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ORIGINAL

Interleukin (IL)-12 gene transduction and its functional expression into human bronchial epithelial cells (BEAS-2B) by adenovirus vector

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Abstract: Interleukin (IL)-12 is known as a cytokine that augments the Th1 type response. Especially in allergic diseases such as a bronchial asthma, IL-12 induced restoration of the balance of the Th1/Th2 type immune response is an attractive strategy. In this study, the functional properties of the human bronchial epithelial cell line (BEAS-2B) transduced by an adenoviral vector encoding the human IL-12 gene were examined.

Adenovirus vectors, AxCAegfp and Ax1CIhp40ip35 were transduced into BEAS -2B cells. Wild and gene-transduced BEAS -2B cells were incubated and the concentrations of IL-12 and IFN- γ produced by co-cultured lymphocytes in the supernatant were measured using ELISA. The expressions of surface adhesion molecules, such as CD54 and CD106 were analyzed using flow cytometry.

The efficiency of transgene expression of BEAS-2B cells was in a multiplicity of infection (MOI)-dependent manner and at an MOI of 30, the efficiency was approximately 80%. The gene-modified BEAS-2B cells produced biologically active IL-12 in dose - and time-dependent manners. IL-12 gene transduction did not significantly affect the expression of adhesion molecules (CD 54, CD106 and HLA-A,B,C) by BEAS-2B cells.

These results suggest that the IL-12 gene may be successfully transduced into human bronchial epithelial cells by adenoviral vector to express IL-12 activity *in vivo*.
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Keywords : *IL-12, BEAS-2B, adenoviral vector*

INTRODUCTION

Bronchial asthma is a disease characterized as a chronic airway inflammation. Among various cells participating in airway inflammation, Th2 lymphocytes (1, 2) which secrete several cytokines including interleukin (IL)-4 and IL-5, predominates over Th1 type cells in bronchial asthma (3). Several studies have reported that Th2 cytokines induce eosinophila and airway hyperresponsiveness in animal models of asthma (4, 5). Thus, Th2 type cytokine plays an important role in the pathogenesis of bronchial asthma.

IL-12 is produced by activated monocyte-macrophages, dendritic cells and other antigen-presenting cells. Bioactive IL-12 directs the differentiation of T cells

Abbreviations : EGFP, enhanced green fluorescence protein ; BEAS/WT, wild type BEAS-2 B cells ; BEAS/EGFP, BEAS-2 B cells transduced with EGFP gene ; BEAS/IL12, wild type BEAS-2B cells transduced with IL-12 gene ; IFN-γ interferon-γ ICAM-1, intracellular adhesion molecule-1 ; VCAM-1, vascular cellular adhesion molecule-1

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into Th1 cytokine-producing cells and is a costimulus of activation of effector Th1 cells while suppressing Th2 generation. IL-12 also stimulates IFN-γ production by these cells. Due to these activities, several approaches using IL-12 have been examined to control bronchial asthma in animal models. Intratracheal administration of IL-12 protein has been shown to be effective in reducing eosinophilia (6). This observation suggested that IL-12 administrated directly to the airway might be a more effective therapeutic strategy for management of bronchial asthma.

Recent attention has focused on the therapeutic use of adenovirus vector for treatment of pulmonary diseases such as cystic fibrosis (7, 8). Although IL-12 gene transfer into the airway was found to be effective in controlling eosinophilia and Th2 immune response in mice (9), little is known about the availability of adenoviral transduction of the IL-12 gene into human bronchial epithelial cells (HBEC) in terms of clinical applications with IL-12 gene therapy.

The cell-cell interaction through the adhesion molecules is important for their functional expressions. In particular, certain adhesion molecules such as CD54 (intracellular adhesion molecule-1, ICAM-1) and CD106 (vascular cell adhesion molecule-1, VCAM-1) are known to play critical roles in the induction of inflammatory and/or allergic reaction, because CD54 is a ligand for neutrophil adhesion and activation and CD106 activates eosinophils. We recently reported that IL-12 gene-modified dendritic cells by the adenoviral vector caused the increased expression of surface antigens such as CD80, CD86 and CD83 (10). Since bronchial epithelial cells were also found to express surface antigens such as CD54 and CD106 (11), It is essential to clarify whether these surface antigens were affected by adenoviral IL-12 gene transduction.

In this study, we observed that the IL-12 gene could be successfully transduced into human bronchial epithelial cells (BEAS-2B) by an adenoviral vector to release bioactive IL-12 without significant changes in the phenotypes.

MATERIALS AND METHODS

Culture of a human bronchial epithelial cell line (BEAS-2B)

A culture of a human bronchial epithelial cell line (BEAS-2B) transformed with SV40 virus was purchased from the American Type Culture Collection (Manassas, VA). The cells at passages 42-46 were plated in 100-mm dishes, and incubated at 37°C, under 5% CO_2 in air for 7-10 days. The LHC 9/RPMI 1640 medium was changed after 24 hr and then every other day as described previously (12).

Preparation of human peripheral blood lymphocytes

Leukocyte concentrates from healthy donors were separated into peripheral blood mononuclear cell (PBMC) by density gradient centrifugation in lymphocyte separation medium. Subsequently, lymphocytes were separated from PBMC by counterflow centrifugal elutriation in a Beckman JE-5.0 rotor (Beckman Instruments, Inc., Fullerton, CA) according to the method described previously (13). The lymphocyte-rich fractions were collected at flow rates of 12-16 ml/min, respectively, at 2,000 rpm. The purity of the lymphocyte fraction was greater than 99%.

Adenoviral vector construction and preparation

The recombinant adenoviruses AxCAegfp and Ax1CIhp40ip35 were used as vectors. AxCAegfp contained an enhanced green fluorescent protein (EGFP) gene. For preparation of AxCAegfp as described previously (10), an Adenovirus Expression Vector Kit (Takara Shuzo Co. Ltd., Kyoto, Japan) was used, in which the adenoviral cosmid, pAxCAwt, was included (14). EGFP cDNA was obtained from pEGFP-N1 (Clontech, Palo Alto, CA) and subcloned into pAxCAwt. This virus was fluorescent. Ax1CIhp40ip35 is a bicistronic vector that expresses IL-12 p40 and p35 subunits simultaneously. It was obtained from RIKEN Gene Bank (Tsukuba, Japan) (10, 15). There was no detectable replication-competent adenovirus or $E1^{+}$ virus in the preparation.

Adenovirus-mediated gene transduction into BEAS-2B cells

When cultured BEAS-2B reached confluence, they were suspended in LHC9/RPMI 1640 medium and plated in 6-well tissue culture plates at 3×10^{5} cells/well. The cells were cultured in LHC9/ RPMI 1640 medium for 24 hr. The adenoviruses AxCAegfp and Ax1CIhp40ip35 were suspended in phosphate-buffered saline (PBS) supplemented with 1% human serum albumin (HSA/PBS). BEAS-2B cells were then washed once with PBS. Next, the HAS/ PBS with adenovirus vector was added to each well and the cells were exposed for 2 hr at 37°C, under 5% CO_2 in air. Following this, the cells were washed with PBS and fed with LHC9/RPMI 1640 medium.

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

The EGFP expression of BEAS-2B transduced with AxCAegfp (BEAS/EGFP) was visualized by fluorescence microscopy using a BX61 Microscope (Olympus, Tokyo, Japan). BEAS/EGFP was photographed using a DP50 digital Camera with Viewfinder Lite software (Olympus, Tokyo, Japan) (Figure. 1A). Expressions of EGFP by BEAS-2B were analyzed quantitatively to evaluate accurate gene-transduction efficiencies. BEAS-2B was washed once, and EGFP expression was quantified by flow cytometry using a FACS Calibur flow cytometer with Cell Quest software (Becton Dickinson, San Jose, CA). EGFP absorbs light energy at 488 nm and emits light at 507 nm, which was detected by the FL 1 detector of the FACS Calibur.

Co-culture system for the gene-transduced BEAS-2B cells and human peripheral blood lymphocytes

To examine the biological activity of IL-12 produced by BEAS/IL12, BEAS -2B cells and human peripheral blood lymphocytes were cultured in a non-contact co-culture system in 6-well plates as described previously (16) Cells were cultured using cell culture inserts (Becton Dickinson, Tokyo, Japan) with a pore size of 0.4 μm and a pore density 1.6×10^6 pores/cm^2. BEAS-2B cells were cultured and were transduced with genes by the method described above, and then were plated at the bottom of a culture-plate well. Human peripheral blood lymphocytes (3.5×10^6 cells) were plated in a culture insert. The culture insert was placed in the well and the cells were incubated with 3.5 ml LHC9/RPMI 1640 at 37°C, under 5% CO_2 in air for 12, 24, 48 and 96 hr. Anti-IL-12 neutralizing antibody was obtained from Endogen (Woburn, MA). 1 μg/ml of this antibody was added at day zero to obtain a final concentration of 10 ng/ml.

Isolation and analysis of mRNA expression

RNA was isolated using the acid guanidinium thiocyanate/phenol-chloroform method (Isogen). IL-12 p40 and p35 mRNA were detected by a one-step RNA PCR kit (TAKARA, Tokyo, Japan). The reverse transcription (RT) reaction was carried out at 50°C for 30 min and at 94°C for 2 min. The PCR reaction cycles were as follows : IL-12p40, denaturation for 30 sec at 85°C, annealing for 30 sec at 46°C, and extension for 30 sec at 72°C, for 36 cycles ; IL-12 p35, denaturation for 30 sec at 94°C, annealing for 30 sec at 57°C, and extension for 60 sec at 72°C, for 25 cycles ; β-actin, denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C, and extension at 72°C for 30 sec, for 30 cycles. The following primers were used : IL-12 p40 sense primer, 5'-TCTAAGCGATTCGCTCCTGC-3' ; IL-12 p40 antisense primer, 5'-AAGCTGCTGGTGTAGTTTTG-3'; IL-12 p35 sense primer, 5'-CTTCACCACTCCCAA AACCTG-3'; IL-12 p35 antisense primer, 5'-AGCTCGTCACTCTGTCAATAG-3'; β-actin sense, 5'-AAGAGAGGCATCCTCACCCT-3'; β-actin antisense, 5'-TACATGGCTGGGGT GTTGAA-3'. PCR products were separated electrophoretically in 1.5% agarose gel, visualized and photographed after ethidium bromide staining.

Measurement of cytokines

Production of IL-12 p70 by BEAS-2B cells and interferon (IFN)-γ produced by human peripheral blood lymphocytes were measured by enzyme-linked immunosorbent assay (ELISA). ELISA for IL-12 p70 was performed based on the manufacturer's instruction (Endogen SP-HIL12, Woburn, MA). IFN-γ was measured using ELISA (Otsuka Pharmaceutical Co., Tokushima, Japan) (17). The sensitivity limits of the ELISA for IL-12 p70 and IFN-γ were 17 pg/ml and 20 pg/ml, respectively.

Flow cytometric measurement of surface antigen expression

The harvested BEAS -2B cells were washed once and incubated for 30 min at 4°C in PBS containing the primary monoclonal antibody (mAb) (2 μg/ml), as indicated below. After washing, cells were indirectly labeled by incubation at 4°C for 30 min with the RPE-Cy5-conjugated F (ab') 2 fragment of rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) (25 μg/ml) and analyzed by flow cytometry. Light energy absorbed at 488 nm by RPE was transferred to Cy5 resulting in light emission at 670 nm. This emission was detected by the FL3 detector of the FACS Calibur. The fluorescent emission of EGFP (maximum emission : 507 nm) and RPE-Cy5 (maximum emission: 670 nm) interfered with each other very little, so that the co-expression of EGFP and the surface adhesion molecule could be clearly distinguished. The following mAbs were used as primary mAbs : Anti-MHC class I was obtained from Immunotech (Marseilles, France). Anti-CD54 and anti-CD106 antibodies were purchased from Pharmingen (San Diego, CA).

Statistical analysis

Analysis of data was performed using Stat-View 5.0 (Abacus Concept, Inc., Berkeley, CA). Experimental results were expressed as means±SEM. The statistical significance of differences between groups was analyzed using the analysis of variance with Fisher

PLSD. Data were considered statistically significant if p values were less than 0.05.

RESULT

Efficiency of adenovirus-mediated gene transduction into BEAS-2B cells

To examine the transduction efficiency of the adenoviral vector to BEAS -2B cells, the EGFP gene was transduced into BEAS -2B cells and used as a marker for gene transduction. The adenovirus AxCAegfp was transduced at multiplicity of infections (MOI) of 10, 30, 60, and 100 for 2 hr at 37°C, under 5% CO_2 in air. The fluorescence from gene-modified cells (BEAS/EGFP) increased markedly in an MOI-dependent manner (Figure 1A). Transduction efficiency and mean fluorescence intensity (MFI) were also increased in an MOI-dependent manner. The efficiency of transgene expression at an MOI of 30 was 85.3% of BEAS-2B cells (Figure 1B),

Determination of IL-12 p40 and p35 mRNA expression by BEAS-2B cells transduced IL-12 gene (BEAS/IL12)

The IL-12 gene-transduction into BEAS-2B cells by adenoviral vector encoding the human IL-12 gene, Ax1CIhp40ip35, was examined next. To confirm IL-12 gene-modification, total RNA was extracted from BEAS-2B cells 24 hr after transduction, and RT-PCR experiments were performed using the specific primers for IL-12 p35 and p40. As shown in Figure 2, non-transduced (BEAS/WT) and EGFP-transduced (BEAS/EGFP) BEAS -2B cells expressed low levels of IL-12 p35 mRNA, but did not express appreciable levels of m IL-12 p40 RNA. These results were consistent with previous findings (18). IL-12 gene-transduced BEAS-2B cells (BEAS/IL12) showed the expression of IL-12 p35 and IL-12 p40 mRNAs.

Determination of IL-12 expression by BEAS/IL12

To examine the dose dependence of MOI, the adenovirus Ax1CIhp40ip35 was transduced at MOI of 10, 30, 60 and 100 for 2 hr at 37°C under 5% CO_2 in air. The culture supernatants were harvested from BEAS-2B cells 48 hr after gene-transduction with Ax1Cihp40ip35. The production of IL-12 p70 from BEAS-2B cells were examined by ELISA specific for IL-12 p70 (Figure 3A). BEAS/IL12 produced IL-12 p70 in an MOI-dependent manner (2.82 ± 0.84 ng/10^6 cells/ MOI of 30). Since the efficiency of the transgene

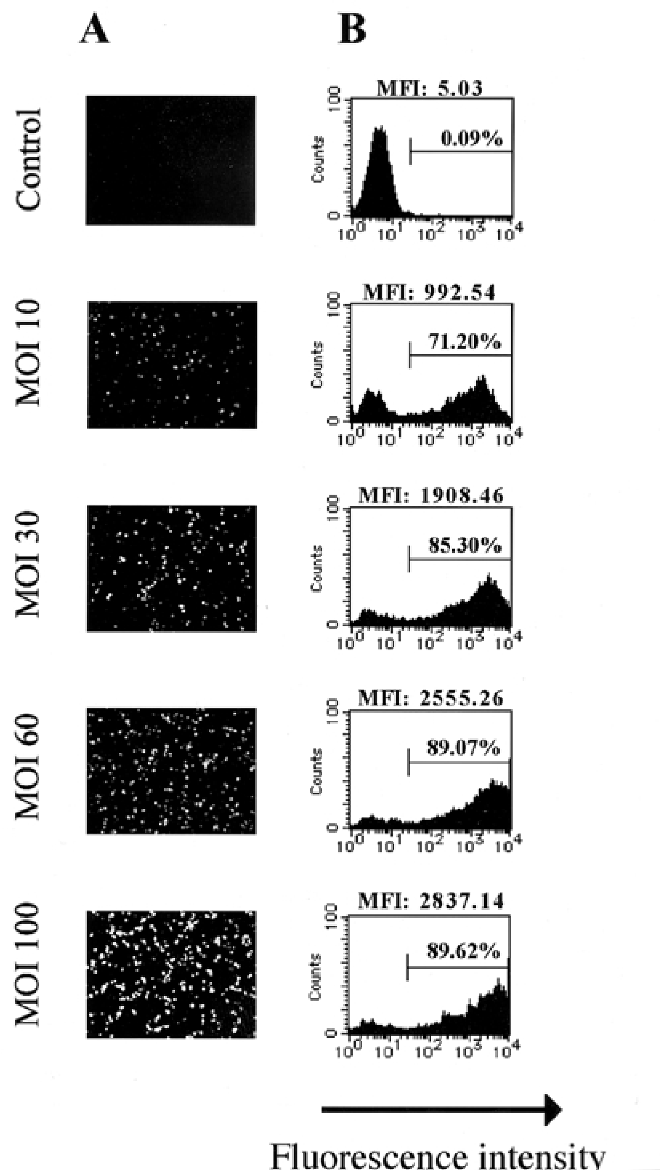

Figure. 1 Expression of EGFP evaluated by flow cytometry for measuring transduction efficiency.
BEAS-2B was transduced with AxCAegfp at MOI of 10, 30, 60, and 100 for 2 hr. (A) : Photograph of fluorescence microscopy (×200) 24 hr after gene transduction into BEAS-2B cells by AxCAegfp. (B) : EGFP expression of BEAS-2B transduced with AxCAegfp by flow cytometry. MFI : Mean fluorescence intensity. Scale bars and the numbers indicated above the scale bars : EGFP-expressing cells and the percentage of gated cells that were EGFP-expressing, respectively. The values are representative for three independent experiments.

expression at an MOI of 30 was 85.3% of BEAS-2B cells (Figure 1B) and BEAS/IL12 produced a large amount of IL-12, the gene transductions were carried out using BEAS -2B cells at an MOI of 30 in the subsequent experiments, except where indicated otherwise.

To examine the time course of IL-12 P70 production, the culture supernatants were also harvested from BEAS/IL12 at 12, 24, 48 and 96 hr and the IL-12

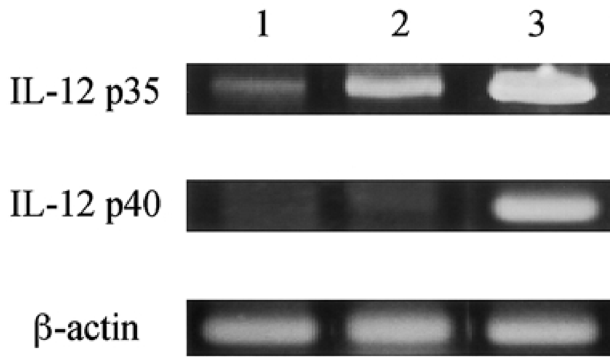

Figure. 2 Expression of IL-12 p40 and p35 mRNA in BEAS-2B cells
Total RNA from BEAS-2B cells was isolated as described in Materials and Methods. One microgram of total RNA was reverse-transcripted into cDNA, and a portion of the product was subjected to PCR using a one-step RNA PCR kit. The resulting material was electrophoresed on a 1.5% agalose gel in 1x TAE, followed by ethidium bromide staining. The values are representative of three independent experiments. Lane 1 : BEAS/WT, Lane 2 : BEAS/EGFP, Lane 3 : BEAS/IL12

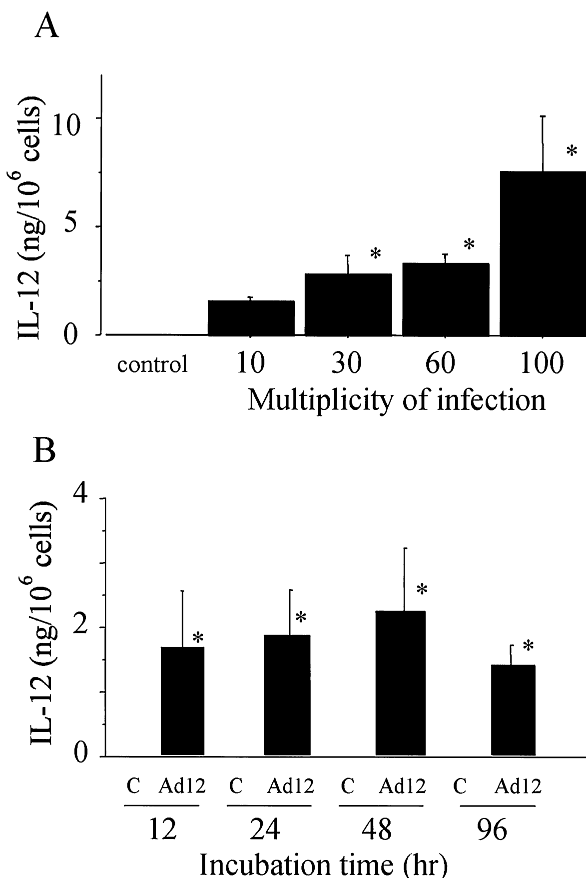

Figure. 3 Production of IL-12 p70 by BEAS-2B cells
BEAS -2B was transduced with AxCAegfp or Ax1CIhp40ip35 for 2 hr. Each BEAS -2Bs was cultured in 24-well culture plates. After incubation, supernatants were collected and the IL-12 p70 concentration was measured using ELISA. Values are means±SEM obtained from four independent experiments (A) : Dose-dependence of IL-12 production by BEAS-2B cells * : p<0.05 compared with non-transduced BEAS-2B cells (control).
(B) : Time course of IL-12 production by BEAS -2B cells. *: p<0.05 compared with non-transduced BEAS-2B cells (control). C : non-transduced BEAS-2B cells, Ad12 : IL-12 gene transduced BEAS-2B cells

concentration was measured by ELISA. As shown in Figure 3B, BEAS/WT and BEAS/EGFP did not produce significant amounts of IL-12 p70. However BEAS/IL12 produced IL-12 p70 constitutively. At the peak at 48 hr, production of IL-12 P70 by BEAS/IL12 was 2.35± 0.85 ng/10^6 cells.

Biological activity of IL-12 produced by BEAS/IL12

Since biologically active IL-12 stimulates IFN-γ production by lymphocytes, we examined IFN-γ production by human lymphocytes incubated with BEAS -2B cells using a non-contact co-culture system. As shown in Table 1, IFN-γ production was increased by lymphocytes cultured with BEAS/IL12 in a time-dependent manner. At 48 hr, production of IFN-γ was 252.1± 150.4 pg/10^6 cells in lymphocytes, but it was not detected in other groups. When IL-12 neutralizing antibody was added to the BEAS/IL12, IFN-γ production by the lymphocytes was completely abolished (Table 1). These findings show that IL-12 p70 by BEAS/IL12 are biologically active to stimulate IFN-γ secretion from the human lymphocytes.

Analysis of surface adhesion molecule expression by BEAS/IL12

Using flow cytometry, we examined whether adenoviral IL-12 gene transduction into BEAS-2B cells affect the expressions of CD54, CD106 and HLA-A,B,C (MHC class I). When the values of the mean fluorescence intensity (MFI) are the means±SEM obtained from three independent experiments, the results showed that the expression of CD54 on BEAS/IL12 (MFI : 397.19±149.00) was similar to that on BEAS/EGFP (MFI : 323.49±211.05) (Figure 4B), and the values for MFI for the gene-modified cells were slightly, but not significantly, higher than that for BEAS/WT MFI (277.79±81.07). Expressions of CD106 and HLA-A,B,C on BEAS/IL12 cells were also similar to those on BEAS/EGFP and BEAS/WT cells (Figure 4C and 4D).

DISCUSSION

The present study showed that IL-12-gene transduction into human bronchial epithelial cells using adenovirus vector was useful to produce biologically active IL-12 without causing a phenotypic change.

Adenoviral vector has been widely used for gene modification of cells without proliferation, because of its high efficiency. In previous studies, adenoviral gene transduction was carried out into human epithelial cells such as gall bladder (19), intestinal (20), respiratory cells (21-23) and bronchial epithelial cells

Table 1. IFN-γ production by human lymphocytes cultured with BEAS-2B cells

	Incubation time (hr)			
	12	24	48	96
BEAS/ IL12	<20	83.2±144.1	252.1±150.4*	329.5±158.3*
BEAS/ IL12+IL-12Ab	<20	<20	<20	<20
BEAS/EGFP	<20	<20	<20	<20
BEAS/WT	<20	<20	<20	<20
Control	<20	<20	<20	<20

Production of IFN-γ by human peripheral blood lymphocytes cultured with BEAS2B cells using a non-contact co-cultured system.
First, 3.5×10^6 human peripheral blood lymphocytes cells were cultured with 3×10^5 gene transduced BEAS-2B cells using a non-contact co-cultured system in 6-well culture plates. After incubation for the indicated times, supernatants were collected and cytokine concentrations were measured by ELISA. Values are means±SEM obtained from three independent experiments. * : $p<0.05$ compared with lymphocytes cultured with medium alone (control).

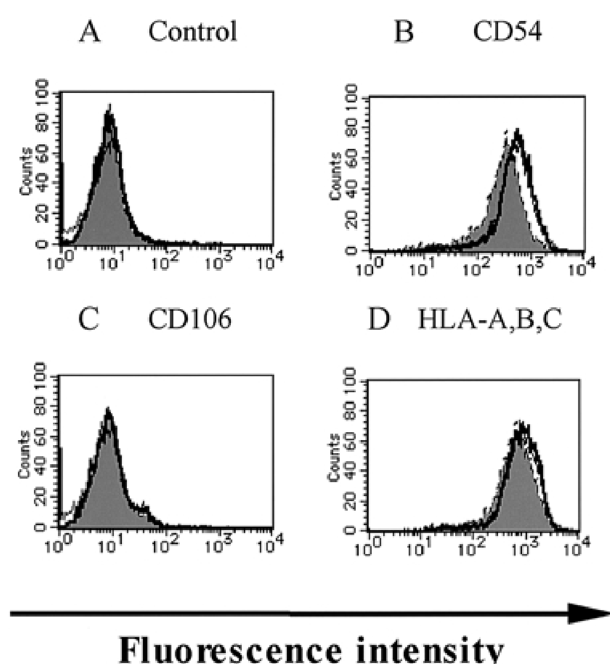

Figure. 4 Comparison of adhesion molecules expressed on BEAS/IL12, BEAS/EGFP and BEAS/WT.
Surface adhesion molecule expressions were measured by flow cytometry. The values are representative of three independent experiments. The histogram shows the expression of surface molecules as follows : (A) : control, (B) : CD54, (C) : CD106, (D) : HLA-A,B,C.
Open histogram with bold line : BEAS/IL12. Open histogram with solid line : BEAS/EGFP. Closed histogram : BEAS/WT. At (B) and (D), the histogram of BEAS/IL12 (bold line) completely overlapped with that of BEAS/EGFP (solid line).

(23-26). The genes used in those studies were lacZ, Iκ-B and CF transmembrane regulator (CFTR) gene, and were efficiently transduced into human bronchial cells by the adenovirus vector to produce the gene products. The findings of those studies raised a question of whether the IL-12 gene could be transduced by adenovirus vector to produce IL-12 heterodimer into human bronchial epithelial cells.

Regarding the transduction efficiency of the adenoviral vector into human epithelial cells, adenoviral transduction to gall bladder epithelial cells resulted in a good efficiency at an MOI of 40 or 100 (17) and its efficiency to intestinal epithelial cells was 80% at an MOI of 10 to 100 (18). In human bronchial epithelial cells (BEAS -2B) transduced with the LacZ gene, the transduction efficiency was 60% to 90% at an MOI of 20 to 100 (23-25). These observations were confirmed and extended by the present findings that transduction efficiency was approximately 80% at an MOI of 30 when the EGFP gene was used as a marker (Figure 1).

First we examined whether the gene-modified BEAS-2B cells by the adenoviral vector could produce IL-12 extracellularly, because we recently found with the same adenoviral vector encoding IL-12 gene that human IL-12 gene-modified dendritic cells produced little IL-12 (10). The present findings clearly showed that IL-12 gene-modified BEAS -2B cell produced significant amounts of IL-12 p70 at greater MOI of 30, and its production was MOI-dependent (Figure 3A). The reason for this difference is unknown, but it might be due to the difference in the transduction efficiency and/or the origin of the target cells used.

There is another question of whether IL-12 produced by gene-modified BEAS -2B cells has a heterodimeric structure, because bioactive IL-12 protein is a heterodimer composed of two subunits (IL-12 p35 and p40) and each subunit does not have biological activity (27, 28). The present findings clearly showed that gene-modified BEAS -2B cells expressed two subunit mRNAs, IL-12 p35 and p40 (Figure 2). On the other hand, biologically active IL-12 is known to induce secretion of IFN-γ by lymphocytes. To confirm the activity of IL-12 produced by IL-12 gene-modified BEAS -2B cells, we measured the ability of lymphocytes co-cultured with BEAS/IL12 to produce IFN-γ.IFN-γ was significantly produced by lymphocytes co-cultured with BEAS/IL12

for more than 48 hr but not with BEAS/EGFP (Figure 4). To our knowledge, this is the first demonstration that human bronchial epithelial cells can be successfully gene-modified by adenovirus vector to produce bioactive IL-12.

A study reported that CD 54 and CD106 expressions were found to be increased in bronchial mucosa of patients with allergic inflammation (29, 30). These findings raise the possibility that augmented expressions of these adhesion molecules are responsible for the development of the allergic inflammation. Recently, adenoviral infection into human bronchial epithelial (A549) cells was found to cause the augmented expression of CD54 (23). This was not the case, how ever, in the present study with gene-modified BEAS-2B cells, because in the present study both BEAS/IL12 and BEAS/EGFP cells did not show a significant increase in the expressions of CD54 in comparison with that of BEAS/WT (Figure 4B). The present findings appeared to be similar to these previous report by Stark *et al*. showing no difference in CD54 expression between adenoviral LacZ gene-modified BEAS-2B cells and the parent cells (23). The reason for this difference in CD54 expression between A549 and BEAS-2B cells is unclear. As a possible explanation, it might be due to the different origins of the cell lines examined. Regarding CD106 expression, human bronchial epithelial cells expressed little (31), and IL-4 IL-5 and TNF-α stimulation increased its expression (30). Little is known, however, about the effect of adenoviral infection on the expression of CD106. We found that there was no difference in the CD106 expression of BEAS-2B cells by adenoviral infection with or without the IL-12 gene (Figure 4C). In addition, there was no change in other phenotypes such as HLA-A,B,C. Thus, the present findings suggested that IL-12 gene transduction with adenoviral vector did not significantly affect the phenotypic changes of BEAS-2B cells.

These results suggest that human bronchial epithelial cells in the airway may be gene-modified with adenoviral vector to produce IL-12 locally for control of allergic inflammatory diseases.

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