

Adenoviral interleukin-12 gene transduction into human bronchial epithelial cells: up-regulation of pro-inflammatory cytokines and its prevention by corticosteroids

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Summary

Background One of the potential effects of IL-12 is to restore Th1/Th2 balance. Therefore, we investigated the possibility of developing a system for local delivery of IL-12 into the airways by examining protein expression in a human bronchial epithelial cell line (BEAS-2B) after adenoviral IL-12 gene transduction. The effects of dexamethasone on the gene-modified cells were also examined.

Methods Adenoviral vectors AxCAegfp and Ax1CIhp40ip35 were used to transduce enhanced green fluorescence protein and IL-12 genes, respectively, into BEAS-2B cells. Wild-type and IL-12 gene-transduced BEAS-2B cells were then incubated with or without dexamethasone, and concentrations of IL-12, IFN- γ , IL-6, IL-8, granulocyte macrophage-colony stimulating factor and chemokines (TARC and RANTES) in the supernatant were measured by ELISA. IL-12 receptor expression was analysed by flow cytometry and RT-PCR.

Results The efficiency of transgene expression in BEAS-2B cells at a multiplicity of infection of 30 was approximately 80%. Gene-modified BEAS-2B cells produced biologically active IL-12, regardless of dexamethasone treatment. While IL-12 gene transduction led to increased production of IL-6 and IL-8 by BEAS-2B cells, expressions of these proteins were suppressed by dexamethasone. Addition of exogenous IL-12 failed to augment BEAS-2B cell IL-6 and IL-8 production, and IL-12 receptor expression by BEAS-2B cells was not detected.

Conclusions Our findings suggest that adenoviral IL-12 gene transduction may be effective in inducing IL-12 expression in the airways, and could be a potential approach in the management of bronchial asthma.

Keywords adenoviral vector, BEAS-2B cell, dexamethasone, gene transduction, human bronchial epithelial cells, IL-12, IL-8, IL-6

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Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways associated with variable restriction of airflow in the lungs. The inflammatory response in asthma is associated with airway hyper-responsiveness and involves a number of different types of cells, including eosinophils, basophils, mast cells, and lymphocytes [1, 2]. In particular, Th2 lymphocytes are known to play an important role in the pathogenesis of bronchial asthma, such that Th2-type cells, which secrete cytokines such as IL-4 and IL-5, predominate over Th1-type cells [3]. Several reports have also shown that Th2 cytokines induce eosinophilia and airway hyper-responsiveness in animal models of asthma [4, 5].

Restoration of the Th1/Th2 balance may be an important factor in the management of bronchial asthma. IL-12 is a candidate cytokine to achieve this balance as it induces production of Th1 cytokines such as IFN- γ . However, previous studies have failed to show the effectiveness of clinical application of IL-12 in bronchial asthma. A recent clinical study using IL-12 administered s.c. to asthma patients showed only limited effects [6], while intratracheal administration of IL-12 protein in a murine asthma model was effective in reducing eosinophilia only after repeat administrations [7]. Therefore, constitutive expression of IL-12 in the local airways may be a key strategy in bronchial asthma management by restoring the Th1/Th2 balance without the side-effects.

One promising strategy to achieve this is adenoviral gene transduction to express IL-12 protein in the local airways. Adenoviral gene transduction is easy to perform and has high transduction efficiency. Indeed, in a murine model of bronchial asthma, IL-12 gene transfer into the airways using

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an adenoviral vector led to reduction in airway eosinophilia and Th2 cytokine production [8]. However, little is known about the effectiveness of adenoviral transduction of IL-12 genes into human bronchial epithelial cells (HBEC).

Several reports have indicated that gene transduction by viruses or viral vectors may have adverse effects. Respiratory syncytial virus induced cytokine production in BEAS-2B cells [9]. Adenoviral Lac Z gene transduction into rat airway epithelial cells caused neurogenic inflammation [10]. Also, Adesanya et al. [11] showed an increased inflammatory response after adenoviral β -gal gene transduction into the rat salivary gland. These previous studies indicated the possibility that adenoviral IL-12 gene transduction may increase the production of inflammatory cytokines and chemokines.

Therefore, to investigate the clinical application of IL-12 gene transduction into HBEC, we examined gene expression and cytokine production in HBEC transduced with IL-12 genes and the effects of dexamethasone on gene-modified HBEC. Our results showed that IL-12 genes could be successfully transduced into a human bronchial epithelial cell line (BEAS-2B) using an adenoviral vector to produce bioactive IL-12. It was also found that pro-inflammatory cytokine production by IL-12 gene-modified BEAS-2B cells was increased, and that this increase could be inhibited by dexamethasone.

Materials and methods

Reagents

Dexamethasone was obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Recombinant human IL-12 (rIL-12) was obtained from Sigma-Aldrich Co. Anti-IL-12 antibody and recombinant human IgG were obtained from Endogen (Woburn, MA, USA) and Monosan (Uden, the Netherlands).

Culture of human bronchial epithelial cell line BEAS-2B

Human bronchial epithelial cell line BEAS-2B, transformed with SV40 virus, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells at passages 42–46 were plated in 100 mm dishes and incubated at 37°C, under 5% CO₂ in air, for 7–10 days. LHC9/RPMI-1640 medium was changed after 24 h and then every other day, as described previously [12].

Preparation of human peripheral blood lymphocytes

Leucocyte concentrates from healthy donors were separated into peripheral blood mononuclear cells (PBMC) by density gradient centrifugation in lymphocyte separation medium. Subsequently, lymphocytes were separated from PBMC by counter flow centrifugal elutriation in a Beckman JE-5.0 rotor (Beckman Instruments, Inc., Fullerton, CA, USA) as described previously [13]. The lymphocyte-rich fractions were collected at flow rates of 12–16 mL/min, respectively, at 150 g. The purity of the lymphocyte fraction was greater than 99%.

Adenovirus vector construction and preparation

The recombinant adenoviruses AxCAegfp and AxCAIhp40ip35 were used as vectors. AxCAegfp contained an enhanced green fluorescent protein (EGFP) gene. For preparation of AxCAegfp, an Adenovirus Expression Vector Kit (Takara Shuzo Co. Ltd., Kyoto, Japan) was used, in which the adenoviral cosmid, pAxCAwt [14], was included as described previously [15]. EGFP cDNA was obtained from pEGFP-N1 (Clontech, Palo Alto, CA, USA) and subcloned into pAxCAwt. Transfection of 293 cells (human embryonic kidney cell line) with pAxCAwt was performed, producing replication-incompetent E1- and E3-deficient adenoviral vectors expressing EGFP. Recombinant virus (AxCAegfp) was prepared by expansion of a single clone generated in 293 cells, and was then purified by limiting dilution. This virus was fluorescent.

Ax1CIhp40ip35 is a bicistronic vector that simultaneously expresses the IL-12p40 and p35 subunits. It was obtained from RIKEN Gene Bank (Tsukuba, Japan) [14, 16]. No replication-competent adenoviruses or E1⁺ viruses were detected in any of the preparations.

Adenovirus-mediated gene transduction into BEAS-2B cells

When cultured BEAS-2B cells reached confluence, cells were suspended in LHC9/RPMI 1640 medium, plated in six-well tissue culture plates at 3×10^5 cells/well, and cultured for 24 h. Adenoviruses AxCAegfp and Ax1CIhp40ip35 were suspended in phosphate-buffered saline (PBS) supplemented with 1% human serum albumin (HSA/PBS). BEAS-2B cells were washed once with PBS, HAS/PBS containing adenoviral vector added to each well, and cells were exposed for 2 h at 37°C, under 5% CO₂ in air. The cells were then washed with PBS and resuspended in LHC9/RPMI-1640 medium. In some experiments, gene transduction was undergone in the presence of 10^{-7} M dexamethasone.

Non-contact co-culture system for gene-transduced BEAS-2B cells and human peripheral blood lymphocytes

Interaction between gene-transduced BEAS-2B cells and human peripheral blood lymphocytes was examined using a non-contact co-culture system in six-well plates, essentially as described previously [17]. Briefly, BEAS-2B cells transduced with IL-12 genes (BEAS/IL-12) were cultured separately from human peripheral blood lymphocytes using cell culture inserts (Becton Dickinson, Tokyo, Japan) with a pore size of 0.4 μ m and a pore density of 1.6×10^6 pores/cm². This allowed the bidirectional diffusion of molecules but not cell migration. Cultures were incubated at 37°C, under 5% CO₂ in air, BEAS-2B cells were transduced as described above, and then plated at the bottom of culture-plate wells. Human peripheral blood lymphocytes (3.5×10^6) were plated in culture inserts placed in the wells and the cells incubated with 3.5 mL LHC9/RPMI for the indicated time periods in the presence or absence of anti-IL-12 antibody or its control IgG.

Determination and flow cytometric measurement of EGFP expression of BEAS-2B cells transduced with AxCAegfp

EGFP expression of BEAS-2B transduced with AxCAegfp (BEAS/EGFP) was visualized by fluorescence microscopy

using a BX61 microscope (Olympus, Tokyo, Japan). BEAS/EGFP cells were photographed using a DP50 digital camera with Viewfinder Lite software (Olympus). Expression of EGFP by BEAS-2B and HBEC cells was analysed quantitatively to evaluate gene-transduction efficiencies accurately. BEAS-2B cells were washed once and EGFP expression was quantified by flow cytometry using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA).

Isolation and analysis of mRNA expression by semi-quantitative RT-PCR

Total RNA was isolated using the acid guanidinium thiocyanate/phenol-chloroform method (Isogen) and RNA concentration was adjusted by a β -actin competitive PCR set (TAKARA, Tokyo, Japan). Expression of IL-12p40, IL-12p35, and IL-12R β 2 was analysed using a one-step RNA PCR kit (TAKARA). The reverse transcription (RT) reaction for IL-12p40 was carried out at 45 °C for 30 min and at 94 °C for 2 min. RT reactions for the other molecules were 50 °C for 30 min and 94 °C for 2 min. PCR reaction cycles were as follows: IL-12p40, denaturation for 30 s at 85 °C, annealing for 30 s at 46 °C, and extension for 30 s at 72 °C; IL-12p35, denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C, and extension for 60 s at 72 °C; β -actin, denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 30 s at 72 °C. For semi-quantitative RT-PCR, PCR cycle number was determined by preliminary examination, such that the cycle numbers gave amplification within the linear range for each primer set. PCR reaction cycles for IL-12R β 2 were as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 60 s at 72 °C, for 35 cycles. The following primers were used: IL-12p40 sense primer, 5'-TCTAAGCGATTCGCTCCTGC-3'; IL-12p40 anti-sense primer, 5'-AAGCTGCTGGTGTAGTTTTG-3'; IL-12p35 sense primer, 5'-CTTCACCACTCCCAAAACCTG-3'; IL-12p35 anti-sense primer, 5'-AGCTCGTCACTCTGTCAA-TAG-3'; IL-12R β 2 sense primer, 5'-AGGGACCTGATGCTATCGGA-3'; IL-12R β 2 anti-sense primer, 5'-CTGGTTCCTGGCCAGGATTC-3'; β -actin sense primer, 5'-AAGAGAGGCATCCTCACCT-3'; and β -actin anti-sense primer, 5'-TACATGGCTGGGGTGTGTGAA-3'. PCR products were separated electrophoretically in 1.5% agarose gels and photographed after ethidium bromide staining.

Measurement of cytokines

Parent and gene-transduced BEAS-2B cells were cultured for indicated time periods and the cell-free supernatants were treated after brief centrifugation. In some experiments, the culture was treated in the presence of dexamethasone (10^{-7} or 10^{-9} M), recombinant IL-12 (10, 100, and 1000 U/mL), anti-IL-12 antibody, and its control IgG (1 μ g/mL). Production of IL-12p70, IL-8, IL-6, granulocyte/macrophage-colony-stimulating factor (GM-CSF), regulated on activation-normal T cell expressed and secreted (RANTES), and thymus- and activation-regulated chemokine (TARC) by BEAS-2B cells were measured by ELISA. IFN- γ produced by human peripheral blood lymphocytes was measured using the same technique. IL-8, IL-6, and IFN- γ ELISA were

performed essentially as described previously [18]. GM-CSF, RANTES and TARC ELISA (R&D, Minneapolis, MN, USA) and IL-12 (Endogen SP-HIL12) were performed according to the manufacturer's instructions. Sensitivity limits of ELISA for IL-12p70, IL-8, IL-6, GM-CSF, IFN- γ , TARC, and RANTES were 17, 20, 20, 7.8, 20, 7.8, and 15.6 pg/mL, respectively.

Flow cytometric measurement of surface IL-12 receptor expression

Anti-IL-12R β 1-fluorescein-labelled antibody (maximum emission 530 nm, detected by the FL1 detector) was obtained from R&D Systems, Inc. Anti-IL-12R β 2-R-PE-conjugated antibody (maximum emission 585 nm, detected by the FL2 detector), anti-CD51-FITC and CD61-FITC antibodies were obtained from BD Pharmingen (San Diego, CA, USA). Harvested BEAS-2B cells were washed once and incubated for 30 min at 4 °C with 10–20 μ g primary monoclonal antibodies and analysed by flow cytometry using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson).

Statistical analysis

Data analysis was performed using Stat-View 5.0 (Abacus Concept, Inc., Berkeley, CA, USA). Experimental results were expressed as mean $\pm$ SEM. The statistical significance of differences between groups was analysed using the analysis of variance with Fisher's test. Differences were considered to be statistically significant if *P*-values were less than 0.05.

Results

Efficiency of adenovirus-mediated gene transduction and IL-12 production in gene-transduced BEAS-2B cells

To examine the transduction efficiency of adenoviral vectors into BEAS-2B cells, we transduced the EGFP gene into BEAS-2B cells as a marker of gene transduction. EGFP gene-modified cells (BEAS/EGFP) were fluorescent (Fig. 1b) compared to non-transduced BEAS-2B (BEAS/WT) cells (Fig. 1a). Flow cytometric histograms showed that the fluorescence intensity of BEAS/EGFP cells was higher than that of BEAS/WT cells (Figs 1c and d). Next, adenoviral vectors AxCAegfp and Ax1Cihp40ip35 were transduced at multiplicity of infection (MOI) of values 10, 30, 60, and 100 for 2 h at 37 °C, under 5% CO₂ in air. Transduction efficiency, mean fluorescence intensity (MFI), and IL-12 production increased in an MOI-dependent manner (Table 1). The efficiency of transgene expression at an MOI of 30 was 83.5% of BEAS-2B cells and IL-12 production was 2.82 ± 0.84 ng/10⁶ cells. Therefore, we used an MOI of 30 for gene transduction into both BEAS-2B cells in all experiments unless otherwise stated.

Biological activity of IL-12 produced by BEAS/IL-12

To examine the biological activity of the IL-12 produced by the BEAS/IL-12 cells, BEAS-2B cells and human peripheral

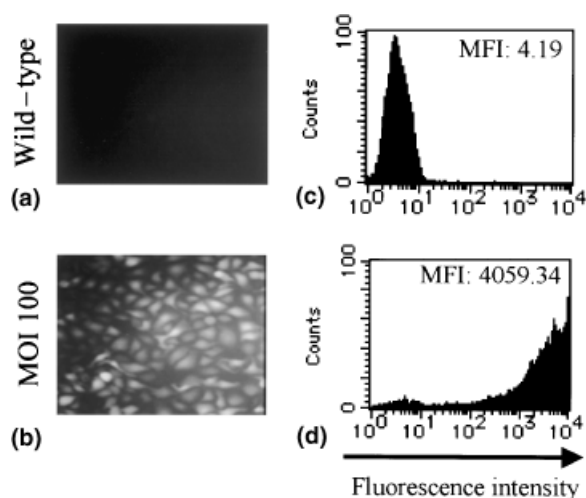


Fig. 1. EGFP expression in BEAS-2B cells after adenoviral EGFP gene transduction. BEAS-2B cells were incubated with AxCaegfp for 2 h. After 24 h, BEAS-2B cells were harvested and analysed for EGFP expression by fluorescence microscopy and flow cytometry. (a) and (b) are fluorescence microscopic images ($\times 400$) of BEAS-2B cells. (a) Wild-type BEAS-2B cells, (b) BEAS-2B cells transduced by AxCaegfp at an MOI of 100. (c) and (d) are EGFP expression levels as analysed by flow cytometry. MFI: mean fluorescence intensity. (c) Wild-type BEAS-2B cells, (d) BEAS-2B cells transduced by AxCaegfp at an MOI of 100.

Table 1. Transduction efficiency and IL-12 production by IL-12 gene-transduced BEAS-2B cells

MOI	Transduction efficiency (%)	MFI	IL-12 production (ng/ 10^6 cells/48 h)
0	0.06	4.19	<20
10	62.77	1860.90	1.53 $\pm$ 0.24
30	83.50	1719.77	2.82 $\pm$.84*
60	92.73	2770.55	3.31 $\pm$ 0.41*
100	96.00	3232.00	7.54 $\pm$ 2.60*

MOI: multiplicity of infection; MFI: mean fluorescence intensity. BEAS-2B cells were transduced with Ax1Clhp40ip35 or AxCaegfp for 2 h. After 24 h incubation, EGFP gene-modified BEAS-2B cells were harvested and transduction efficiency and MFI analysed by flow cytometry. Data represent three independent experiments. IL-12 gene-modified BEAS-2B cells were cultured and supernatants were collected at 48 h. IL-12 concentrations were measured by ELISA. Data are mean $\pm$ SEM from three independent experiments.

* $P < 0.05$ compared to non-transduced cells (i.e. MOI = 0).

blood lymphocytes were cultured in a non-contact co-culture system. Lymphocytes were cultured with BEAS-2B cells for 12, 24, 48, and 96 h, and IFN- γ levels were measured by ELISA. As shown in Fig. 2, IFN- γ production by lymphocytes cultured with BEAS/IL12 cells increased in a time-dependent manner. At 48 h, lymphocyte IFN- γ production was 252.1 ± 150.4 pg/ 10^6 cells. However, wild-type BEAS-2B cells (BEAS/WT) did not induce IFN- γ production in lymphocytes. Addition of anti-IL-12 antibody neutralized IFN- γ production but control IgG antibody did not. These findings showed that the IL-12 produced by the BEAS/IL12 cells was biologically active.

Determination of cytokine production by BEAS/IL-12

BEAS-2B cells are known to produce IL-8, GM-CSF, and IL-6 constitutively. It has also been shown recently that HBEC

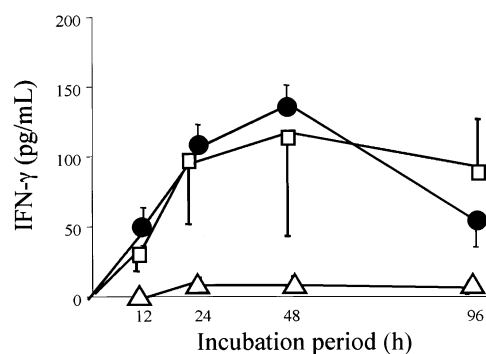


Fig. 2. Production of IFN- γ by human peripheral blood lymphocytes cultured with BEAS-2B cells using a non-contact co-culture system. Human peripheral blood lymphocytes (3.5×10^5) were cultured with 3×10^5 gene-transduced BEAS-2B cells using a non-contact co-culture system in six-well culture plates. After incubation for the indicated hours, supernatants were collected and cytokine concentrations were measured by ELISA. Values are means $\pm$ SEM of results from three independent experiments. ●: lymphocytes with BEAS/IL-12 cells. □: lymphocytes with BEAS/IL-12 cells in the presence of control antibody. △: lymphocytes with BEAS/IL-12 cells in the presence of anti-IL-12 antibody.

Table 2. Production of cytokines by gene-modified BEAS-2B cells

Time (h)	Cells	Cytokines (pg/ 10^6 cells)				
		IL-8	IL-6	GM-CSF	RANTES	TARC
48	BEAS/WT	1485 $\pm$ 662	366 $\pm$ 159	20 $\pm$ 28	<7.8	<15.6
	BEAS/EGFP	1945 $\pm$ 407	457 $\pm$ 209	10 $\pm$ 14	<7.8	<15.6
	BEAS/IL12	4718 $\pm$ 1623*	921 $\pm$ 381*	70 $\pm$ 56	<7.8	<15.6
96	BEAS/WT	3057 $\pm$ 1743	602 $\pm$ 206	14 $\pm$ 19	<7.8	<15.6
	BEAS/EGFP	3959 $\pm$ 1653	716 $\pm$ 234	27 $\pm$ 38	<7.8	<15.6
	BEAS/IL12	8717 $\pm$ 3727*	1274 $\pm$ 336*	110 $\pm$ 71*	<7.8	<15.6

GM-CSF: granulocyte/macrophage-colony-stimulating factor; RANTES: regulated on activation-normal T cell expressed and secreted; TARC: thymus- and activation-regulated chemokine. BEAS-2B cells were transduced with Ax1Clhp40ip35 or AxCaegfp at an MOI of 30 for 2 h. Each group of gene-transduced BEAS-2B cells was cultured in 24-well culture plates. Supernatants were collected at the indicated times, and cytokine levels were measured by ELISA. Data are mean $\pm$ SEM from three independent experiments.

* $P < 0.05$ compared to BEAS/WT.

produce RANTES and TARC. Therefore, we examined whether IL-12 gene transduction altered the production of these cytokines by BEAS-2B cells. BEAS/WT, BEAS/EGFP, and BEAS/IL-12 cells were cultured for 48 and 96 h and cytokine levels were measured by ELISA. As shown in Table 2, production of IL-8 and IL-6 by BEAS/IL-12 cells was significantly higher than that by BEAS/WT cells at 48 and 96 h. Similarly, GM-CSF production by BEAS/IL-12 cells was higher than that by BEAS/WT cells, but this difference did not reach statistical significance. RANTES and TARC were not detected in either BEAS/WT or BEAS/IL-12 cells.

Analysis of an autocrine mechanism of IL-12 production in BEAS/IL-12 cells

It is possible that cytokine production by BEAS/IL-12 was due to an autocrine mechanism involving the IL-12 produced

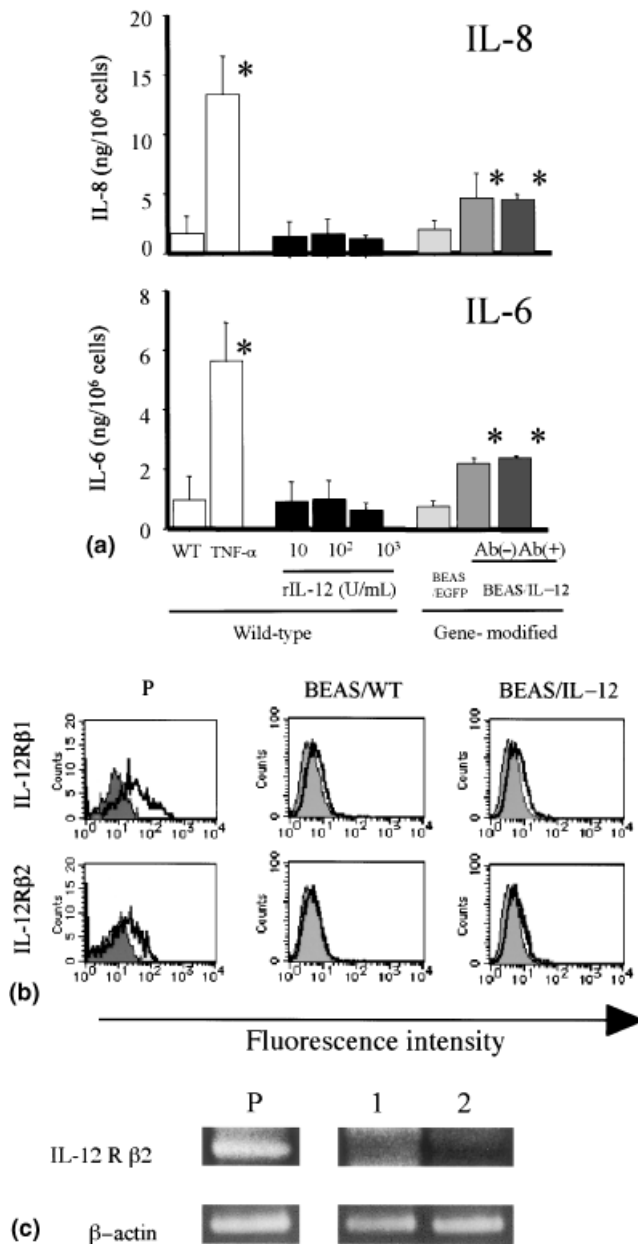


Fig. 3. (a) IL-8 and IL-6 production by BEAS-2B cells with exogenous IL-12 and by BEAS/IL-12 cells with anti-IL-12 antibody. BEAS-2B cells were incubated with recombinant IL-12 of 10, 100, and 1000 U/mL for 48 h. BEAS/IL-12 cells were incubated with anti-IL-12 antibody for 48 h. Supernatants were collected and cytokine concentrations were measured by ELISA. Data are mean $\pm$ SEM from three independent experiments. Open bar: wild-type BEAS-2B cells; closed bar: BEAS-2B cells in the presence of rIL-12; dotted bar: BEAS/EGFP; shaded bar: BEAS/IL-12; Ab: anti-IL-12 antibody. * $P < 0.05$ compared to wild-type BEAS-2B cells (WT). (b) Comparison of IL-12 receptor expression by BEAS/IL-12 and BEAS/WT cells. Expression of IL-12 receptor $\beta 1$ and $\beta 2$ was analysed by flow cytometry. Data represent three independent experiments. Closed histogram: control; open histogram: IL-12 receptor $\beta 1$ and $\beta 2$ expressing cells; P: positive control (lymphocytes cultured with IL-18 of 100 ng/mL for 72 h). (c) IL-12R $\beta 2$ expression by RT-PCR. RT-PCR was performed using an RNA-PCR kit as described in the Materials and methods. PCR products were electrophoresed in 1.5% agarose gels. Lane P: positive control (human lymphocytes cultured with LHC9/RPMI); lane 1: BEAS/WT cells; lane 2: BEAS/IL-12 cells. Data represent three independent experiments.

by BEAS/IL-12 cells. To examine this hypothesis, we first examined the cytokine production pattern by BEAS-2B cells stimulated with exogenous rIL-12. As shown in Fig. 3a, IL-8

and IL-6 levels were increased by the addition of TNF- α (open bar) but not by rIL-12 (closed bar). To examine the possible contribution of produced IL-12 to cytokine production, we blocked endogenous IL-12 by anti-IL-12 neutralizing antibody. As shown in Fig. 3a, IL-8 and IL-6 levels were not increased in BEAS/EGFP cells (dotted bar) but were increased in BEAS/IL-12 cells (shaded bar). The addition of anti-IL-12 neutralizing antibody to BEAS/IL-12 cells did not affect IL-8 and IL-6 production (shaded bar). These results suggest that exogenous or endogenous IL-12 is not responsible for the augmented cytokine production by BEAS-2B cells.

We also analysed IL-12 receptor $\beta 1$ and $\beta 2$ expression by BEAS/WT and BEAS/IL-12 cells by flow cytometry and RT-PCR. Flow cytometric analysis failed to detect expression of IL-12 receptor $\beta 1$ and $\beta 2$ on BEAS/IL-12 and BEAS/WT cells, but detected on human lymphocytes stimulated with IL-18 (Fig. 3b). Similarly, IL-12 receptor $\beta 2$ mRNA expression was not observed in BEAS/WT and BEAS/IL-12 cells by RT-PCR (Fig. 3c). These results suggested that BEAS-2B cells did not express IL-12 receptor, such that increased pro-inflammatory cytokine production is not mediated through an autocrine mechanism involving the IL-12 receptor.

Effect of dexamethasone on pro-inflammatory cytokine production by BEAS/IL-12

We also examined whether dexamethasone treatment could inhibit IL-12 gene transduction-dependent cytokine production. Several reports have shown that corticosteroids inhibit IL-8 and IL-6 secretion and mRNA accumulation in HBEC [19], and that augmentation of inflammatory cytokines after adenoviral vector transduction was decreased by dexamethasone [11,20]. BEAS/IL-12 cells were cultured in the presence or absence of dexamethasone (10^{-7} or 10^{-9} M) for 48 or 96 h, and cytokine levels were measured by ELISA. As shown in Table 3, dexamethasone was effective in suppressing the production of IL-8 and IL-6, and the magnitude was greater at 10^{-7} M.

Efficiency of adenoviral gene transduction into BEAS-2B cells in the presence of dexamethasone

The inhibitory effect of dexamethasone on the production of pro-inflammatory cytokines was dramatic. Therefore, we

Table 3. IL-8 and IL-6 production by IL-12 gene-modified BEAS-2B cells treated in the presence of dexamethasone

Dex	IL-8 (ng/10 ⁶ cells)		IL-6 (ng/10 ⁶ cells)	
	48 h	96 h	48 h	96 h
(-)	6.55 $\pm$ 2.23	11.36 $\pm$ 0.78	1.09 $\pm$ 0.46	1.38 $\pm$ 0.42
10^{-7} M	4.15 $\pm$ 2.42	5.01 $\pm$ 1.77*	0.38 $\pm$ 0.18*	0.47 $\pm$ 0.38*
10^{-9} M	5.65 $\pm$ 1.73	6.69 $\pm$ 1.36*	0.70 $\pm$ 0.09	0.82 $\pm$ 0.38

BEAS-2B cells were transduced with Ax1C1hp40ip35 at an MOI of 30 for 2 h. Then these cells were cultured with medium alone, 10^{-7} M dexamethasone or 10^{-9} M dexamethasone in 24-well culture plates. After incubation for 48 or 96 h, supernatants were collected and cytokine concentrations were measured by ELISA. Values are means $\pm$ SEM of data from three independent experiments. Dex: dexamethasone.

* $P < 0.05$ compared to BEAS/IL-12 cells cultured without dexamethasone.

examined whether dexamethasone inhibited the adenoviral gene transduction that allowed IL-12 production. The efficiency of transgene expression from BEAS-2B cells was found to be similar in the presence or absence of dexamethasone (Fig. 4a). At an MOI of 30, the efficiency of gene-modified cells treated with 10^{-7} M dexamethasone was 83.6% compared to 85.3% without dexamethasone (Fig. 4a).

In addition, to test the possibility that dexamethasone affected the expression of attachment molecules required for adenovirus infection into BEAS-2B cells, we examined the expression of CD51 and CD61, involved in viral internalization and infection, on BEAS-2B cells cultured with or without dexamethasone. As shown in Fig. 4b, CD51 and CD61 were constitutively expressed on BEAS-2B cells, with MFI values of CD51 and CD61 on BEAS-2B cells being 49.32 ± 6.67 and 23.65 ± 1.57 , respectively. Expression of CD51 and CD61 was not affected by dexamethasone (MFI values of 48.99 ± 4.29 and 20.58 ± 2.55 , respectively).

Production of IL-12 by IL-12 gene-transduced BEAS-2B cells in the presence of dexamethasone

Next, we examined the production of IL-12 by BEAS/IL-12 at an MOI of 30 in the presence or absence of dexamethasone. We first followed the time-course of IL-12 production by BEAS/IL-12 cells in the presence of dexamethasone (10^{-7} M). Culture supernatants were harvested from BEAS/IL-12 cells at 12, 24, 48 or 96 h and IL-12 production was examined by IL-12p70-specific ELISA (Fig. 5a). While BEAS/WT did not produce IL-12, BEAS/IL-12 cells produced large amounts of IL-12 (2.35 ± 0.85 ng/ 10^6 cells/48 h). Treatment of BEAS/IL-12 cells with 10^{-7} M dexamethasone did not affect IL-12 production at any time-point.

We then compared IL-12 mRNA expression levels. Total RNA was extracted from BEAS-2B cells culture for 24 h after transduction in the presence or absence of dexamethasone (10^{-7} M) and semi-quantitative RT-PCR was performed using primers specific for IL-12p35 and p40. Positive control data (Lane P) demonstrated that the cycle numbers used were within the linear range for each primer set (Fig. 5b). IL-12p35 and p40 mRNAs were not detected in BEAS/WT cells, while BEAS/IL-12 and BEAS/IL-12 cells treated with 10^{-7} M dexamethasone for 24 h showed similar expression levels.

Finally, we examined the bioactivity of IL-12 produced by BEAS/IL-12 in the presence of dexamethasone. As shown in Fig. 5c, dexamethasone of 10^{-7} M significantly inhibited IFN- γ production by lymphocytes co-cultured with BEAS/IL-12. Dexamethasone of 10^{-9} M also inhibited them, but the magnitude was low and statistical significance was observed only at 24 h.

Discussion

Our results showed that HBEC could be efficiently gene-modified by an adenoviral vector to produce IL-12. The gene-modified cells showed an enhanced production of pro-inflammatory cytokines, but dexamethasone treatment significantly inhibited that production while not affecting IL-12 levels.

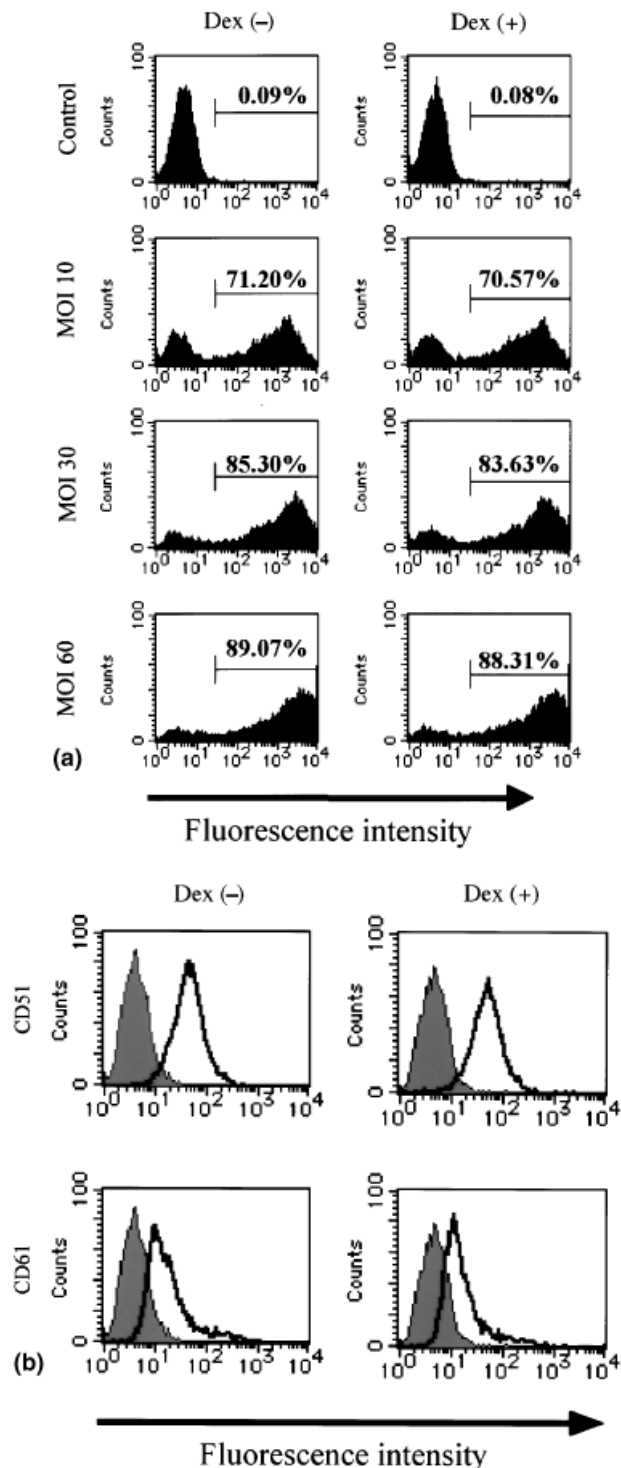


Fig. 4. (a) EGFP expression as analysed by flow cytometry for assessment of transduction efficiency in the presence or absence of dexamethasone. BEAS-2B cells were transduced with AxCaegfp at MOI values of 10, 30, and 60 for 2 h. Histograms represent EGFP expression levels, as measured by flow cytometry, of BEAS-2B cells transduced with AxCaegfp, with or without dexamethasone (10^{-7} M). Scale bars indicate EGFP-expressing cells and associated numbers at the top of the scale bars indicate percentage of gated cells that expressed EGFP. MOI: multiplicity of infection. Data represent three independent experiments. (b) Integrins $\alpha_v\beta_3$ (CD51) and $\alpha_v\beta_5$ (CD61) expression on BEAS-2B cells. BEAS-2B cells were incubated with or without dexamethasone (10^{-7} M) for 24 h, harvested, and analysed by flow cytometry. Closed histogram: control; open histogram: CD51 and CD61 expressing cells.

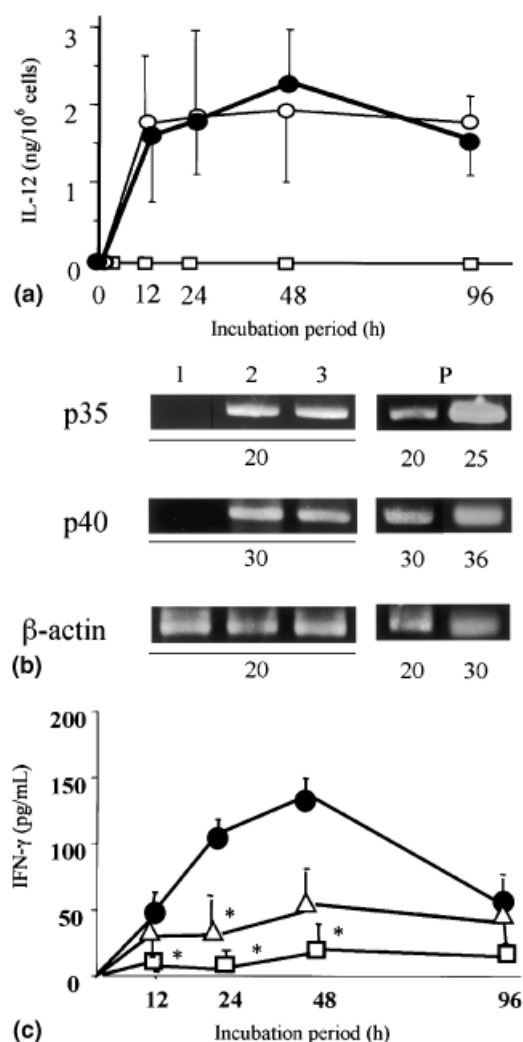


Fig. 5. (a) Production of IL-12 by BEAS-2B cells in the presence or absence of dexamethasone. BEAS-2B cells were transduced with Ax1-CIhp40ip35 vectors at an MOI of 30 for 2 h and cultured in 24-well culture plates for the indicated times in the presence or absence of dexamethasone (10^{-7} M). Supernatants were then collected and IL-12 concentration was measured by ELISA. Values are means $\pm$ SEM of data from five independent experiments. □: BEAS/WT cells; ●: BEAS/IL-12 cells; ○: BEAS/IL-12 cells treated with 10^{-7} M dexamethasone. (b) Semi-quantitative RT-PCR. Total RNA from BEAS-2B cells was isolated as described in Materials and methods, and adjusted to the same concentration using the β -actin competitive PCR set. RT-PCR was performed using a one-step RNA PCR kit. The resulting material was electrophoresed on a 1.5% agarose gel in 1 $\times$ TAE and stained by ethidium bromide. Data represent three independent experiments. Numbers under the panels indicate the number of PCR cycles. Lane 1: BEAS/WT cells; lane 2: BEAS/IL-12 cells; lane 3: BEAS/IL-12 cells treated with 10^{-7} M dexamethasone; lane P: positive control for IL-12p35, p40 and β -actin with different cycle numbers. (c) Production of IFN- γ by lymphocytes co-cultured with BEAS-2B cells in the presence or absence of dexamethasone (10^{-7} or 10^{-9} M). BEAS-2B cells were transduced with Ax1CIhp40ip35 vectors at an MOI of 30 for 2 h and cultured with human lymphocytes using a non-contact co-culturing system for the indicated times. Supernatants were then collected and IFN- γ concentration was measured by ELISA. Values are means $\pm$ SEM of data from five independent experiments. ●: lymphocytes with BEAS/IL-12 cells; □: lymphocytes with BEAS/IL-12 cells in the presence of 10^{-7} M dexamethasone; △: lymphocytes with BEAS/IL-12 cells in the presence of 10^{-9} M dexamethasone. * $P < 0.05$ compared to lymphocytes with BEAS/IL-12 cells.

As IL-12 is known to play a major role in regulating Th1/Th2 balance, constitutive expression of IL-12 *in situ* is an attractive strategy for the management of Th2-type inflam-

mation such as bronchial asthma. In previous reports, adenoviral gene transduction has been carried out on human respiratory [21–23] and bronchial epithelial [23–26] cells. In these reports, lacZ, I κ -B, and CF transmembrane regulator (CFTR) genes were successfully transduced into human bronchial cells using adenoviral vectors, and the corresponding proteins were expressed. In the present study, IL-12 produced by gene-modified cells was detected and induced the IFN- γ production by lymphocytes. To our knowledge, the present study is the first to demonstrate that bioactive IL-12 can be successfully produced by HBEC following adenoviral IL-12 gene transduction (Table 1 and Fig. 2).

With regard to the adverse effects of adenoviral IL-12 gene transduction, we found that IL-12 gene modified cells showed augmented production of pro-inflammatory cytokines (Table 2). As previous reports using adenoviral vectors observed no increases in IL-6 or IL-8 production [25], we hypothesized that the IL-12 produced by BEAS/IL-12 cells may have directly affected cytokine production through binding BEAS-2B cell IL-12 receptors. However, our experiments showed that the addition of recombinant IL-12 did not affect BEAS-2B cell cytokine secretion (Fig. 3a), and that BEAS-2B cells did not express IL-12 receptor molecules (Figs 3b and c). These results suggested that augmentation of inflammatory cytokine production did not involve an autocrine mechanism. Thus, while the mechanism underlying the increased inflammatory cytokine production might be due to the activation of an intracellular signal transduction pathway involving IL-12, the exact mechanism remains unclear and requires further study.

With regard to the clinical application for management of Th2-mediated disease, IL-12 gene transduction by adenoviral vector alone may not be suitable for control of the disease, because of the risk of increased allergic inflammation due to augmented inflammatory cytokine production by IL-12 gene-transduced HBEC. To overcome possible adverse effects, we examined the role of steroids such as dexamethasone. Previous reports have shown that corticosteroids inhibit the secretion of IL-8 and IL-6, as well as the accumulation of their mRNA transcripts in HBEC [19]. As expected, production of inflammatory cytokines from BEAS/IL-12 cells was significantly inhibited by dexamethasone (Table 3). The present data suggest that combination use with IL-12 gene transduction and dexamethasone could be a potential approach to overcome the adverse effects that could arise from enhanced production of pro-inflammatory cytokines.

The IL-12 gene transduction in combination use of corticosteroids may be of little value if IL-12 gene transduction and IL-12 expression could be suppressed by the concomitant dexamethasone. Interestingly, the adenoviral gene transduction into HBEC was not affected by dexamethasone (Figs 4 and 5). When adenovirus transduced into HBEC, the virus particles first adhere to cell-surface coxsackievirus and adenovirus receptor (CAR) molecules [27] and are then internalized into the cells via integrins $\alpha_v\beta_3$ (CD51) and $\alpha_v\beta_5$ (CD61) [28,29]. The expression of CD51 and CD61 was not increased by dexamethasone (Fig. 4b). Moreover, dexamethasone did not change the transduction efficiency. Present findings suggested that expression of these molecules participating in the viral transduction are stable in the presence of dexamethasone. We also found that IL-12

production remains unaffected by dexamethasone. This mechanism remains unclear, but our findings showed that expression of IL-12p35 and p40 mRNA was not inhibited by dexamethasone (Fig. 5b). Thus, IL-12 mRNA expression might escape dexamethasone inhibition by being under the control of an adenoviral promoter.

Produced IL-12 by gene-modified HBEC may restore Th1/Th2 balance directly through various mechanisms such as the differentiation of T cells into Th1 cytokine-producing cells and a co-stimulus of activation of effector Th1 cells while suppressing Th2 generation [30]. In addition, IL-12 induced IFN- γ production in lymphocytes. The present study showed that IFN- γ induction was inhibited by dexamethasone, but the magnitude of inhibitory effects for IFN- γ production was lower than that for IL-6 and IL-8 production. Therefore, the effect of IFN- γ induced by IL-12 could be expected even in the presence of dexamethasone.

Interestingly, production of RANTES and TARC was not affected by IL-12 gene transduction (Table 2). In previous reports, RANTES was produced by BEAS-2B cells stimulated with TNF- α or INF- γ [31, 32], and was increased by influenza virus A [33] or RS virus [34,35] infection of airway epithelial cells. Likewise, previous studies showed that TARC was also produced by BEAS-2B cells [36, 37]. The present findings suggest that IL-12 gene transduction may not augment infiltration of eosinophils and Th2 cells, through overproduction of these chemokines, and this is the first to demonstrate the effects of gene transduction on the production of these chemokines by BEAS-2B cells.

In summary, BEAS-2B cells were successfully transduced with IL-12 gene to produce bioactive IL-12 dependent on the transduction efficiency of the adenoviral vector. IL-12 gene transduction augmented pro-inflammatory cytokines, but dexamethasone could inhibit them without suppression of IL-12. Adenoviral IL-12 gene transduction into HBEC is a potential approach to express active IL-12 in the local airways. For clinical application, further study is warranted to clarify the *in vivo* effect of IL-12 gene transduction in combination with corticosteroids, such as that on Th1 cell development [38].

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