expression, normalized to CD31, was determined by real-time PCR 16 hours after TNF-α stimulation. C. TM protein expression was determined by Western blot analysis 16 hours after TNF-α stimulation. Representative blots are shown on the left and densitometric analysis with normalization to actin is shown on the right. Par = parthenolide. Values in all figures are the mean ± SEM of n=3 experiments (n=5-6 for control groups). * p ≤0.02 for 8 µM parthenolide compared to 2 µM parthenolide or control groups stimulated with TNF-α. # p ≤0.03 for AdIκBsr compared to AdNull or control groups stimulated with TNF-α.

Figure 2. Effect of blocking NF-κB activation on IL-1β and endotoxin-induced TM repression. HUVEC were pretreated with 8 µM parthenolide or transduced or either AdNull or AdIκBsr (MOI =100pfu/cell) prior to stimulation ±10 ng/mL human IL-1β (A) or 10 µg/mL bacterial endotoxin (B) for 16h. TM gene expression, normalized to CD31, was determined by real-time PCR. Values shown are the mean ± SEM of n =3 experiments (n =6 for control groups). * p <0.01 for parthenolide compared to control groups stimulated with IL-1β or endotoxin. # p ≤0.001 for AdIκBsr compared to AdNull or control groups stimulated with IL-1β or endotoxin.
**Figure 3.** Effect of blocking NF-κB activation on in vivo TNF-α-induced TM repression. The lungs of mice were transduced with no virus or $10^9$ pfu of either AdNull or AdIkBsr via intra-tracheal instillation. 4 days later they were challenged with either PBS or 20 µg human TNF-α via intra-tracheal instillation. A. Pulmonary NF-κB activity measured in whole lung nuclear extracts by ELISA. *p < 0.007 for AdIkBsr versus no virus or AdNull controls challenged with TNF-α. B. Pulmonary TM gene expression in whole lung extracts determined by real-time PCR in. # p < 0.04 for AdIkBsr versus no virus and AdNull controls challenged with TNF-α. All values are the mean ± SEM of n =3 experiments. C. Photomicrographs of TM immunostaining 16h after exposure to either PBS or TNF-α.

**Figure 4.** Binding of NF-κB to the TM promoter. HUVEC were stimulated ± 80 ng/mL human TNF-α for 1h. A. EMSA was performed on nuclear extracts with probes containing either the sequence of a potential non-canonical NF-κB binding site within the TM promoter (left panel) or the classic NF-κB consensus sequence (right panel). Competition with 100-fold excess of unlabelled oligonucleotides failed to eliminate non-specific DNA-protein complexes (solid arrows) but did reduce intensity of p50/p65-DNA complexes (open arrow) induced by TNF-α. B. Chromatin immunoprecipitation was performed using primers/probe specific for either the TM (left panel) or tissue factor (right panel) promoters and antibodies specific for various NF-κB subunits. Values are the mean ± SEM for n=4 experiments. P =NS for paired vehicle and TNF-α groups except where indicated.
Figure 5. Binding of Ets transcription factors to the TM promoter. HUVEC were stimulated ± 80 ng/mL human TNF-α for 16h. A. Representative EMSA performed with a primers/probe containing the sequence of the TNF-α response element within the TM promoter. Pre-incubation of HUVEC nuclear extracts with unlabelled oligonucleotides containing Ets binding sequences from the stromelysin promoter (EtsSTR), but not a non-specific (NS) oligonucleotide containing the potential non-canonical NF-κB binding site, competitively reduced the observed the intensity of the dominant DNA-protein complex (solid arrow). B. Chromatin immunoprecipitation was performed using a probe specific for the TM promoter and antibodies specific for various Ets species. Specificity for the TNF-α response element within the TM promoter was confirmed by lack of enrichment above background using primers and probe to a sequence ~18 kb upstream of the TM promoter (data not shown). Values are the mean ± SEM for n=4 experiments. P =NS for paired vehicle and TNF-α groups.

Figure 6. Change in p300 association with the TM promoter following TNF-α stimulation. Chromatin immunoprecipitation was performed in HUVEC stimulated ± 80 ng/mL human TNF-α for 1 h using an antibody recognizing p300 and primers/probes specific for either the TM (left panel) or tissue factor (right panel) promoters. Values are the mean ± SEM for n=3 experiments. * p =0.04 for AdIκBsr versus AdNull groups.

Figure 7. Effect of p300 over-expression on TNF-α–induced TM repression. HUVEC were transduced with either AdNull, Adp300 or AdIκBsr (MOI =100 pfu/cell) prior to
stimulation ± 100 ng/mL human TNF-α. A. TM gene expression, normalized to CD31, was determined by real-time PCR 16 hours after TNF-α stimulation. B. Activated protein C formation was determined by in situ chromogenic assay 24 hours after TNF-α stimulation. Values are the mean ± SEM of n=3 experiments. # p <0.004 for Adp300 versus AdNull and control groups stimulated with TNF-α. *p <0.01 for AdlkBsr versus AdNull and control groups stimulated with TNF-α.
Figure 1.
Figure 2.

A.

B.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

A. TM Gene Expression (% of Vehicle Control)

B. Protein C Activation (O.D./mm/kg Protein)
Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated via activation of nuclear factor-kappa B

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