

Inhibition of Endothelial Cell Activation by Adenovirus-mediated Expression of I κ B α , an Inhibitor of the Transcription Factor NF- κ B

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Summary

During the inflammatory response, endothelial cells (EC) transiently upregulate a set of genes encoding, among others, cell adhesion molecules and chemotactic cytokines that together mediate the interaction of the endothelium with cells of the immune system. Gene upregulation is mediated predominantly at the transcriptional level and in many cases involves the transcription factor nuclear factor (NF) κ B. We have tested the concept of inhibiting the inflammatory response by overexpression of a specific inhibitor of NF- κ B, I κ B α . A recombinant adenovirus expressing I κ B α was constructed (rAd.I κ B α) and used to infect EC of human and porcine origin. Ectopic expression of I κ B α resulted in marked, and in some cases complete, reduction of the expression of several markers of EC activation, including vascular cell adhesion molecule 1, interleukins 1, 6, 8, and tissue factor. Overexpressed I κ B α inhibited NF- κ B specifically since (a) in electrophoretic mobility shift assay, NF- κ B but not AP-1 binding activity was inhibited, and (b) von Willebrand factor and prostacyclin secretion that occur independently of NF- κ B, remained unaffected. Functional studies of leukocyte adhesion demonstrated strong inhibition of HL-60 adhesion to I κ B α -expressing EC. These findings suggest that NF- κ B could be an attractive target for therapeutic intervention in a variety of inflammatory diseases, including xenograft rejection.

Regulation of gene expression at the transcriptional level is governed by the presence of distinct transcription factors binding to specific sites in the promoter region, and in some cases other regions, of the respective gene. Prominent among transcriptional activators for inducible genes in general is the transcription factor nuclear factor (NF)¹ κ B. NF- κ B is involved in the transient expression of many different genes in a variety of cell types and in response to diverse physiological and pathological stimuli. Examples include the expression of IL-2, TCR, I κ light chain, MHC-1, β -IFN, G-CSF, GM-CSF, proenkephalin, angiotensinogen, and acute phase genes, the majority of which are involved in immune- and host-defense functions (for reviews, see references 1–4). In endothelial cells (EC), genes that are dependent on NF- κ B include, among oth-

ers, IL-1, -6, and -8, monocyte chemoattractant protein 1, E-selectin, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), inducible nitric oxide synthase and tissue factor. Deletion mutagenesis and reporter gene analysis have demonstrated that NF- κ B is necessary, although usually not alone sufficient, for the expression of these genes.

NF- κ B represents a family of proteins comprising different members, including Rel (c-Rel; 5), RelA (p65; 6–8), RelB (9, 10), NF- κ B1 (p50; 11–13), and NF- κ B2 (p52; 14–17). They form homo- or heterodimers with different affinities for variants of a decameric consensus binding site (GGGRNNYYCC, where *R* indicates A or G, *Y* indicates C or T, and *N* indicates any base). The prototype NF- κ B is composed of the p50/p65 subunits, however, in EC it is primarily the p65/c-Rel heterodimer that has been implicated in regulatory functions. Several genes relevant for EC contain an atypical NF- κ B binding site with the sequence HGGRNNYYCC (where *H* indicates A, C, or T), suggesting a certain degree of cell type specificity of the otherwise ubiquitous NF- κ B family (18).

Regulation of NF- κ B occurs through complex formation of the heterodimer with a cytoplasmic inhibitor, I κ B α (19–

¹Abbreviations used in this paper: EC, endothelial cells; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein EC; moi, multiplicity of infection; NF, nuclear factor; NLS, nuclear localization sequence; PAEC, porcine aortic EC; rAd.I κ B α , recombinant adenovirus expressing I κ B α ; TLCK, L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanone-hydrochlorid; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor.

22). I κ B α retains the p65-containing NF- κ B complex in the cytoplasm by masking its nuclear localization sequence (23, 24). Upon appropriate stimulation, e.g., by IL-1 α , TNF- α , or LPS, I κ B α is phosphorylated on two serine residues, S32 and S36 (25, 26). This phosphorylation renders I κ B α susceptible to proteolytic degradation via the ubiquitin-proteasome pathway (27, 28), resulting in release and nuclear translocation of NF- κ B. Resynthesis of I κ B α occurs within 60 min through transcriptional activation by NF- κ B p65 and c-Rel (29–33).

In addition to retaining NF- κ B in the cytoplasm, a second function has been demonstrated for I κ B α : it can prevent in vitro DNA binding of NF- κ B, namely of the p65 and c-Rel subunits (19, 34). Taking advantage of this attribute, we have constructed a recombinant adenovirus that expresses I κ B α (rAd.I κ B α) fused to a nuclear localization sequence (NLS). Targeting of the ectopically expressed I κ B α to the nucleus should prevent its degradation by the cellular (cytoplasmic) degradation machinery and allow inhibition of NF- κ B binding to its DNA target sequence(s). As described here, infection of EC with rAd.I κ B α results in successful inhibition of inflammatory cytokine-induced gene expression, procoagulant activity, and leukocyte adhesion to EC in vitro.

Materials and Methods

EC Culture. Enzymatic isolation and culture of EC from porcine aorta was done as described (35). Porcine aortic EC (PAEC) were grown on gelatine-coated cell culture flasks (Nunc, Roskilde, Denmark) in complete DMEM supplemented with 10% FCS and glutamine. Human umbilical vein EC (HUVEC) were purchased from Technoclone (Vienna, Austria) and grown in medium 199 supplemented with 20% bovine calf serum, EC growth factor, and heparin. For stimulation, 500 U/ml human recombinant IL-1 α , 200 U/ml human recombinant TNF- α (both from Genzyme Corp., Cambridge, MA), 500 ng/ml bacterial LPS, or 3 U/ml human thrombin (Sigma Chemical Co., St. Louis, MO) was used.

Construction of Recombinant Adenovirus. Construction of recombinant adenovirus was done as described (36). The first ATG of the porcine I κ B α cDNA (22) was replaced by a BamHI restriction site using PCR. A double-stranded oligonucleotide (NLS-1; 5'-CTAGAGTCGACATGCCCAAGAAGAAGAGGAAGGTGGGCGCG-3') encoding an initiator methionine followed by the SV40 large T Ag NLS and three glycine residues as a flexible spacer was ligated into the newly generated BamHI site. The construct was sequenced to exclude possible errors generated during the amplification procedure, ligated into the vector pACCMV-pLpASR+ (36), and cotransfected together with pJM17, a plasmid containing the adenoviral genome with a deletion in the E1 region (37) into 293 cells (Fig. 1 A). Clones obtained after subcloning on 293 cells were tested for I κ B α expression by Western blotting using an anti-MAD-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1,500. Purification of a large batch of the recombinant adenovirus was done by two consecutive cesium chloride centrifugations, as described (38).

Infection of EC. Postconfluent PAEC were washed once with complete PBS and incubated at a multiplicity of infection (moi) of 1,000 with rAd.I κ B α or control adenovirus (DL-312; 39) in

PBS. After 30 min at 37°C, the adenovirus was washed off and fresh growth medium added. Cells were maintained for 3 d more until assayed. HUVEC were treated identically, except that a 10-fold lower amount of adenovirus was used (moi of 100).

RNA Isolation and Northern Blotting. RNA was extracted from PAEC, fractionated on formaldehyde/agarose gels, transferred to Hybond N membranes (Amersham Corp., Arlington Heights, IL) and hybridized to random-primed cDNA probes as described (40). Two oligonucleotides, one complementary to NLS-1 (see above) and one complementary to position 92–118 (22) in the 5' untranslated region of porcine I κ B α (5'UTR; 5'-ATGAGGGC-TTGGGCGCTGCTCCCTAGGT-3') were used as probes to distinguish between endogenous and adenovirus-expressed I κ B α . Equivalent loading and transfer of RNA was confirmed by ethidium bromide staining of the gel and of the membrane after transfer.

Immunostaining. Paraformaldehyde-fixed, NP-40 permeabilized cells were stained for I κ B α using an anti-MAD-3 antibody at a dilution of 1:1,000 and a peroxidase-conjugated donkey anti-rabbit second antibody (Amersham Corp.). Detection was performed using a metal-enhanced diaminobenzidine substrate kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol.

Western Blotting. Cells were lysed in Laemmli buffer and analyzed by SDS-PAGE on 12.5% Minigels (Mini-Protean II; Bio-Rad Laboratories, Richmond, CA). For separation of the phosphorylated and nonphosphorylated forms of I κ B α , samples were run on 30-cm gels (10%). I κ B α was detected using the anti-MAD-3 antibody at a dilution of 1:1,500 and peroxidase-conjugated donkey anti-rabbit second antibody followed by enhanced chemiluminescence (ECL) detection (Amersham Corp.).

Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear proteins were extracted from PAEC, either infected with control virus or rAd.I κ B α , stimulated with 500 ng/ml TNF- α for 1 h or from nonstimulated cells, as described (41). The double-stranded oligonucleotides BS-2 representing a NF- κ B binding site from the porcine I κ B α promoter (5'-AATTCGGCTTGAAATTC-CCCAGCG-3'; 22), and the oligonucleotide TFAP-1 containing the tandem AP-1 site from the tissue factor promoter (5'-AATGGGTTGAATCACGGTGAATCAGCCCTTGCAGG-3'; 42) were labeled by filling in the EcoRI overhangs with Klenow enzyme in the presence of radioactive nucleoside triphosphates and 0.2 ng (100,000 cpm) used per lane in electrophoretic mobility shift assay (EMSA). The resulting complexes were separated on 5% polyacrylamide gels.

Cell ELISA. HUVEC were grown in 96-well microtiter plates and cell ELISA performed essentially as described (43). Briefly, cells were fixed with glutaraldehyde and blocked overnight in PBS/5% BSA. Expression of VCAM-1 was determined using a monoclonal anti-VCAM-1 antibody at a 1:500 dilution (BBA5; British Biotechnology Ltd., Cowley, United Kingdom) and peroxidase-coupled goat anti-mouse second antibody (Amersham Corp.), followed by *o*-nitrophenylene-diamine detection. Equal numbers of cells in the assay were determined by staining with sulforhodamine B (Sigma Chemical Co.).

Determination of Procoagulant Activity. PAEC were stimulated for 6 h with 500 ng/ml LPS, transferred to Eppendorf tubes, collected by centrifugation, and resuspended in clotting buffer (12 mM Na-acetate, 7 mM Na-barbital, 130 mM NaCl, pH, 7.4). A 100- μ l cell suspension was combined with 100 μ l pig plasma (Sigma Chemical Co.) and 100 μ l of 20 mM CaCl₂, and incubated at 37°C. Time until clot formation was recorded and transformed into thromboplastin units using a standard curve.

Determination of von Willebrand Factor Secretion. HUVEC grown

in 24-well plates were stimulated with 3 U/ml thrombin for 30 min. Supernatants were harvested and assayed for von Willebrand factor (vWF) using the Imubind vWF sandwich ELISA (American Diagnostica Inc., Greenwich, CT). Toxicity was controlled

by assaying lactate dehydrogenase release (CytoTox96, Promega Corp., Madison, WI).

Cell Adhesion Assay. HUVEC grown in 24-well microtiter plates were infected with rAd.I κ B α or control virus. 3 d later, cells were stimulated with 500 U/ml IL-1 α (Genzyme Corp.) for 4 h or left unstimulated. HL-60 cells were labeled with CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Inc., Eugene, OR) at a concentration of 5 μ M for 20 min at 37°C, as described by the manufacturer. Labeled HL-60 cells (10^6) were added to the HUVEC cultures in a total volume of 0.4 ml and incubated for 30 min at 37°C. After two washes with PBS deficient, adhered HL-60 cells were quantitated by measuring the fluorescence using 490- (excitation) and 520-nm (emission) filters.

Determination of Prostacyclin. Prostacyclin (PGI $_2$) was determined by measuring the concentration of its hydrolysis product 6-keto-prostaglandin F $_{1\alpha}$ by enzymeimmunoassay according to the manufacturer's protocol (Amersham Corp.).

Results

Construction and Characterization of a rAd.I κ B α . Cotransfection of I κ B α , cloned into the adenovirus transfer vector pACCMVpLpASR+, together with the plasmid pJM17 into 293 cells (Fig. 1 A) resulted in the generation of recombinant adenovirus. After subcloning of the virus pool, individual clones were obtained and infected 293 cells were tested by Western blotting for I κ B α expression. All 15 clones tested expressed an immunoreactive protein of the expected size of I κ B α . One clone (clone 15) was expanded further and used in the subsequent experiments.

After infection of PAEC with the recombinant adenovirus, increasing expression of I κ B α was observed up to day 3, with no further increase at day 4, as determined by Western blotting (Fig. 1 B). The amounts expressed were estimated to be about 50 times higher as compared to the endogenous protein (data not shown). Stimulation of the infected cells at day 3 with LPS for 1 h did not detectably decrease I κ B α levels. In contrast, in noninfected EC the endogenous protein was rapidly degraded followed by resynthesis (Fig. 1 C), in accordance with published observations (44). To determine whether addition of the NLS affects the ability of I κ B α to be phosphorylated, rAd.I κ B α -infected cells were treated at day 3 after infection with LPS or with the phosphatase inhibitor okadaic acid, and analyzed by high resolution SDS-PAGE and Western blotting to resolve the phosphorylated and nonphosphorylated forms of I κ B α . Treatment with okadaic acid resulted in the conversion of \sim 50% of I κ B α into the phosphorylated form within 45 min, whereas LPS stimulation did not lead to generation of detectable amounts of phosphorylated I κ B α (Fig. 1 D). These results indicate that overexpressed, NLS-tagged I κ B α can still be phosphorylated, but only by strong nonphysiological stimuli (see Discussion). Treatment with the protease inhibitor L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochlorid (TLCK) alone in the absence of additional stimuli also leads to I κ B α phosphorylation, possibly due to activation of the EC by the alkylating properties of TLCK.

To achieve expression of I κ B α in 90–100% of EC, a

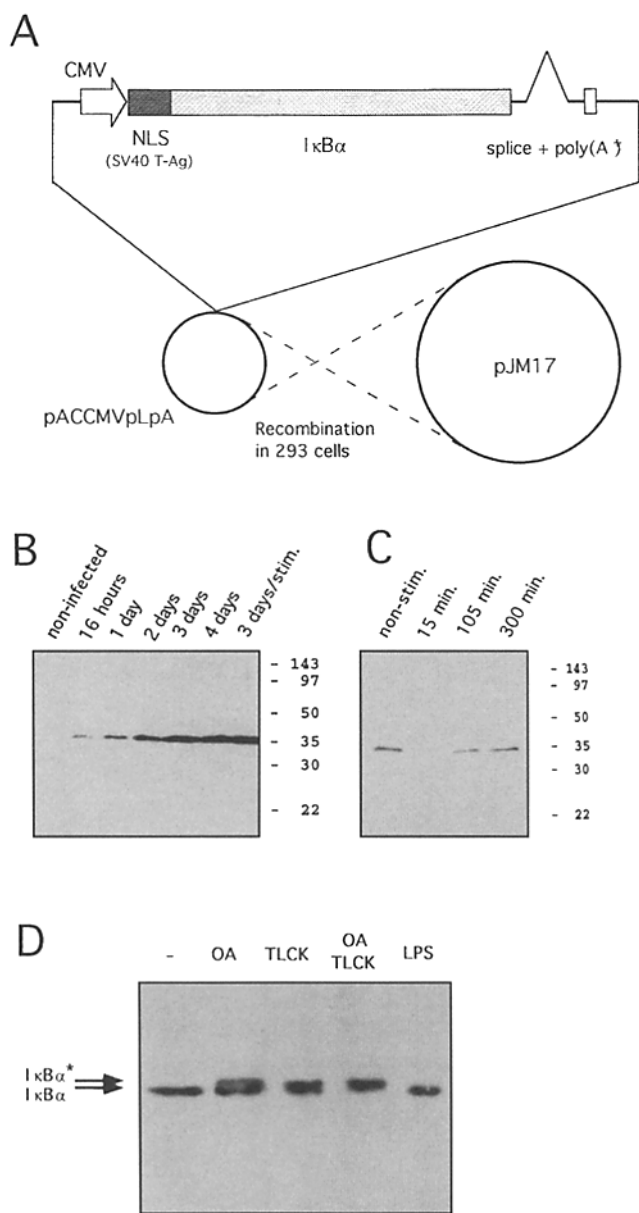


Figure 1. Construction and analysis of recombinant adenovirus. (A) Schematic representation. (B) Kinetics of adenovirus-mediated I κ B α expression. PAEC were infected with the rAd.I κ B α and, after the indicated periods of time, analyzed by SDS-PAGE (on minigels) and Western blotting using an anti-I κ B α antibody. In the last lane, cells were treated with TNF- α for 1 h at day 3 after infection. Numbers on the right indicate molecular weight (kD). (C) Endogenous I κ B α is degraded and resynthesized in noninfected EC after stimulation with TNF- α . Cells were analyzed as in B with the anti-I κ B α antibody after the indicated periods of time. (D) I κ B α -expressing cells were treated with the phosphatase inhibitor okadaic acid (OA), a protease inhibitor (TLCK), or with LPS for 45 min and analyzed by high resolution SDS-PAGE and Western blotting. (I κ B α *) Position of the phosphorylated form of I κ B α . Exposure times using ECL detection were 30 s in B and D and 10 min in C.

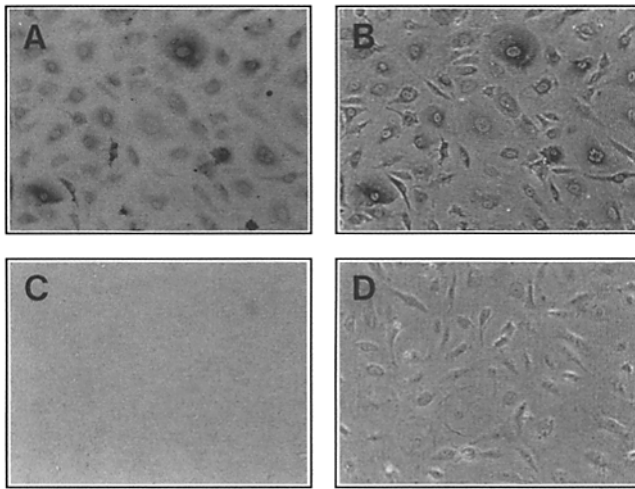


Figure 2. Immunoperoxidase staining of HUVEC infected with rAd.IκBα. 3 d after infection, cells were stained with an anti-IκBα antibody followed by immunoperoxidase detection. (A and B) rAd.IκBα-infected; (C and D) control virus infected; (A and C) without phase contrast; (B and D) with phase contrast.

moi of 100 had to be used for HUVEC, as determined by immunoperoxidase staining (Fig. 2). In contrast, a moi of 1,000 was needed to obtain the same percentage of PAEC staining positive for IκBα, indicating a significantly lower infectivity of the (human) adenovirus towards porcine cells (not shown). However, the localization of IκBα appeared to be heterogeneous: although IκBα was detected in the nucleus, most cells also expressed the protein to varying amounts in the cytoplasm. Since the SV40 TAG NLS has been successfully transferred to a number of heterologous proteins in the past, the partial nuclear transport of NLS-IκBα might be due to inefficient recognition of the NLS, possibly because of steric hindrance.

Northern blot analysis showed high levels of IκBα mRNA in rAd.IκBα-infected, but only small amounts corresponding to the endogenous gene in control virus-infected PAEC (Fig. 3 A). To distinguish whether increased IκBα mRNA expression is of endogenous or viral origin, hybridization was performed with an oligonucleotide specific for a sequence (NLS) present only in the adenovirus-expressed but not in the endogenous IκBα. A specific band was detected only in rAd.IκBα-infected, but not in noninfected or control virus-infected cells (Fig. 3 B). In turn, an oligonucleotide directed against the 5'-untranslated region of IκBα (a region that is not present in the rAd.IκBα expression construct) recognized only the endogenous IκBα. Consistent with previous findings demonstrating the requirement of NF-κB for IκBα expression (22), endogenous IκBα was downregulated in rAd.IκBα-infected cells, whereas it was upregulated in PAEC after stimulation with LPS (Fig. 3 C).

Inhibition of DNA Binding of NF-κB. The consequences of adenovirus-mediated overexpression of IκBα on NF-κB are shown in EMSA. Nuclear extracts from either noninfected, nonstimulated or stimulated PAEC, from control virus-infected/stimulated and from rAd.IκBα-infected/

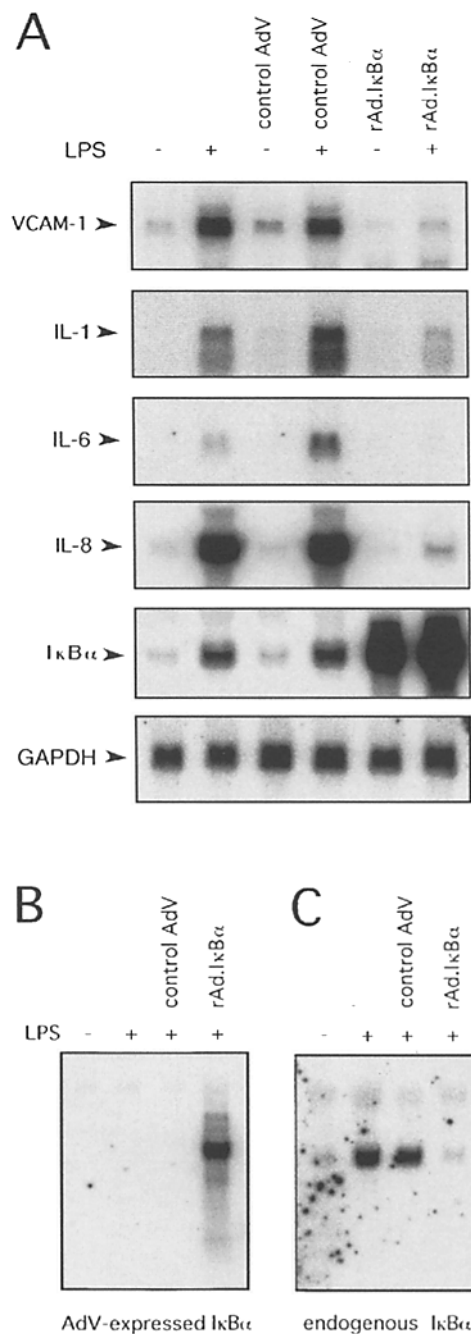


Figure 3. Northern blot analysis of rAd.IκBα-infected cells. PAEC were infected with either the rAd.IκBα or a control adenovirus and left unstimulated or stimulated with LPS as indicated above each lane. Total RNA was isolated and probed for (A) VCAM-1, IL-1, IL-6, IL-8, and IκBα expression as indicated. (B) IκBα expressed from the recombinant adenovirus was detected by hybridization with an oligonucleotide recognizing a sequence in the expression construct. (C) Endogenous IκBα was detected by hybridization with an oligonucleotide recognizing a sequence present in the 5'-untranslated region of IκBα that had been removed from the expression construct. Equal loading and transfer of RNAs was controlled by ethidium bromide staining of the gel and of the membrane after transfer, and by hybridization with a GAPDH probe.

stimulated cells were analyzed (Fig. 4). Whereas noninfected, as well as control virus-infected cells showed strong inducible activity of NF- κ B, no detectable binding of the transcription factor was observed in rAd.I κ B α -infected cells, with NF- κ B levels even below the basal amounts of noninfected, nonstimulated cells. The inhibitory effect was specific for NF- κ B, since binding of AP-1-like factors to their respective site remained unaffected.

Inhibition of Gene Expression and Leukocyte Adhesion. Since several genes that are characteristic for the inflammatory response in EC contain functional NF- κ B binding sites in their promoter regions, we have analyzed the levels of some of these genes by Northern blotting in nonstimulated or stimulated, either noninfected, rAd.I κ B α - or control virus-infected EC. VCAM-1, IL-1 α , IL-6, and IL-8 were inducible by LPS in noninfected and control virus-infected cells, but showed between 90 and 98% reduction of their steady-state mRNA levels in rAd.I κ B α -infected cells (Fig. 3 A). In some cases (IL-1 and IL-6), induction by the control virus was observed, indicating some effect of adenovirus infection itself on the EC (45). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were not affected by I κ B α expression (Fig. 3 A).

We also determined the expression of VCAM-1 by cell ELISA. The inhibitory effect of I κ B α on VCAM-1 mRNA was reflected on the protein level, with reduction of VCAM-1 to basal levels (Fig. 5 A). Using a clotting assay as a measure-

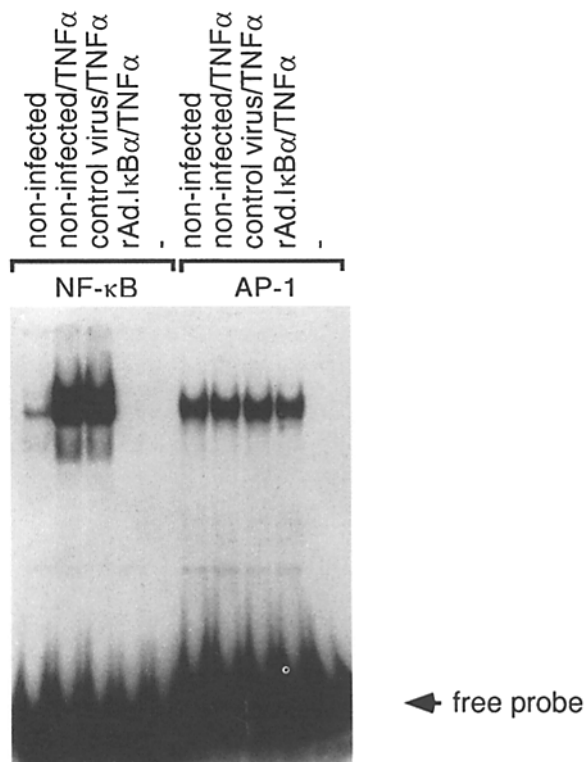


Figure 4. EMSA. PAEC were infected with either rAd.I κ B α or a control adenovirus and left unstimulated or stimulated with LPS as indicated above each lane. Nuclear extracts were prepared and analyzed by EMSA using double-stranded oligonucleotides containing either NF- κ B or AP-1 binding sites.

ment for tissue factor expression (42), virtually complete inhibition was observed (Fig. 5 B). In contrast, thrombin-induced levels of vWF, a protein that is constitutively synthesized and its secretion regulated by release from intracellular storages, remained unaffected (Fig. 5 C). In addition, the LPS-induced release of prostacyclin was similar in rAd.I κ B α -infected, noninfected, and control virus-infected cells, demonstrating that LPS signaling pathways that are distinct from NF- κ B activation are not affected.

Binding of human promyelocytic HL-60 cells to activated endothelium is mediated by several cell adhesion molecules on the EC surface, including VCAM-1 (46, 47). As expected, repression of VCAM-1 by I κ B α (and also of E-selectin and intercellular adhesion molecule 1; Wrighton, C.J., A. McShea, R. de Martin, and F.H. Buch, unpublished observations) inhibited adhesion of HL-60 cells to IL-1-stimulated HUVEC by 94% (Fig. 6).

Discussion

The current model of NF- κ B/I κ B α views this transcription factor and its inhibitor as an autoregulatory system for the control of transient gene expression. In quiescent, nonstimulated cells, complex formation with I κ B α prevents nuclear translocation of NF- κ B. After stimulation of the cells, activation of NF- κ B directly causes the expression of I κ B α , which in turn leads to the termination of NF- κ B activity and NF- κ B-dependent gene transcription a few hours after stimulation (29–33, 48). By premature expression of I κ B α , the balance between the two factors would therefore be shifted towards the “inactive” state.

Antioxidants have been used in vitro to inhibit NF- κ B in a variety of cell types, however, this class of compounds has pleiotropic effects on various other cellular functions (49). A more specific approach has utilized double-stranded phosphorothionate oligonucleotides as transcription factor “decoys,” but high concentrations of the oligonucleotides were needed to achieve inhibition in the entire cell population (50). Recently, inhibition of nuclear translocation of NF- κ B1 was demonstrated after treatment of cells with a cell-permeable peptide containing the NF- κ B1 NLS (51). In transient transfection experiments using promoter-reporter genes, cotransfection of I κ B α resulted in significantly reduced expression from the ELAM-1 as well as from an artificial NF- κ B-dependent promoter (44, 52). To assay the inhibitory effect of I κ B α on the expression of the endogenous genes and to allow biochemical analysis, it is necessary to utilize a highly efficient transfection system. Adenovirus-mediated gene transfer meets this criterion: several groups have demonstrated that, using recombinant adenovirus, up to 100% of the cells, e.g., EC, smooth muscle cells, and hepatocytes, can be infected in vitro and in vivo (53–56). Likewise, using a recombinant adenovirus, we have been able to express I κ B α in 90–100% of human and porcine EC. I κ B α was demonstrated to be expressed from the recombinant adenovirus, since: (a) control virus infection did not lead to increased I κ B α expression (Fig. 3 A);

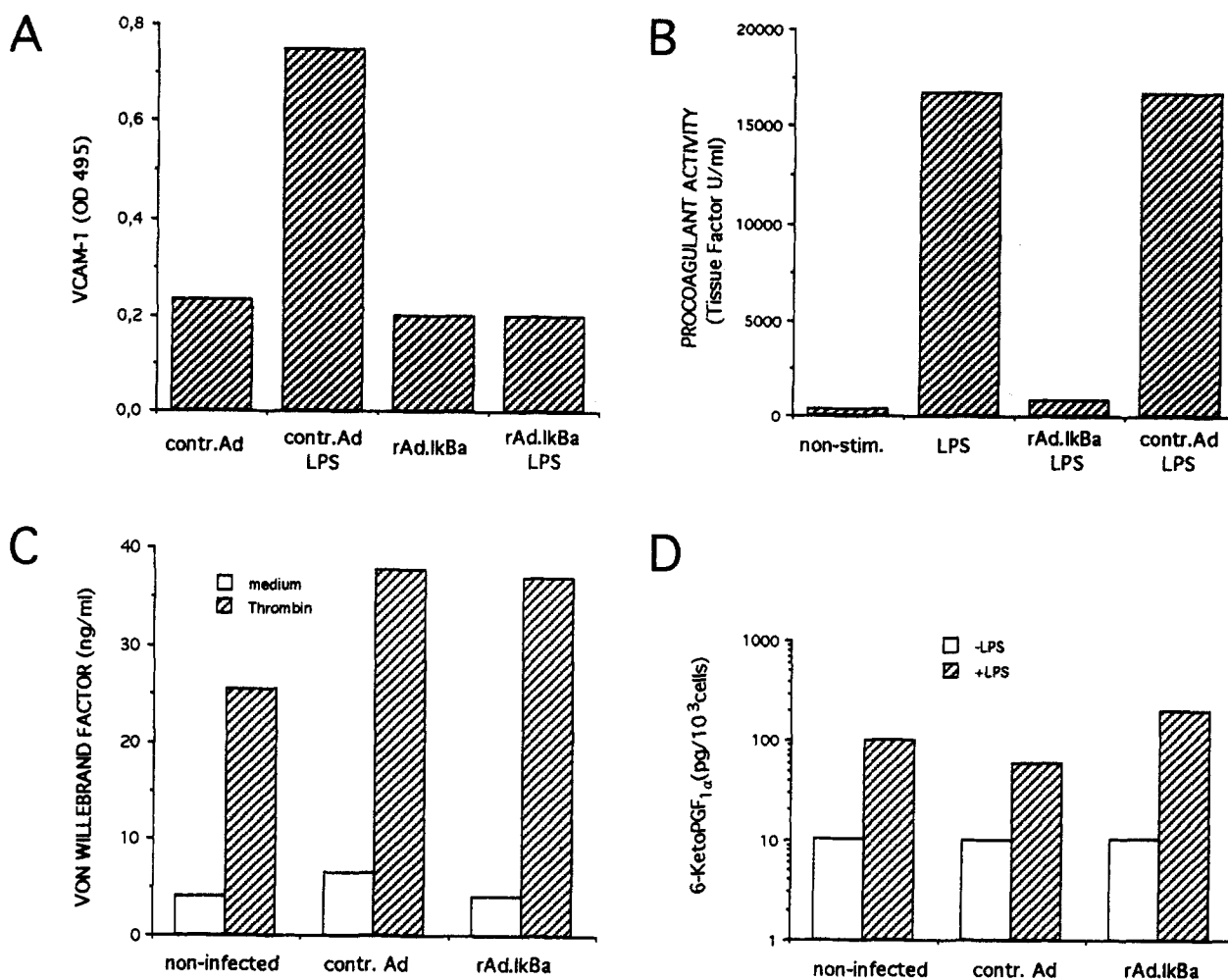


Figure 5. Determination of VCAM-1, tissue factor, von Willebrand factor, and prostacyclin in $\text{I}\kappa\text{B}\alpha$ adenovirus-infected cells. (A) HUVEC were infected with either the rAd.I $\kappa\text{B}\alpha$ or a control adenovirus and left unstimulated or stimulated as indicated. After 8 h, a cell ELISA using an anti-VCAM-1 antibody was performed. (B) PAEC were treated as above and procoagulant activity determined in a standard clotting assay. Clotting times were converted into tissue factor units using a standard curve. (C) HUVEC were treated as in (A) and vWF secretion determined by ELISA. (D) PAEC were treated as in (A), stimulated with LPS for 12 h, and prostacyclin measured by EIA.

(b) an oligonucleotide directed against the NLS of the $\text{I}\kappa\text{B}\alpha$ fusion gene detected a specific band exclusively in RNA from rAd.I $\kappa\text{B}\alpha$ -infected cells (Fig. 3 B); and (c) NLS-tagged $\text{I}\kappa\text{B}\alpha$ shown in the Western blot (Fig. 1 B) can be distinguished by its larger size from the endogenous gene product detected after long exposure (not shown). We have noted that an approximately 10-fold higher virus titer is necessary to achieve similar amounts of porcine cells to become infected as compared to human EC, suggesting partial species incompatibilities between the virus and its receptor on porcine cells.

As shown by Western blot, rAd.I $\kappa\text{B}\alpha$ -infected cells expressed high levels of $\text{I}\kappa\text{B}\alpha$, the amounts exceeding the endogenous levels by approximately 50-fold. Typically, endogenous $\text{I}\kappa\text{B}\alpha$ is rapidly degraded within minutes after stimulation of cells with agents that lead to NF- κB activation. This process has been demonstrated to require two distinct steps: first, the phosphorylation of $\text{I}\kappa\text{B}\alpha$ by a possibly very specific, yet unidentified kinase; and second, proteolytic degradation of $\text{I}\kappa\text{B}\alpha$ via the ubiquitin-proteasome pathway

(27, 28). In contrast, upon stimulation of rAd.I $\kappa\text{B}\alpha$ -infected cells, the exogenous $\text{I}\kappa\text{B}\alpha$ protein was not detectably degraded. This could only partially be due to the nuclear localization of the protein, since considerable amounts of $\text{I}\kappa\text{B}\alpha$ were found in the cytoplasm. To determine whether overexpressed NLS- $\text{I}\kappa\text{B}\alpha$ could still be phosphorylated, we treated rAd.I $\kappa\text{B}\alpha$ -infected cells with LPS and with the phosphatase inhibitor okadaic acid, and determined the amount of $\text{I}\kappa\text{B}\alpha$ phosphorylation by high resolution SDS-PAGE. Treatment with okadaic acid, but not with LPS, resulted in conversion of significant amounts of $\text{I}\kappa\text{B}\alpha$ into the phosphorylated form (Fig. 1 D). The most likely explanation for this is that, although adenovirus-expressed $\text{I}\kappa\text{B}\alpha$ can be phosphorylated, phosphorylation by LPS is limiting, sufficient for the phosphorylation of endogenous levels of $\text{I}\kappa\text{B}\alpha$ but not for the approximately 50-fold higher amounts generated by the recombinant adenovirus. These low levels of phospho- $\text{I}\kappa\text{B}\alpha$ were not detectable because of its short half-life. Attempts to stabilize the phosphorylated $\text{I}\kappa\text{B}\alpha$ by preventing its degradation us-

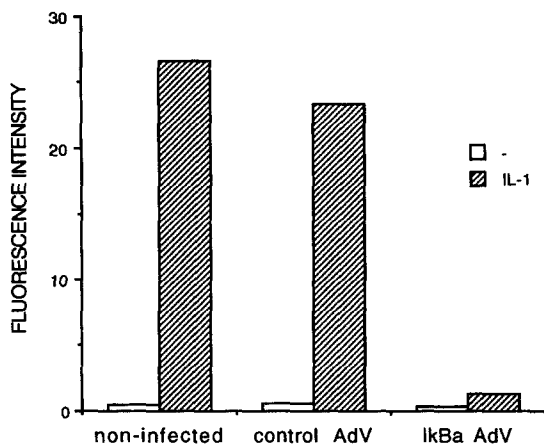


Figure 6. Cell adhesion assay. HL-60 adhesion to noninfected, rAd.IκBα- or control adenovirus-infected HUVEC was determined. (Open bars) Unstimulated EC; (hatched bars) EC stimulated with 500 U/ml IL-1α for 4 h. Numbers of adhered HL-60 cells are expressed as relative fluorescence after labeling with the fluorescent dye CFMFA.

ing the protease inhibitor TLCK were masked by the fact that in EC, TLCK alone already lead to significant IκBα phosphorylation. Alternatively, okadaic acid could activate a kinase(s) distinct from those that normally phosphorylate(s) IκBα under physiological conditions. Further experiments will be necessary to distinguish between these possibilities.

Nuclear extracts from rAd.IκBα-infected, stimulated cells lacked any detectable NF-κB binding activity, even less than in noninfected, unstimulated cells. Concomitantly, LPS-induced levels of VCAM-1, IL-1, and IL-8 mRNA, as well as VCAM-1 and procoagulant activity (expressed as units per milliliter tissue factor), were reduced by up to 98%. The difference between an apparently complete lack of NF-κB activity and still partial mRNA expression indicates that either: (a) a portion of noninfected cells is responsible for the low overall mRNA expression that occurs independently in each cell, whereas in EMSA, the high amounts of IκBα from the (pooled) nuclei of infected cells would also inhibit NF-κB from the portion of noninfected cells; or (b) a basal level of transcription can occur independently of NF-κB. In rAd.IκBα-infected cells, tissue factor expression measured as procoagulant activity was reduced to basal levels. However, in this assay, responses other than tissue factor expression, e.g., downregulation of thrombomodulin, might contribute to procoagulant activity.

EC activation can be distinguished between two mechanistically distinct responses: rapid events ("type I activation") such as translocation of P-selectin to the cell surface that occur independently of protein synthesis, and late events (type II activation) that occur over hours and require mRNA and protein synthesis (57). vWF, a marker for type I activation in EC, is released from Weibel-Palade bodies after stimulation of EC independently of gene transcription. In contrast to the inhibition of those genes mentioned above, IκBα overexpression did not affect the thrombin-induced vWF secretion, demonstrating that IκBα does not interfere with at least some aspects of type I activation. In addition,

the LPS-stimulated secretion of prostacyclin remained unaffected, indicating that NF-κB-independent branches of the LPS signaling pathway are not influenced by IκBα.

In some cases, however, we have noted that adenovirus infection per se has an effect on EC activation: IL-1α and IL-6 mRNA levels were significantly enhanced in control virus-infected as compared to noninfected cells, whereas the expression of other genes tested was not affected. In addition, infection with the control adenovirus slightly stimulated vWF secretion (Wrighton, C., unpublished observation). The molecular basis for this differential susceptibility of IL-1α and IL-6 towards adenovirus infection remains to be established. It is consistent with previous observations in the literature (45), describing an inflammatory reaction in response to adenovirus. Moreover, enhanced serum levels of IL-6 have been attributed to the release of the preformed cytokine in response to adenovirus infection in a clinical study with recombinant adenovirus expressing the cystic fibrosis transmembrane conductance regulator (58). In contrast, our experiments suggest that enhanced IL-6 mRNA levels could contribute to increased IL-6 production after adenovirus infection. It is, therefore, conceivable that coinfection with rAd.IκBα would suppress the inflammatory reaction encountered with other recombinant adenoviruses used in ongoing gene therapy experiments. Studies to evaluate this possibility are underway.

The inhibition of cell adhesion molecule expression by IκBα was reflected by inhibition of HL-60 adhesion to EC in vitro. In vivo, under flow conditions, the interaction between immune cells and the endothelium is more complex, involving an initial weak adhesion (rolling) that precedes firm adhesion and transmigration. Lymphocyte rolling has been demonstrated to involve members of the selectin family (59), whereas sticking and emigration is mediated by members of the immunoglobulin family on the EC surface interacting with integrins on the leukocytes (60). In addition, chemotactic and activating cytokines secreted by the EC (activating for the leukocyte as well as for the EC themselves) contribute to the inflammatory cascade. However, as demonstrated by the inhibition of expression of IL-8 and other cytokines by IκBα, NF-κB appears to be a common mediator not only for cell adhesion molecules, but also for chemotactic and activating cytokines involved in the inflammatory reaction, suggesting that the strategy of inhibiting this transcription factor would also function in vivo. Supportive of the concept of targeting NF-κB to prevent the inflammatory reaction are the recent findings that glucocorticoids, the most potent antiinflammatory drugs available, act at least in part by stimulating the expression of IκBα, thereby downregulating NF-κB activity (61, 62).

To date, it is not clear whether adenovirus-mediated gene transfer as currently practiced will become a tool that can be used clinically on a routine basis. In xenotransplantation studies, where an inflammatory reaction appears to play a considerable role in the rejection process, perfusion of isolated organs with rAd.IκBα before transplantation could be feasible, though several aspects of biosafety, of duration of expression of the foreign gene and others need to

be carefully evaluated. However, in the case of I κ B α , adenovirus methodology offers the possibility to extend our studies to animal models of inflammation. Should these experiments confirm the in vitro data, other efforts to inhibit

NF- κ B, possibly via small synthetic molecules, could be developed into a promising strategy for the clinical treatment of a wide range of inflammatory diseases.

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