

Simultaneous Stimulation with TGF- β 1 and TNF- α Induces Epithelial Mesenchymal Transition in Bronchial Epithelial Cells

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Key Words

Airway remodeling • Epithelial mesenchymal transition • TGF- β 1 • TNF- α

Abstract

Background: Airway remodeling is an important feature of chronic airway disease, but the mechanisms involved remain unclear. Recently, epithelial mesenchymal transition (EMT) was reported to be associated with tissue fibrosis. TGF- β 1, which is a potent inducer of EMT, is thought to be related to the pathogenesis of airway remodeling. We investigated whether TGF- β 1 and/or TNF- α induce EMT in bronchial epithelial cells. **Methods:** Cultured BEAS-2B cells and primary normal human bronchial epithelial cells (NHBE) were treated with TGF- β 1 and/or TNF- α . Morphological changes and the expression of EMT-related markers were evaluated by immunocytochemical staining. Expressions of EMT-related markers, extracellular matrix (ECM) components (collagen type I and versican), and TGF- β receptors I, II, and III were analyzed by quantitative RT-PCR. Migration was evaluated using the Boyden chamber technique. **Results:** The TGF- β 1-induced EMT in BEAS-2B cells was demonstrated on the basis of morphological changes and the downregulation of E-cadherin. Costimulation with TNF- α enhanced the TGF- β 1-induced morphological changes and increased vimentin expression.

Treatment with TGF- β 1 increased the expression of collagen type I and versican. EMT induced with TGF- β 1 plus TNF- α promoted cell migration. Stimulation of NHBE with TGF- β 1 led to EMT. **Conclusion:** TGF- β 1 induced EMT in BEAS-2B cells, and costimulation with TNF- α enhanced the EMT. As a result of the EMT process, BEAS-2B cells acquired functions of mesenchymal cells. In addition, TGF- β 1 treatment induced EMT in NHBE as shown by changes in EMT-related markers. Bronchial epithelial cells might contribute to airway remodeling through EMT.

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Introduction

Bronchial asthma and chronic obstructive pulmonary disease (COPD) are common chronic inflammatory diseases of the airway. An important feature of these airway diseases is airway remodeling. Airway remodeling is defined as a thickening and reconstruction of the airways, i.e. fibrosis, deposition of the extracellular matrix (ECM), smooth muscle hypertrophy, and increased angiogenesis [1–3]. Airway remodeling is thought to correlate with the severity of the disease, i.e. refractoriness to corticosteroid therapy [4] and reduced lung function [5], in asthmatics. However, the mechanisms of airway remodeling and the

origin of the mesenchymal cells that are involved in airway remodeling are poorly understood.

Epithelial mesenchymal transition (EMT) is a biological process whereby epithelial cells lose their polarity and acquire a mesenchymal phenotype. EMT is reported to play important roles in normal development, tissue fibrosis, and metastasis. TGF- β is the most potent known inducer of EMT and is thought to contribute to tissue fibrosis, including renal [6], hepatic [7], and lung fibrosis [8]. The concentration of TGF- β is increased in the bronchoalveolar lavage fluid of asthmatics [9]. The increase in TGF- β -expressing cells within the bronchial mucosa correlates with the thickness of the airway basement membrane (BM) and the severity of the asthma [10, 11]. TGF- β and its mRNA are increased in bronchiolar epithelium from COPD patients [12]. These findings suggest that TGF- β plays important roles in the pathogenesis of both asthma and COPD.

Additionally, there is evidence demonstrating that TNF- α contributes to the pathogenesis of airway disease. Expression levels of both TNF- α mRNA and protein are increased in the airways of asthmatics [13, 14]. TNF- α is involved in the pathogenesis of severe refractory asthma, including the induction of glucocorticoid resistance [15] and airway neutrophilia [16]. There is a possibility that TNF- α , in addition to TGF- β , is involved in airway remodeling.

To elucidate the source of mesenchymal cells involved in normal and abnormal airway repair, we focused on the bronchial epithelium. We hypothesized that mesenchymal cells might be derived from airway epithelial cells through EMT. We thus investigated the TGF- β induction of EMT in bronchial epithelial cells and their acquisition of mesenchymal functions. We also examined the effect of TNF- α on EMT and the synergistic effect of these factors.

Methods

Cells and Cell Culture

Immortalized human bronchial epithelial (BEAS-2B) cells were obtained from the European Collection of Cell Cultures (Porton Down, UK). Primary normal human bronchial epithelial cells (NHBE) were purchased from BioWhittaker, Inc. (Walkersville, Md., USA). Cells were grown in culture dishes coated with collagen type I (Iwaki, Tokyo, Japan). The cells were cultured on serum-free epithelial growth medium (BEGM; Cambrex, Walkersville, Md., USA) supplemented with Bullet Kit (Cambrex) to contain 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μ g/ml hydrocortisone, 10 μ g/ml transferrin, 0.5 μ g/ml epinephrine, 5 μ g/ml insulin, 50 μ g/ml bovine pituitary extract, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50 μ g/ml genta-

micin, and 0.1 ng/ml amphotericin B at 37°C in a humidified atmosphere of 5% CO₂. Plates with 50–60% confluence were used for the following experiments. The cells were stimulated with 5 ng/ml TGF- β 1 (Sigma, St. Louis, Mo., USA) and/or 10 ng/ml TNF- α (Sigma) for 2–5 days.

Quantitative RT-PCR

Total RNA was extracted from BEAS-2B cells or NHBE cells cultured for 48 h using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The total RNA concentration was determined using a SmartSpec 3000 (Bio-Rad, Hercules, Calif., USA) and its purity was checked by the A₂₆₀/A₂₈₀ ratio. cDNA was synthesized from 1 μ g total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) in accordance with the manufacturer's instructions. After preparing a mixture of 1 μ g total RNA, 1 μ l 10-mM dNTP Mix (10 mM each of dATP, dGTP, dCTP, and dTTP), and 1 μ l oligo(dT)₂₀, it was incubated at 65°C for 5 min. Subsequently, SuperScript III Reverse Transcriptase was added, followed by incubation at 65°C for 60 min and quenching of the reaction by heating at 70°C for 15 min. Quantification of the mRNA level was performed using Mx-3000P (Stratagene, La Jolla, Calif., USA) and QuantiTect SYBR Green PCR (Qiagen) according to the manufacturers' protocols. Briefly, PCR consisted of incubation for 10 min at 95°C to activate HotStar Taq DNA polymerase, 60 s of annealing at 55°C, and 30 s of elongation at 72°C. Specific primer sets for E-cadherin; vimentin; collagen I; versican; TGF- β type I, II, and III receptors, and GAPDH were designed as follows:

E-cadherin: (5')-CCCATCAGCTGCCCCAGAAAATGAA, (3')-AAACAGGATGGCTGAAGGTGACAG; vimentin: (5')-GACAATGCGTCTCTGGCAGCTCTT, (3')-AAGAACCTGCAGGAGGCAGAAAGAA; collagen I: (5')-CTGGTCCCCAAGGCTTCAAGG, (3')-GCTGTTGGTGCTAAGGGTGAAG; versican: (5')-GGTATAGCCCCATCTTCCATTTCC, (3')-TCCTGGTTGGGTCTCCAATTC; TGF- β receptor type I: (5')-GGTCTTGC-CCATCTTCACAT, (3')-TTGCTCCAAACCACAGAGTG; TGF- β receptor type II: (5')-TTTCCACCTGTGACAACCA, (3')-CTGGAAGATGCTGCTTCTCC; TGF- β receptor type III: (5')-GCCTTGATGGAGAGCTTCAC, (3')-CCACATCCACCA-CAAGTCTG, and GAPDH: (5')-GGTGAAGGTCGGAGTCA-ACGCA, (3')-TCTTCCAGGAGGAGCGAGATCCCTG.

GAPDH was used as the internal control.

Immunocytochemistry

After stimulation with the designed cytokines for 72 h, cells were washed twice with cold phosphate-buffered saline and fixed with acetone-methanol at 60:40 for 7 min.

Immunocytochemistry was performed with an Envision+ kit, HRP (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The cells were incubated for 5 min with 0.03% hydrogen peroxide containing sodium azide to inactivate the horseradish peroxidase. Next, the cells were incubated with primary antibodies for 60 min. The primary antibodies used were rabbit monoclonal anti-pan-keratin antibody (Cell Signaling, Danvers, Mass., USA) at a 1:50 dilution and mouse monoclonal anti-vimentin antibody (Sigma-Aldrich, St. Louis, Mo., USA) at a 1:50 dilution. Next, the cells were incubated for 30 min with peroxidase-labeled polymer conjugated to anti-mouse or anti-rabbit immunoglobulin (Dako). The reaction was visualized using diaminobenzidine (Dako). The slides were counterstained with Mayer's hematoxylin (Wako, Tokyo, Japan).

In place of the primary antibodies, negative-control rabbit immunoglobulin fraction (Dako) and mouse IgG1 (Dako) were used as negative controls.

Migration

After treatment of the cells with TGF- β 1 and/or TNF- α for 72 h, those stimuli were eliminated overnight to avoid possible effects of TGF- β 1 or TNF- α on migration. Migration ability was assessed using a modified Boyden chamber technique [17]. Briefly, the cells (1×10^6 /ml) were loaded into the upper well, and a chemoattractant, i.e. fibronectin (5 μ g/ml), was placed in the lower well of the chamber. The wells were separated by an 8- μ m-pore filter coated with 0.1% gelatin (Bio-Rad). The chamber was then incubated at 37°C in a moist atmosphere with 5% CO₂. After 6 h of incubation, the cells on the top of the filter were removed by scraping. The filter was then fixed, stained with Diff-Quik (Sysmex Co., Kobe, Japan), and mounted on a glass. Migration was assessed by counting the number of cells in 5 high-power fields using a light microscope. Replica experiments were performed using separate cultures of cells under separate conditions.

Measurement of TGF- β 1 and TNF- α in Cell Culture Supernatants

BEAS-2B and NHBE cells were cultured in BEGM for 3 days. TGF- β 1 was measured in the culture supernatants using a Quantikine Human TGF- β 1 ELISA kit (R&D Systems, Minneapolis, Minn., USA). The samples were acidified with HCl to measure the biologically active TGF- β 1. After assay of the active TGF- β 1, the acidified samples were neutralized with 1.2 N NaOH/0.5 M HEPES. The concentration of TNF- α was then measured using a Quantikine Human TNF- α /TNFSF1A kit (R&D Systems), according to the manufacturer's protocol.

Statistics

All statistical analyses were performed using Statcel2 software (OMS Publishing, Inc., Saitama, Japan). The results are expressed as means $\pm$ SEM. Multivariate comparisons were analyzed using ANOVA with Bonferroni's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Morphological Studies

To assess whether TGF- β 1 induces morphological changes in human bronchial cell line BEAS-2B, cultures with 50–60% confluence were treated with and without TGF- β 1 (5 ng/ml) for 72 h.

The untreated control cells displayed a cobblestone-like shape and were attached to each other (fig. 1a). In contrast, treated cells were elongated and showed a loss of cell-cell contacts (fig. 1b). We also investigated TNF- α for possible effects on the cells' morphology. Simultaneous stimulation with TGF- β 1 and TNF- α (10 ng/ml) caused more significant changes compared to treatment with TGF- β 1 alone (fig. 1c). Stimulation with TNF- α alone

showed no differences from the control cells (fig. 1d). Treatment with either TGF- β 1 or TNF- α inhibited the proliferation of BEAS-2B cells [18], so the treated cells were less confluent than the control cells. In order to avoid any effect of the reduced confluence of the treated cells on the morphological changes, the duration of the stimulation was extended by 5 days. The cells treated with both TGF- β 1 and TNF- α showed a spindle-shaped morphology (fig. 1g), and the morphological changes were more apparent compared to the TGF- β 1-treated cells (fig. 1f). Untreated control cells and cells stimulated with TNF- α alone showed no changes (fig. 1d, h).

E-Cadherin, Pan-Keratin, and Vimentin Expression

We then examined whether the morphological changes correlated with the expression of epithelial and mesenchymal markers determined by quantitative RT-PCR and immunocytochemistry.

BEAS-2B cells treated with TGF- β 1 for 48 h showed a decreased expression of E-cadherin mRNA (fig. 2a) and a slight, but not significant, increase in the expression of vimentin mRNA (fig. 2b). Costimulation with TGF- β 1 and TNF- α further enhanced the expression of vimentin mRNA. There were no differences in the expression of mRNA for E-cadherin and vimentin between the control cells and TNF- α -treated cells (fig. 2). In immunostaining experiments, almost all of the control cells were pan-keratin-positive (fig. 3a). TGF- β 1 treatment caused partial conversion of the pan-keratin-positive cells to negative cells and costimulation with TGF- β 1 and TNF- α markedly enhanced that conversion to negative cells compared to stimulation with TGF- β 1 only. Correspondingly, the expression of vimentin was upregulated by TGF- β 1 treatment, and simultaneous stimulation with TNF- α enhanced that change.

We next investigated whether NHBE cells also show changes in the expression of E-cadherin and vimentin in response to stimulation with TGF- β 1. E-cadherin expression was decreased in the same way as was seen with BEAS-2B cells (fig. 2c), whereas vimentin expression by NHBE was increased by treatment with TGF- β 1 alone (fig. 2d) and the addition of TNF- α did not result in an enhancement of that change.

Cell Migration

To evaluate the physiological significance of EMT, we assessed the migratory ability of treated cells using the Boyden blind-well chamber technique. BEAS-2B cells inherently show chemotactic activity [19]. As shown in figure 4, there was no difference in the number of migrating

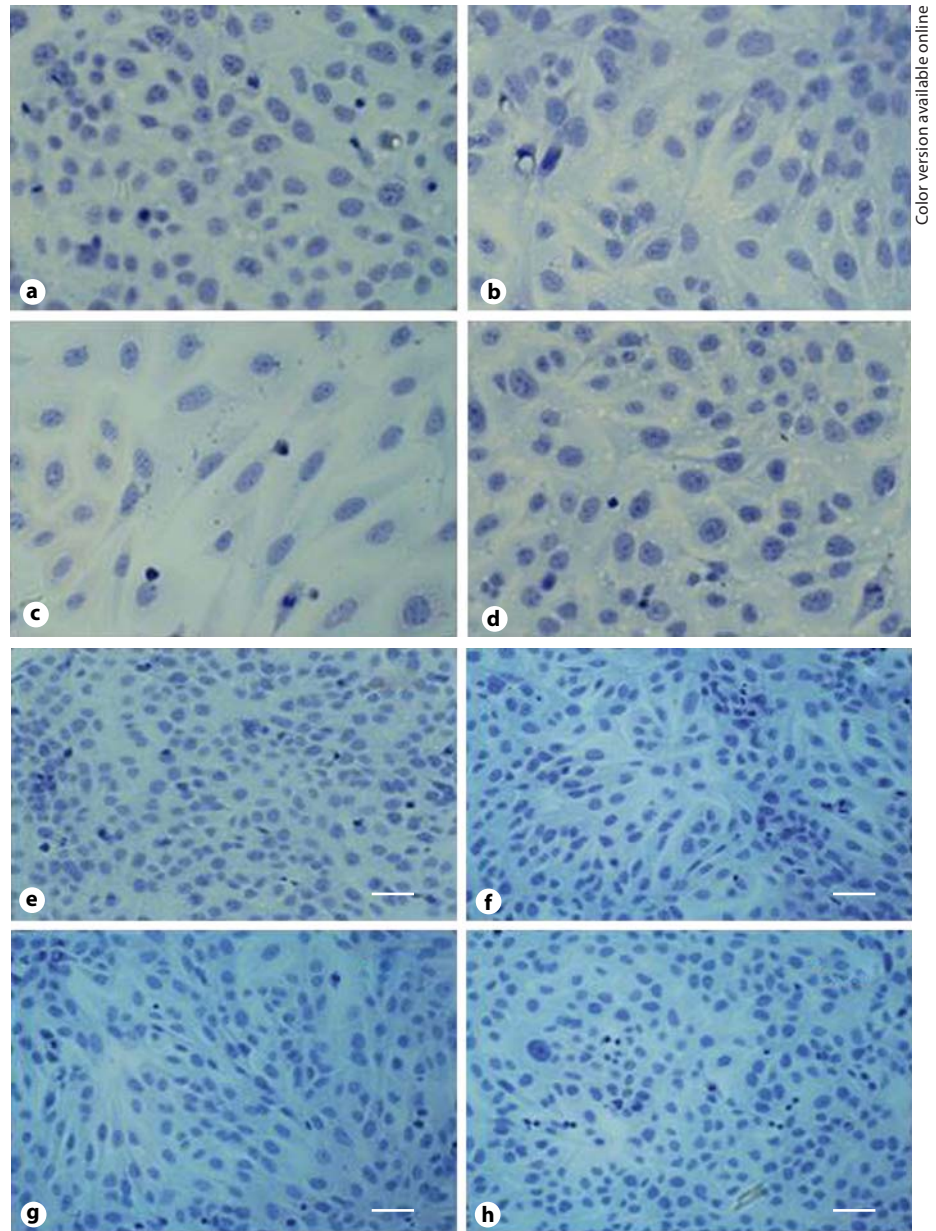


Fig. 1. Morphological changes in BEAS-2B cells. Hematoxylin staining was performed after 72 h (**a–d**) and 5 days (**e–h**) of culture under the following conditions: control condition (**a, e**), stimulation with 5 ng/ml TGF- β 1 (**b, f**), stimulation with 5 ng/ml TGF- β 1 and 10 ng/ml TNF- α (**c, g**), and stimulation with 10 ng/ml TNF- α (**d, h**). **e–h** Scale bars = 40 μ m.

cells between the control and cells treated with TGF- β 1 alone or TNF- α alone. However, the cells treated with both TGF- β 1 and TNF- α showed greater migration compared to the control.

Expression of ECM Genes

To examine the properties of EMT cells derived from BEAS-2B cells, the expressions of 2 ECM components, i.e. collagen type I and versican, were measured. Although TGF- β 1 enhanced the expression of both collagen type I

and versican, costimulation with TNF- α did not further enhance their expression (fig. 5).

Concentrations of TGF- β or TNF- α in Cultured Cell Supernatants

We examined the autocrine release of TGF- β or TNF- α in cultured cells. The baseline concentrations of TGF- β 1 in the BEAS-2B and NHBE cell supernatants were higher than in medium only. However, the concentration of TGF- β 1 in the NHBE cell supernatant was extremely

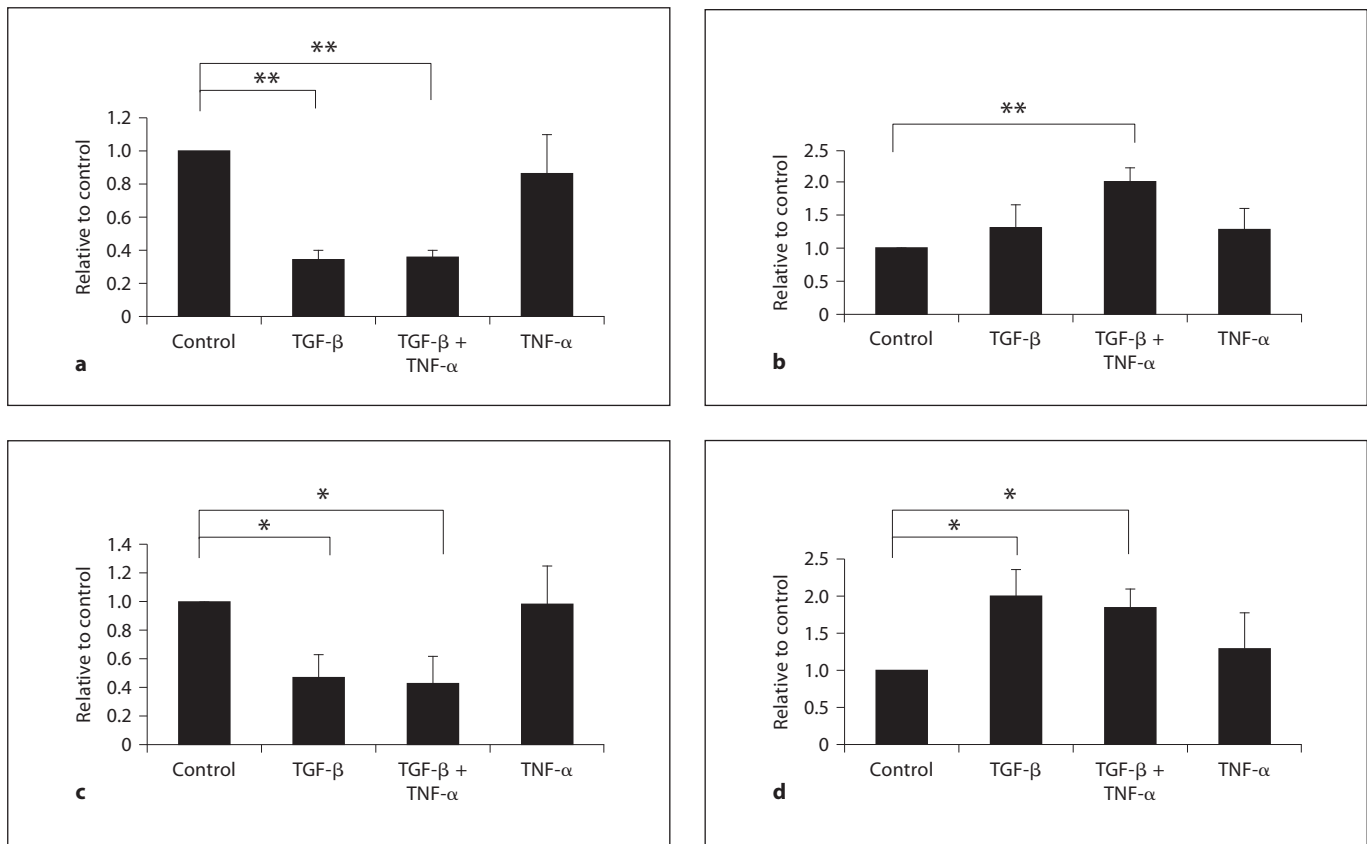


Fig. 2. EMT-related marker expression. Real time PCR was performed on samples after 48 h of incubation. **a** Expression of epithelial cell marker E-cadherin by BEAS-2B cells. **b** Expression of mesenchymal cell marker vimentin by BEAS-2B cells. **c** Expression

sion of E-cadherin by NHBE. **d** Expression of vimentin by NHBE. The mRNA level is reported as the E-cadherin/GADPH or vimentin/GADPH ratio normalized to the control. * $p < 0.05$, ** $p < 0.01$.

low (fig. 6a). There was no difference between the concentrations of TNF- α in the medium only, NHBE cell supernatant, and BEAS-2B cell supernatant (fig. 6b).

Expressions of TGF- β Receptors I, II, and III on BEAS-2B Cells

TGF- β 1 increased the BEAS-2B expression of TGF- β receptor I, but not of TGF- β receptor II or III. TNF- α did not affect the expressions of TGF- β receptor I, II, or III (fig. 7).

Discussion

In this study, we demonstrated that TGF- β 1 induces EMT in human bronchial epithelial cell lines BEAS-2B and NHBE, as shown by morphological and EMT-related marker changes. Furthermore, we showed that TNF- α

enhances TGF- β 1-induced EMT in BEAS-2B cells. Additionally, as a result of EMT, BEAS-2B cells acquired the functions of mesenchymal cells, such as migration and the production of ECM components.

Through EMT, epithelial cells undergo a conversion from epithelial markers to mesenchymal markers as well as cytoskeletal rearrangements that are essential for migration. In this study, TGF- β 1 treatment did not increase vimentin expression or the capacity of BEAS-2B cells for migration. However, cells treated with both TGF- β 1 and TNF- α showed increased migration. These results suggest that TGF- β 1 induced partial EMT in BEAS-2B cells, and that TNF- α enhanced the EMT. Unlike the case of BEAS-2B cells, TGF- β 1 increased vimentin expression by NHBE. This discrepancy is presumed to have been due to the differences between these immortalized and primary cell lines. In the present study, BEAS-2B cells secreted TGF- β 1 in the control culture in which BEAS-2B cells

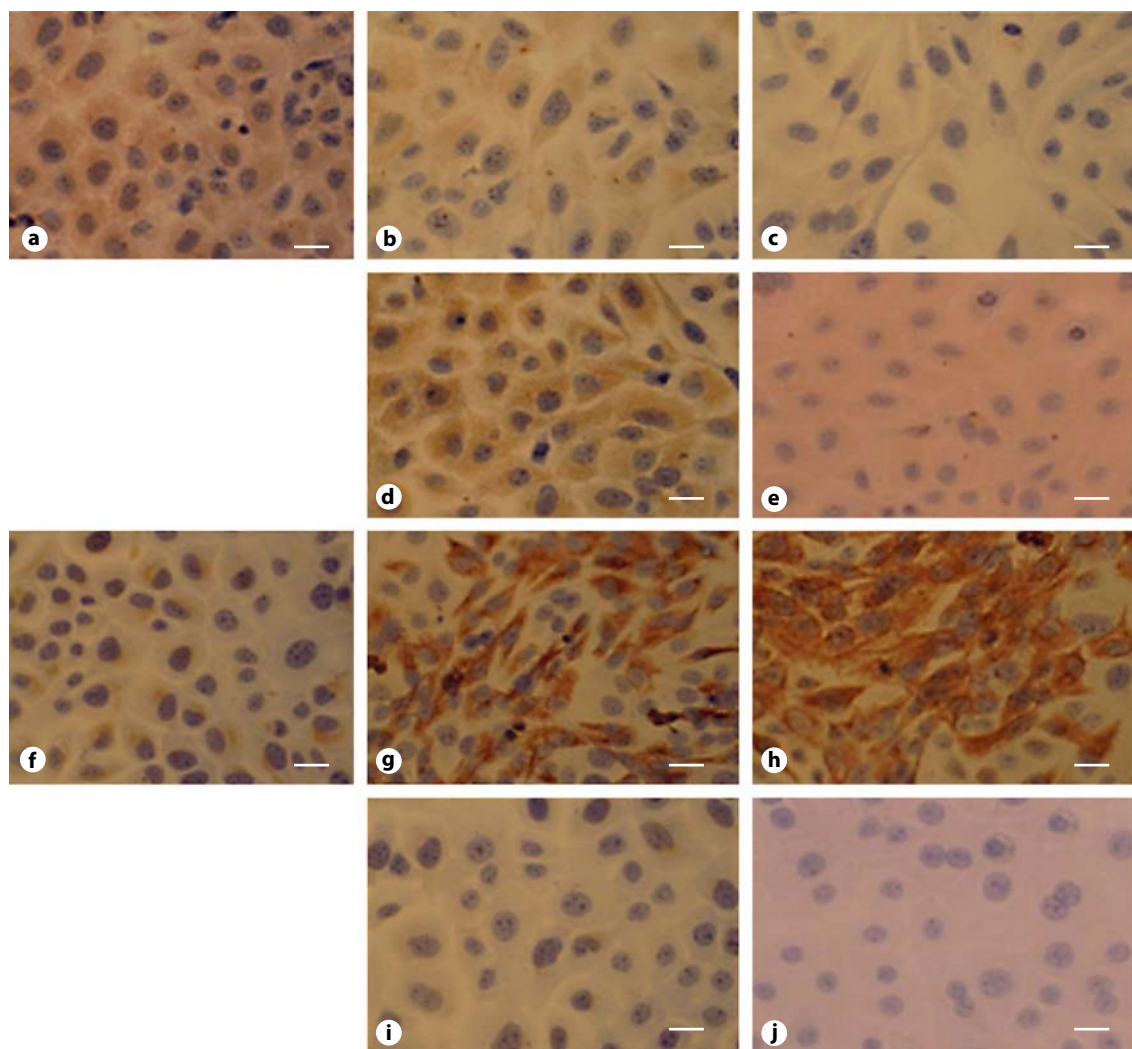


Fig. 3. Immunostaining of BEAS-2B cells cultured under various conditions for 72 h. Pan-keratin immunostaining of BEAS-2B cells: control condition (a), stimulation with 5 ng/ml TGF- β 1 (b), stimulation with 5 ng/ml TGF- β 1 and 10 ng/ml TNF- α (c), stimulation with 10 ng/ml TNF- α (d), and negative control (e). Vi-

mentin immunostaining of BEAS-2B cells: control condition (f), stimulation with 5 ng/ml TGF- β 1 (g), stimulation with 5 ng/ml TGF- β 1 and 10 ng/ml TNF- α (h), stimulation with 10 ng/ml TNF- α (i), and negative control (j). Scale bars = 20 μ m.

were exposed to a low concentration of TGF- β 1. This might have induced the different response to TGF- β 1.

Airway remodeling is a recognized pathological feature of airway diseases such as bronchial asthma and COPD [4]. Subepithelial fibrosis correlates with the severity of bronchial asthma [10], and small airway fibrosis is one of the pathogenetic features of COPD [20].

Several studies have shown that fibroblasts and myofibroblasts play important roles in the development of fibrosis, but their origin is not clear. Some reports have indicated the possibility that mesenchymal cells come

from resident fibroblasts stimulated by cytokines [21] and from circulating fibrocytes acting as progenitor cells of myofibroblasts [22, 23].

EMT is an important physiological phenomenon in normal development, fibrosis, and cancer pathology [24]. TGF- β has been reported to be a potent inducer of EMT [25]. Interestingly, TGF- β is increased in diseases which involve airway fibrosis, such as asthma and COPD [9, 26]. Anti-TGF- β 1 antibody suppressed airway remodeling in a murine model of asthma [27]. It is hard to induce peribronchial fibrosis in Smad3-deficient mice [28]. Airway

Fig. 4. BEAS-2B migration assay. Migration assay was performed using a Boyden chamber assay system. Fibronectin (5 μ g/ml) was used as the chemoattractant. Cells were incubated under various conditions for 72 h. After the removal of the stimulus overnight, assay was performed. Cell migration was expressed as a percentage of the control. ** $p < 0.01$.

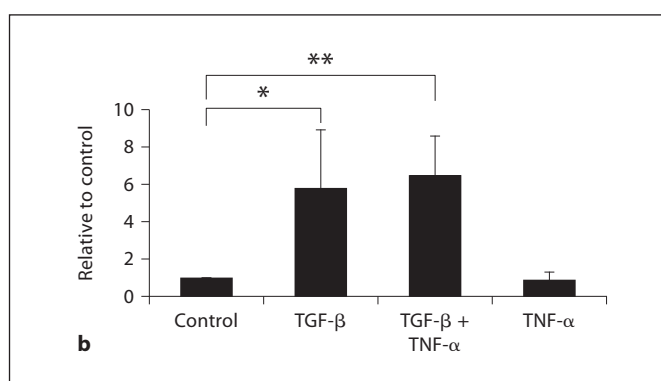
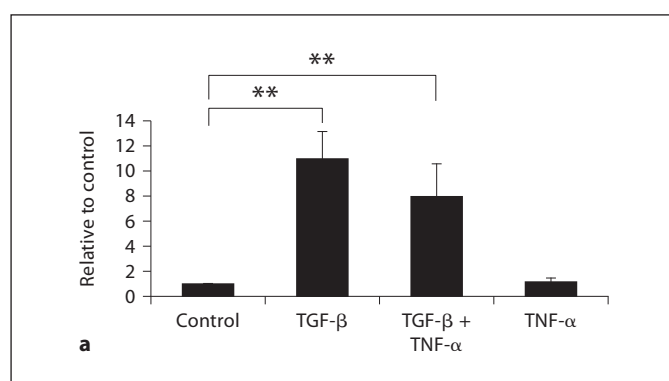
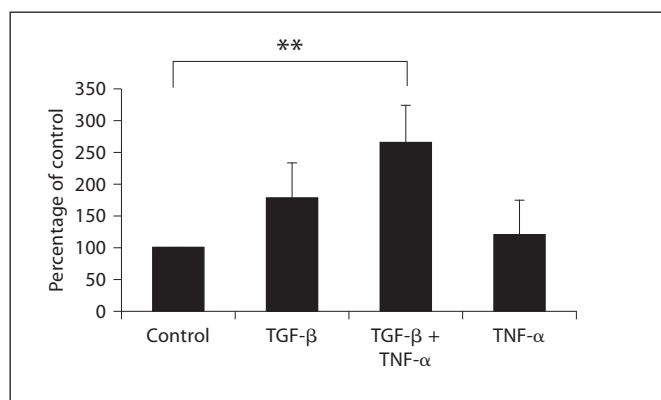


Fig. 5. ECM-related gene expression. RT-PCR analysis of ECM-related genes was performed on samples of BEAS-2B cells after 48 h of incubation under various conditions. **a** Expression of mRNA for collagen I. **b** Expression of mRNA for versican. The mRNA level is reported as the collagen I/GADPH or versican/GADPH ratio normalized to the control. * $p < 0.05$, ** $p < 0.01$.

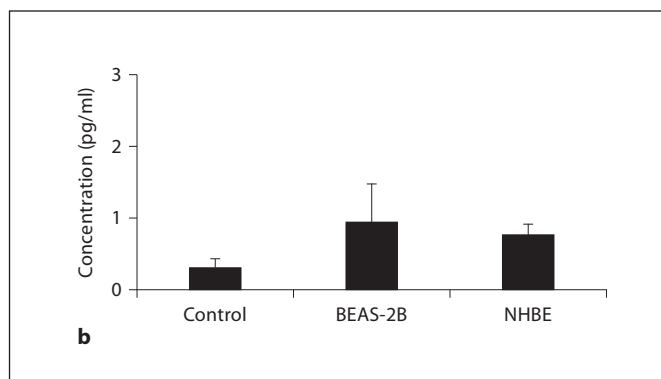
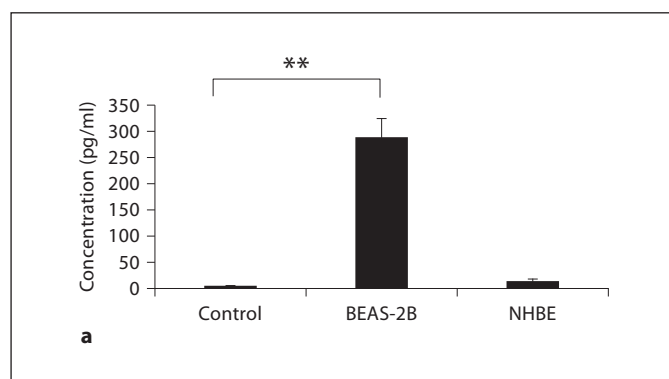


Fig. 6. Concentrations of TGF- β 1 and TNF- α . The concentrations of TGF- β 1 and TNF- α were measured by ELISA. **a** Concentration of TGF- β 1 in the supernatant of cultured cells (pg/ml). **b** Concentration of TNF- α in the supernatant of cultured cells (pg/ml). ** $p < 0.01$.

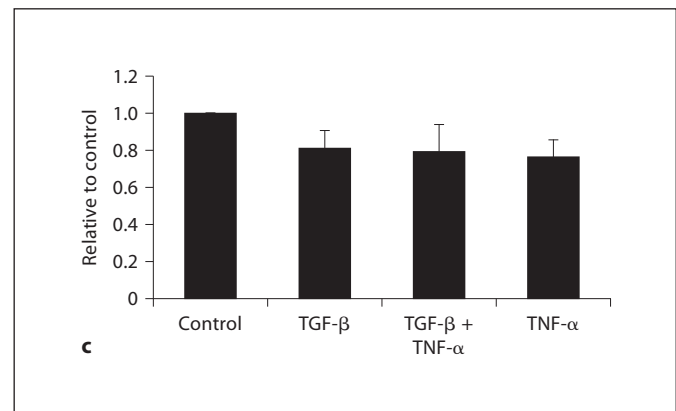
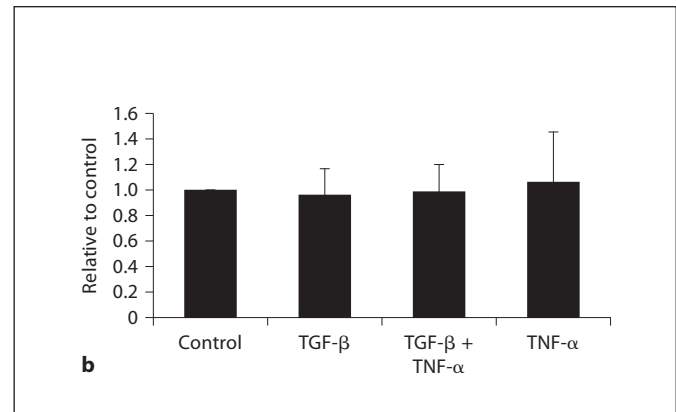
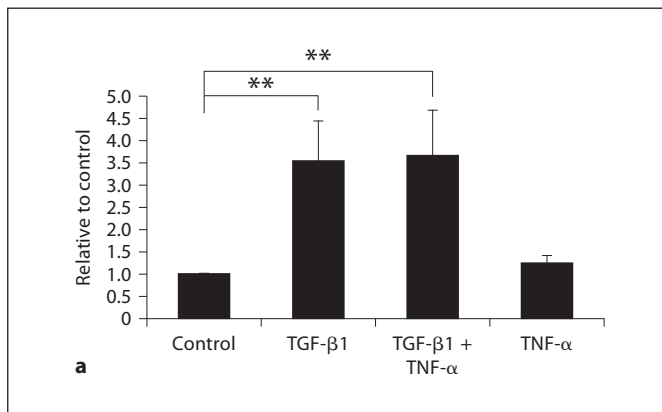


Fig. 7. Expression of TGF- β receptors I, II, and III. Real-time PCR was performed on samples of BEAS-2B cells after 48 h of incubation under various conditions. **a** Expression of mRNA for TGF- β receptor I. **b** Expression of mRNA for TGF- β receptor II. **c** Expression of mRNA for TGF- β receptor III. The mRNA level is reported as the ratio of TGF- β receptor I, II, and III and GAPDH normalized to the control. ** $p < 0.01$.

remodeling does not occur in all cases of asthma. This suggests that genetic factors may contribute to a propensity for airway remodeling. Some TGF- β single-nucleotide polymorphisms are reported to be involved in asthma and, especially, the presence of single-nucleotide polymorphism +915G/C in the TGF- β gene may predispose to airway fibrosis [29].

These reports support the concept that TGF- β is associated with the development of fibrosis by inducing EMT to fibroblasts and myofibroblasts in asthma and COPD.

TNF- α is another important cytokine in the pathogenesis of airway disease. The level of TNF- α in bronchoalveolar lavage fluid and the expression of TNF- α mRNA in airway biopsy specimens are increased in severe asthma patients [13, 14]. Clinical trials showed that anti-TNF- α therapy was effective in severe asthma patients [30, 31]. Moreover, TNF- α overexpression in a murine model induced airspace enlargement, the loss of small airspaces, and increased collagen, which are features of COPD [32]. Those reports thus suggested that TNF- α might be involved in airway remodeling. Here, we

showed that TNF- α is involved in airway fibrosis through EMT by enhancing the effects of TGF- β .

Some ECM proteins have been reported to regulate EMT. Collagen type I promoted EMT in alveolar epithelial cells A549 [33], and fibronectin regulated EMT in murine alveolar epithelial cells ex vivo [34]. Deposition of such ECM components as collagen types I, III, and IV as well as fibronectin, lumican, and versican was increased in the subepithelial fibrosis of patients with bronchial asthma [35–37]. The BM prevented epithelial cells from contacting the ECM in vivo and inhibited EMT [38]. MMP-2 and MMP-9 broke down collagen type I and laminin, the main components of the BM, and were upregulated in EMT in renal tubular epithelial cells [39]. In this context, disruption of the BM and production of ECM could be surmised to be triggers for EMT.

Molloy et al. [40] reported that BMP4 induced EMT in BEAS-2B cells. BMP4 is a member of the TGF superfamily, but the intracellular transmission molecule Smad, which mediates the signal response, is different. The molecular response to BMP and the response to TGF- β oc-

cur due to signaling through Smads 1, 5, and 8 and Smads 2 and 3, respectively [41–43].

TGF- β binds directly to TGF- β receptor II and makes a complex with TGF- β receptor I. Then TGF- β receptor I is phosphorylated by receptor II and phosphorylates Smads 2 and 3.

In the present study, TGF- β 1 increased the expression of TGF- β receptor I, but not of receptor II or III. TNF- α had no effect on the upregulation of receptor I by TGF- β 1.

Stimulation by TGF- β phosphorylated Smad 3, but did not affect Smads 1, 5, and 8. Stimulation by TNF- α did not cause the phosphorylation of Smad 3 or of Smads 1, 5 and 8 (data not shown). There are non-Smad pathways that are common to TGF- β and to BMPs. They may include a mitogen-activated protein kinase pathway including ERK, P38, and Jun N-terminal kinase [41].

The pathogenesis of airway remodeling is complicated and much remains unclear. This is the first report demonstrating that TGF- β 1 induces EMT and that TNF- α enhances TGF- β 1-induced EMT. Further studies are necessary to better understand the mechanisms of EMT in bronchial epithelial cells.

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