Mechanism of the Tumor Necrosis Factor α-mediated Induction of Endothelial Tissue Factor*

(Received for publication, March 7, 1995, and in revised form, July 31, 1995)

Angelika Bierhaus†, Youming Zhang‡, Youhua Deng§, Nigel Mackman¶, Peter Quehenberger†, Michael Haase‡, Thomas Luther*, Martin Müller*, Hubert Böhret†, Johannes Greet†, Eike Martini§, Patrick A. Baeuerle*, Rüdiger Walldherr*, Walter Kisiel**, Reinhard Ziegler†, David M. Stern‡, and Peter P. Nawroth§§

From the †Departments of Medicine, Pathology, and Anesthesiology, University of Heidelberg, Heidelberg 69115, Germany, the *Department of Immunology, Scripps Research Institute, La Jolla, California 92037, the ¶Department of Pathology, Technical University of Dresden, Dresden 01307, Germany, the **Department of Pathology, University of New Mexico, Albuquerque, New Mexico 87131, and the §§Department of Physiology, Columbia University, New York, New York 10032

This study examines the regulation of the human tissue factor (TF) promotor in vitro and in vivo. Transient transfections were performed in bovine aortic endothelial cells to investigate the role of two fundamentally different AP-1 sites and a closely located NF-κB site in the human TF promotor. The NF-κB site is functionally active, since overexpression of NF-κB(p65) resulted in induction of TF mRNA and activity. Promotor analysis showed that NF-κB induction was dependent on the integrity of the region from base pair –188 to –181. Overexpression of Jun/Fos resulted in TF induction of transcription and procoagulant activity. Functional studies revealed that the proximal AP-1 site, but not the distal, was inducible by Jun/Fos heterodimers. The distal AP-1 site, which has a G → A switch at position 4, was inducible by Jun homodimers. Electrophoretic mobility shift assays, using extracts of tumor necrosis factor α (TNF-α)-stimulated bovine aortic endothelial cells, demonstrated TNF-α-inducible binding to the proximal AP-1 site, comprising Jun/Fos heterodimers. At the distal AP-1 site, only minor induction of binding activity, characterized as proteins of the Jun and ATF family, was observed. Consistently, this site only marginally participates in TNF-α induction. Functional studies with TF promotor plasmids confirmed that deletion of both the proximal AP-1 or the NF-κB site decreased TNF-α-mediated TF induction to a higher extent than loss of the distal AP-1 site. However, integrity of both AP-1 sites and the NF-κB site was required for optimal TNF-α stimulation. The relevance of these in vitro data was confirmed in vivo in a mouse tumor model. Expression plasmids for a dominant negative Jun mutant or IκB were packaged in liposomes. When either mutated Jun or IκB were injected intravenously 48 h before TNF-α, a reduction in TNF-α-mediated TF expression in the tumor endothelial cells was observed. Simultaneously, fibrinogen deposition decreased and free blood flow could be restored. Thus, TNF-α-induced up-regulation of endothelial cell TF depends on a concerted action of members of the bZIP and NF-κB family.

Unstimulated endothelial cells express no tissue factor (TF) in vitro and in vivo (1–6). Recent studies show that TF expression in vitro can be induced by TNF-α (7–11). In vivo, however, expression of TF has only been shown in selected vascular beds: in the splenic endothelium in a septicemia model (12) and in tumors such as Meth-A and Kaposi sarcomas (13, 14). The human TF promotor has been characterized, and its function has been extensively studied in monocytes/macrophages (15–18). The porcine TF promotor has been described in endothelial cells (11). The data available from the human TF promotor suggest that two AP-1 and the NF-κB site are central in the endotxin-dependent regulation of TF expression (16–18). In contrast, the study of the porcine TF promotor shows that mainly the induction of NF-κB is responsible for lipopolysaccharide- and TNF-α-mediated induction of endothelial TF expression (11).

The discrepancy in the role of AP-1 in the human and porcine TF promotor might be due to differences in the sequence composition of the AP-1 binding sites of the various species. Sequence alignments revealed important differences between the AP-1 sites of the different species (11). The porcine TF promotor has two non-canonical AP-1 sites that differ from the defined AP-1 consensus sequence in one central base (11). Non-canonical AP-1 sites have been reported of being weak binding sites (19). In contrast, the proximal AP-1 site in the human (and the mouse) TF promotor contains the core of the consensus sequence (11, 15, 16) and thereby represents a high affinity site for AP-1 binding. This indicates that different AP-1-like proteins may be involved in the regulation of TF expression in vivo.

* This work was in part supported by Deutsche Forschungsgemeinschaft Grant Na-138/2-2 (to P. P. N.), and a grant from the Verein zur Förderung der Medizinischen Wissenschaft Grant Na-138/2-2 (to P. P. N.), Mildred Scheel Stiftung Grant W 1683). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† A Heisenberg Scholar of the Deutsche Forschungsgemeinschaft. To whom correspondence should be addressed: Dept. of Medicine I, Bergheimer Str. 58, D69115 Heidelberg, Germany. Tel.: 49-6221-566006; Fax: 49-6221-564696.

The abbreviations used are: TF, tissue factor; TNF-α, tumor necrosis factor α; BAEC, bovine aortic endothelial cells; PAEC, porcine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; Meth-A sarcoma, methylcholanthrene-A-induced sarcoma cells; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; TBE, Tris-borate-EDTA; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; AP-1, activator protein 1; ATF-2, activating transcription factor-2; CAT, chloramphenicol acetyltransferase; β-Gal, β-galactosidase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; bp, base pair(s); DEPC, diethyl pyrocarbonate; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase.
different species. If the porcine model (11) would be relevant for human disease, then blocking of NF-κB activation would provide a powerful way to prevent excess TF expression in human disease. If the human model (15–18) is relevant, then inhibition of NF-κB activation might lead to increased c-Fos transcription (20, 21) and thereby to AP-1-mediated TF induction. To resolve this issue, we studied the role of both AP-1 sites and their cooperative action with the NF-κB site in the human TF promoter.

A number of homo- and heterodimers can recognize AP-1 sites (22–25) but exhibit different affinities for different motifs (26). These proteins have been termed bZIP family (27) due to their ability to dimerize via an α-helical leucine “zipper.” The members of the Jun subfamily c-jun, J unB, and J unD are highly homologous in their dimerization and binding domains and can compete for the same AP-1 sites (28). The transactivating capacities of c-jun and J unD are dramatically increased in combination with c-Fos (29, 30). The functional homologues of c-Fos, Fra-1, Fra-2, and FosB can also dimerize with Jun proteins (30–33). In addition, members of the ATF/CREB family like ATF-2, ATF-3, and ATF-4 (but not ATF-1) are capable of binding to proteins of the AP-1 transcription factor family (25, 26, 34). Thus, it was our hypothesis that the distal non-canonical AP-1 site in the human TF promotor and the proximal canonical AP-1 site bind different members of the AP-1 bZIP family.

MATERIALS AND METHODS

Reagents—Reagents were obtained as follows: DMEM, RPMI 1640, HEPES buffer solution, l-glutamine, penicillin-streptomycin mixture, and PBS, pH 7.4, were from BioWhittaker, Walkersville, MD. FCS, DOPAT, DNase I (RNase-free), proteinase K, and the DIG nucleic acid detection kit were from Boehringer, Mannheim, Germany. Barbitol buffer was purchased from KabiVitrum, Stockholm, Sweden. [α-32P]dCTP (3000 Ci/mmol at 10 Ci/ml), [γ-32P]dATP (3000 Ci/mmol at 10 Ci/ml), [α-35S]methionine (10000 Ci/mmol), Hybond-N nylon filter, and Hyperfilm x-ray films were obtained from Amersham, Buckinghamshire, England. Poly(d-dc) was purchased from Sigma, Deisenhofen, Germany. Rabbit reticulocyte lysate and recombinant AP-1 (c-jun, human) were purchased from Promega. Anti-p50 (sc-114X), anti-p65 (sc-109X), and anti-c-Rel (sc-70X) polyclonal antibodies, anti-c-jun (sc-45X; specific for c-jun, non-cross-reactive with other Jun family members), and anti-junD (sc-74X; no c-jun or junD cross-reactivity) were purchased from Santa Cruz, Santa Cruz, CA. TNF-α (10 units/ml) was a gift from Dr. E. Sadler, Washington University, St. Louis, MO (40).

Plasmids—The SV-40-driven luciferase control plasmid pGL2-control, the promoterless plasmid pGL2-basic, the β-galactosidase control plasmid pSV-β-gal, and the chloramphenicol transferase control vector pCAT-control were obtained from Promega. pSP18 was purchased from Boehringer Mannheim, Germany. The TF promoter mutants pHTF(-278)Luc (A1-A2-N), pHTF(-278)Luc (A2-N), pHTF(-278)Luc (A2-N). (35) were used as a reporter construct to examine the effects of point mutations. The pHTF(-278)Luc (A1-A2-N) was prepared by inserting the polymerase chain reaction product of a synthetic oligonucleotide into the SmaI site of pGL2-basic, yielded the mutant pHTF(-278)Luc (A2-N), and then subcloned into pGL2-basic. The pHTF(-278)Luc (A1-A2-N) was then used to prepare a series of point mutations in the region of the pHTF(-278)Luc (A1-A2-N) by using the method of Heidelberger et al. (35).

The HEPES buffer solution, L-glutamine, penicillin-streptomycin mixture, HEPES buffer solution, and forskolin were purchased from KabiVitrum, Stockholm, Sweden. [α-32P]UTP (3000 Ci/mmol at 10 Ci/ml), [γ-32P]dATP (3000 Ci/mmol at 10 Ci/ml), [α-35S]methionine (10000 Ci/mmol), Hybond-N nylon filter, and Hyperfilm x-ray films were obtained from Amersham, Buckinghamshire, England. Poly(d-dc) was purchased from Sigma, Deisenhofen, Germany. Rabbit reticulocyte lysate and recombinant AP-1 (c-jun, human) were purchased from Promega. Anti-p50 (sc-114X), anti-p65 (sc-109X), and anti-c-Rel (sc-70X) polyclonal antibodies, anti-c-jun (sc-45X; specific for c-jun, non-cross-reactive with other Jun family members), and anti-junD (sc-74X; no c-jun or junD cross-reactivity) were purchased from Santa Cruz, Santa Cruz, CA. TNF-α (10 units/ml) was a gift from Dr. E. Sadler, Washington University, St. Louis, MO (40).

Meth-tRNA was a gift of Dr. Xanthopoulus (Karolinska Institute, Stockholm, Sweden); other plasmids were obtained from ATCC.

Tissue Culture—Bovine aortic endothelial cells (BAEC) were cultured in DMEM supplemented with 10% FCS as described previously (41, 42) and characterized as endothelial cells by the expression of von Willebrand factor, thrombomodulin, low basal levels of TF, and morphology of the cells. Cells were passaged every 6–10 days without showing gross morphological changes until passage 15. Passages 4–8 were used. All experiments were performed with cells that had been confluent for 3–5 days. For transfection experiments, cells growing in the logarithmic phase were used.

Methylcholanthrene-A-induced (Meth-A) sarcoma cells were a gift of Dr. D. Bohmann (EMBL, Heidelberg, Germany) and were cultured in RPMI 1640, 10% FCS, 100 units/ml penicillin, 100 units/ml streptomycin as described elsewhere (14).

Determination of Tissue Factor (TF) Activity by One-stage Clotting Assay—One-stage clotting assays were performed as described (1). After washing, cells were removed non-enzymatically by scraping in barbitol buffer, pH 7.6, collected by centrifugation for 5 min at 1000 rpm, and resuspended in 100 μl of the same buffer. After addition of 100 μl of citrated bovine plasma 100 μl of citrated bovine plasma, and 100 μl of 25 mM CaCl2 solution (Behring, Marburg, Germany) the samples were incubated at 37°C. The time from addition of CaCl2 to the first defined fibrin strand was determined. TF activity was calculated by comparing the measured clotting time with a standard curve made with known amounts of TF (1). The measured amount of TF was expressed as picograms of TF/106 cells ± S.D. All experiments were performed at least three times, and each experiment was done in triplicates.

Determination of Tissue Factor (TF) Activity by Monitoring the Hydrolysis of S2222 Synthetic Peptide Substrate—TF activity was also assessed by monitoring the hydrolysis of the synthetic peptide substrate Bz-Ile-Glu-Gly-Arg-MCA (S2222, KabiVitrum, Stockholm, Sweden) as described previously (43). Cells were harvested in PBS, pH 7.4, and incubated with 30 μl of human factor VII (final concentration 35 μg/ml) (44) and 10 μl of factor X (final concentration 200 μg/ml) (44) in the presence of 10 mM CaCl2. Aliquots (30 μl) were added to 470 μl of 50 mM Tris, pH 7.9, 175 mM NaCl, 5 mM EDTA, and 0.1% BSA. Factor Xa formation was assessed using 100 μl of S2222 added to the entire 0.5-ml sample. Hydrolysis was monitored at room temperature by measuring the change in absorbance at 405 nm, using a Beckmann DU 7400 spectrophotometer (Beckmann, Dreieich, Germany). Factor Xa (final concentration 20 μg/ml) (44) served as control. Each experiment was repeated three times.

Statistical Analysis—Data were analyzed with the aid of SIGMA PLOT software (Jandel Scientific). Levels of significance were determined by Student’s t test. Any p value of 0.05 and below was considered to be significant. For calculation of p values in Fig. 1c, the Mann-Whitney U test was used.

Nuclear Run-on Transcription Assay—Nuclear run-on transcription assays were performed essentially according to the procedure of Green and Ziff (45) as described elsewhere (46–48). Bovine aortic endothelial cells were harvested from 2 × 106 cells after 42 h of transfection. Run-on reactions were performed in 0.7 mM KCI, 50 mM MgCl2, 50 mM Tris-HCl, pH 8.0, 25 mM DTT, 1 mM EDTA in the presence of 250 μCi of [α-35S]UTP (3000 Ci/mmol) and incubated for 30 min at 30°C. The synthesized mRNA was recovered by incubation with RNase A for 5 min at 30°C, treated with proteinase K (10 mg/ml), and extracted with 0.45-μm Millipore filters (type HA). The RNA was collected by ethanol precipitation and redissolved in 300 μl of DEPC-H2O, 1 μl was counted, and equal numbers of Cerenkov counts were made up to 2 ml of hybridization solution and added to the previously prepared slot blot filters. Hybridization was performed without prehybridization in 50% formamide, 5 × SSC, 5 × Denhardt’s solution at 50°C for 2 days. Filters were washed at room temperature for 10 min in 2 × SSC and once for 10 min at 60°C in 1 × SSC. Blots were exposed to Amersham Hyperfilms for 1–4 weeks at –80°C with intensifying screens. The density of autoradiographic signals was quantitated using a Beckman DU 7400 densitometer (Beckman, Dreieich, Germany). For details of hybridization and washing, see Materials and Methods. For hybridization, see Materials and Methods. For hybridization, see Materials and Methods. For hybridization, see Materials and Methods. For hybridization, see Materials and Methods. For hybridization, see Materials and Methods.

Northern Blot Analysis—Northern blots were essentially as described previously (46, 47). Tumor tissue was broken down and extracted under liquid nitrogen and homogenized in an Ultrafrosix (Wheaton, Millville, NJ), and total RNA was purified by the guanidine isothiocyanate-cesium chloride method (49). RNA concentrations were determined spectrophotometrically, and 20 μg of RNA/ane were separated onto an 1.1%
agarose-formaldehyde gel and transferred to Hybond-N-Nylon membranes (Amersham, Braunschweig, Germany) according to standard methods (50). A mouse tissue factor cDNA fragment (bp 721 to bp 1043) (14, 48) was labeled with [α-32P]dCTP to a specific activity >10^6 cpm/μg DNA by the random prime technique (51). Filters were prehybridized for 1 h and hybridized for 12–16 h at 65°C in 50 ml PIPE, pH 6.8, 200 mM NaCl, 20 ml Na$_2$BO$_3$, 30 ml Na$_2$EDTA, 0.1 ml salmon sperm/μl, 50 μg of yeast RNA/ml (47), washed > 15 × 3 SSC, 5% SDS, and exposed to Hybond-N Hyperfilms (Amersham, Braunschweig, Germany) for 5 days.

Electrophoretic Mobility Shift Assay—For electrophoretic mobility shift assays (EMSA), nuclear proteins were harvested by the method of Andrews (52) and harvested by the method of Bradford (53). When organ tissue was used, the above protocol was followed. When recombinant proteins produced in rabbit reticulocyte lysates (Promega) were included in the reaction, the poly(dI-dC) concentration was increased to 0.05 μg/ml. Reactions were incubated for 10 min on ice, the supernatant was discarded, and cells were incubated for 42 h. Cells were harvested in the appropriate buffers.

Plasmid DNA used in transfections was isolated by alkaline lysis, followed by CsCl equilibrium centrifugation (50). For promoter studies 0.5 μg of luciferase promoter constructs/ml of medium were transcribed. To correct for variability in transfection efficiency 0.15 μg of pSV-β-Gal plasmid/ml of medium were included. For transactivation experiments, 0.25 μg/ml pSV-c-jun, pBK280(c-Fos), NF-κB(65), IκB, or mutated c-jun were cotransfected with 0.5 μg of luciferase containing promoter constructs. Reactions were set up with PCAT-control (serving as mock control) to give the final DNA concentration of 1.4 μg/ml medium. Cell extracts were prepared by lysis in 25 ml Tris phosphate, pH 7.8, 2 ml 12-diaminomucleic acid (DNAse-free), 1.3 mM MgCl$_2$, 0.01 mM EDTA, 20 mM NaCl, 20 mM Na$_2$PO$_4$, 30 mM NaHPO$_4$, 1 mM EDTA, 5% SDS, 50 μg/ml salmon sperm/ml, 50 μg/ml yeast tRNA/ml (47), washed 3 × 50 ml of each RNA was used. Efficient translation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was monitored in a parallel reaction using [35-S]Methionine as substrate. 0.3–10 μl (approximately 0.15–5 μg) of in vitro translated proteins were used in EMSA. Unprogrammed lysate served as control.

In Situ Hybridization—In situ hybridization was performed as described previously (13, 14, 48). After three washes with 150 ml NaCl, 100 ml Tris-HCl, pH 7.5, 20 ml of PBS incubated for 14 days at 4°C. Tissues were fixed in 4% formaldehyde. A fixed section was incubated with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.

In Vivo Transfection—In vivo transfection was performed as described previously (13, 14, 48). After cutting, sections were incubated with anti-mouse TF antibodies (48) for 1 h at room temperature with a peroxidase-conjugated second antibody. After washing color development was performed with 3-aminio-9-ethylcarbazole and H$_2$O$_2$. Antibody titration was performed and the specificity of the antibodies was confirmed using immunofluorescence staining anti-fibrin-fibrinogen antibodies (fluorescein conjugate, Cappel, West Chester, PA) were used in a 1:8 dilution (14). Sections were incubated with the antibody for 45 min at room temperature, followed by three washes with PBS. 10 cuts from at least three different experiments were analyzed independently.
Mechanism of TNFα-mediated Tissue Factor Induction

Tissue Factor Is Induced by Overexpression of Proteins of J un/Fos and NF-κB—Cultured bovine aortic endothelial cells (BAEC) were transiently transfected with plasmids overexpressing c-j un and c-Fos or NF-κB(p65). Transient transfection with c-j un/Fos(AP-1), NF-κB(p65), or both transcription factors together resulted in enhanced expression of TF compared to mock transfected cells (expressing the bacterial CAT). TF was studied at several levels. (i) Nuclear run-on assays (Fig. 1a) revealed that induction of transcription of the TF gene occurred. (ii) The biological activity was characterized by its factor VII-dependent activation of factor X (synthetic substrate assay; Fig. 1b) and by a coagulant assay (one-stage clotting time; Fig. 1c). Since almost no factor X activation was observed in the absence of factor VII (data not shown), most of the coagulant activity induced is TF. But it cannot be absolutely excluded that other proteins involved in activation of coagulation are also inducible by overexpression of AP-1 or NF-κB(p65). When BAEC were cotransfected with AP-1 and NF-κB(p65), an additive effect in TF induction was observed in all systems tested (Fig. 1).

Structural analysis of the human TF promotor has demonstrated two linked AP-1 sites, a proximal canonical site at −210 (A2), a distal non-canonical site at −223 (A1), and an NF-κB site (N) at position −188 (15–18). The availability of promoter constructs (Table I) allows for definition of the areas involved in activation of TF by AP-1 or NF-κB(p65) (Fig. 2). The plasmids containing both AP-1 sites responded to c-j un/Fos overexpression in the presence or absence of the NF-κB site (Fig. 2a). However, deletion of the NF-κB binding site reduced the induction by c-j un/Fos (Fig. 2a), suggesting that endogenously present proteins (presumably p65/c-Rel) capable to bind to this site, act in concert with AP-1. To test whether inducible c-j un/Fos was dependent on the presence of endogenous NF-κB, we cotransfected BAEC with plasmids overexpressing IκB. Overexpression of IκB reduced the c-j un/Fos induction as long as the NF-κB site was present (Fig. 2c).

When BAEC were transfected with a plasmid overexpressing NF-κB(p65) (Fig. 2b), induction was maximal, when both AP-1 sites and the NF-κB site were present. NF-κB(p65) inducibility was reduced, but not lost, when the AP-1 sites were deleted (Fig. 2b). Deletion of the NF-κB site resulted in loss of inducibility by NF-κB(p65). To test, whether optimal NF-κB induction was equally dependent on the presence of endogenous AP-1, we cotransfected BAEC with plasmids overexpressing NF-κB(p65) and the J un-specific inhibitor mutated J un. This negatively dominant c-j un point mutant (39) is able to dimerize with other members of the AP-1/bZIP family, however, it is unable to bind to the DNA recognition site. Since c-Fos does not bind to DNA at all, due to its failure to homodimerize (31), overexpression of mutated J un leads to a significant reduction of J un/Fos heterodimer binding. When NF-κB(p65) and mutated J un were cotransfected, a reduction of the NF-κB(p65)-mediated stimulation was seen (Fig. 2c). Thus AP-1 and NF-κB(p65) act in concert in inducing TF (Figs. 1 and 2), which seems to be dependent on the presence of DNA sequences that other proteins involved in activation of coagulation are also inducible by overexpression of AP-1 or NF-κB(p65). Cells were transiently transfected with the respective plasmids, harvested after 42 h, and assayed for factor X activation in the presence of factor VII (35 μg/ml). The generation of factor X was measured spectrophotometrically over a period of 15 min. S2222 served as substrate as described under "Materials and Methods." Two experiments were performed in triplicates with identical results. One typical experiment is shown. c. BAEC were transiently transfected with CAT (mock control), c-j un, c-Fos, and/or NF-κB(p65) overexpressing plasmids. Nuclear run-on experiments were performed as described under "Materials and Methods" to allow in vitro synthesis of [α-32P]UTP-labeled mRNA. This was hybridized against filters onto which CDNA's for TF (top), Meth-IRNA (bottom), pSPT18, and TNFα (negative controls, data not shown) had been fixed. b. activation of factor X by BAEC transfected with CAT (mock control), c-j un, c-Fos, and/or NF-κB(p65). Cells were transiently transfected with the respective plasmids, harvested after 42 h, and assayed for factor X activation in the presence of factor VII (35 μg/ml). The generation of factor X was measured spectrophotometrically over a period of 15 min. S2222 served as substrate as described under "Materials and Methods." Two experiments were performed in triplicates with identical results. One typical experiment is shown. c. BAEC were transiently transfected with CAT (mock control), c-j un, c-Fos, and/or NF-κB(p65), harvested 42 h after transfection, and assayed for procoagulant activity as described under "Materials and Methods." TF activity of each sample was determined based on comparison with a standard curve established with known amounts of recombinant TF. The data ± S.D. represent the mean of three independent experiments performed in triplicate (p values: control versus J un/Fos = 0.008; control versus NF-κB = 0.009; control versus J un/Fos/NF-κB = 0.009).
Characterization of the Proteins Binding to Both AP-1 Sites—

To analyze the complexes I and II that were observed at the inducibility of mutants containing either the proximal (A2) or the distal (A1) AP-1 site, EMSA were performed to compare the binding capacity for both AP-1 sites (Fig. 5c). The proximal AP-1 site (A2) (Fig. 5c) was only weakly, C-Jun homodimers, while TNFα-stimulated cells (Fig. 5a, lane 1 versus lane 2), in TNFα-induced cells (Fig. 5a, lane 3 versus lane 4) and in cells cotransfected with C-Jun/Fos (AP-1) (Fig. 5a, lane 6 versus lane 7). Specificity of binding was demonstrated, since binding of C-Jun/Fos programmed lysate resulted in a similar shift (Fig. 5a, lane 8). In addition, cold consensus AP-1 oligonucleotides inhibited the shift seen after TNFα stimulation (Fig. 5a, lane 5).

In contrast, in nuclear extracts of BAEC three complexes (marked I, II, and III) were observed at the non-canonical distal AP-1 (Fig. 5b). When excretion of the films was extended for up to 8 days, a weak band, marked as complex I, occurred (Fig. 5b). The weak binding and its migration in the gel confirmed the results shown in Fig. 3b, i.e. this band is due to weak binding of J un/Fos heterodimers.

Complex II (Fig. 5b) does not represent AP-1 heterodimers based on the following criteria. (i) The bands in control extracts (Fig. 5b, lane 1) and extracts from cells overexpressing J un/Fos (Fig. 5b, lane 6) were only weakly (Fig. 5b, lanes 2 and 7) inhibited in the presence of mutated J un. (ii) Complex II seen after TNFα induction (Fig. 5b, lane 3) was not competed by a 100-fold molar excess of cold AP-1 consensus oligonucleotides (Fig. 5b, lane 5) or by mutated J un (Fig. 5b, lane 4), suggesting differences between control and TNFα-stimulated cells. These data further indicate that complex II does not contain C-Fos or Fos-related proteins; however, they do not exclude the involvement of other members of the bZIP family, i.e. members of the ATF family (see Fig. 6b and Table II), which are able to bind DNA and therefore are only minorly influenced by mutated J un. (iii) Complex II migrated more rapidly (Fig. 5b) than the complex formed with C-Jun homodimers (Fig. 4b).

Since complex III (Fig. 5b) was not at all competed by unlabelled consensus AP-1 oligonucleotides (see below) and also present in unprogrammed rabbit reticuloocyte lysate (Fig. 3b), it is regarded as nonspecific as depicted in Fig. 3b.

To analyze the complexes I and II that were observed at the distal non-canonical AP site (Fig. 5b), characterization with supershifting antibodies was performed (Fig. 6a). The upper

**Mechanism of TNFα-mediated Tissue Factor Induction**

**TABLE I**

| plasmid: | other names: | description |
|---------|-------------|-------------|
| A1-A2-N | phTFF(-278)lac | Mackman et al, 1990 (18); Mackman et al 1991 (19) |
| A1-A2-N | phTFFM2(-278) | Mackman et al, 1990 (18); Mackman et al 1991 (19) |
| A1-A2-N | phTFFM3(-278) | Mackman et al, 1990 (18); Mackman et al 1991 (19) |
| A1-A2-N | phTFFM4(-278) | Mackman et al, 1990 (18); Mackman et al 1991 (19) |
| A1-A2-N | oligonucleotide region -236 to -213 cloned into the Sma I site | |
| A1-A2-N | oligonucleotide region -212 to -189 cloned into the Sma I site | |
| A1-N | oligonucleotide region -195 to -171 cloned into the Sma I site | |
| N | phTFF(-111) | Mackman et al, 1990 (18); Mackman et al 1991 (19) |

The organization of the TF promoter, which was cloned in front of the reporter gene luciferase, is given in the upper part of the table (15, 16). The positions of the two AP-1 sites and the NF-κB site along with the TATA box and the luciferase transcription start are indicated. The S'-boundaries of the various TF promoter plasmids, which sequentially remove these transcription factors (row 1), published names of the constructs (row 2), and a short description of the cloning strategy (row 3) are shown.
Mechanism of TNFα-mediated Tissue Factor Induction

Fig. 2. Functional analysis of the TF promotor demonstrates activation of TF expression by c-Jun/Fos(AP-1) and NF-κB(p65).

BAEC cells were cotransfected with the TF promotor plasmids [A1-A2-N], [A1-A2], or [N] (1 μg/ml medium) (see "Materials and Methods") and c-Jun, c-Fos, NF-κB(p65), mutated Jun, and/or l-bgal (0.5 μg each/ml medium) and cultivated for 42 h. After harvest, luciferase activity was determined in each sample and normalized for transfection efficiency to the amount of β-galactosidase expressed by the plasmid pSV-β-gal (Promega). The normalized data are expressed as relative Luc units and represent the mean of three independent experiments ± S.D. performed in triplicate. a, Cotransfection of the TF promotor by j un/Fos/AP-1; j un/Fos (AP-1) overexpression induced TF as long as AP-1 sites were present in the TF promotor constructs. b, Cotransfection of the TF promotor by NF-κB(p65); overexpression of NF-κB(p65)-induced TF as long as the NF-κB site was present in the TF promotor constructs. c, The TF promotor plasmid [A1-A2-N] was cotransfected with c-Jun, c-Fos, or NF-κB(p65) and the specific inhibitor of the opposite transcription factor; the j un/Fos (AP-1)-dependent transactivation could be partly suppressed by the NF-κB-specific inhibitor I-κBα, while the AP-1-specific inhibitor mutated j un partly suppressed transactivation by NF-κB(p65).

gel shift band (complex I; Fig. 6a, lane 1) was reduced in the presence of pan-j un antibodies (Fig. 6a, lane 2) and anti-j unD antibodies (Fig. 6a, lane 4) and suppressed in the presence of anti-c-Jun (Fig. 6a, lane 3) and anti-ATF-2 antibodies (Fig. 6a, lane 7). In addition, anti-c-Fos (Fig. 6a, lane 5) and anti-ATF-1 antibodies (Fig. 6a, lane 6) slightly decreased the shift. Complex II was suppressed when anti-ATF-2 antibodies were included in the reaction (Fig. 6a, lane 6) and reduced in the presence of pan-j un antibodies (Fig. 6a, lanes 2 and 3), while anti-j unD, anti-c-Fos, and anti-ATF-1 antibodies did not affect binding (Fig. 6a, lanes 4-6). Thus complex I and II also consist of different members of the AP-1/bZIP family. Intensity of the lower band (complex III), previously characterized as nonspecific (Fig. 5b), was not affected by any of the antibodies.

To further confirm this hypothesis, recombinant j un and recombinant ATF-2 (0.5 μg each), produced as inclusion bodies in Escherichia coli and able to heterodimerize, were used in binding reactions and their migration was compared to the complexes seen in extracts of control and TNFα stimulated cells (Fig. 6b). Consistent with the above data j un homodimers bound to the distal non-canonical AP site (Fig. 6, panel a, lane 8, and panel b, lane 3) forming complexes that migrated in the gel at the same position as complex I (Fig. 6, panel a, lane 1, and panel b, lane 2). The faster migrating ATF-2 homodimers demonstrated stronger binding to the distal non-canonical AP-1 site (Fig. 6b, lane 4) than j un homodimers (Fig. 6b, lane 5). When equimolar amounts of recombinant ATF-2 and recombinant j un were coinubated in the binding reaction, a slightly faster migrating complex was observed (Fig. 6b, lane 5). No significant binding was observed, when programmed j un/Fos lysate was included in the binding reaction (Fig. 6b, lane 6). The observed binding, seen in lane 6 is also present in unprogrammed lysate (Figs. 3b and 4b) and therefore regarded as nonspecific. To further characterize the TNFα-inducible
complexes, a 500-fold molar excess of unlabeled AP-1 oligonucleotide probes containing the proximal (a) or the distal (b) AP-1 site were incubated with recombinant J un, included as reagent body in E. coli. The amount of recombinant J un, included in the binding reaction, is shown above the lanes (control lane 1 shows 2.5 μl (approximately 1.25 μg) of c-J un/Fos programmed lysate). The mobility of the formed complexes was analyzed on 4% nondenaturing polyacrylamide gels. Arrows indicate the specific AP-1 binding. Nonspecific reactions are marked by brackets: c, BAEC were cotransfected with the TF promoter plasmids [A1], [A2], or [A1-A2] (1 μg) and a control plasmid (CAT = "mock") or c-J un (0.5 μg) and cultivated for 42 h. After harvest, luciferase activity was determined and normalized for transfection efficiency to the amount of β-galactosidase expressed by the plasmid pSV-β-gal (Promega). The normalized data are expressed as relative Luc units and represent the mean of three independent experiments ± S.D. performed in triplicates. The inducibility of the various TF promoter plasmids by c-J un homodimers is shown. The level of basal expression (transfected with CAT as control) is indicated with Basal.

At the proximal canonical AP-1 site TNFα induces binding of different proteins to the proximal (A2) and the distal AP-1 site (A1) of the human TF promoter: BAEC were transiently transfected with CAT (= "mock"), mutated J un, or c-J un and c-Fos (AP-1) overexpressing plasmids and cultivated for 42 h. Where indicated, TNFα (1 nM) was added 1 h before harvest. Nuclear extracts (10 μg/binding reaction) were prepared as described under Materials and Methods and assayed in EMSA for binding to the proximal (a) or the distal (b) AP-1 site. To confirm AP-1 binding, TNFα-induced nuclear extract was competed with a 160-fold molar excess of cold consensus AP-1 (lane 5). In addition, a parallel binding reaction was performed with 10 μl of J un/Fos programmed lysate (lane 8), a, the AP-1 complex binding to the canonical proximal AP-1 site is indicated with an arrow b, the complexes binding to the distal noncanonical AP-1 site are termed I, II, and III and indicated by arrows (see Results).
Mechanism of TNFα-mediated Tissue Factor Induction

with antibodies directed against members of the ATF family (Fig. 7c, lanes 6 and 7). Consistent with the above results (Fig. 4a), no binding was observed with 0.3 μg of j un homodimers (Fig. 7c, lane 8). Therefore, the TNFα-induced binding to the proximal canonical AP-1 site comprises J un/Fos heterodimers. Although c-j un-specific antibodies did not result in

TABLE II

Sequences of double-stranded synthetic oligonucleotides used in this study

| Source          | Sequence: 5’ - 3’ |
|-----------------|-------------------|
| Consensus AP-1  | CTAGTGATGAGTCAGCCCGATC |
| AP-1 distal (TF)| TGCGTGGCCGGGTGAATCATCGG |
| AP-1 proximal (TF)| GGGAGGTCACCTTGGACGGGTC |
| Consensus NF-κB | AGGTAGGGACACCTCCAGCC |
| NF-κB (TF)      | AGGTCCCGGAGTTCTACGAGGA |

Fig. 6. Characterization of complexes I, II, and III, formed at the distal AP-1 site (A1). a, initial characterization of the nuclear complexes I, II, and III formed at the distal AP-1 site (Table II) derived from the human TF promotor indicates that complex I and complex II contain members of the J un and ATF family; complex III did not react with the antibodies used and was defined as nonspecific (see “Results”). Characterization was performed six times, using three different nuclear extract preparations, with identical results. One typical experiment is shown. 10 μg of nuclear extract were included in each binding reaction: lane 1, 1 nM TNFα (1 nM, 2 h); lane 2, 1 nM TNFα + 2.5 μg of anti-pan-Jun antibodies; lane 3, 1 nM TNFα + 0.5 μg of anti-c-Jun antibodies; lane 4, 1 nM TNFα + 2.5 μg of anti-c-Fos antibodies; lane 5, 1 nM TNFα + 2.5 μg of anti-ATF-1 antibodies; lane 6, 1 nM TNFα + 2.5 μg of anti-ATF-2 antibodies; lane 8, 0.3 μg of recombinant c-Jun; lane 9, 10 μl of c-Jun/Fos programmed lysate. Antibodies were added directly before addition of the 32P-labeled distal AP-1 oligonucleotides. Complexes I and II and the nonspecific complex III are indicated by arrows.

b. Characterization of the complexes bound to the distal AP-1 site (part II)

| Source  | Sequence: 5’ - 3’ |
|---------|-------------------|
| Consensus AP-1  | CTAGTGATGAGTCAGCCCGATC |
| AP-1 distal (TF)| TGCGTGGCCGGGTGAATCATCGG |
| AP-1 proximal (TF)| GGGAGGTCACCTTGGACGGGTC |
| Consensus NF-κB | AGGTAGGGACACCTCCAGCC |
| NF-κB (TF)      | AGGTCCCGGAGTTCTACGAGGA |

Fig. 6. Characterization of complexes I, II, and III, formed at the distal AP-1 site (A1). a, initial characterization of the nuclear complexes I, II, and III formed at the distal AP-1 site (Table II) derived from the human TF promotor indicates that complex I and complex II contain members of the J un and ATF family; complex III did not react with the antibodies used and was defined as nonspecific (see “Results”). Characterization was performed six times, using three different nuclear extract preparations, with identical results. One typical experiment is shown. 10 μg of nuclear extract were included in each binding reaction: lane 1, 1 nM TNFα (1 nM, 2 h); lane 2, 1 nM TNFα + 2.5 μg of anti-pan-Jun antibodies; lane 3, 1 nM TNFα + 0.5 μg of anti-c-Jun antibodies; lane 4, 1 nM TNFα + 2.5 μg of anti-c-Fos antibodies; lane 5, 1 nM TNFα + 2.5 μg of anti-ATF-1 antibodies; lane 6, 1 nM TNFα + 2.5 μg of anti-ATF-2 antibodies; lane 8, 0.3 μg of recombinant c-Jun; lane 9, 10 μl of c-Jun/Fos programmed lysate. Antibodies were added directly before addition of the 32P-labeled distal AP-1 oligonucleotides. Complexes I and II and the nonspecific complex III are indicated by arrows. b, binding of recombinant J un, recombinant ATF-2, recombinant J un/ATF-2 heterodimers, and J un/Fos programmed lysate to the distal non-canonical AP-1 site compared to binding of TNFα-induced nuclear proteins (10 μg/reaction). The experiment was performed three times with identical results. One typical experiment is shown: lanes 1 and 2 represent cellular extract from control (lane 1) or TNFα (1 nM, 2 h) treated cells (lane 2). Lanes 3–6 represent EMSA of the distal AP-1 site (A1) incubated with various recombinant members of the AP-1/bZIP family. Lane 1, control; lane 2, 1 nM TNFα (1 nM, 2 h); lane 3, 0.5 μg of J un homodimers; lane 4, 0.5 μg of ATF-2 homodimers; lane 5, 0.25 μg of J un and 0.25 μg of ATF-2, coincubated for 30 min at room temperature before addition to the binding reaction; lane 6, 8 μl of J un/Fos programmed lysate; lane 7, 1 nM TNFα + 500-fold molar excess of unlabeled AP-1 consensus oligonucleotides. The nuclear extract-derived complexes I, II, and III are indicated by arrows. c, time course of the TNFα-inducible complex II, binding to the distal AP-1 site (A1). Nuclear extracts were prepared from BAEC induced for various times (0–6 h) with TNFα (1 nM) (lanes 1–8). To demonstrate that the observed bands were not due to the oligonucleotide preparation used, a reaction without nuclear extract was included (lane 9). 10 μg of nuclear extract were used in each binding reaction. DNA-protein complexes were analyzed on native 4% polyacrylamide gels. The very weak binding of complex I, the TNFα-inducible complex II, and the nonspecific complex III are indicated by arrows.
supershifted or reduced binding, c-Jun might contribute to the observed complexes, since it might be possible that the anti-c-Jun antibody fails to recognize c-Jun in Jun/Fos heterodimers.\textsuperscript{3}

\textbf{FIG. 7.} **TNF\textsubscript{a} induces time- and dose-dependent binding of AP-1 to the proximal AP-1 site (A2) of the human TF promoter.** a, time course of AP-1 binding to the proximal AP-1 site (A2) of the human TF promoter. Nuclear extracts were prepared from BAEC induced for various times (0–3 h) with TNF\textsubscript{a} (1 nM) (lanes 1–7). 10 \( \mu \)g of nuclear extract were included in each binding reaction. DNA-protein complexes were analyzed on 4% native polyacrylamide gels. EMSA detected AP-1 binding to the proximal AP-1 site (Table II) derived from the human TF promoter. The TNF\textsubscript{a}-inducible AP-1 complex is indicated with an arrow. Specificity of binding was ascertained by competing with 160-fold molar excess of cold AP-1 consensus oligonucleotides (Table II) included in the binding reaction (lane 8).

b, dose response of AP-1 binding to the proximal AP-1 site (A2). BAEC were stimulated with various doses of TNF\textsubscript{a} (0 pM to 1000 pM) for 30 min (lanes 1–6). Nuclear extracts were prepared, and 10 \( \mu \)g of this extract were included in each binding reaction and analyzed as above. The TNF\textsubscript{a}-inducible AP-1 complex (JunD/Fos) is indicated with an arrow. Specificity of binding was ascertained by competing with 160-fold molar excess of cold AP-1 consensus oligonucleotides (Table II) included in the binding reaction (lane 7).

c, characterization of the complex bound to the proximal AP-1 site (A2) after TNF\textsubscript{a} induction. Characterization was performed two times, using two different nuclear extract preparations, with identical results. One typical experiment is shown. 10 \( \mu \)g of nuclear extract were included in each binding reaction: lane 1, 1 nM TNF\textsubscript{a} (1 nM, 1 h); lane 2, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-pan-Jun antibodies; lane 3, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-c-jun antibodies; lane 4, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-\( \beta \)-catenin antibodies; lane 5, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-c-Fos antibodies; lane 6, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-ATF-1 antibodies; lane 7, 1 nM TNF\textsubscript{a} + 2.5 \( \mu \)g of anti-ATF-2 antibodies; lane 8, 0.3 \( \mu \)g of recombinant c-Jun; lane 9, 10 \( \mu \)l of c-jun/Fos programmed lysate. Antibodies were added directly before addition of the 32P-labeled proximal AP-1 oligonucleotide. The TNF\textsubscript{a}-inducible complex is indicated with an arrow.

\textsuperscript{3} N. Mackman, personal communication.
Fig. 8. TNFα induces tissue factor expression by a concerted action of AP-1/bZIP- and NF-κB-like proteins. Functional analysis of TF expression in unstimulated and TNFα-induced BAEC. BAEC were transfected with various TF promoter plasmids (Table I; for detail see "Materials and Methods") for 36 h before TNFα (1 nM) was added for 6 h, where indicated. After harvest luciferase activity was determined in the cell lysates and normalized for transfection efficiency to the amount of β-galactosidase activity expressed by the control plasmid pSV-β-Gal (Promega). Corrected values were expressed as relative luciferase units. The results represent the mean of at least three independent experiments ± S.D. that were performed in triplicate. a, functional analysis of TF expression in unstimulated BAEC compared with TF expression in TNFα-stimulated BAEC; the mean of six independent experiments ± S.D. performed in triplicate is shown. b, the inducibility by TNFα relating to basal expression is shown. The level of basal expression is indicated with B. c, to directly demonstrate the role of NF-κB(p65) in TNFα-mediated TF induction, various TF promoter plasmids (Table I) were cotransfected with plasmids overexpressing CAT (mock) or the NF-κB(p65) specific inhibitor IκBα and cultivated for 36 h before TNFα (1 nM) was added to the cells for 6 h. After harvest luciferase activity and transfection efficiency were determined as above. The data represent the mean of three different experiments performed in triplicate. d, to directly demonstrate the role of NF-κB(p65) in TNFα-mediated TF induction, various TF promoter plasmids (Table I) were cotransfected with plasmids overexpressing CAT (mock) or mutated Jun and cultivated for 36 h before TNFα (1 nM) was added to the cells for 6 h. Data were obtained from three different experiments ± S.D. performed in triplicate.

shown). The p65-containing faster migrating complex was induced between 5 min and 3 h (data not shown). Taken together, these data indicate that activation of the human TF promoter follows a kinetic that includes first activation of NF-κB(p65/c-Rel), followed by activation of Jun/Fos heterodimers that bind to the proximal AP-1 site. While NF-κB(p65/c-Rel) activation declines, the distal AP-1 site exhibits maximal binding of AP-1/bZIP proteins, characterized to contain Jun and ATF proteins. This might explain, why TF transcription is still enhanced after 4–6 h (Refs 9 and 10 and data not shown), even TNFα-induced NF-κB(p65/c-Rel) activation has already dropped to base-line level.

TNFα Mediates Induction of Endothelial TF by a Concerted Action of the AP-1 and NF-κB Sites—To confirm that different members of the AP-1/bZIP and NF-κB family functionally act on the human TF promoter and contribute to TF induction by TNFα, transient transfections of BAEC with TF promoter mutants (Fig. 8) were performed. When BAEC were cotransfected with TNFα, promoter activity compared to basal expression was highest, when the proximal AP-1 (A2) and the NF-κB site were present (Fig. 8, a and b). Loss of the proximal AP-1 site or the NF-κB site resulted in significantly decreased TF induction (Fig. 8, a and b). Loss of the non-canonical distal AP-1 site had a less prominent effect, as expected (Fig. 8, a and b). Promoter mutants with only the proximal AP-1 site (A2) doped in front of the minimal promoter were still inducible by TNFα, while the distal AP-1 site (A1) alone was unable to confer TF induction (Fig. 8, a and b). The proximal AP-1 significantly contributed to TF basal expression (Fig. 8a). Therefore mutants that contained the NF-κB, but not the proximal AP-1 site, were more inducible by TNFα than mutants comprising the proximal AP-1 site (Fig. 8b); however, the overall TF expression of mutants without proximal AP-1 was lower (Fig. 8a). Highest TF expression was observed only when both AP-1 sites and the NF-κB site were intact, consistent with participation of all three sites in regulation of TF transcription (Fig. 8a). The TNFα-induced activity of the construct A1-A2-N, spanning the complete promoter, is higher (396 Luc units) than the added activity of the constructs containing each element alone (A1 = 50, A2 = 125, N = 106 Luc units; total 281 Luc units). Thus, these elements act in concert on the human TF promoter in mediating TNFα-induced TF transcription.

The concept that both AP-1 sites and the NF-κB site act in concert was further supported by studies overexpressing specific inhibitors of NF-κB(p65)/IκBα or AP-1/bZIP (mutated Jun). Overexpression of IκBα reduced TNFα-mediated TF induction as long as the NF-κB site was present (Fig. 8c). Overexpression of mutated Jun reduced TF induction by TNFα as long as the proximal AP-1 site was present (Fig. 8d).

TNFα-mediated TF Induction Is Dependent on AP-1/bZIP and NF-κB Proteins in Vivo—No data are available to support the concept of AP-1/bZIP- and NF-κB-mediated TF induction in vivo. Since in the human and the mouse TF promoter the same sequences are found for the distal and the proximal AP-1, as well as for the NF-κB recognition motif, we used a mouse model to study the dependence of TNFα-mediated TF expression of endothelial cells on Jun and NF-κB in vivo. In this model, TNFα induces the expression of TF and fibrin formation on
endothelial cells of the tumor vasculature (14, 64). 3 h after intravenous injection of TNFα, TF expression was induced, based on in situ hybridization (Fig. 9a) and immunohistochemistry (Fig. 9b). TF was expressed by subendothelial structures including tumor cells and also by endothelial cells. With respect to the topic of this study, we focused on endothelial cells. More than 150 vessels were evaluated in this part of the study.

When animals were treated by intravenous somatic gene transfer with mutated Jun 24 h prior to TNFα injection, a decrease in the endothelial response to TNFα was observed by in situ hybridization (Fig. 9a) and immunohistology (Fig. 9b) compared to vector-transfected animals. Thus, by blocking the interaction of Jun with other members of the bZIP family by somatic gene transfer with a plasmid overexpressing mutated Jun, endothelial TF induction could be partially reduced (Fig. 9, a and b). Similar data were obtained when a plasmid overexpressing I-κB was used (Fig. 9, a and b). Mutated Jun and I-κB both reduced the inducibility of TF in endothelial cells in this tumor model in vivo. The tumor model was further used to examine the functional effect of TF; when the fibrin/fibrinogen deposition in response to TNFα was studied in animals perfused with 30–40 ml of PBS (see “Materials and Methods”), a reduction by mutated Jun and I-κB was demonstrated in some, but not all vessels (Fig. 9c). Thus TF expression in vivo is under the control of AP-1/bZIP and NF-κB-like proteins.

Successful transfection with mutated Jun or I-κB was monitored in EMSA of tumor tissue (Fig. 10). Tumors derived from animals transfected with vector DNA prior to TNFα had a stronger AP-1 binding activity than tumors derived from animals transfected with mutated Jun (Fig. 10, top left). Consequently, tumors from I-κB transfected animals demonstrated reduced NF-κB binding activity at the TF derived NF-κB site (Fig. 10, top right) compared to vector controls. In addition, Northern blot of mRNA, derived from whole tumors, showed

**Fig. 9. AP-1/bZIP proteins and NF-κB control TF expression in vivo.** Meth-A sarcoma (10⁶ cells/animal) were implanted into C3H mice. After the tumors reached an average size of 0.5 cm, intravenous somatic gene transfer was performed with plasmids overexpressing a vector control, mutated Jun, or I-κB. 12 days after planting the tumors, mice received PBS (control) or 5 µg of TNFα/animal for 3 h. Mice were sacrificed and perfused with 30–40 ml of PBS by intracardiac injection of PBS into the left ventricle. Tumors were harvested and TF transcription (a, in situ hybridization), TF antigen (b, immunohistochemistry), and fibrin/fibrinogen deposition (c, immunofluorescence) was evidenced in the tissue. a, in situ hybridization with a mouse TF-specific riboprobe (see “Materials and Methods”) in control (top) and TNFα (bottom) treated animals, transfected with vector control (left), mutated Jun (middle), or I-κB (right). Magnification, ×160. b, immunohistochemistry using an anti-mouse TF antibody (see “Materials and Methods”) in control (top) and TNFα (bottom) treated animals, transfected with vector control (left), mutated Jun (middle) or I-κB (right). Magnification, ×160. c, immunofluorescence of fibrin/fibrinogen deposition in control (top) and TNFα (bottom) treated animals, transfected with vector control (left), mutated Jun (middle), or I-κB (right). Magnification, ×40.
Mechanism of TNFα-mediated Tissue Factor Induction

Tissue factor (TF) is a potent initiator of the coagulation cascade (1, 2, 4, 65, 66) and normally is not expressed by quiescent endothelial cells (1, 3, 6, 11, 12). In vitro data showed induction of TF synthesis in endothelial cells by inflammatory mediators such as endotoxin, phorbol esters, oxygen-free radicals, or cytokines (7–11, 67–70). Recently members of the NF-κB and the AP-1/BZIP family have been reported to be involved in the lipopolysaccharide- and cytokine-mediated TF induction in monocytes (15–18, 62) and porcine endothelial cells (11). It has been more difficult to show endothelial TF in vivo (3, 67); however, recent studies demonstrate that in selected areas of the vascular bed activators of the host response or TNFα lead to the synthesis and expression of TF (12–14, 71). This study addresses the molecular mechanisms that underlie the regulation of the human TF promotor in response to the proinflammatory cytokine TNFα.

We used bovine aortic endothelial cells (BAEC), which exhibit lower basal AP-1 and NF-κB activity than porcine (PAEC) or human (HUVEC) endothelial cells, and the human TF promoter. A striking difference between the human and the porcine TF promoter is seen at the proximal AP-1 site, which resembles a canonical high affinity site in the human TF promoter (15, 16), while it is a low affinity non-canonical site in the porcine promotor, due to a G → A switch at position 4 of the AP-1 heptamer (11). Thus, the proximal AP-1 site of the human

Figure 10. Efficiency of intravenous somatic gene transfer. Transfection efficiency was monitored in EMSA (top) and Northern blot (bottom). Top I, EMSA of tumor nuclear extracts, derived from mice transfected with vector control (left) or mutated Jun (middle) before application of TNFα. EMSA were performed with the proximal AP-1 site (A2) of the human TF promotor. AP-1 binding was confirmed by suppressing the observed shift in TNFα-induced vector controls by a 160-fold molar excess of unlabeled AP-1 consensus competitor (right). Top II, EMSA of tumor nuclear extracts, derived from mice transfected with vector control (left) or I-κB (middle) before application of TNFα. EMSA were performed with the TF-derived NF-κB site. NF-κB binding was confirmed by suppressing the observed shift in TNFα-stimulated vector controls by a 160-fold molar excess of unlabeled NF-κB consensus competitor (right). Bottom I, Northern blot: total mRNA of tumors from TNFα-treated animals, transfected with vector control (left, 1) or mutated Jun (right, 3) was hybridized against tissue factor (TF; top) or GAPDH (bottom) specific DNA probes. Bottom II, Northern blot: total mRNA of tumors from TNFα-treated animals, transfected with vector control (left, 1) or I-κB (right, 3) was hybridized against tissue factor (TF; top) or GAPDH (bottom) specific DNA probes.

Decreased TF mRNA levels, when the animals had been transfected with mutated Jun or I-κB prior to TNFα application (Fig. 10, bottom). However, the suppression observed was only partial, since (i) members of the Jun and ATF family are less responsive to inhibition by overexpression of mutated Jun than c-Fos, (ii) the in vivo involvement of other transcription factors cannot be excluded, and (iii) transfection did not reach all cells.

To give a picture of the overall efficiency of transfection, microbeads were used for measuring blood flow of the whole organ, avoiding potential artifacts due to selection of a single area in histological studies. When microbeads were injected into animals, a high number of beads was present in tumors of animals not treated with TNFα (Fig. 11). The number of beads reflecting tumor perfusion was clearly decreased after TNFα injection with previous somatic gene transfer with vector DNA (Fig. 11). This indicated that TNFα treatment resulted in loss of free blood flow, potentially due to TF-mediated microvascular thrombosis. Therefore this method adds to the histological study by providing data about the effect of I-κB and mutated Jun on the whole organ. Gene transfer with I-κB or mutated Jun partially reversed this effect of TNFα (Fig. 11). Hence blocking TF on the transcriptional level reduced not only TF induction by TNFα, but also reduced the fibrin/fibrinogen deposition and restored free blood flow.

**DISCUSSION**

Somatic gene transfer with mutated Jun or I-κB restores the free blood flow in tumors treated with TNFα. 106 Meth-A sarcoma cells were planted intracutaneously into mice. Somatic gene transfer and TNFα application was performed as described in Fig. 9. 3 h after intravenous injection of 5 μg of TNFα, mice were anesthetized, microspheres were injected into the left ventricle for 10–20 s (see “Materials and Methods”), and mice were sacrificed thereafter. Tumor tissue was harvested and microspheres counted microscopically (see “Materials and Methods”). The free blood flow is shown, evidenced by the number of latex particles per gram of tumor tissue. A, tumors before TNFα application; B, tumors after TNFα, pretreated with mutated Jun; C, tumors after TNFα, pretreated with I-κB; D, tumors after TNFα, pretreated with mutated Jun and I-κB; E, tumors after TNFα.
promoter is more prominently involved in the TNF-α-mediated up-regulation of TF than it is in the porcine promoter and, as a consequence, in addition to NF-κB activation, AP-1 activation may be relevant for human disease.

In transient transfection studies, the highest TNF-α inducibility was only observed when the NF-κB (p65/c-Rel) site was present in the TF promoter plasmids. Enhanced NF-κB (p65/c-Rel) binding to its TF-derived motif was detectable within 5 min after TNF-α stimulation and rapidly down-regulated after 1 h (data not shown). This fast activation of NF-κB (p65/c-Rel) reflects that TF mRNA can be rapidly induced in the absence of protein synthesis; therefore, TF has been classified as an immediate early gene (72). The dependence of TF induction by inflammatory mediators on NF-κB (p65/c-Rel) activation may thus be explained by the fact that induction is transient, since it has been recently reported that increased NF-κB levels lead to increased expression of the inhibitor IκB (73, 74), followed by NF-κB inactivation. This might prompt the activated endothelial cells to return to a quiescent state.

Therefore, the existence of increased TF mRNA levels in HUVEC and BAEC 4–6 h after TNF-α stimulation (9, 10) cannot be explained solely on the basis of NF-κB activation and demands the involvement of other inducible transcription factors. Functional studies demonstrated that optimal TF induction by TNF-α was also mediated by both AP-1 sites in the human TF promoter (Fig. 8). EMSA revealed that the TNF-α-inducible complex bound to the canonical proximal AP-1 site of the human TF promoter consisted mainly of NFκB (p65/c-Rel) (Fig. 7c); however, it cannot be excluded that other members of the Jun family are also involved. Highly vascularized organs (spleen, lung, intestine, ovary, and brain) express high levels of NFκB (30). While the expression of c-jun and J un B is rapidly up-regulated by various stimuli, J un D is only modestly induced by growth factors and phorbol esters (26, 30, 75, 76). Transactivation by J un D homodimers is significant lower than by c-jun homodimers (30). In cooperation with c-Fos, however, J un D has transactivation capacities similar to those of c-jun (29, 30).

The results displayed here demonstrate that in cultured endothelial cells TNF-α induces J un D/Fos heterodimers that recognize the proximal AP-1 site of the human TF promoter and thereby activates TF transcription. In this respect the human and the porcine system differ significantly. Binding of J un D/ Fos-containing complexes to the proximal AP-1 site is already detected 30 min after TNF-α stimulation. This rapid response excludes newly synthesized J un D or Fos and indicates the rapid activation of preexisting proteins. This availability of J un D/Fos heterodimers therefore is a limiting factor. Consistently, the canonical proximal AP-1 alone was not able to confer high TNF-α-mediated induction in transient transfection experiments and required the presence of the NFκB (p65/c-Rel) site (11, 15, 16, 18) for optimal TF expression. These data imply that the disposal of J un D/Fos heterodimers is not sufficient for maximal induction by TNF-α and need to recruit NFκB (p65/c-Rel) nuclear binding activity. Since NFκB (p65/c-Rel) translocation into the nucleus precedes J un D/Fos activation only by 20 min, one might speculate that binding of one transcription factor facilitates binding of the other.

The proximal high affinity AP-1 site of the human TF promoter, which is missing in the porcine TF promoter, is of particular importance with respect to therapeutic interventions. A great variety of antioxidative agents has been reported to suppress activation of NFκB in vitro and in vivo (77) and therefore might potentially be used for reducing TF activity under certain pathophysiological conditions. However, recent studies elucidated that changes in the cellular redox system by radical scavengers suppress very fast NFκB, but at the same time induce time-dependent AP-1 activation (J un/ Fos) (20, 21). Antioxidative conditions strongly induce c-Fos, which can form reactive heterodimers with preexisting J un homodimers (20, 21). As pointed out before, tissues with high endothelial portions contain constitutively high amounts of J un D homodimers (30) and are therefore primed to generate large amounts of J un D/Fos heterodimers under antioxidative therapy.

To define the role of the non-canonical distal AP-1 site in human TF regulation is more difficult. This site is a low affinity site for AP-1 binding and resembles the two non-canonical AP-1 sites of the porcine TF promoter (11). In accordance with these data, several independent approaches demonstrated that J un homodimers, but not J un/Fos heterodimers, bind to this site (Figs. 3, 4, and 6). Differences in the structure of the DNA binding domains for J un homodimers and J un/Fos heterodimers have been described (78); therefore, it seems likely that a G–A switch at position 4 of this site facilitates J un binding and excludes significant Jun/Fos binding. Furthermore, specific properties of the regions outside the defined AP-1 binding sites might be responsible for preference in binding of the various homo- and heterodimer complexes (79). EMSA demonstrated (Fig. 6, a–c) that TNF-α also induced protein complexes that were different from J un homodimers. These complexes have been characterized to contain J un and ATF family proteins (Fig. 6, a–c). In contrast, Moll et al. (11) recently reported constitutive binding of c-jun, J un D, and possibly Fra2 complexes to the porcine TF promoter-derived non-canonical AP-1 sites. Since basal AP-1 binding activity is low in BAEC compared to PAEC, this might explain why the study presented here detected TNF-α-inducible binding at the non-canonical distal AP-1 site of the human TF promoter. Consistent with our observations, Donovan-Peluso and co-workers mentioned that in THP-1 cells large differences between the distal and the proximal AP-1 site were detected in EMSA, which indicate the involvement of different heterodimers (17). This finding differs from previous observations in HUVEC, where J un homodimer and J un/Fos heterodimer binding occurs at the distal and the proximal AP-1 site (47, 63). This might be due to (i) a greater availability of J un homodimers, (ii) a different composition of the complexes induced, or (iii) to species differences in HUVEC versus BAEC. The low affinity distal AP-1 site of the human TF promoter only marginally participates in TNF-α-induced TF expression, consistent with the data described for the two low affinity AP-1 sites in the porcine TF promoter (11). However, the non-canonical AP-1 site significantly supports NFκB-mediated TF induction, even when the proximal AP-1 is deleted (Fig. 8). Since recently a cooperative action of ATF proteins and NFκB family members has been demonstrated (80), one might speculate that proteins bound to the distal AP-1 site support and facilitate NFκB activity. Therefore a set of different transcription factors has to be activated at the same time before endothelial TF is successfully induced.

The in vivo data presented (Fig. 9–11) support this concept. Intravenous somatic gene transfer with plasmids overexpressing IκB or mutated J un reduce TF induction in vascular endothelial cells of the tumor. They also decrease deposition of fibrin/fibrinogen. The antibody used does not discriminate between fibrin and fibrinogen. Therefore, the animals were perfused with 30–40 ml of PBS (see “Materials and Methods”) prior to harvest of the organs to remove non-clotted material. The reactive material represents at least in part fibrin, since we observed striking differences in fibrin/fibrinogen deposition between the different animal groups corresponding to the perfusion studies with microbeads (the later ones giving a better view of the overall efficiency of IκB and mutated J un). How-
NF-κ-mediated activation of endothelial TF transcription occurs in vitro and in vivo by members of the NF-κB and AP-1/bZIP family.

Acknowledgment—We thank Dr. D. Bohmann for the cDNA of mutated Jun, Dr. J. E. Sadler for the human tissue factor cDNA probe, and Dr. S. M. Mann for the antisense oligonucleotide. We thank Dr. D. Bohmann for the cDNA of mukB and AP-1 in the animal model used.”

References

1. Nawroth, P. P., Stern, D. M., Kasis, W., and Bach, R. (1985) Thromb. Res. 39, 677–691
2. Bach, R. (1988) CRC Crit. Rev. Biochem. 23, 339–368
3. Wilcox, J. N., Smith, K. M., Schwartz, S. M., and Gordon, D. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 2839–2843
4. Edgington, T. S., Mackman, N., Fan, S. T., and Ruf, W. (1992) Nouv. Rev. Fr. Hematol. 34 (suppl.), 15–27
5. Mackman, N., Sawdey, M. S., Keaton, M. R., and Loskutoff, D. J. (1993) Am. J. Pathol. 143, 76–84
6. Solberg, S., Ostensen, W. B., Larsen, T., and Sorlie, D. (1990) Blood Coagul. Fibrinol. 1, 595–600
7. Weibelsta, M. C., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S., and Gimbrone, M. A., Jr. (1990) Proc. Natl. Acad. Sci. U.S.A. 83, 4533–4537
8. Nawroth, P. P., and Stern, D. M. (1986) J. Exp. Med. 163, 740–745
9. Conway, E. M., Bach, R., Rosenberg, R. D., and Konigsberg, W. H. (1989) J. Exp. Med. 174, 1893–1896
10. Hofer-Warbinek, R., Wagner, E., Winkler, H., Bach, F. H., and Hofer, E. (1995) J. Biol. Chem. 270, 3849–3857
11. Drake, T. A., Cheng, J. B., Chang, A., and Taylor, F. B., Jr. (1993) Am. J. Pathol. 142, 1458–1470
12. Zang, Y., Badmann, H., Emmer, C., van Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
13. Zhang, Y., Badmann, H., Emmer, C., van Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
14. Mangel, N., McCall, J. P., and Sibatani, R. (1995) Clin. Invest. 95, in press
15. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1995) J. Exp. Med. 188, 51–59
16. Mangel, N., McCall, J. P., and Sibatani, R. (1995) Clin. Invest. 95, in press
17. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
18. Mangel, N., McCall, J. P., and Sibatani, R. (1995) Clin. Invest. 95, in press
19. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
20. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
21. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
22. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
23. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
24. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
25. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59
26. Badmann, H., Emmer, C., von Lunzen, J., von Stemmler, D., Kern, P., Dietrich, M., and Nawroth, P. P. (1999) J. Pathol. 188, 51–59