Eur Respir J 2001; 18: 801–809
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European Respiratory Journal
ISSN 0903-1936

Anti-inflammatory effect of adenovirus-mediated IκBα overexpression in respiratory epithelial cells

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Anti-inflammatory effect of adenovirus-mediated IkBα overexpression in respiratory epithelial cells. G.Y. Park, S. Lee, K.H. Park, C.T. Lee, Y.W. Kim, S.K. Han, Y.S. Shim, C.G. Yoo. ©ERS Journals Ltd 2001.

ABSTRACT: Many studies into basic biological characteristics of inflammation and tissue injury have implicated pro-inflammatory cytokine-mediated tissue injury in the pathogenesis of inflammatory lung diseases. Because transcription of most pro-inflammatory cytokines is dependent on the activation of nuclear factor (NF)-κB, NF-κB could be a good potential target to suppress the cytokine cascade. Cytokine-induced activation of NF-κB requires phosphorylation and subsequent degradation of IκBa. Therefore, the blocking NF-κB activation by IκBα could inhibit the pro-inflammatory cytokine-induced tissue injury.

To evaluate whether blocking of NF-κB activation shows an anti-inflammatory effect, this study investigated the effect of adenovirus-mediated overexpression of IκBα super-repressor (IκBα-SR) on the pro-inflammatory cytokine expression in respiratory epithelial cells.

The transduction efficiency of adenovirus was >90% in both A549 and NCI-H157 cells. Ad5IκBα-SR-transduced cells expressed high levels of IκBα-SR, which was resistant to tumour necrosis factor (TNF)-α-induced degradation. Adenovirus-mediated overexpression of IκBα-SR blocked cytokine-induced nuclear translocation of p65 and NF-κB deoxyribonucleic acid binding activity without affecting total cellular expression level of NF-κB. Ad5IκBα-SR transduction suppressed cytokine-induced interleukin-8 and TNF-α expressions at both ribonucleic acid and protein levels.

These results suggest that blocking the nuclear factor-κB pathway by adenovirus-mediated overexpression of IκBα-super-repressor shows an effective anti-inflammatory effect in respiratory epithelial cells.

Eur Respir J 2001; 18: 801–809.

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Keywords: Adenovirus
interleukin-8
IκBα super-repressor
nuclear factor-κB
respiratory epithelial cells
tumour necrosis factor-α

Received: November 28 2000
Accepted after revision June 1 2001

This work was supported by the 1999 National Research and Development Programme (Ministry of Science and Technology).

Nuclear factor (NF)-κB is an ubiquitous inducible transcription factor involved in immune, inflammatory, stress and developmental processes. It is sequestered in the cytoplasm in an inactive state by association with the inhibitory molecule IκBα. NF-κB is rapidly activated in response to various stimuli, including viral infection, lipopolysaccharide (LPS), ultraviolet irradiation, and pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β [1–3]. TNF-α leads to the sequential activation of the downstream NF-κB-inducing kinase (NIK) and the recently isolated TNF-α-inducible IκB kinase complex (IKK) [4–8]. When activated, IKK directly phosphorylates Ser^{32} and Ser^{36} of IκBα, triggering ubiquitination at Lys^{21} and Lys^{22}, and rapid degradation of IκBα in 26S proteasome [1–3]. This process liberates NF-κB, allowing it to translocate to the nucleus. In the nucleus, NF-κB binds to its cognate κB site and transactivates the downstream genes.

In inflammatory lung diseases, such as acute lung injury (ALI), pro-inflammatory cytokines, especially TNF-α and IL-1β, are known to be involved in tissue damage [9]. Pro-inflammatory cytokines function in redundant and overlapping ways through the cytokine "cascade" or "network". As most genes for inflammatory mediators (such as TNF-α, IL-2, IL-6, IL-8, lymphotoxin, granulocyte macrophage-colony stimulating factor (GM-CSF), β-interferon, and adhesion molecules) have κB site in the 5′ flanking region [1–3], the transcription of most pro-inflammatory cytokine genes are regulated by NF-κB activation. Therefore, NF-κB could be a good potential target to suppress the cytokine cascade.

NF-κB activation can be inhibited by blocking various points in the NF-κB/IκB pathway. One method of NF-κB inhibition is overexpression of IκBα. The overexpression of wild-type IκBα partially blocked NF-κB responsive gene expression *in vitro* [10, 11]. Since wild type IκBα is degraded in response to inflammatory mediators which are present at the site of inflammation, overexpression of wild type IκBα would not be effective in blocking NF-κB activation. In order to block the activation of NF-κB effectively,

IκBα should be resistant to degradation. Since inducible phosphorylation of IκBα at Ser^{32} and Ser^{36} is a critical step in the degradation of IκBα, IκBα mutants in which Ser^{32} and Ser^{36} are substituted with nonphosphorylatable alanine (IκBα super-repressor, IκBα-SR) are resistant to degradation.

The present study investigated the effect of blocking the activation of NF-κB by adenovirus-mediated overexpression of IκBα-SR on the pro-inflammatory cytokine expression in respiratory epithelial cells to evaluate the therapeutic potential of Ad5IκBα-SR in inflammatory lung diseases.

Materials and methods

Cells

A549 and NCI-H157 human respiratory epithelial cell lines were maintained in a monolayer in Roswell Park Memorial Institute (RPMI) 1640 containing 10% foetal bovine serum (FBS), penicillin (60 $\mu g \cdot mL^{-1}$), and streptomycin (100 $\mu g \cdot mL^{-1}$) at 37°C under 5% carbon dioxide (CO_2).

Study design

Adenovirus vectors expressing IκBα-SR (Ad5IκBα-SR) and β-galactosidase (Ad5LacZ) were constructed. The transduction efficiency of adenovirus vector, its effect on the cell viability, and the stability of IκBα-SR were evaluated. The effect of adenovirus-mediated overexpression of IκBα-SR on the NF-κB activity and the pro-inflammatory cytokine expressions in respiratory epithelial cells were evaluated.

Methods

Transduction of adenoviruses. An adenovirus vector expressing IκBα-SR was constructed as previously described [12]. A recombinant adenovirus expressing β-galactosidase gene was used as a control virus. Cells were transduced at multiplicities of infection (moi) of 20 by adenovirus vector in serum free RPMI for 1 h with gentle shaking and then washed with phosphate buffered saline (PBS) and incubated with growth medium at 37°C, 5% CO_2 until use.

Analysis of cell viability. Cell viability was measured by a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT solution was added to cells in 96-well plates to the final concentration of 0.5 $mg \cdot mL^{-1}$, and cells were incubated at 37°C for 4 h. After removing culture media, 50 μL of dimethyl sulphoxide (DMSO) was added, and the optical density of each well was read at 590 nm.

Electrophoretic mobility shift assays. NF-κB deoxyribonucleic acid (DNA) binding activity was assessed as described previously [13]. Briefly, nuclear extracts were incubated for 20 min at room temperature with radiolabelled NF-κB consensus sequence in the κ light chain enhancer in B-cells (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). In competition experiments, 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. In supershift experiments, anti-p65 or anti-p50 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and allowed to react for 45 min at room temperature. DNA-protein complexes were resolved on 4% non-denaturing polyacrylamide gel. Gels were dried and autoradiographed at -70°C.

Western blot analysis. Cytoplasmic, nuclear, and whole cell extracts were prepared as described previously [13]. Twenty micrograms of protein was resolved on 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. The membranes were blocked with 5% skim milk-PBS/0.1% Tween 20 for 1 h prior to overnight incubation at room temperature with rabbit polyclonal anti-p65 or anti-IκBα antibodies from Santa Cruz Biotechnology, diluted 1:1000 in 5% skim milk-PBS/0.1% Tween 20. Membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Promega, Madison, WI, USA), diluted 1:2000 in 5% skim milk-PBS/0.1% Tween 20 for 1 h. Following successive washes, membranes were developed with an enhanced chemoluminescence (ECL) kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunofluorescent staining for nuclear factor-κB. Cells grown in 2-well chamber slides were fixed and permeabilized as described previously [13]. Cells were incubated with rabbit polyclonal anti-p65 antibody, diluted 1:100 in 1% bovine serum albumin (BSA), for 30 min, and then incubated with rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin-G antibody (Jackson Immuno-Research, West Grove, PA, USA), diluted 1:100 in 1% BSA, for 30 min. Slides were analysed using a fluorescent light microscope.

Northern blot analysis. Total cellular ribonucleic acid (RNA) was isolated using TRIZOL Reagent (Gibco BRL, Gaithersburg, MD, USA). Equal amounts of total RNA (20 $\mu g \cdot lane^{-1}$) from each sample were loaded into each lane of 1.0% agarose/2% formaldehyde gel, and capillary transferred to a nylon membrane. The human complementary DNA (cDNA) for TNF-α and IL-8 (ATCC, Rockville, MD, USA) were radiolabelled with [α-^{32}P]deoxycytidine triphosphate (dCTP) using a random priming kit (Stratagene, La Jolla, CA, USA). After prehybridizing the membranes for 2 h at 45°C in hybridization buffer, radiolabelled cDNA probe (1×10^6 $cpm \cdot mL^{-1}$ final concentration) was added and incubated overnight at 45°C. The membranes were exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA) with intensifying screen for up to 5 days at -70°C.

Interleukin-8 enzyme-linked immunosorbent assay. Cells (1×10^4) were grown in 96-well culture plates in equal numbers. The supernatants were collected and stored at -70°C until being analysed. IL-8

concentrations were quantitated using enzyme-linked immunosorbent assay (ELISA) kit (R & D System, Minneapolis, MN, USA) according to the manufacturer's specifications.

Statistical analysis

All experiments were carried more than three times. The cell viability data were shown as mean±SD percentage of control of three different experiments. The ELISA data were reported as means±SD of three different experiments. Paired t-tests were used for comparisons and a value of $p<0.05$ was considered as significant.

Results

Adenovirus efficiently transduces IκBα super-repressor gene into respiratory epithelial cells

The transduction efficiency of adenovirus vector in A549 cells was evaluated using a different adenovirus vector encoding β-galactosidase (Ad5LacZ) moi and observing the subsequent β-galactosidase staining. More than 90% of cells expressed β-galactosidase at the virus dose of 20 moi (fig. 1). To assess the adenovirus-mediated expression of IκBα-SR, Western blot analysis was performed with extracts of cells 48 h after infection with Ad5IκBα-SR. The exogenous IκBα-SR was heavily expressed compared to cells infected with Ad5LacZ (fig. 2a). To see the intracellular distribution of IκBα-SR, Western blot analysis was performed with cytoplasmic and nuclear extracts from Ad5LacZ or Ad5IκBα-SR transduced cells. While the endogenous IκBα was located mainly in the cytoplasm, some of the overexpressed IκBα-SR translocated to the nucleus (fig. 2b). In accordance with this observation, most cells expressed IκBα-SR in varying amounts both in the cytoplasm and nucleus in immunofluorescent staining (fig. 2c).

Adenovirus transduction does not affect cell survival

Excessive adenovirus infection itself may be cytotoxic. To evaluate this possibility, A549 and NCI-H157 cells were infected with Ad5LacZ or Ad5IκBα-SR. Cell viability was evaluated by MTT assay 24 and 48 h after infection. Cell viability did not change up to 48 h after infection, in both Ad5LacZ (A549: 96±5 at 24 h, 98±7 at 48 h; NCI-H157: 98±7 at 24 h, 93±9 at 48 h) and Ad5IκBα-SR (A549: 84±9 at 24 h, 84±6 at 48 h; NCI-H157: 94±9 at 24 h, 90±7 at 48 h) infected cells at adenovirus dose of 20 moi (data are shown as mean percentage of control±SD).

IκBα super-repressor is resistant to tumour necrosis factor-α-induced degradation

Since various pro-inflammatory cytokines are produced during an inflammatory process, exogenous IκBα should be resistant to cytokine-induced degradation in order to block NF-κB activation effectively at the site of inflammation. To investigate the stability of IκBα-SR, time dependent degradation of IκBα in response to TNF-α stimulation was determined by Western blot analysis after transduction with Ad5LacZ or Ad5IκBα-SR. In Ad5LacZ infected cells, endogenous IκBα was completely degraded in 30 min, followed by resynthesis in 60 min in both A549 and NCI-H157 cells (fig. 3a). In contrast,

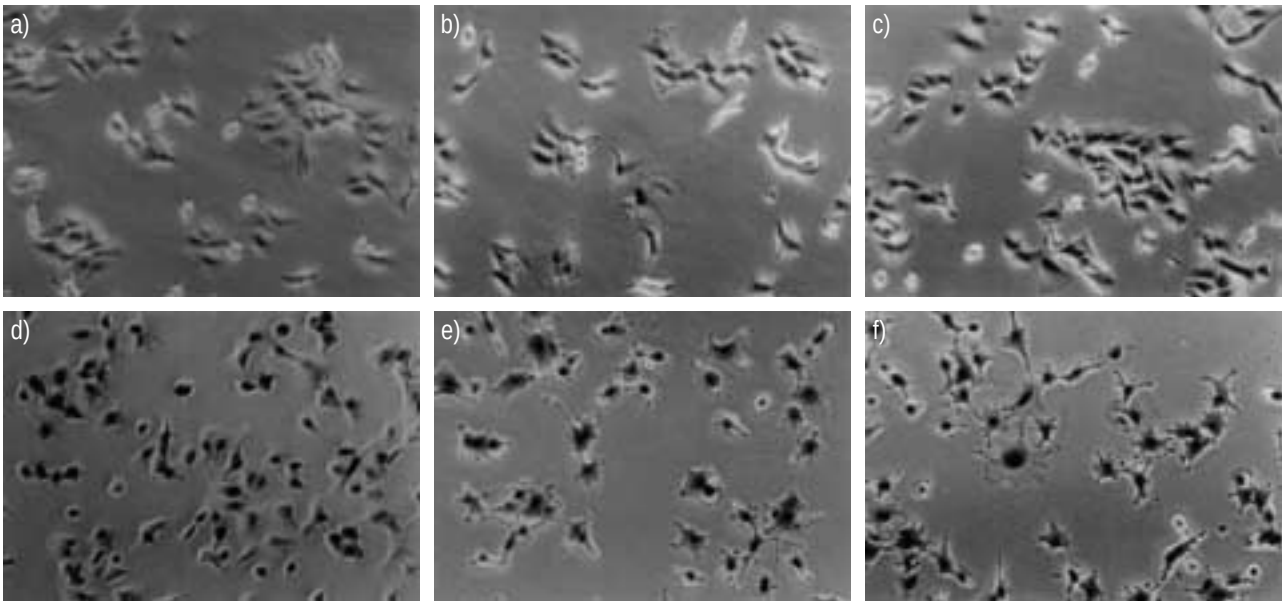

Fig. 1. – Transduction efficiency of adenovirus vector. A549 cells were infected with various doses of Ad5LacZ and stained for β-galactosidase 48 h after infection. a) Control; b) 5 multiples of infection (moi) Ad5LacZ; c) 10 moi; d) 20 moi; e) 50 moi; f) 100 moi.

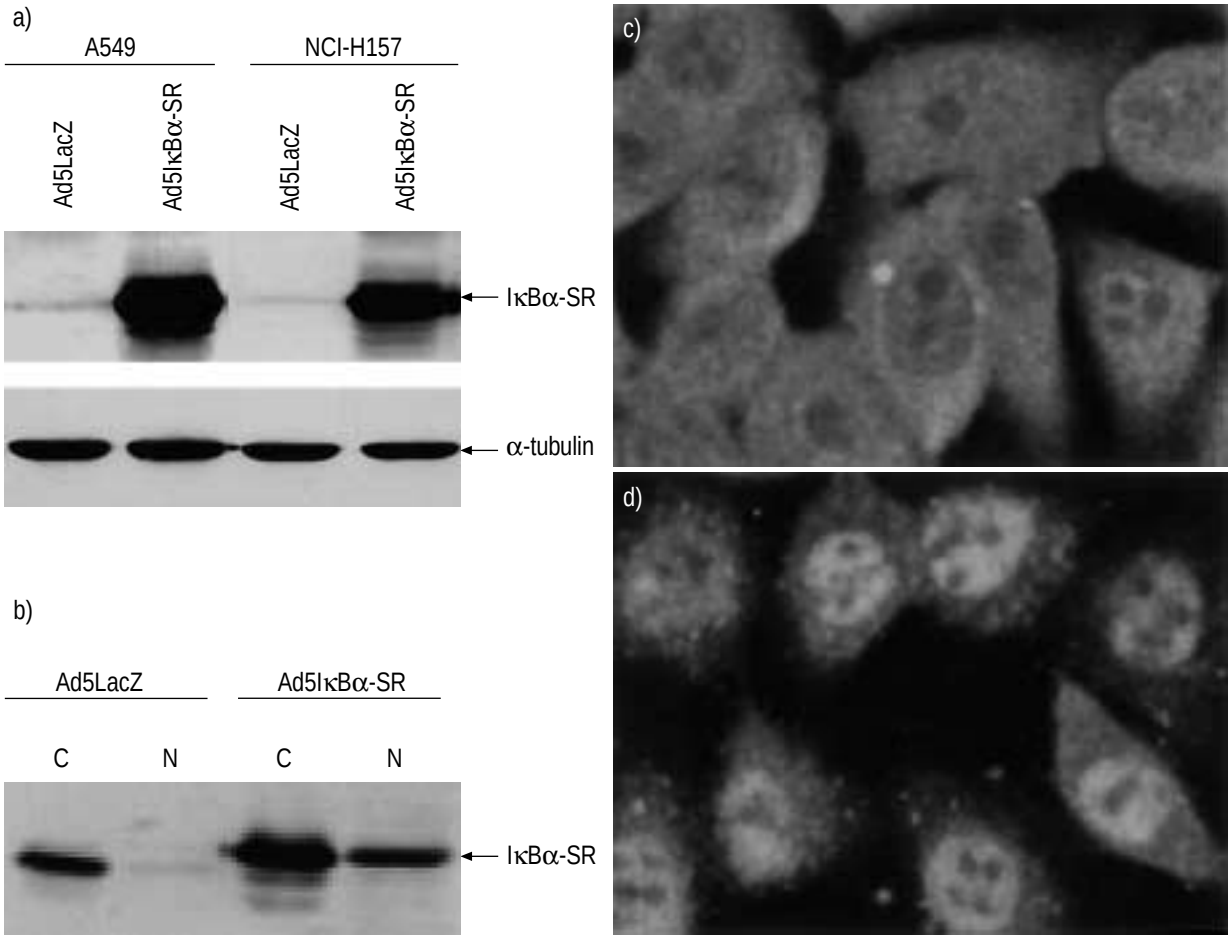

Fig. 2. – Overexpression and subcellular localization of exogenous IκBα super-repressor (IκBα-SR) using adenoviral vectors in respiratory epithelial cells. a) Adenovirus-mediated overexpression of IκBα-SR. A549 and NCI-H157 cells were infected with Ad5LacZ or Ad5IκBα-SR at 20 multiples of infection (moi). Forty-eight hours after infection, whole cell extracts were separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and IκBα was detected by rabbit polyclonal IκBα antibody. b) Subcellular localization of exogenous IκBα-SR. A549 cells were infected with Ad5LacZ or Ad5IκBα-SR. Cytoplasmic (C) and nuclear (N) extracts were assayed for IκBα by Western blot analysis 48 h after infection. c) and d) Cytoplasmic and nuclear localization of exogenous IκBα-SR. A549 cells were infected with c) Ad5LacZ or d) Ad5IκBα-SR. Immunofluorescent staining for IκBα was performed using IκBα antibody followed by rhodamine-conjugated detection antibody.

stimulation of Ad5IκBα-SR infected cells with TNF-α did not detectably decrease IκBα- SR protein levels (fig. 3b).

Adenovirus-mediated gene transfer of IκBα super-repressor blocks tumour necrosis factor-α-induced nuclear factor-κB activation

The high level of nondegradable exogenous IκBα-SR expression in Ad5IκBα-SR infected cells suggests a potential inhibitory effect of IκBα-SR on the NF-κB activity. To test this possibility, NF-κB activity in Ad5LacZ and Ad5IκBα-SR infected cells was measured in response to TNF-α stimulation. NF-κB activity was assayed by two approaches: first, by measuring the NF-κB-DNA binding activity by electrophoretic mobility shift assay (EMSA) and second by assessing the nuclear translocation of NF-κB subunit p65. To evaluate the effect of overexpression of IκBα-SR on the NF-κB-DNA binding activity, Ad5LacZ and Ad5IκBα-SR infected cells were stimulated with TNF-α (5 $ng \cdot mL^{-1}$) for 30 min, and nuclear extracts were subjected to EMSA with κB site DNA probe. In Ad5LacZ-transduced cells, NF-κB-DNA binding activity increased in response to TNF-α stimulation (fig. 4a). In contrast, this TNF-α-induced increase in NF-κB-DNA binding activity was completely blocked in Ad5IκBα-SR infected cells (fig. 4a). When 50-fold molar excess of unlabelled double-stranded NF-κB oligonucleotide was added to the binding reaction, the retarded band disappeared, suggesting the specificity of binding. Supershift assay showed the presence of p50 and p65 subunits of NF-κB. To confirm further that the nuclear translocation of transcriptionally active NF-κB subunit p65 was reduced by Ad5IκBα-SR transduction, cytoplasmic and nuclear extracts were assayed by Western blot analysis for changes in relative abundance of p65. In Ad5LacZ infected cells, the majority of p65 was located in the cytoplasmic fraction under basal state (fig. 4b). After 30 min of TNF-α stimulation, a

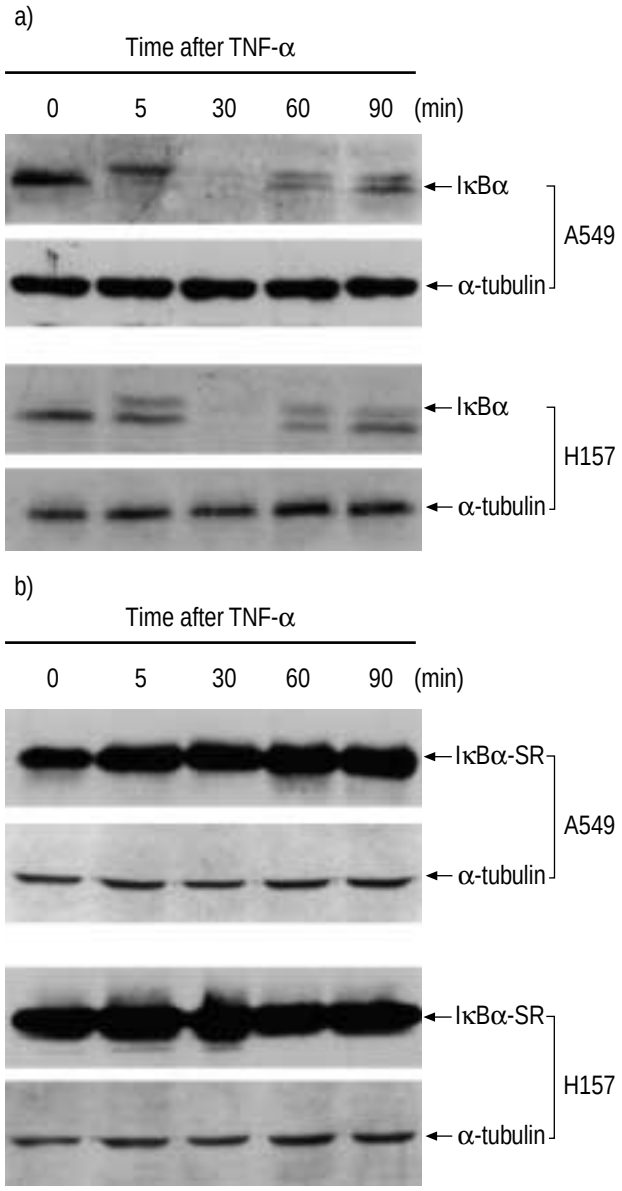

Fig. 3. – Exogenous IκBα super-repressor (IκBα-SR) is resistant to tumour necrosis factor (TNF)-α-induced degradation. A549 and NCI-H157 cells were infected with a) Ad5LacZ or b) Ad5IκBα-SR at 20 multiples of infection (moi) for 48 h, and then stimulated with TNF-α (5 ng·mL^{-1}) for 5, 30, 60, and 90 min. Whole cell extracts were assayed for IκBα by Western blot analysis.

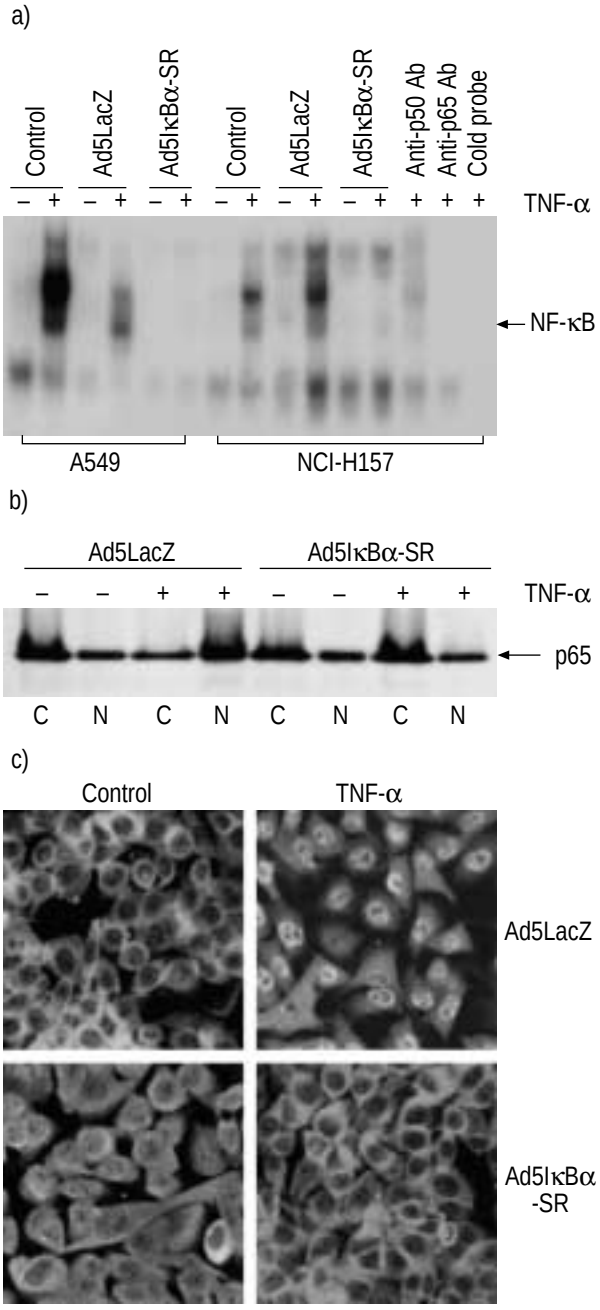

Fig. 4. – Adenovirus-mediated overexpression of IκBα super-repressor (IκBα-SR) blocks tumour necrosis factor (TNF)-α-induced activation of nuclear factor (NF)-κB. a) Effect of overexpression of IκBα-SR on NF-κB deoxyribonucleic acid (DNA) binding activity. A549 and NCI-H157 cells were infected with Ad5LacZ or Ad5IκBα-SR for 48 h, and then incubated for 30 min in the presence or absence of TNF-α (5 ng·mL^{-1}). Nuclear extracts were subjected to electrophoretic mobility shift assay with κB site DNA probe. b) Effect of overexpression of IκBα-SR on blocking of TNF-α-induced nuclear translocation of NF-κB subunit p65. Ad5LacZ or Ad5IκBα-SR-infected A549 cells were treated with media alone or TNF-α (5 ng·mL^{-1}) for 30 min and then cytoplasmic and nuclear extract were prepared. Cytoplasmic (C) and nuclear (N) extracts were analysed for the presence of p65 by Western blot analysis. c) Effect of overexpression of IκBα-SR on subcellular localization of p65. Ad5LacZ- or Ad5IκBα-SR-infected A549 cells were treated with media alone (control) or TNF-α (5 ng·mL^{-1}) for 30 min and then fixed and permeabilized for 5 min. Immunofluorescent staining for p65 was performed using anti-p65 antibody followed by a rhodamine-conjugated antibody.

decrease in cytoplasmic p65 was noted with concomitant increase in nuclear p65. In contrast, nuclear p65 abundance was not observed in Ad5 IκBα-SR infected cells by TNF-α stimulation (fig. 4b). The subcellular localization of p65, with or without TNF-α stimulation, was also investigated by immunofluorescent staining. In Ad5LacZ infected cells, there was a strong nuclear staining of p65 after 30 min of TNF-α stimulation compared to the cytoplasmic distribution in unstimulated cells. This nuclear translocation of p65 by TNF-α was blocked in Ad5IκBα-SR infected cells, as demonstrated by the cytoplasmic staining pattern (fig. 4c). These results support the

conclusion that Ad5IκBα-SR transduction suppresses NF-κB activation following stimulation with TNF-α.

Adenovirus-mediated gene transfer of IκBα super-repressor blocks pro-inflammatory cytokine expression

Since most inflammatory cytokine genes that play an important role in the inflammatory response contain functional NF-κB binding sites in their promoter regions, the effect of inhibition of NF-κB activation by adenovirus-mediated overexpression of IκBα-SR on the expression of inflammatory cytokines, both at mRNA and protein levels, have been analysed. At first, TNF-α-induced IL-8 and IL-1β-induced TNF-α mRNA expressions were evaluated by Northern blot analysis in nontreated, and Ad5LacZ- and Ad5IκBα-SR-infected cells. In nontreated control

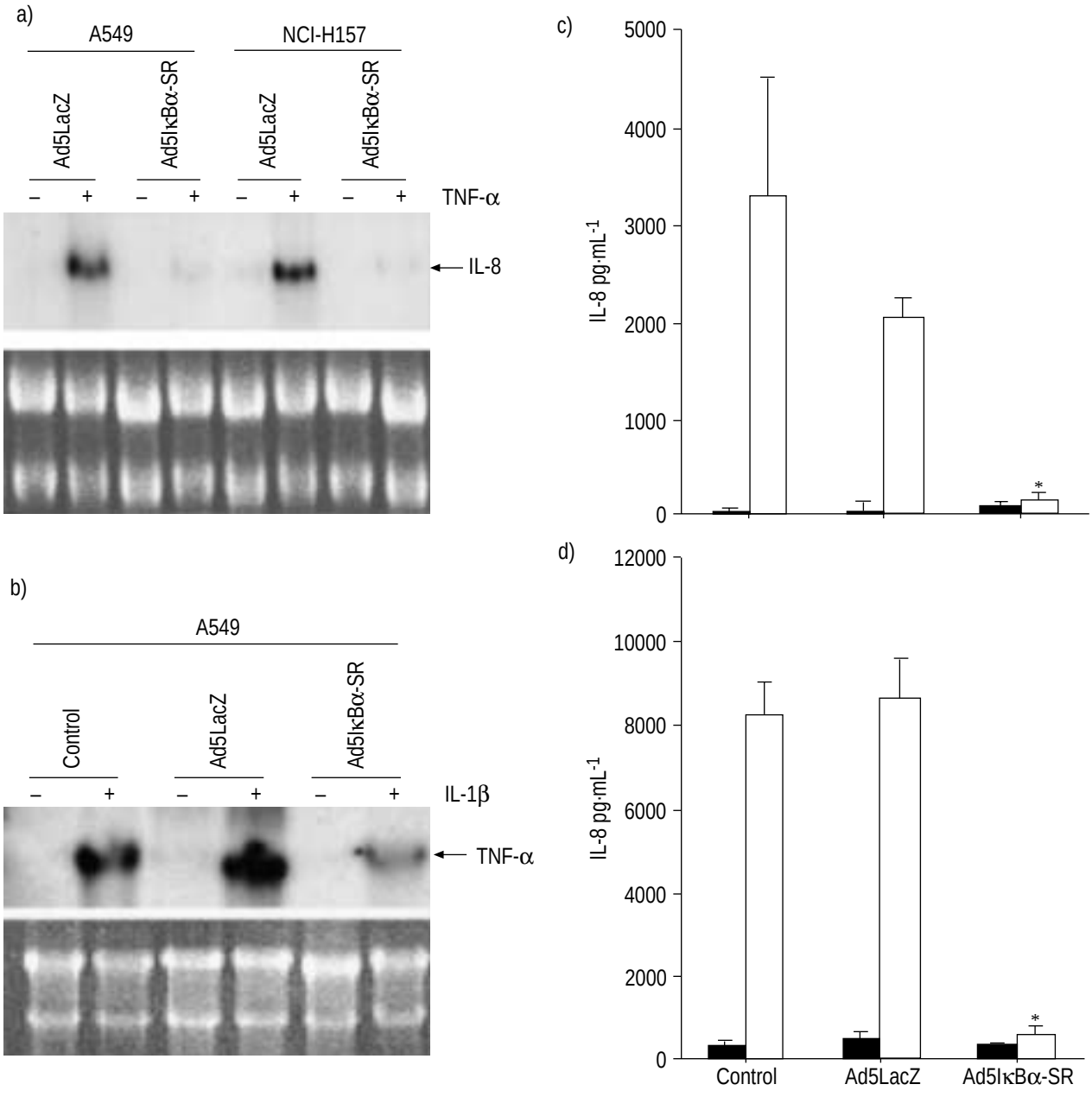

Fig. 5. – Adenovirus-mediated overexpression of IκBα super-repressor (IκBα-SR) blocks interleukin (IL)-1β- and tumour necrosis factor (TNF)-induced pro-inflammatory cytokine expression. a) and b) Effect of exogenous IκBα-SR on pro-inflammatory cytokine messenger ribonucleic acid (mRNA) expression. A549 and NCI-H157 cells were infected with Ad5LacZ or Ad5IκBα-SR, then stimulated with a) TNF-α (5 ng·mL^{-1}) and b) IL-1β (5 ng·mL^{-1}) for 4 h. IL-1β-induced TNF-α mRNA, and TNF-α-induced IL-8 mRNA expressions were assayed by Northern blot analysis. c) and d) Effect of exogenous IκBα-SR on the production of IL-8. Ad5LacZ- or Ad5IκBα-SR-infected cells were stimulated with TNF-α for 18 h. Concentrations of IL-8 in supernatant fluid were quantitated in c) A549 cells and d) NCI-H157 cells, by enzyme-linked immunosorbent assay (ELISA). ■: nonstimulated cells; □: cells stimulated with TNF-α (5 ng·mL^{-1} for 18 h). Data are presented as mean±SD of three different experiments. *: $p<0.05$ *versus* Ad5LacZ.

and Ad5LacZ-infected cells, both TNF-α-induced IL-8 and IL-1β-induced TNF-α mRNA expressions were significantly increased 4 h after TNF-α or IL-1β stimulation, which was completely inhibited in Ad5IκBα-SR-infected cells (fig. 5a). To evaluate the effect of IκBα-SR overexpression on the production of TNF-α-induced IL-8 protein, IL-8 protein concentration was measured by ELISA in culture supernatant, 18 h after stimulation with TNF-α. The production of IL-8 was significantly increased by TNF-α stimulation in Ad5LacZ-infected cells. Ad5IκBα-SR transduction completely blocked TNF-α-induced IL-8 production in both A549 and NCI-H157 cells (fig. 5b).

Discussion

The present study investigated the effect of overexpression of IκBα-SR, using an adenovirus vector, on the pro-inflammatory cytokine expression in respiratory epithelial cells. It was found that adenovirus-mediated overexpression of IκBα-SR suppressed the cytokine-induced activation of NF-κB. Ad5IκBα-SR transduction suppressed cytokine-induced IL-8 and TNF-α expressions at both RNA and protein levels. These results suggest that blocking the NF-κB pathway by adenovirus-mediated overexpression of IκBα-SR shows an effective anti-inflammatory effect in respiratory epithelial cells.

The present study constructed an adenovirus vector-expressing nondegradable IκBα to block NF-κB activation. Adenovirus vectors have advantages over other viral vector systems. Because an adenovirus does not integrate into chromosomal DNA, it poses a reduced risk for cellular transformation caused by insertional mutagenesis or activation of endogenous retroviruses. An adenovirus also has the capacity for expression of relatively large DNA inserts (up to 7 kb), the ability to be propagated easily and purified to high titres, an extremely broad host range, and the availability of a large number of vectors containing different promoters [14]. In addition, in contrast to retroviral vectors, which can express transduced genes only in proliferating cells with relatively low efficiency, adenovirus vectors can express their gene products with much higher efficiency in dividing, nondividing, or slowly proliferating cells, without prolonged *in vitro* culture or selection [15, 16]. To assay the inhibitory effect of IκBα on the expression of endogenous genes, and to allow biochemical analysis, it is necessary to utilize a highly efficient transfection system. Adenovirus-mediated gene transfer meets this criterion [17–20]. In this study, the transduction efficiency was >90% and IκBα-SR was highly expressed in both A549 and NCI-H157 cells. Adenoviral gene therapy is particularly applicable to the respiratory system because of its easy accessibility by bronchoscopy or inhalation.

There is evidence that IκBα, which is resynthesized after degradation, can enter the nucleus and remove NF-κB from DNA. The inactive NF-κB-IκBα complex is then transported back into the cytoplasm, thereby completing a cycle of activation and inactivation of NF-κB [21–23]. In the present study, some of the over-expressed IκBα-SR translocated to the nucleus whereas the endogenous IκBα remained in the cytoplasm. This observation suggests that over-expressed IκBα-SR suppressed the function of activated NF-κB in addition to sequestering NF-κB in the cytoplasm.

Excessive adenovirus infection itself is cytotoxic. In addition, overexpression of IκBα-SR alone resulted in increased cytotoxicity in lung cancer cells [24]. To evaluate whether the decreased pro-inflammatory cytokine production after transduction with Ad5IκBα-SR may be due to a cytotoxic effect of adenovirus infection, cell viability was assessed by MTT assay at 24 and 48 h after adenovirus infection. Neither Ad5LacZ nor Ad5IκBα-SR transduction decreased cell viabilities of A549 or NCI-H157 cells, up to 48 h, at a dose of 20 moi, minimizing the possibility of a cytotoxic effect of Ad5IκBα-SR in these experiments. This is not consistent with a previous study by BATRA *et al*. [24], which showed a marked decrease in cell viability by Ad5IκBα-SR transduction. One factor that may contribute to this discrepancy is the dose of adenovirus. In contrast to 20 moi used in the present study, cells were transduced at 100 moi in the BATRA *et al*. [24] study.

Respiratory epithelial cells are the main target of injury in various inflammatory lung diseases. In addition, respiratory epithelial cells are actively involved in initiating and maintaining inflammation by producing pro-inflammatory mediators. This inflammatory process delays the normal epithelial cell repair following lung injury, which is critical for restoration of lung function. For these reasons, suppression of the inflammatory response in respiratory epithelial cells seems to be a prerequisite for accelerating the repair process in inflammatory lung diseases. In this study, both TNF-α-induced IL-8 and IL-1β-induced TNF-α expressions were inhibited at the mRNA and protein levels in Ad5IκBα-SR-infected respiratory epithelial cells compared to Ad5LacZ-infected cells. Because TNF-α and IL-1β are both chemotactic and mitogenic for lung fibroblasts and stimulate collagen synthesis by these cells [25], the inhibition of NF-κB activation with Ad5IκBα-SR may be an effective gene therapy for various inflammatory lung diseases, by suppressing both inflammation and fibrosis. In animal studies, intravenous somatic gene transfer with IκBα reduced endotoxin-induced activation of inflammatory mediators and increased survival [26]. However, there are many problems that need to be solved before gene therapy can be widely used in clinical settings. First, an efficient method of delivering the adenoviral vector into the respiratory epithelial cells should be developed. In addition, host immunological responses against viral vector proteins need to be modulated to allow long-term exogene expression [27, 28].

In diverse cell types, NF-κB has been shown to regulate the apoptotic program, either as essential for the induction of apoptosis or, perhaps more commonly, as blockers of apoptosis. This depends on the specific cell type and the type of inducer. For example, in respiratory epithelial cells, the inhibition of NF-κB activation by Ad5IκBα-SR made NCI-H157 cells more susceptible to TNF-α-induced apoptosis [12], but not in A549 cells (data not shown).

Therefore, TNF-α, which is released in large amounts from inflammatory cells, may induce apoptosis in cells with adenovirus-mediated overexpression of IκBα-SR at the regional inflammation site. This may decrease the usefulness of Ad5IκBα-SR as a therapeutic tool to manage the excessive inflammation. The development of specific and safe NF-κB inhibitors, which does not impair NF-κB dependent normal cell functions, would be necessary for the effective suppression of inflammation.

In conclusion, the present data suggest the feasibility of adenovirus-mediated overexpression of IκBα super-repressor to suppress the inflammatory response in respiratory epithelial cells. Because there is no evidence that adenovirus-mediated overexpression of IκBα super-repressor would be effective *in vivo*, additional studies are needed to determine the effectiveness of this approach in lung inflammation *in vivo*.

Acknowledgements. The authors thank A.S. Baldwin (University of North Carolina, Chapel Hill, NC, USA) for providing IκBα-SR cDNA, and R.D. Gerard (University of Texas Southwestern Medical Center, Dallas, TX) for providing pAC-CMVpLpA and pJM17. The authors also thank S. Kim for proofreading this manuscript.

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