Role of NFκB in the Mortality of Sepsis

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Abstract

Binding activity for nuclear factor kappa B (NFκB) consensus probes was studied in nuclear extracts from peripheral blood mononuclear cells of 15 septic patients (10 surviving and 5 not surviving). Nonsurvivors could be distinguished from survivors by an increase in NFκB binding activity during the observation period (P < 0.001). The increase in NFκB binding activity was comparable to the APACHE-II score as a predictor of outcome. Intravenous somatic gene transfer with an expression plasmid coding for IκBα was used to investigate microsomal coagulation in a mouse model of endotoxemia. In this model, increased NFκB binding activity was present after injection of LPS. Intravenous somatic gene transfer with IκBα was used before LPS attenuated renal NFκB binding activity and increased survival. Endothelial cells and monocytes/macrophages were the major target cells for somatic gene transfer, transfected with an average transfection efficiency of 20–35%. Tissue factor, a gene under regulatory control of NFκB, was induced by LPS. Somatic gene transfer with a reporter plasmid containing the functional tissue factor promoter demonstrated NFκB-dependent stimulation by LPS. Intravenous somatic gene transfer with IκBα reduced LPS-induced renal tissue factor expression, activation of the plasmatic coagulation system (decrease of thrombin–antithrombin III complexes) and renal fibrin/fibrinogen deposition. Somatic gene transfer with an expression plasmid with tissue factor cDNA in the antisense direction (in contrast to sense or vector alone) also increased survival. Furthermore, antisense tissue factor decreased renal tissue factor expression and the activation of the plasmatic coagulation system. (J. Clin. Invest. 1997. 100:972–985.) Key words: sepsis • NFκB • tissue factor • coagulation

Introduction

LPS released by gram-negative bacteria is the most frequent cause of septic shock, affecting ~ 400,000 patients in the United States annually (1). The host response to LPS and in-
members of the NFκB family are likely candidates responsible for this effect. This study demonstrates that nuclear extracts from PBMC harvested from nonsurviving septic patients show increased nuclear binding activity of NFκB to NFκB consensus oligonucleotide probes compared with survivors. In an endotoxemia model of LPS-treated mice, suppression of NFκB activation by intravenous somatic gene transfer with an IκB-overexpressing plasmid attenuated the LPS-mediated induction of tissue factor and increased survival. These data indicate a direct role for protein complexes activating expression of NFκB-regulated genes in the acute response to sepsis and endotoxemia.

Methods

Patients

A description of the patients, including age, sex, underlying disease, and the daily APACHE-II scores, is given in Table I. The patients presented were treated in the Department of Visceral-Thoracic-Vascular Surgery in Dresden, Germany (T.Z. and H.D.S.). The study was approved by the ethics committee of the University of Dresden, Germany, according to the guidelines of the Helsinki declaration.

Animal model

Female BALB/c mice, aged 10–12 wk, 18–20 g (Charles River Wiga, Sulzfeld, Germany) were injected intraperitoneally with a mixture of E. coli LPS (0111:B4; Sigma Chemie, Deisenhofen, Germany; 1.75 μg in 0.1 ml sterile PBS; pH 7.4) and d-galactosamine (Sigma Chemie; 15 mg in 0.1 ml sterile PBS), in order to sensitize them to the lethal effects of LPS (51, 52). Mortality was monitored after 4, 8, 12, 16, 20, and 24 h. No further mortality was seen after 24 h.

Plasmids

The pXT1 vector (Stratagene, Heidelberg, Germany) was used for constructing the tissue factor expression system. Murine tissue factor cDNA was generously provided by Dr. Nathans (Johns Hopkins University, Baltimore, MD; 53). The full-length mouse tissue factor cDNA was inserted into the XbaI site of the pXT1 vector from 3’ to 5’ for the tissue factor antisense construct (pXTF-as) or from 5’ to 3’ for the tissue factor sense construct (pXTF-s) (44) (Fig. 1). The pGLITF₂-galactosidase (LUC) expression plasmid, driven by the tissue factor promoter fragment (bp –278 to bp +121, pl4) (54) was constructed by inserting the fragment into the multiple-cloning site of pGL₂-Luc (Promega, Heidelberg, Germany), from which the SV₆gal promoter was removed (Fig. 1). The promoterless Luc-control vector pGL₂-basic (Promega) served as control. The IκBα (pRC/CMVMAD-3wt) expression plasmid (55) was provided by Dr. P.A. Baeuerle (Tularik Inc., San Francisco, CA). The mutated jun plasmid pDB7, derived from the point mutant MutI4 (56), was generously provided by Dr. D. Bohmann (EMBL, Heidelberg, Germany). The β-galactosidase control plasmid pSV-β-Gal was obtained from Promega.

Preparation of human PBMC

Human PBMC were separated after venipuncture from 20 ml whole blood, anticoagulated with 3.8% sodium citrate (9:1, vol/vol), by centrifugation on Ficoll Paque® (Pharmacia, Freiburg, Germany) according to the manufacturer’s instructions. PBMC fractions were analyzed microscopically and independently counted by two investigators (A.B. and T.I.). Before electrophoretic mobility shift assays (EMSA), the amount of PBMC was adjusted to 2 × 10⁶ PBMC/ml.

Preparation of murine PBMC

Murine PBMC were separated using a similar method as for human PBMC isolation. For each time point, 5 ml whole blood was collected and pooled from 10 mice, anticoagulated with 3.8% sodium citrate (9:1, vol/vol), loaded carefully onto 5 ml of a Ficoll Paque® Plus gradient (Pharmacia) and centrifuged at 500 g without brakes at room temperature for 30 min according to the manufacturer’s instructions. The mononuclear band was aspirated, washed with PBS, and analyzed microscopically by two investigators (F.Q. and A.B.). 10⁶ PBMC were seeded onto gelatin-coated glass chamber slides (Nunc, Naperville, IL), and incubated overnight in RPMI medium containing 10% FCS, before immunohistochemistry was performed.

EMSA

For EMSA, nuclear proteins were harvested by the method of Andrews (57). 2 × 10⁶ isolated PBMC were lysed in 400 μl cold buffer A (10 mM Hepes-KOH, pH 7.9, at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Nuclear extracts were prepared by Dounce homogenization (10 strokes) and centrifugation at 1,000 g for 5 min at 4°C. 50 μg of nuclear proteins was incubated with 20 ng of each NFκB consensus oligonucleotide in 10 μl cold buffer A containing 5 mM DTT, 50 mM KCl, 1 mM MgCl₂, 100 μM each dATP, dCTP, dGTP, and 0.5 μCi [γ-32P]dATP. EMSA was performed using a Multiprobe gel shift system (Pharmacia) and centrifuged at 500 g without brakes at room temperature for 20 min. The gels were dried and autoradiographed using Kodak X-OMAT MF film. For competition experiments, a 100-fold excess of unlabelled oligonucleotide was added.
Tissue Factor Expression System

- pXT1, vector plasmid; S, sense tissue factor expression plasmid pXTF-s; AS, antisense tissue factor expression plasmid pXTF-as; LTR, Moloney murine leukemia virus long terminal repeats; Neo, neomycin resistance; TK, herpes simplex thymidine kinase promoter; TF, mouse tissue factor. (a) Tissue factor expression system. pXT1, vector plasmid; S, sense tissue factor expression plasmid pXTF-s; AS, antisense tissue factor expression plasmid pXTF-as; LTR, Moloney murine leukemia virus long terminal repeats; Neo, neomycin resistance; TK, herpes simplex thymidine kinase promoter; TF, mouse tissue factor. (b) Luc expression system. SV<sub>as</sub>, SV<sub>as</sub> promoter; Er, SV<sub>as</sub> enhancer; TF-P, tissue factor promoter; Amp', β-lactamase; pA, synthetic poly(A) signal.

Luciferase Expression System

- pGL<sub>2</sub>-Basic
- pGL<sub>2</sub>-Luc
- pGLUFL<sub>2</sub>

Figure 1. Scheme of expression plasmids. (a) Tissue factor expression system. pXT1, vector plasmid; S, sense tissue factor expression plasmid pXTF-s; AS, antisense tissue factor expression plasmid pXTF-as; LTR, Moloney murine leukemia virus long terminal repeats; Neo, neomycin resistance; TK, herpes simplex thymidine kinase promoter; TF, mouse tissue factor. (b) Luc expression system. SV<sub>as</sub>, SV<sub>as</sub> promoter; Er, SV<sub>as</sub> enhancer; TF-P, tissue factor promoter; Amp', β-lactamase; pA, synthetic poly(A) signal.

Luc activities were determined as previously described (30, 44). Kidneys were harvested after perfusion with 30 ml PBS, and homogenized with a homogenizer (IKA-Werk; Janke & Kunkel GmbH, Staufen, Germany) for 30 s in lysis buffer (Promega) containing 25 mM Tris, pH 7.8, with H<sub>2</sub>O<sub>2</sub>, 2 mM EDTA; 2 mM DTT 10% glycerol; 1% Triton X-100. After brief centrifugation, the supernatant was collected and the Luc activity was measured with the Luciferase Assay System (Promega) using a luminometer (LB 9501; Lumat, Berthold, Germany) (61, 62). Data were expressed as Luc activity per gram of tissue.

Detection of transfected Luc antigen

Preparation of kidneys. For immunocytochemistry, mouse kidneys were harvested after the animal had been perfused extensively with 30 ml of PBS to remove unclotted material. Samples were instantaneously frozen in isopentane, cooled in liquid N<sub>2</sub>, cryostat sections of mouse kidneys were fixed in acetone for 10 min and then incubated for 5 min in 0.6% H<sub>2</sub>O<sub>2</sub> in methanol for blocking endogenous peroxidase activity.

Preparation of PBMC. To perform immunocytochemistry, PBMC were fixed in 4% paraformaldehyde in PBS. After washing with PBS, the endogenous peroxidase activity was blocked by incubating in 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 15 min.

Detection of Luc antigen. Before adding the anti-Luc antibody (Promega), the sections were incubated in 10% goat serum (Promega) and then incubated with anti-Luc antibody in Tris-HCl buffer 4°C overnight. An anti-rabbit IgG conjugated with peroxidase was used as second antibody. The color reaction was performed with Sigma Fast DAB substrate (3,3′-diaminobenzidine tetrahydrochloride; Sigma Chemie). Hematoxylin (Sigma Chemie) was used for counterstaining.

Oligonucleotides were labeled to a specific activity >5 x 10<sup>6</sup> cpm/μg DNA using T4-polynucleotide kinase (Promega). Binding of NFκB was performed in 10 mM HEPES, pH 7.5, 0.5 mM EDTA, 100 mM KCl, 2 mM DTT, 2% glycerol, 4% Ficoll 400, 0.25% NP-40, 1 mg/ml BSA (DANS free), and 0.1 μg/μl poly dI/dC in a total of 20 μl as described essentially by Pahl et al. (60). 1 ng of labeled oligonucleotides (~50,000 cpm) was added to 10 μg of nuclear extract and incubated at room temperature for 20 min in the appropriate binding buffer. Protein–DNA complexes were separated from the free DNA probe by electrophoresis through 5% native polyacrylamide gels containing 2.5% glycerol and 0.5% TBE. The gels were run at room temperature with 30 mA for ~2.5 h. Gels were dried under vacuum on Whatmann D-81 paper (Schleicher and Schüll, Dassel, Germany) and exposed for 12–48 h to Amersham Hyperfilms at ~−80°C with intensifying screens. Specificity of binding was ascertained by competition with a 1:100 molar excess of cold consensus oligonucleotides and by characterization with specific polyclonal antibodies (Santa Cruz, Heidelberg, Germany).

Densitometric quantification of EMSA autoradiograms

Signals obtained in EMSA were quantitated using a Scan-Pack Personal Densitometer (Pharmacia). The determination of the signal area to be measured and the quantitative evaluation were performed independently by two different investigators (A.B. and T.I.). The mean of both measurements was taken for statistical analysis.

Intravenous somatic gene transfer

Intravenous somatic gene transfer was performed as described previously (30, 44). 50 μg of plasmid DNA (pGL2-Luc, control plasmid pGL2-basic, murine tissue factor expression plasmids, pXT1-control vector, IκBα or mutated Jun expression plasmids) was mixed with 150 μg DOTAP (Boehringer Mannheim Biochemicals, Mannheim, Germany). The first injection was performed 7 d before administration of LPS. The second injection was given 2 d before administration of LPS. Each injection was given via the tail vein. The total volume of the DNA–liposome complex was 250 ml.

Luc assay

Luc activities were determined as previously described (30, 44). Kidneys were harvested after perfusion with 30 ml PBS, and homogenized with a homogenizer (IKA-Werk; Janke & Kunkel GmbH, Staufen, Germany) for 30 s in lysis buffer (Promega) containing 25 mM Tris, pH 7.8, with H<sub>2</sub>O<sub>2</sub>, 2 mM EDTA; 2 mM DTT 10% glycerol; 1% Triton X-100. After brief centrifugation, the supernatant was collected and the Luc activity was measured with the Luciferase Assay System (Promega) using a luminometer (LB 9501; Lumat, Berthold, Germany) (61, 62). Data were expressed as Luc activity per gram of tissue.

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Preparation of PBMC. To perform immunocytochemistry, PBMC were fixed in 4% paraformaldehyde in PBS. After washing with PBS, the endogenous peroxidase activity was blocked by incubating in 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 15 min.

Detection of Luc antigen. Before adding the anti-Luc antibody (Promega), the sections were incubated in 10% goat serum Tris-HCl buffer (pH 7.5, 150 mM NaCl, 0.3% Triton X-100) for 30 min at room temperature and then incubated with the anti-Luc antibody in Tris-HCl buffer 4°C overnight. An anti-rabbit IgG conjugated with peroxidase was used as secondary antibody. The color reaction was performed with Sigma Fast DAB substrate (3,3′-diaminobenzidine tetrahydrochloride; Sigma Chemie). Hematoxylin (Sigma Chemie) was used for counterstaining.
counterstaining. Negative controls included omission of the first or second antibodies and substitution of the first antibody by nonspecific antibodies (data not shown).

Detection of tissue factor antigen
Sections of mouse kidneys were fixed in 4% paraformaldehyde dissolved in PBS, pH 7.4. After washing two times with PBS, sections were incubated with anti-mouse tissue factor antibodies described earlier (44) for 2 h at room temperature in 100 mM Tris-HCl buffer (pH 7.5, 150 mM NaCl, 0.3% Triton X-100). After washing, sections were incubated with the second antibody (goat anti-rat IgG conjugated with peroxidase) for 1 h at room temperature. Color development was performed with AEC (3-amino-9-ethyl-carbazole; Sigma Chemie) and H2O2 (44, 63). Negative controls included omission of the first or second antibodies and substitution of the first antibody by nonspecific antibodies (data not shown).

Immunofluorescence
For immunofluorescence studies, the sections of extensively perfused kidneys (see above) were fixed with acetone for 10 min and incubated with anti-fibrin/fibrinogen antibody (fluorescein-conjugate; Cappel Laboratories, West Chester, PA) diluted (1:80) with PBS containing NaBr for 45 min at room temperature (44).

In situ hybridization
In situ hybridization for tissue factor was performed as previously described (30, 44, 64). Briefly, antisense and sense single-strand cRNAs were synthesized from a mouse tissue factor cDNA fragment (721–1,043 bp) subcloned into pGEM2 (Promega) in the presence of digoxigenin-labeled UTP (Boehringer Mannheim Biochemicals). These probes were characterized using Northern blots (data not shown). The kidney sections were fixed in 4% paraformaldehyde in PBS, pH 7.4, acetylated for 15 min at room temperature in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine HCl plus 0.9% NaCl, pH 8.0, and incubated with proteinase K (100 mg/ml) for 10 min. Sections were prehybridized for 2 h at room temperature with hybridization buffer containing 0.6 M NaCl, 1 mM EDTA, 10 mM Tris HCl, pH 7.6, 10% dextran sulfate, 0.25% SDS, 100 mM DTT, 50 μg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 1× Denhardt’s solution, 5% dextran, and 50% (vol/vol) deionized formamide. After prehybridization, hybridization solution containing 5–10 ng cRNA probe was applied to each section, followed by incubation in a moist chamber for 16 h at 37°C. Samples were then incubated with 20 μg/ml RNase A (Boehringer Mannheim Biochemicals) for 30 min at 37°C, followed by washing once with 2× SSC plus 50% formamide for 30 min at room temperature and twice with 0.2× SSC for 30 min at 50°C. For immunological detection, the DIG Nucleic Acid Detection Kit (Boehringer Mannheim Biochemicals) was used according to the manufacturer’s instructions. In each section, a consecutive cut was stained as control with the sense riboprobe, which did not show a specific signal (data not shown).

Determination of thrombin–antithrombin III complexes (TAT)
1 h before drawing the blood by heart puncture from mice, 200 U heparin (Sigma Chemie) was injected via the tail vein. Plasma was prepared by centrifugation of the blood at 3,000 rpm for 10 min. 50 μl of the supernatant was used to measure TAT. The TAT assay was performed according to the manufacturer’s instructions (Behring, Marburg, Germany).

Statistical analysis
All values are given as mean, with the bars showing standard deviations. The means of groups were compared by ANOVA using the Newman-Keul’s test to correct for multiple comparisons. P < 0.05 was considered to be statistically significant. Discrimination analysis was performed with the aid of the program StatView (Abacus Concepts Inc., Berkeley, CA).

Results

NFκB binding activity in nuclear extracts of PBMC from septic patients. Members of the NFκB family are believed to mediate central events in inflammatory disease. To study the significance of NFκB activation in vivo, 15 septic patients (10 survivors, 5 nonsurvivors) were graded by the APACHE-II score (Table I). NFκB binding activity was determined in nuclear extracts of PBMC by EMSA. Autoradiograms were analyzed by laser densitometry, and the intensity of the gel shift band on day 1 was assigned a value of 100% for comparison with subsequent samples up to day 14 (Table II). All samples from each patient were run simultaneously on the same gel. During the course of the disease, the average NFκB binding activity was lower in survivors than in nonsurvivors (Fig. 2, a and b). Representative data of one nonsurvivor (patient 12) from days 1 to 10 showed a striking increase in nuclear binding activity (Fig. 2b, right lane). All patients in whom NFκB binding activity exceeded 200% (compared with day 1) died. This increase did not occur in survivors (Fig. 2 b, left). The proteins binding to the NFκB consensus motif were characterized as members of the NFκB family based on the following criteria: competition with an unlabeled NFκB consensus oligonucleotide (but not AP-1 consensus oligonucleotides, data not shown) and interaction with antibodies directed against proteins of the NFκB family (Fig. 2 c). Most of the binding activity was characterized as NFκB p50 and NFκB p65, while NFκB p52 and c-rel represented only a minor portion of the binding proteins (Fig. 2 c). No reduction of NFκB binding activity was observed in the

Table II. NFκB Binding Activity in Patients with Sepsis

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The NFκB signals observed in EMSA of nuclear extracts from PBMC were evaluated by laser densitometry. For EMSA, 10 μg of nuclear extract derived from isolated PBMC was incubated with the NFκB consensus oligonucleotide. The value obtained on day 1 was defined as 100%. All other values were calculated as the percentage of day 1. The data shown represent the mean of two independent measurements. *D1, day 1, etc.
The 

NFκB binding activity was lower in survivors with a lower APACHE-II score than in nonsurvivors with a higher APACHE-II score (Fig. 3). When the variable score was split by outcome, a significantly higher NFκB binding activity was observed in nonsurvivors than in survivors. Discriminate analysis (Table III) demonstrated that the predictive power of the APACHE-II score was 82%, while the predictive power of PBMC NFκB binding activity was 85%. Both prognostic results did not show significant differences in their predictive power (Newman-Keul's test, \( P > 0.1 \)). Outcome was determined by the severity of the disease and the course of NFκB binding activity. The absolute value of the signal observed on day 1 did not correlate with outcome.

**Figure 2.** Time course of NFκB binding activity in PBMC of septic patients. (a) Time course of NFκB binding activity determined in 10 μg nuclear extracts of PBMC. Each EMSA signal obtained at the time points indicated (Tables I and II) was quantitated by laser densitometry. Signal intensity was determined at each day for all patients surviving (n = 10) or not surviving (n = 5) and the mean±SD is given for survivors (open bars) and nonsurvivors (filled bars). (b) EMSA of nuclear extract prepared from PBMC isolated from a patient surviving (patient 3) and a patient not surviving (patient 12). 10 μg of nuclear extract was incubated with the NFκB consensus oligonucleotide. The specific NFκB complex is indicated by arrows. (c) Characterization of NFκB binding activity. 10 μg nuclear extract from PBMC of a patient surviving (patient 6, day 1, left) and a patient not surviving (patient 15, day 5, right) was incubated with the NFκB consensus oligonucleotide in the presence of 2.5 μg of the following antibodies: first lane, control IgG; second lane, anti-p50; third lane, anti-p65; fourth lane, anti-p52; fifth lane, anti-c-rel; sixth lane, anti-rel B; seventh lane, binding was competed with a 160-fold excess of unlabeled NFκB consensus oligonucleotide. The inducible NFκB complex is indicated by arrows. A second band, not observed in EMSA without antibodies (compared with b), is probably due to unspecific reactions of the IgG with the NFκB binding motif. Similar results have been obtained with PBMC isolated from other patients.
Mouse LPS model. The data shown are suggestive of an important role of NFκB activation in the outcome of sepsis. However, the clinical situation is complex and does not allow exact analysis of the role of NFκB activation on survival. Our goal was to determine whether preventing activation of NFκB would influence the outcome of experimentally induced endotoxemia in mice treated with E. coli LPS 011:B4 in the presence of galactosamine (51, 52). In this animal model, a time-dependent increase in NFκB binding activity occurred in kidneys and in isolated PBMC (Fig. 4, a and b), when a lethal dose of LPS was applied. When a nonlethal amount of LPS was injected, only a marginal increase in NFκB binding activity was detected by EMSA (data not shown). Therefore, activation of NFκB in kidneys and in PBMC seems to be comparable in the patient study and in the animal model used.

The experimental approach selected to prevent NFκB activation in endotoxemia was intravenous somatic gene transfer with a plasmid overexpressing IκBα (30, 44). To establish an optimal protocol for transfection, somatic gene transfer efficiency experiments were performed with the SV40 promoter driven Luc plasmid pGL2-Luc and the promoterless control vector pGL2-basic (Fig. 5). After intravenous administration of the plasmid in DOTAP, reporter gene expression was studied in kidneys, one of the key target tissues of LPS. Luc activity in renal extracts increased steadily after administration of the construct, reaching a maximum between 24 and 48 h (Fig. 5a). When the first pulse of plasmid was given 7 d ahead, followed by a second one 5 d later, we found a severalfold higher expression of the reporter plasmid than with a single injection (30, 44). When serial sections of kidney were studied after intravenous somatic gene transfer using pGL2-Luc, a significant positive staining (20–35%) was observed in glomerular endothelial cells, arterial and arteriolar endothelial cells, and peritubular capillary endothelial cells (Fig. 5b, left, and Table IV). Other renal cells, such as tubular epithelial cells, were negative. Only negative staining results were obtained in animals transfected with the control plasmid pGL2-basic (Fig. 5b, right, and Table IV). A similar transfection efficiency was found in isolated PBMC (Fig. 5c and Table IV).

Role of NFκB in LPS-mediated lethality and activation of coagulation. When mice were treated by intravenous somatic gene transfer with IκBα (or vector pXT1 alone) 5 d and 24 h before administration of LPS, a significant reduction in mortality was observed (Fig. 6). None of the animals died after 24 h. Thus, the mortality caused by LPS in this animal model was in part dependent on NFκB, since inhibition of NFκB by its inhibitor IκBα increased the number of surviving animals. These data confirm that activation of NFκB binding activity in LPS-treated animals might play a central role in setting motion-effect mechanisms relevant to the outcome of endotoxemia.

Table III. Predictive Power of NFκB Binding Activity and APACHE-II Score

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<tr>
<th>Discriminant score</th>
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<th>Nonsurvivors (group 2)</th>
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<tr>
<td>APACHE-II score</td>
<td>&gt; 14</td>
<td>83%</td>
<td>79%</td>
</tr>
<tr>
<td>NFκB binding activity</td>
<td>&gt; 137</td>
<td>89%</td>
<td>74%</td>
</tr>
</tbody>
</table>

Discriminant analysis of APACHE-II score and NFκB binding activity in PBMC of survivors and nonsurvivors for each analysis point, starting at the day of diagnosis.
Using the above established protocol of intravenous somatic gene transfer, IκBα was effective in suppressing LPS-mediated NFκB translocation to the nucleus (Fig. 7, a and b). When animals were pretreated with control vector pGL2-basic alone, a strong induction of NFκB binding activity by LPS was observed in EMSA from renal extracts prepared as described in Methods. Pretreatment with IκBα (but not mutated jun) reduced the LPS-mediated NFκB activation. Similar data were obtained using the NFκB consensus motif (Fig. 7a) or the NFκB binding region derived from the tissue factor promoter (Fig. 7b).

Tissue factor, the central activator of the coagulation mechanism, is known to be involved in the lethal effects of LPS. Tissue factor transcription in vitro and in vivo is under control of NFκB and AP-1 (12–15, 30–34). Treatment of mice with LPS resulted in induction of renal NFκB binding activity to the tissue factor NFκB motif (Fig. 7b) and induction of renal tissue factor antigen expression (Fig. 8, A and B). When intravenous somatic gene transfer with IκBα was performed, an attenuation of LPS-mediated renal tissue factor antigen expression compared with the vector control pXT1 was evident (Fig. 8, C vs. B). Thus, IκB pretreatment reduced NFκB binding activity to the NFκB motif in the tissue factor promoter and thereby
Role of NFκB in the Mortality of Sepsis

Figure 7. Effect of gene transfer with IkBα on LPS-mediated NFκB activation. Nuclear extracts from kidneys were prepared 8 h after injection of 1.75 μg LPS and 15 mg d-galactosamine. 10 μg of nuclear extract was incubated with the NFκB consensus sequence (a) or the NFκB motif derived from the tissue factor promoter (b). Shown is the effect of somatic gene transfer with either the control vector pXT1, IkBα, or mutated jun on NFκB binding activity. The specificity of the binding reaction was shown by competition with a 160-fold molar excess of unlabeled NFκB consensus oligonucleotide. The experiment was repeated independently with three kidneys from three animals with similar results.

Table IV. Quantification of Cells Expressing Luc after Intravenous Somatic Gene Transfer

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell type</th>
<th>% positive cells (pGL₂-Luc expression vector transfected)</th>
<th>% positive cells (pGL₂-basic control vector transfected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Glomerular endothelial cells</td>
<td>25–35%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Arterial and arteriolar endothelial cells</td>
<td>20–30%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Peritubular capillary endothelial cells</td>
<td>25–30%</td>
<td>0</td>
</tr>
<tr>
<td>Blood</td>
<td>PBMC</td>
<td>30–40%</td>
<td>0</td>
</tr>
</tbody>
</table>

Intravenous somatic gene transfer with the pGL₂-Luc expression plasmid or the promoterless vector pGL₂-basic was performed and Luc expression was demonstrated using an anti-Luc antibody as described in Methods. Cells not indicated above were luciferase negative. The data were obtained from 10 sections for each of 10 mice. Similar results were obtained from three independent transfections.

Reduced expression of the gene product. Accordingly, somatic gene transfer with the sense construct pXTF-s did further increase the amount of tissue factor antigen detected after LPS induction (Fig. 8 D), while pretreatment with the tissue factor antisense construct pXTF-as significantly decreased LPS-mediated tissue factor expression (Fig. 8 E).

Tissue factor has been shown to mediate fibrin deposition in a mouse tumor model (30, 44). Consistent with the data shown in Fig. 7 b and Fig. 8, a reduction in LPS-mediated renal fibrin/fibrinogen deposition was also observed (Fig. 9), when mice were pretreated with IkBα (Fig. 9 C) or the antisense tissue factor plasmid pXTF-as (Fig. 9 E). Extensive in vivo perfusion of the animal with buffer before harvesting the organs ensured that all the unclotted material was removed before staining. Hence, despite the lack of antibody specificity for fibrin, the immunoreactive material is likely to represent in large part fibrin (see also difference of control kidneys vs. LPS-treated kidneys; Fig. 9, A and B) (30, 44).

Figure 6. Effect of gene transfer with IkBα on LPS-mediated mortality. Survival was determined in mice treated with 1.75 μg LPS and 15 mg d-galactosamine (see Methods). 5 d and 24 h before LPS injection mice were treated with the IkBα expression plasmid (n = 32) or control vector pXT1 (n = 32) by intravenous somatic gene transfer (see Methods). The data give the percentage of surviving animals vs. time. No deaths occurred after 24 h. The difference between IkBα-treated animals and animals that had received the control vector pXT1 was statistically significant on the basis of P < 0.05. The experiment was repeated three times with identical results.
Using the above established protocol of intravenous gene transfer with IκBα, we studied whether the LPS-mediated increase in transcriptional activity of the tissue factor promoter-Luc plasmid pGLTF4 was dependent on NFκB. Mice were pretreated with the control vector pXT1 or IκBα/H9251 together with pGLTF4. Compared with pXT1-transfected mice, attenuation of tissue factor promoter activity after LPS treatment was observed in IκBα/H9260-pretreated mice (Fig. 11b). Thus, LPS-induced activation of the transcriptional activity of the tissue factor promoter is in part dependent on LPS-mediated NFκB activation.

**Role of tissue factor in LPS-mediated lethality and activation of coagulation.** Previous data have shown that neutralization of the tissue factor pathway increases survival and blocks activation of coagulation after LPS (45–47, 65). It is conceivable that preformed cryptic tissue factor is responsible for the LPS-mediated activation of the coagulation mechanism. Another possibility is that the enhanced vascular permeability during sepsis results in exposure of blood components. To study whether NFκB-dependent activation of tissue factor transcription is involved in the lethal effect of LPS, we performed intravenous somatic gene transfer with tissue factor antisense plasmids and compared the effect on survival with tissue factor sense plasmids or pXT1 vector alone. Intravenous somatic gene transfer with tissue factor cDNA in the antisense direction has been shown to reduce TNFα-mediated tissue factor expression, activation of the coagulation mechanism, and fibrin deposition in a mouse tumor model (44). The tissue factor antisense plasmid pXTF-as significantly increased the number of surviving animals (Fig. 12a), while the tissue factor sense plasmid pXTF-s reduced survival compared with the control vector pXT1. The effect of tissue factor antisense was dependent on the dose of LPS used (Fig. 12b). No effect was present at very high LPS doses.

Treatment with the tissue factor antisense construct reduced LPS-mediated tissue factor transcription (Fig. 13F) and tissue factor antigen induction (Fig. 8E) as well as fibrin/fibrinogen deposition (Fig. 9E) in the kidneys. Furthermore, decreased formation of TAT was observed in tissue factor antisense–pretreated animals (Fig. 14). Thus, induction of tissue factor

![Figure 8. Effect of gene transfer with IκBα and tissue factor expression plasmids on the LPS-mediated tissue factor antigen expression. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg β-galactosamine) and stained with an anti–tissue factor antibody. Animals were pretreated by intravenous gene transfer with the control vector pXT1, IκBα, or tissue factor expression plasmids. A, pXT1 vector; B, pXT1 vector plus LPS; C, IκBα plus LPS; D, tissue factor sense (pXTF-s) plus LPS; and E, tissue factor antisense (pXTF-as) plus LPS. Four kidneys from three independent experiments were analyzed. From each kidney, at least five sections were stained with similar results.](image-url)
Role of NF\textsuperscript{κB} in the Mortality of Sepsis

Factor transcription and expression is involved in the lethal effect of LPS and activation of coagulation.

Discussion

Most of the data describing a role of NF\textsuperscript{κB} in inflammatory disease are derived from in vitro studies. We used sepsis and LPS-induced endotoxemia as models to study the role of NF\textsuperscript{κB} activation in vivo. Fatal outcome in patients with sepsis was predicted by an increase in NF\textsuperscript{κB} binding activity PBMC after day 1. All patients with an NF\textsuperscript{κB} binding activity exceeding 200% of day 1 died. These data support the concept that NF\textsuperscript{κB} activation might be an important event in clinical sepsis. However, the number of patients studied was too small to allow for a final statement. In addition, a correlation between the APACHE-II score and the NF\textsuperscript{κB} binding activity of circulating cells cannot prove a causal relationship even in a larger clinical trial.

To verify a causal relationship between NF\textsuperscript{κB} activation and endotoxemia, intravenous somatic gene transfer with an expression plasmid coding for I\textsuperscript{κB} (the inhibitor of NF\textsuperscript{κB}) was tested as a tool for analyzing the role of NF\textsuperscript{κB} in a standard mouse model (51, 52), in which the addition of d-galacto-
tosamine conditioned the mice to mortality 8–12 h after LPS administration. This model allowed us to clearly define the role of NFκB activation in endotoxemia-mediated lethality. As shown previously (30, 44), intravenous somatic gene transfer can be used to affect biological responses of circulating blood cells and resting cells, including endothelial cells. Intravenous somatic gene transfer with an IκBα expression plasmid reduced LPS-mediated NFκB activation in renal extracts and increased survival after LPS. The experiments provide evidence that NFκB partly mediates LPS-induced mortality. However, they do not prove that the LPS effect itself is dependent only on this transcription factor. The only partial prevention of mortality could be due to several reasons: (a) involvement of other transcription factors; (b) non–transcription-dependent mediators; and (c) a low transfection efficiency by intravenous somatic gene transfer (∼20–40%, Table IV). IκBα preferentially inhibits NFκB p50 and p65. The characterization of the proteins binding to the NFκB consensus motif also revealed the presence of other members of the NFκB family (Fig. 2 c).

Thus, a complete inhibition of NFκB cannot be expected by gene transfer with IκBα. However, intravenous somatic gene transfer was at least partially effective as evidenced by the inhibition of NFκB translocation shown in EMSAs of renal extracts (Fig. 7). Therefore, this method may be used to further delineate the possible mechanism of endotoxemia at the level of the transcription factors involved. It has to be noted that an animal model of LPS-mediated lethality reproduces only partly human septicemia. Although the animal model cannot directly be transferred to the clinical situation, it provides a rational basis for the hypothesis that NFκB activation observed in patients has a predictive value in determining the outcome of sepsis.

Data shown represent percent survival vs. time. No deaths occurred after 24 h. The experiment was repeated three times with similar results. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of \( P < 0.05 \). (b) Dose dependence. Mice were treated with the tissue factor expression plasmids (Sense, \( n = 32 \); Antisense, \( n = 32 \); or pXT1, \( n = 12 \)) 5 d and 24 h before administration of LPS. Shown is the survival of the 12 animals 24 h after LPS administration. The difference between LPS-treated animals that had received the sense tissue factor construct pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of \( P < 0.05 \) as long as the LPS dose did not exceed 1.75 μg per mouse.
Gene transfer with IkBα was not effective when given simultaneously with or after LPS. Thus, IkBα has to be expressed before LPS is injected, which is compatible with NFκB-dependent gene induction after LPS administration. The hypothesis is that gene transfer has to affect the cell before an amplification limb releases mediators critical in the pathophysiology of endotoxemia. This might explain the lack of effectiveness of TNFα neutralizing antibodies in clinical trials. If this hypothesis is correct, then one has to assume that NFκB activation may not only occur in cells stimulated by LPS, but also in neighboring cells stimulated by mediators released from target cells of LPS. Future studies will have to determine which cells are indirectly affected by LPS. However, since we were able to show that endothelial cells and monocytes/macrophages were transfected by intravenous somatic gene transfer (Fig. 5), these cells might be most probably the critical targets for the action of LPS.

Antibodies against TNFα have been shown to improve survival of animals in endotoxemia models, however, they failed to significantly improve survival in patients with septicemia. An important difference between animal studies and human disease is the time point of intervention. In animal studies, TNFα neutralizing antibodies are applied much earlier in the course of the disease than in humans sepsis, in which patients have been ill for some time before a diagnosis is made. Since timing of administration and dose of TNFα neutralizing antibodies are critical for efficient reduction of endotoxia-dependent mortality in animals (66), different timing of TNFα neutralizing antibodies might account for the conflicting results obtained in animal models and clinical trials. Cytokines other than TNFα might also contribute to septicemia-mediated NFκB activation and subsequent gene expression. Cytokine activation of NFκB lasts in vitro for only short time; therefore, we hypothesize that yet unknown mechanisms perpetuate NFκB activation and NFκB-dependent gene expression observed in prolonged inflammatory response. In contrast to the short-lasting NFκB activation induced by TNFα, we observed recently that ligands of the TNFα-inducible cellular receptor RAGE mediate perpetuated NFκB activation (Bierhaus, A., and P.P. Nawroth, unpublished observations). Thus, blocking TNFα by neutralizing antibodies might only in part inhibit LPS-dependent NFκB activation and therefore fail to inhibit pathways of NFκB activation downstream of TNFα. Furthermore, it has been reported that TNFα antibodies specifically inhibited fibrinolysis and thereby enhanced the risk for microvascular thrombosis (23). Thus, in the course of human sepsis side effects of TNFα neutralizing antibodies might cause long-term complications and mortality, which are not evident in time-defined animal models.

Tissue factor has been described previously as an impor-

Figure 13. Effect of tissue factor expression plasmids on LPS-mediated renal tissue factor transcription. Kidneys were harvested 8 h after treatment with LPS (1.75 μg LPS, 15 mg d-galactosamine) and analyzed by in situ hybridization for the presence of tissue factor transcription. Animals were pretreated by intravenous somatic gene transfer with the control vector pXT1 and the tissue factor sense and antisense expression plasmids. A, pXT1 vector; B, tissue factor sense (pXTF-s); C, tissue factor antisense (pXTF-as); D, pXT1 vector plus LPS; E, tissue factor sense (pXTF-s) plus LPS; F, tissue factor antisense (pXTF-s) plus LPS. Four kidneys from three independent experiments were analyzed. From each kidney, at least five sections were stained with similar results.
Experiment the difference between LPS-treated animals that had received the sense tissue factor plasmid pXTF-s or the control vector pXT1 compared with animals pretreated with the antisense tissue factor plasmid pXTF-as was statistically significant on the basis of $P < 0.05$.

**Figure 14.** Effect of tissue factor on LPS-induced TAT formation. TAT were determined in mice 8 h after treatment with 1.75 µg LPS and 15 mg ng-galactosamine. Mice were pretreated by intravenous somatic gene transfer with control vector pXT1 (black bar, n = 5), tissue factor sense (pXTF-s; open bar, n = 5), or tissue factor antisense (pXTF-as; hatched bar, n = 5). The data represent the mean of three independent experiments ± SD.

Tissue factor is not the only mediator involved in LPS-mediated mortality, since antisense tissue factor prevented only part of the LPS effect. One explanation is the rather low transfection efficiency of intravenous somatic gene transfer. Another explanation is that other genes controlled by NFκB, such as cytokines, endothelin-1, leukocyte adhesion molecules, and others, are also involved in endotoxemia. Moreover, mediators not dependent on protein synthesis might also be involved. Thus, the patient study and the animal study complement each other in demonstrating that LPS-mediated activation of the transcription factor NFκB plays a central role in human disease and in an animal model of endotoxemia. Intravenous somatic gene transfer with hκBα can be used to analyze the contribution of NFκB to endotoxemia. Further applications of this approach may include ischemia–reperfusion and transplant rejection.

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**References**


