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INHERITED DISEASE ***RESEARCH ARTICLE***

Anti-inflammatory gene therapy directed at the airway epithelium

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Cystic fibrosis (CF) is characterised by chronic airway inflammation. Pro-inflammatory mediators in the lung are regulated by the transcription factor nuclear factor kappa B (NFκB). We have assessed the effect of adenovirus and liposome-mediated overexpression of the NFκB inhibitor IκBα, as well as liposome-mediated transfection with oligonucleotides resembling NFκB consensus binding sites (decoys) in a cystic fibrosis airway epithelial cell line (CFTE). Electrophoretic mobility shift assays (EMSA) were used to assess NFκB activity and secretion of the pro-inflammatory cytokine interleukin-8 (IL-8) was measured by ELISA. At a MOI of 30, Ad-IκBα significantly decreased IL-8 secretion to 60% and 43% of control unstimulated and TNF-α stimulated cells, respectively. At this MOI, approximately 70% of cells are transduced. EMSA showed an approximately 50% decrease in NFκB activation. Liposome-mediated transfection of IκBα did not reduce IL-8 secretion, probably due to low transfection efficiency (approximately 5% of cells). Liposome-mediated transfection of CFTE cells with rhodamine-labeled decoy oligonucleotides indicated a transfection efficiency close to 100%. TNF-α stimulated IL-8 secretion was reduced by approximately 40% using this approach. EMSA confirmed a significant decrease of NFκB activation. Decoy oligonucleotides may be a promising approach for reduction of NFκB-mediated pulmonary inflammation. Gene Therapy (2000) **7,** 306–313.

Keywords: *NFκB; inflammation; gene therapy*

Introduction

Cystic fibrosis (CF) is characterised by chronic airway inflammation. The inflammation is neutrophil predominated, and while these cells clearly have a key role in the killing of micro-organisms,[1] uncontrolled release of their cytotoxic contents leads to mucus hypersecretion and progressive lung damage. This cycle ultimately contributes to the morbidity and mortality in the vast majority of patients.[2,3]

Persistent bacterial colonisation, specifically by *Pseudomonas aeruginosa* (PA), is a characteristic feature of the CF lung, and may be the primary stimulus for neutrophil infiltration.[4] Thus, PA infection has been shown directly to stimulate secretion of the pro-inflammatory cytokine interleukin-8 (IL-8), a major neutrophil chemoattractant, in the airways,[5] which is produced by several cell types, including epithelial cells. Recently we have shown, that the degree of IL-8 stimulation in primary airway epithelial cells is directly related to the number of PA bound to the cell surface.[6]

Cytokine secretion is predominantly regulated at the level of gene transcription. The transcription factor nuclear factor kappa B (NFκB) is a central regulator of inflammatory and immune responses,[7,8] and belongs to the REL-family of transcription factors.[9] Various homo- and heterodimer isoforms have been identified, but the heterodimer consisting of the REL proteins p50 and p65 (Rel A) appears to be most prominent in the regulation of the inflammatory response.[10] NFκB is constitutively expressed in most cell types, but in unstimulated cells it is retained in an inactive form in the cytoplasm, because of its association with inhibitory proteins (IκBs), of which several are known (α, β, γ, ϵ);[11] IκBα is the best studied member of this family.

NFκB is activated by a large number of stimuli, including micro-organisms, cytokines and oxidative stress. These stimuli lead to phosphorylation and subsequent degradation of the IκB proteins,[12,13] which is followed by the rapid translocation of NFκB from the cytoplasm into the nucleus where it binds to consensus binding sites in the promoter region of its target genes.[14] Recently, it has been shown that PA infection of both non-CF and CF respiratory epithelial cells activates NFκB.[15,16] NFκB activation is also important in asthma, highlighted in a recent report showing that NFκB activation is increased in sputum and biopsies of asthmatics when compared with healthy controls.[17] Further, many commonly prescribed anti-inflammatory drugs, such as glucocorticoids and aspirin decrease NFκB activity.[18] Recently, it was shown that NFκB activation can also be altered through gene therapy based interventions. Thus, IκBα overexpression, or transfection with NFκB decoy consensus binding sites decreased NFκB activity in endothelial and stromal cells and monocytes *in vitro*.[19–21] This has been extended to animal models of inflammation such as ischemia-reperfusion injury and septic shock.[22,23]

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In this study we have determined the anti-inflammatory effect of NFκB decoys and IκBα overexpression in a cystic fibrosis airway cell line and demonstrate for the first time their anti-inflammatory effect in airway epithelial cells.

Results

IL-8 secretion following adenovirus- and liposome-mediated IκBα overexpression

CFTE cells show basal secretion of IL-8 and this can be stimulated further by TNF-α (Figure 1). CFTE cells were transduced with adenovirus expressing IκBα or a control virus expressing β-galactosidase using MOIs of 0.3, 3 and 30. Expression of vector-specific IκBα mRNA was confirmed through RT-PCR (data not shown). Adenovirus infection increased basal IL-8 secretion approximately two-fold (Figure 1a). Ad-IκBα transduction at a MOI of 30 reduced IL-8 secretion by approximately 60% when compared with Ad-β transduction ($P < 0.01$), but did not reduce IL-8 secretion below the levels secreted from uninfected cells (Figure 1a). TNF-α stimulation increased IL-8 secretion of uninfected cells about seven-fold (Figure 1b), but there was no further increase in IL-8 secretion due to virus infection. IL-8 secretion in Ad-IκBα transduced cells was significantly reduced ($P < 0.01$) when compared with either Ad-β transduced or uninfected cells.

In contrast, liposome-mediated transfection produced no significant decrease in IL-8 secretion either in unstimulated or TNF-α stimulated cells (Figure 2). However, the presence of plasmid-derived IκBα mRNA could be demonstrated by RT-PCR (Figure 3). Transfection efficiency of virus- and liposome-mediated gene transfer in this system was assessed using β-galactosidase expression (Figure 4). The number of X-gal-positive cells after liposome transfection (4.6% ± 1.7, $n = 3000$) was comparable to that achieved using the lowest virus titer (MOI 0.3; 2.2% ± 0.4, $n = 3000$). At an MOI of 30, 68.0% ± 4.0 ($n = 3000$) of cells were transfected. Thus, for IκBα overexpression to produce a significant reduction in IL-8 secretion *in vitro* more than 15% of cells need to be transduced or transfected.

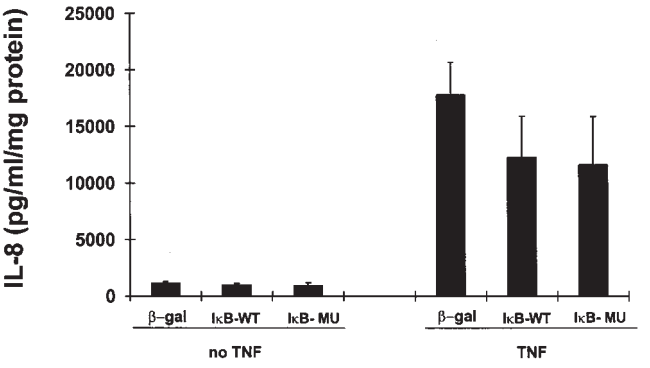

Figure 2 *IL-8 secretion after liposome-mediated IκBα overexpression. Cells were transfected with wild-type (IκBα-WT) or mutant (IκBα-MU) plasmid complexed to DC-cholesterol/DOPE, or with a β-galactosidase expressing control plasmid. 48 h after transfection the cells were stimulated with TNF-α for 1 h or remained unstimulated. Secreted IL-8 was measured 6 h after stimulation. Error bars indicate s.e.m. n = 8 for all experiments.*

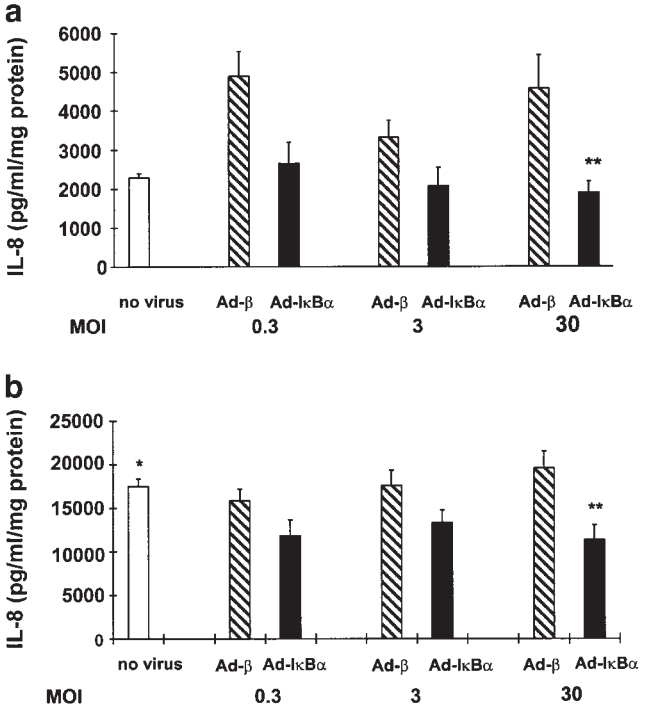

Figure 1 *IL-8 secretion after adenovirus-mediated IκBα overexpression. CFTE cells were transfected with adenovirus expressing IκBα (Ad-IκBα) or a β-galactosidase expressing control virus (Ad-β) at multiplicity of infection units (MOI) of 0.3, 3 and 30. 48 h after transfection cells were either unstimulated (a) or stimulated with TNF-α (b) for 1 h. IL-8 secretion was determined 6 h after stimulation. Error bars indicate s.e.m. n = 10–15 for all experiments, ** = $P < 0.01$ compared with respective Ad-β control; * = < 0.01 compared with no virus control (open bars).*

EMSA following adenovirus-mediated IκBα overexpression

EMSAs using a NFκB consensus oligonucleotide result in the appearance of multiple bands. To determine which of these protein complexes is relevant to an inflammatory state, we stimulated CFTE cells with TNF-α for 1 h and determined subsequent NFκB activity. As shown in Figure 5a only NFκB/DNA complexes I and II were

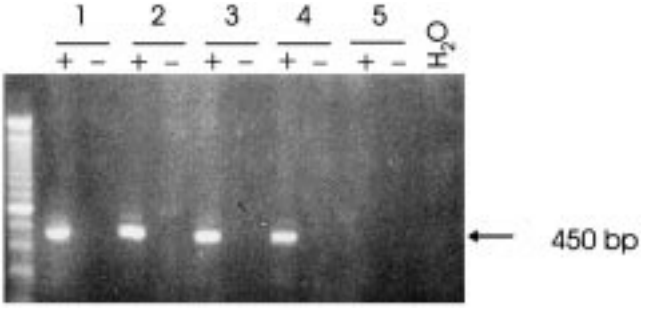

Figure 3 *RT-PCR following liposome-mediated IκBα overexpression. CFTE cells were transfected with wild-type (lanes 1 and 2) or S32/36A mutant (lanes 3 and 4) IκBα cDNA complexed to DC-Cholesterol/DOPE or a control plasmid expressing β-galactosidase (lane 5). 48 h after transfection total RNA was prepared and RT-PCR carried out to detect recombinant IκBα RNA. For all samples a reaction with (+) and without (–) reverse transcriptase was carried out. Arrow indicates expected 450 bp band. Left hand lane contains size marker (100 bp ladder).*

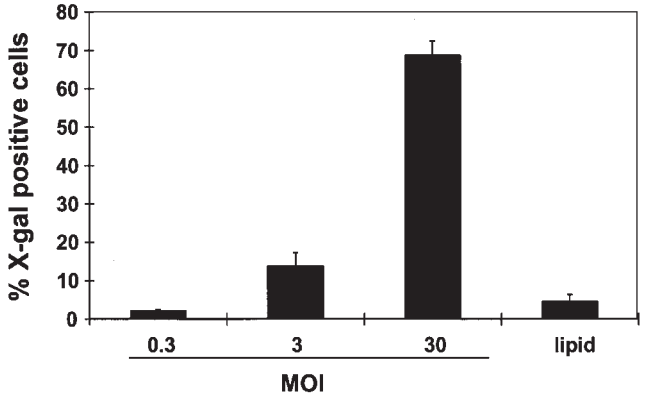

Figure 4 *Transduction and transfection efficiency. CFTE cells were transduced with adenovirus expressing β-galactosidase at MOIs of 0.3, 3 and 30 or with a β-galactosidase expression plasmid complexed to DC-Cholesterol/DOPE for 48 h. β-Galactosidase expression was determined by X-gal assays. Error bars indicate s.e.m., n = 10 optical fields (3000 cells).*

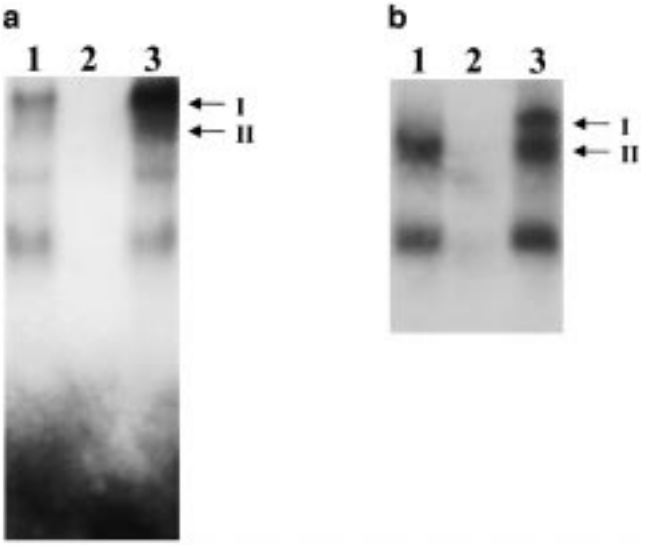

Figure 5 *Electrophoretic mobility shift assays. (a) CFTE cells remained unstimulated (lane 1) or were stimulated with TNF-α (lane 3). One hour after stimulation NFκB activity was measured in cell extracts. (b) C57Bl/6 control (lane 1) or mice infected with Pseudomonas aeurginosa (lane 3). NFκB activity was determined in lung protein extracts 2 h after infection. The specificity of the DNA/protein binding was determined in reactions using unlabeled specific competitor (lane 2, both assays). NFκB/DNA complexes labeled as I and II, increase after TNF-α and Pseudomonas aeurginosa stimulation.*

increased. Infection of wild-type C57Bl/6 with *Pseudomonas aeruginosa* also resulted in increases of complexes I and II, 2 h after infection (Figure 5b). These bands were therefore used as indicators of NFκB activity induced by an inflammatory stimulus. Transduction of CFTE cells with Ad-IκBα produced an approximately 50% decrease in complexes I and II after TNF-α stimulation (Figure 6) in keeping with the IL-8 data noted above.

Transfection efficiency of decoy oligonucleotides complexed to liposomes

Liposome-mediated transfection efficiency of decoy oligonucleotides was determined using rhodamine-labeled decoys. One tenth of the decoys (0.56 μg) were labeled

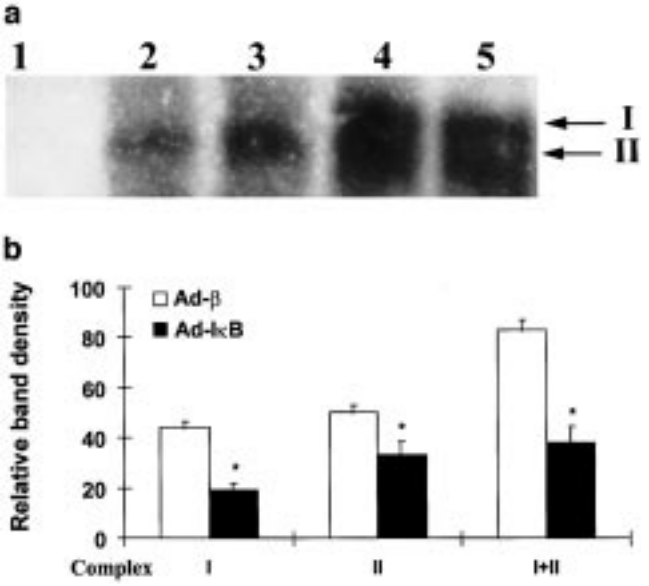

Figure 6 *EMSA quantification after adenovirus-mediated IκBα overexpression. (a) CFTE cells were transduced with Ad-IκBα (lanes 2 and 3) or a β-galactosidase expressing control virus (lanes 4 and 5) at an MOI of 30. 48 h after transduction, cells were stimulated with TNF-α for 1 h, after which protein extracts were prepared. NFκB/DNA complexes I + II are indicated. The specificity of the DNA/protein binding was determined in reactions using unlabeled specific competitor (lane 1). (b) NFκB complexes I + II were quantified (n = 6). Error bars indicate s.e.m., * = $P < 0.05$ compared with respective Ad-β control.*

and mixed with 5.04 μg of unlabeled decoys before complexing with DC-cholesterol/DOPE. A representative field of CFTE cells is shown in Figure 7, with fluorescent signal visible in the nucleus and cytoplasm, including the perinuclear region. Quantitative analysis of the fluorescence in nucleus and cytoplasm showed a 100% (mean fluorescence 4.4 ± 0.8 *versus* 0.4 ± 0.06 in controls, $n = 15$, $P < 0.005$) and 86% (mean fluorescence 3.4 ± 0.6 *versus* 0.4 ± 0.07 in controls, $n = 15$, $P < 0.005$) transfection efficiency, respectively. For comparison Cos 7 cells show a mean fluorescent signal approximately five-fold higher in the nucleus and three-fold higher in the cytoplasm than CFTE cells (data not shown), consistent with the characteristically higher transfection efficiency of Cos 7 cells.

IL-8 secretion and EMSA following decoy transfection

Transfection of CFTE cells with scrambled control oligonucleotides complexed to liposomes increased IL-8 secretion about 2.5-fold over untransfected levels (Figure 8). A small, but non-significant increase following transfection was also seen in the presence of TNF-α. Transfection with NFκB decoy oligonucleotides resulted in an approximately 40% significant ($P < 0.05$) decrease of IL-8 secretion after TNF-α stimulation, but did not reduce basal secretion (Figure 8). Transfection of cells with NFκB decoy, but not scrambled oligonucleotides, reduced NFκB activity in EMSA assays by over 90% in TNF-α stimulated and unstimulated cells (Figure 9).

Discussion

We have shown that secretion of the pro-inflammatory cytokine IL-8, a representative marker of inflammation,

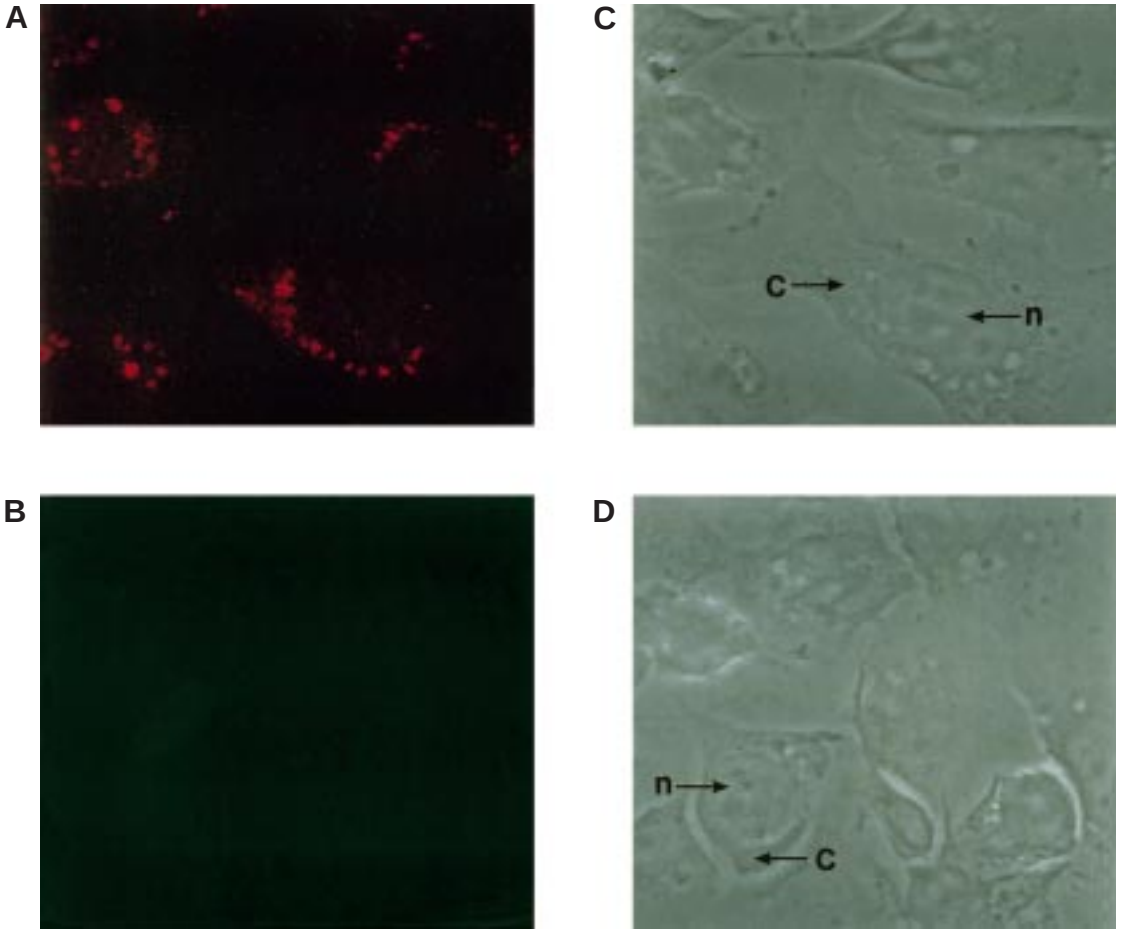

Figure 7 *Liposome-mediated transfection of decoy oligonucleotides. CFTE cells were transfected with decoys complexed to DC-Cholesterol/DOPE for 24 h. One tenth (0.56 μg per six-well plate) of the decoys was end-labeled with tetramethylrhodamine-5-UTP. Fluorescence was determined using confocal microscopy (left panel, 100×) and was visible in cytoplasm (c), nucleus (n) and the perinuclear region of transfected (A), but not control cells (B). Corresponding bright field images (C and D).*

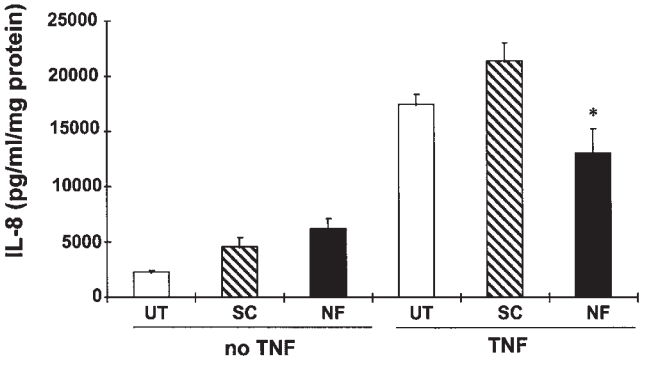

Figure 8 *IL-8 secretion after liposome-mediated transfection with NFκB decoys. CFTE cells were transfected with NFκB decoys (NF) or scrambled (SC) oligonucleotides complexed to DC-Cholesterol/DOPE or remained untransfected (UT). Twenty-four h after transfection cells remained unstimulated or were stimulated with TNF-α for 1 h. IL-8 secretion was measured 6 h after stimulation (n = 6 each experiment). Error bars indicate s.e.m., * = $P < 0.05$ compared with scrambled oligonucleotide.*

can be attenuated by decreasing NFκB activity in a cystic fibrosis airway epithelial cell line. Adenovirus-mediated, but not liposome-mediated IκBα overexpression significantly reduced IL-8 secretion and NFκB activity. Transfection with decoy/liposome complexes also significantly decreased TNF-α stimulated IL-8 secretion and NFκB activation.

NFκB is a central regulator of inflammation in the airways and other organs[24] and presents an attractive target for anti-inflammatory lung gene therapy. In CF, excessive secretion of IL-8 and hence neutrophil infiltration, contribute dramatically to the chronic lung injury. A potential source of IL-8 in the airways is the respiratory epithelium,[25,26] which although at present a challenge for gene transfer, is ultimately likely to be an appropriate target for anti-inflammatory gene therapy, because of its accessibility. This study was designed to provide proof-of-principle that IL-8 secretion can be attenuated by decreasing NFκB activation in a simulated inflammatory environment generated in cystic fibrosis airway epithelial cells. Adenovirus-mediated overexpression of IκBα reduced IL-8 secretion and NFκB activity in a dose-dependent fashion. We have shown that a significant proportion of cells need to be transduced to achieve this anti-inflammatory effect. However, despite their success

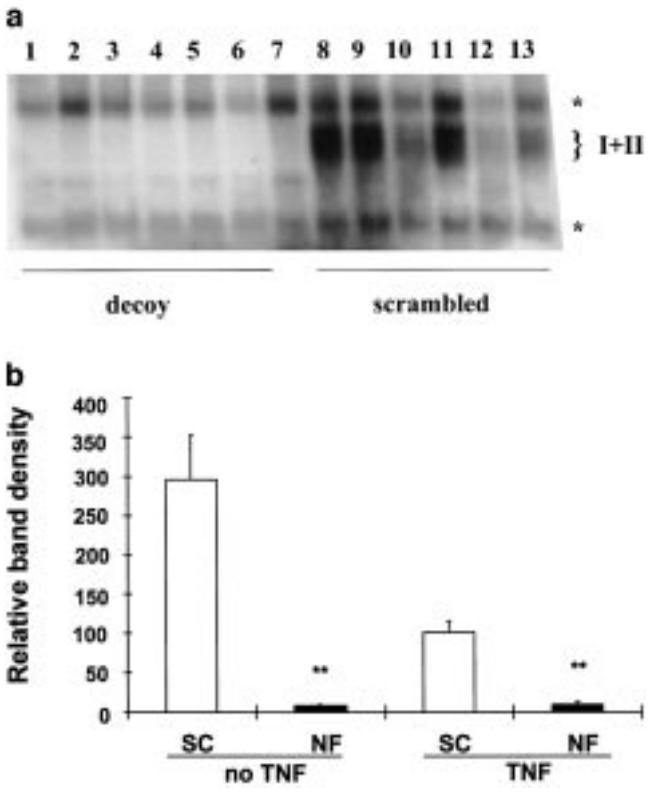

Figure 9 *EMSA after NFκB decoy transfection. (a) CFTE cells were transfected with NFκB decoy (lanes 1–7) or scrambled oligonucleotides (lanes 8–13). Twenty-four h after transfection, cells were stimulated with TNF-α for 1 h, after which protein extracts were prepared. NFκB/DNA complexes I + II are indicated. The specificity of the DNA/protein binding was determined in reactions using unlabeled specific competitor (lane 7). The asterisks mark non-specific bands. (b) Quantification of NFκB/DNA complexes I + II in NFκB decoy (NF) and scrambled oligonucleotide (SC) transfected cells, either unstimulated or TNF-α stimulated (n = 6). Error bars indicate s.e.m. ** = P < 0.01 compared with scrambled oligonucleotides.*

in vitro, the use of adenovirus-mediated IκBα overexpression might be limited *in vivo*. Even though adenoviruses possess a natural tropism for the lung, the virus does not transfect airway epithelium efficiently,[27] and thus IκBα overexpression may not be achievable *in vivo*. In addition, virus infection, particularly at high titers induces inflammation, here seen as a two-fold increase in IL-8 production when compared with uninfected controls. However, in the presence of TNF-α which may mimic the chronic inflammatory environment in a CF lung, virus infection does not further increase IL-8 secretion.

Liposome-mediated IκBα overexpression did not decrease IL-8 secretion *in vitro*, most likely due to insufficient transfection efficiency. Thus, under optimised conditions less than 5% of CFTE cells expressed the β-galactosidase reporter gene. Similar efficiencies have been reported for other cell lines using labeled plasmid DNA/liposome complexes.[28] Liposome-mediated transfection of the lung epithelium has also been shown to be an inefficient process,[29] although recent reports indicate ways in which this may be augmented.[30] However, it is unlikely that liposome-mediated IκBα overexpression will achieve a reduction of NFκB activity *in vivo*. Apart from IκBα, several other inhibitors for NFκB (IκBβ, IκBε) have recently been cloned.[11] The relative contribution of these in controlling particularly chronic inflammation remains to be established.

Transfection with rhodamine-labeled decoys complexed to liposomes resulted in transfection efficiencies close to 100% in CFTE cells, when approximately 10^6 oligonucleotides per cell were added, although we did not assess the minimum number of oligonucleotides needed per cell. Liposome-mediated decoy transfection decreased TNF-α stimulated IL-8 secretion by about 40% and NFκB activity by over 90%. The discrepant reduction of NFκB is likely a reflection of decoys present in the cytoplasm and nucleus which can act as specific competitors during the electrophoretic mobility shift assay. It is unclear why decoys did not reduce basal IL-8 secretion in CFTE cells. One possibility is that basal IL-8 secretion relates to a different transcription pathway. Thus transcription factors involved in regulating the basal expression might not bind to the same promoter consensus binding sites as proteins regulating the stimulated secretion and might therefore not recognize the same decoy molecules. However, in an inflamed lung IL-8 secretion and NFκB activation are likely to be stimulated through TNF-α-related pathways and thus susceptible to decoy inhibition.

Decoy/liposome complexes may overcome some of the limitations associated with virus- and liposome-mediated IκBα transfection *in vivo*. In contrast to the relatively poor transfection efficiency of plasmids when complexed to liposomes, the decoy oligonucleotide/liposome complexes used in the study transfect cytoplasm and nucleus of CFTE and Cos 7 cells very efficiently. It has recently been demonstrated that there is a marked difference in the extent and kinetics of nuclear uptake between oligonucleotides and plasmids.[31] Oligonucleotides accumulate in the nucleus of transfected cells within hours of transfection, whereas plasmids can only be detected in the nucleus after overnight incubation. Cell division may not be required for intranuclear uptake of oligonucleotides, since they enter the nucleus rapidly and their accumulation is temperature and energy-independent.[29,32] In contrast, plasmid uptake into the nucleus appears to be enhanced through cell division[33] and this process is energy- and temperature-dependent.[34] In contrast to IκBα overexpression, decoys do not require protein expression, a further potential advantage. Thus, the decoy approach warrants evaluation in animal models of pulmonary inflammation.

In conclusion, we have demonstrated that NFκB activity and IL-8 secretion can be decreased in a CF airway epithelial cell line through overexpression of IκBα or NFκB decoy transfection. Anti-inflammatory lung gene therapy based on nucleotide gene transfer may offer promise for inflammatory diseases of the lung.

Materials and methods

Cell culture

Tracheal epithelial cells derived from a ΔF508 homozygote cystic fibrosis patient (CFTE) and green monkey kidney cells (Cos 7) were grown in MEM and DMEM supplemented with 10% fetal calf serum and 1% streptomycin/ampicillin (Gibco BRL, Paisley, UK), respectively, and passaged under routine tissue culture

procedures. In studies of tumor necrosis factor alpha stimulation (TNF-α; Genzyme Corporation, Cambridge, MA, USA) 200 units/ml medium were added for 1 h, after which the medium was replaced with unconditioned medium.

Adenovirus and plasmid preparation

The adenovirus IκBα (AdIκBα) has been previously described.[18] The virus has an E1 deletion and carries the porcine IκBα cDNA in combination with the SV40 large T antigen nuclear localization signal (NLS). An E1-deleted adenovirus expressing nuclear localized β-galactosidase[35] was used as a control virus. Purification of the recombinant adenovirus was carried out using two consecutive cesium chloride centrifugations, as previously described.[36] The wild-type human inhibitor kappa B alpha cDNA (IκBα) and a mutant carrying the S32/36A mutation to increase its stability[37] were blunt-ended and cloned into the *Sma*I site of the eukaryotic expression plasmid pCI (Promega, Southampton, UK), which uses the CMV immediate–early promoter/enhancer region to regulate expression.

Decoy oligonucleotides

Phosphorothioated, HPLC purified oligonucleotides (Genosys Biotechnologies, London, UK) included the following sequences (NFκB consensus sequences are shown in bold letters): NFκB decoy: 5′-CCT TGA A**GG GAT TTC CC**T CC-3′, 3′-GGA ACT T**CC CTA AAG GG**A GG-5′, scrambled decoy: 5′-TTG CCG TAC CTG ACT TAG CC-3′, 3′-AAC GGC ATG GAC TGA ATC GG-3′. The synthetic oligonucleotides were washed in sterile TE buffer (10 mM tris(hydroxy-methyl)-aminomethane, 1 mM ethylenediamine-tetraacetic acid). The supernatant was purified over NAP10 columns (Pharmacia, St Albans, UK) and quantified spectrophotometrically. The single-stranded oligonucleotides (3 nM) were annealed for 2 h with decreasing temperatures from 80°C to 25°C. For some experiments the double-stranded NFκB decoys were 3′hydroxy end-labeled with tetramethylrhodamine-5-UTP (Boehringer, Mannheim, Germany) using terminal transferase (Boehringer).

Cell transduction and transfection

Transductions and transfections were carried out on semi-confluent cells, grown in six-well tissue culture plates (7 cm^2) in the absence of serum for 18–20 h, after which the medium was replaced with normal growth medium. For the adenovirus transductions, cells were infected with doses ranging from 1.1×10^5 to 1.1×10^7 plaque forming units (p.f.u.) per 6-cm well, corresponding to multiplicity of infection units (MOI) of 0.3 to 30. Decoy oligonucleotides were complexed to the cationic lipid DC-cholesterol:DOPE[38] at a ratio of 1:3 (5.6 μg oligonucleotides:16.8 μg lipid) in Opti-MEM (Sigma, Poole, UK). For immunohistochemistry cells were grown on glass coverslips. After transfection with rhodamine-labeled or unlabeled oligonucleotides cells were acid-washed (0.2 M acetic acid, 0.5 M NaCl in PBS) for 5 min at 4°C, washed in PBS and fixed in 4% paraformaldehyde for 30 min. Subsequently the coverslips were mounted and fluorescence was determined using confocal microscopy in the nucleus and cytoplasm of 15 individual cells.

Measurements of secreted IL-8 through ELISA

Ad-IκBα transduced cells were stimulated with TNF-α 48 h after virus infection and secreted IL-8 was measured in stimulated and unstimulated cells 6 h after stimulation, using a standard ELISA (Genzyme Corporation). Twenty-four hours following the start of transfection with decoys, the cells were stimulated with TNF-α or remained unstimulated and secreted IL-8 was assayed as described above.

Electrophoretic mobility shift assays (EMSA)

Protein lysates were prepared as previously described.[39] In brief, cells were scraped into the surrounding medium and pelleted at 4°C (5 min, 1100 *g*). The pellets were washed in ice-cold phosphate-buffered saline (PBS) and lysed in 30 μl extraction buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 20% glycerol, 1% NP40, 0.5 mM EDTA, 1 mM EGTA, 0.5 mM DTT) for 30 min on ice. Cell debris was removed through centrifugation (5 min, 14 000 *g*) and the protein concentration in the supernatant was determined by a modified Folin-Lowry method.[40] The gel shift assay system from Promega was used and EMSAs were carried out according to the manufacturer's recommendations. An oligonucleotide probe containing the κ-light chain enhancer NFκB consensus binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end-labeled with ^{32}P-ATP (Amersham, Little Chalfont, UK). Ten micrograms of cellular proteins were used for each reaction. Proteins were resolved on 5% non-denaturing polyacrylamide gels at 10 mA for 3 h. Subsequently, gels were dried (80°C, 2 h) and exposed with intensifying screens to Kodak XAR-5 film (Sigma) for 24 h at –80°C. Quantification of NFκB complexes was carried out by densitometry.

Pseudomonas aeruginosa infection of mice

Pseudomonas aeruginosa serotype 0:1 (a non-mucoid piliated laboratory strain) was grown overnight in tryptone soya broth (Unipath, Basingstoke, UK). Bacteria were pelleted by centrifugation for 10 min at 1100 *g* and resuspended in 1 ml of PBS. Bacterial concentration was quantified by spectophotometry, with an OD_{260} of 0.1 corresponding to 2.5×10^8/ml bacteria, as previously described.[41] C57Bl/6 mice were infected with 10^7 bacteria in 100 μl PBS through nasal instillation as previously described.[42]

Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using the RNesy Mini kit (Qiagen), following the manufacturer's recommendations. The samples were treated with 1 unit DNase (Pharmacia)/μg DNA. Reverse transcription was performed using 3 μg RNA, 0.5 mM of each dNTP, 100 μM random hexamer primers (Pharmacia), 300 units RNA guard (Pharmacia), 10 mM dithiothreitol (DTT), 5 × RT-buffer (BRL) and 200 units Superscript reverse transcriptase (BRL) in a total volume of 20 μl. The reaction was incubated at 37°C for 1 h and heat-inactivated for 10 min at 95°C. RT-PCR reactions were performed with primers specific for the IκBα cDNA, which amplify a 450 bp cDNA specific product (forward primer: 5′-AGT CTC GAA CTT AAG CTG-3′, reverse primer 5′-ATC ACT TCC ATG GTC AGT-3′) using 150 ng cDNA, 200 μM dNTP, 50 ng of each primer, 1.5 mM $MgCl_2$, 10 × PCR buffer (BRL) and 1 unit Taq polymerase in a total volume

of 20 μl. Thermal cycling was carried out for 30 cycles (20 s 92°C, 30 s 55°C, 60 s 72°C). Negative controls without reverse transcriptase were performed for all samples. PCR products were separated on 2% agarose gels.

X-gal assay

Cells were washed with phosphate buffer (0.1 M, pH 7.3) and fixed for 15 min (0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in 0.1 M phosphate buffer, pH 7.3). After fixation, cells were washed three times for 20 min in wash buffer (2 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate in 0.1 M phosphate buffer, pH 7.3). To visualize β-galactosidase expression, cells were stained at 37°C for 1 h in X-gal staining solution (1 mg/ml 4-bromo-4-chloro-3-indolyl-D-galacto-pyranoside (X-gal, Gibco BRL) in di-methyl formamide, 5 mM potassium ferrocyanide (Sigma), 5 mM potassium ferricyanide (Sigma) in wash buffer). A total of 3000 cells in 10 optical fields were counted in two independent experiments.

Statistical analysis

Values are expressed as mean ± standard error of the mean (s.e.m.) for convenience. The Mann–Whitney test was used to compare means and the null hypothesis rejected at $P < 0.05$.

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