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Regulation of procollagen I (α1) by interleukin-4 in human bronchial fibroblasts: a possible role in airway remodelling in asthma

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Summary

Background In bronchial mucosa, T cells are in close association with fibroblasts. This cell contact raises the possibility of cross-talk between the two cell types through cytokines, such as interleukin-4 (IL-4).

Objective We postulated that IL-4 may modulate collagen synthesis and degradation in the fibroblasts of asthmatics.

Methods Bronchial fibroblasts from asthmatics (BAF) and normal controls (BNF) were stimulated with IL-4. Procollagen I gene expression and protein production were measured by real-time PCR, RT-PCR, and radioimmunoassay. The effect of IL-4 on the regulation of procollagen I (α_1) promoter was studied through transient cell transfections. The implication of Sp1 and AP-1 in regulating IL-4-induced procollagen I (α_1) production was determined. The effect of IL-4 on metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) production and gene expression was evaluated.

Results Following IL-4 stimulation, there was a significant increase in the expression of mRNA of procollagen I (α_1) by human bronchial fibroblasts of asthmatics and controls. IL-4 has a dose–response effect on mRNA, with a maximal effect at 5 ng/mL, as determined by real-time PCR. The maximal increase in procollagen I (α_1) was observed at 6 h after IL-4 stimulation in both BNF and BAF. BAFs have a greater increase in the procollagen I (α_1)/β_2 microglobulin ratio after 6 h of IL-4 stimulation ($4.1 \times 10^{-2} \pm 0.03$ to $20.8 \times 10^{-2} \pm 0.1$) compared with BNF ($2.9 \times 10^{-2} \pm 0.006$ to $9.2 \times 10^{-2} \pm 0.08$) ($P = 0.001$). In transient transfection experiments, IL-4 increased promoter activity by threefold in BAF and BNF. Sp1 was up-regulated after IL-4 stimulation and AP-1 was down-regulated as shown by electrophoretic mobility shift assay. IL-4 decreased MMP-2 protein and mRNA levels, and did not alter TIMP-2 production.

Conclusions IL-4 positively regulates procollagen I (α_1) transcription by direct promoter activation and increases the TIMP-2/MMP-2 ratio, thereby supporting the profibrotic effect of this cytokine. Thus, this study emphasizes that IL-4 may be considered as a link between inflammation and collagen deposition observed in asthmatic airways.

Keywords airway remodelling, asthma, bronchial fibroblasts, collagen promoter, metalloproteinases, transcription factors

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Introduction

Allergic asthma is a bronchial disease characterized by chronic airway inflammation and alteration of the bronchial wall structure [1]. Asthmatic inflammation is believed to trigger an injury–repair cycle in the bronchial wall leading to airway remodelling [2]. This airway remodelling includes epithelial changes, thickening underneath the basement membrane, hyperplasia and hypertrophy of the bronchial smooth muscle and extracellular matrix (ECM) protein deposition [3].

The components of ECM like collagen, elastin, fibronectin, and proteoglycan contribute not only to the mechanical properties of the mucosa but also modulate various cell functions such as migration, proliferation, and differentiation [4, 5]. In asthma, matrix components undergo changes [6]. These changes within the asthmatic airways could be due to an increase in the synthesis or a decrease in the degradation of the matrix components.

The integrity of the ECM requires equilibrium between synthesis and subsequent degradation by metalloproteinases (MMPs). MMPs are involved in several diseases that lead to the destruction or disorganization of the matrix [7]. Their

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activities are modulated by specific inhibitors termed tissue inhibitors of metalloproteinases (TIMPs) [8]. These inhibitors can act on all active MMPs via reversible non-covalent binding to form a 1 : 1 complex [9]. The breakdown in this equilibrium, and the quantity and activity of MMPs and their inhibitors may play a key role in the accumulation of collagen. MMP-9 is present in higher concentrations in bronchoalveolar lavage (BAL) and in the culture supernatants of alveolar macrophages isolated from asthmatics [10]. However, the functions of MMPs and their inhibitors in asthma need to be studied. We have shown that bronchial fibroblasts from asthmatic subjects are less able to degrade collagen compared with fibroblasts from normal subjects. These cells produce less MMP-2, a 72-kDa gelatinase that cleaves type IV collagen and degrades denatured collagen and collagen fragments, and have less collagen phagocytic activity than fibroblasts from healthy controls [11].

IL-4 is a cytokine that plays a role in the Th2 response and is produced by T lymphocytes, eosinophils, and mast cells [12]. It has a broad spectrum of activity against haematopoietic and non-haematopoietic cells. Bronchial epithelial cells express IL-4 receptors and stimulation of these cells by IL-4 induces the production of IL-8, MCP-1, and IL-1 antagonist receptors [13]. Fibroblasts also express IL-4 receptors [14]. It has been shown that IL-4 and IL-13 induce the production of cytokines (IL-6, IL-11, and GM-CSF) by pulmonary fibroblast lines [15]. This cytokine also promotes the differentiation of synovial fibroblasts to myofibroblasts [16]. Over-expression of IL-13 in a mouse model induces sub-epithelial fibrosis and smooth muscle proliferation, suggesting that IL-13 may play a role in the airway remodelling observed in asthma [17]. The fibrogenic role of IL-4 has been documented in the skin. This cytokine stimulates collagen synthesis by dermal fibroblasts. Its effect is twice as powerful as TGFβ [18]. IL-4 also increases collagen and fibronectin synthesis by synovial fibroblasts [19]. Moreover, in addition to stimulating collagen synthesis, IL-4 inhibits the induction of MMPs [20] and increases the expression of TIMP-2 in dermal fibroblasts [21], which support the profibrotic effect of this cytokine.

Studies with animal models have revealed important effects of IL-4 and IL-13 on the structural cells of the airways, which may account for some of the remodelling observed in asthma [22]. An increased number of cells expressing mRNA and protein for IL-4 has been reported in bronchial biopsies from asthmatic subjects compared with non-asthmatic controls [23]. The major source of IL-4 in the asthmatic airways is $CD3^+$ T cells [24]. IL-4Rα expression is increased in epithelial and endothelial layers and in the sub-epithelial area in bronchial biopsies obtained from asthmatics compared with control subjects [13]. The increased amount of IL-4 found in the asthmatic airways may directly stimulate structural cells to produce more ECM components.

In this study, we have investigated the effect of IL-4 on procollagen I (α_1) synthesis and MMP-related degradation pathways in bronchial fibroblasts from both healthy and asthmatic donors. We have found that human IL-4 positively regulates procollagen I (α_1) transcription by direct promoter activation. IL-4 induces an up-regulation of Sp1 and a down-regulation of AP-1. Moreover, IL-4 increases the TIMP-2/MMP-2 ratio, thereby supporting the profibrotic effect of this cytokine. This study provides new insights into the mechanisms that govern collagen alteration observed in asthma and emphasizes that IL-4 could be considered as a potential link between inflammation and remodelling in asthma.

Materials and methods

Bronchial fibroblast cell culture

Primary fibroblasts were isolated from bronchial biopsies of asthmatic ($n = 7$) and healthy ($n = 5$) subjects. The asthmatic subjects fulfilling the American Thoracic Society criteria for asthma [25] were recruited from the Asthma Clinic at the Laval Hospital (Quebec, QC, Canada). They used only an inhaled β_2-agonist on demand. None used inhaled or systemic corticosteroids. All asthmatic patients were atopic non-smokers. Healthy subjects were non-atopic non-smokers with no history of asthma or systemic diseases. Bronchial biopsies were obtained by bronchoscopy from asthmatic and healthy subjects. The Ethic Committee of Laval Hospital approved the study and all subjects gave written informed consent.

Isolated fibroblasts were characterized by immunofluorescence and flow cytometry using a mouse anti-vimentin and a mouse anti-human fibroblast antigen Ab-1 antibody (Calbiochem, San Diego, CA, USA) that shows no cross-reactivity with epithelial cells, endothelial cells, smooth muscle cells, or other cell types. This identification confirmed the purity of bronchial fibroblast cell culture as described previously [26, 27].

Fibroblasts were used in passages 3–4. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, Ont., Canada) supplemented with 10% heat-inactivated fetal calf serum (Medicorp, Montreal, QC, Canada), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin at 37 °C in the presence of 5% CO_2. Sub-confluent bronchial fibroblasts were stimulated with human recombinant IL-4 (Cedarlane Laboratories, Ont., Canada) at different concentrations (2.5–10 ng/mL). An Sp1 inhibitor, mithramycin (25–200 nM) (Sigma, Oakville, Ont., Canada), was also added in some experiments 18 h before IL-4 stimulation. An AP-1 inhibitor, curcumin (5–40 μM) (Sigma), was also added in some experiments 30 min before stimulation.

RNA extraction and RT-PCR

Confluent bronchial fibroblasts were incubated with or without IL-4 at various times and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, Ont., Canada) following the manufacturer's protocol. Concentrations of total RNA were estimated by a fluorescent dye with the Ribogreen RNA quantification reagent (Molecular Probes Inc., Eugene, OR, USA). RNA was analysed by RT-PCR amplification. In all, 500 ng of total RNA per sample was denaturated at 65 °C for 10 min and incubated at 37 °C for 60 min, with the following mix: 0.5 mM dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.25 μg/mL hexamer pd(N)6 (Amersham Pharmacia Biotech), 0.2 U RT-MMLV (Invitrogen), and first-strand buffer (Invitrogen). One microlitre of each cDNA was then mixed with 50 pmol of each primer (sense and antisense) in the presence of PCR buffer (Invitrogen), 0.2 mM dNTP, 1.25 U of

Table 1. Sequences and predicted sizes of primers used for PCR analysis

Molecules	Primers	Predicted size of the amplicon (bp)
COL1A1	5′-TGA ATC TGG ACG TGA GGG GG-3′ sense,	647
	5′-CAC GGT CAC GAA CCA CAT TGG CAT-3′ antisense	
MMP-2	5′-GTG CTG AAG GAC ACA CTA AAG AAG A-3′ sense,	580
	5′-TTG CCA TCC TTC TCA AAG TTG TAG G-3′ antisense	
TIMP-2	5′-AAA CGA CAT TTA TGG CAA CCC TAT C-3′ sense	405
	5′-ACA GGA GCC GTC ACT TCT CTT GAT G-3′ antisense	
GAPDH	5′-ATG CAA CGG ATT TGG TCG TAT-3′ sense	221
	5′-TCT CGC TCC TGG AAG ATG GTG-3′ antisense	

Taq DNA polymerase (Promega, Madison, WI, USA), and 1.25 mM $MgCl_2$. The cDNAs were denaturated for 2 min at 95 °C followed by 30 cycles of 1 min denaturation at 95 °C, 30 s annealing at 56 °C, and 1 min elongation at 72 °C. A final elongation period of 10 min was performed. The different sets of primers used to amplify procollagen I (α1), MMP-2, TIMP-2, and GAPDH cDNAs specifically are presented in Table 1 [28, 29]. After amplification, samples were analysed on a 2% agarose gel. A picture of the gels was taken, and the relative intensities of the PCR products were measured by digitalized image on a Macintosh computer using the public domain program NIH image. We used DNA sequencing to ensure that the amplified sequences were the relevant ones.

Real-time PCR

Quantification of the mRNA message coding for β_2 microglobulin (forward 5′-GAT GAG TAT GCC TGC CGT GTG-3; reverse 5′-CAA TCC AAA TGC GGC ATC T-3′) and procollagen I (α_1) was performed using Lightcycler technology (Roche Diagnostics, Mannheim, Germany) following reverse transcriptase as previously described [30]. PCR reactions were performed in a volume of 20 μL containing 1 μL of cDNA, 0.3 μM of each primer and 10 μL of QuantiTect™ SYBR® Green PCR containing DNA polymerase, dNTP mix, buffer, $MgCl_2$ and fluorescent dyes (Qiagen, Mississauga, Ont., Canada). The PCR protocol consisted of three programs: denaturation, amplification, and melting curve analysis for product identification. The denaturation and amplification conditions for β_2 microglobulin were 95 °C for 15 min followed by 45 cycles of PCR. Each cycle included denaturation at 95 °C for 20 s, annealing of 20 s at 57 °C, and extension of 20 s at 72 °C. The temperature transition rate was 20 °C/s, except when heating at 72 °C, when it was 5 °C/s. Fluorescence was measured at the end of every cycle to allow quantification of cDNA. After amplification, a melting curve was obtained by slowly heating at 95 °C with fluorescence detection every 0.2 °C following the normal cycle. Using standard curves, the amount of copy numbers of procollagen gene in each sample was calculated and normalized to β_2 microglobulin in the same sample.

Measurement of procollagen I protein production in culture supernatant

Procollagen I was measured with a radioimmunoassay kit (Pharmacia and Upjohn, Don Mills, Ont., Canada) as we have reported previously [26].

Measurement of MMP-2 and TIMP-2 protein production in culture supernatant

MMP-2 and TIMP-2 were measured by ELISA using the BIOTRAK™ system (Amersham, Oakville, Ont., Canada) as previously described [11]. The number and the viability of cells were evaluated at the end of IL-4 stimulation.

Plasmid construct and transient transfections

The wild-type procollagen I (α_1) promoter (−174/+42) plasmid, p204-CAT, was kindly provided by Dr J. Varga (University of Illinois, Chicago, IL, USA) [31]. The CAT gene was removed from the construct by digestion with *Bam*H1 and *Kpn*1 and replaced with the *Kpn*1/*Bam*H1 fragment from pGL3basic (Promega), which contained the luciferase reporter gene, giving the p204-Luc. A promoter-less construct, composed of Bluescript KS^+ plasmid containing the luciferase reporter gene (pLuc), was used in parallel transfection as a control.

Transient transfections Bronchial fibroblasts (1×10^6) were incubated with 10 μg DNA and 60 μL of Superfect reagent (Qiagen) for 24 h. Transfected cells were then plated at 200 000 cells/well in fresh medium and incubated for 24 h with or without 5 ng/mL of human recombinant IL-4 and in some experiments with mithramycin (an Sp1 inhibitor) and with curcumin (an AP-1 inhibitor). After incubation, cells were lysed in lysis buffer (1 ×: 25 mM tris-phosphate pH 7.8, 2 mM DTT, 10% glycerol, 1% Triton X-100) and luciferase activity was measured with a Dynex 96-well plate luminometer (Dynex Technologies Inc., Chantilly, VA, USA). All transfection experiments were repeated at least three times.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

Nuclear proteins from IL-4-stimulated or untreated bronchial fibroblasts were extracted 0 min and 30 min post-stimulation, according to the method described in Schreiber et al. [32]. Briefly, 1×10^6 cells were washed twice in phosphate-buffered saline. The pellet was then suspended in 1 × buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and left on ice for 15 min. Nonidet NP-40 (10%) was added to the pellet and mixed vigorously for 10 s. After centrifugation, the nuclear pellet was suspended in ice-cold 1 × buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and shaken vigorously for 15 min at 4 °C. The

nuclear extracts were then centrifuged and the supernatants were frozen at −70 °C. Concentrations of nuclear proteins were determined by the Bio-Rad protein assay (BioRad, Mississauga, Ont., Canada).

Probe labelling for EMSA Double-stranded DNA probe corresponding to Sp1 (5′-ATT CGA TCG GGG CGG GAG C-3′) and AP-1 (5′-CGC TTG ATG ACT CAG CCG GAA-3′) consensus sequences were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The double-stranded DNA probes (50 ng) were end-labelled at 37 °C for 30 min with 15 μCi of ^{32}P-γ-ATP (Mandel Scientific Co. Ltd., Guelph, Ont., Canada) and 10 U of T4 polynucleotide kinase in a kinase buffer (Amersham Pharmacia Biotech). Reactions were stopped with 40 mM of EDTA pH 7.0. The labelled probes were extracted with phenol–chloroform and purified on a Sephadex G50 column (Amersham Pharmacia Biotech) in a final volume of 100 μL.

EMSA gel Each binding reaction used 2.5 μg of nuclear extract, 0.5 ng of labelled probe, 2 μg of polydIdC (Amersham Pharmacia Biotech), 50 μg of BSA, 10 mM of Hepes pH 7.9, 4% glycerol, 1% Ficoll, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 25 mM NaCl. The mix was incubated at room temperature for 20 min. Competition experiments were performed in the presence of a 100-fold molar excess of unlabelled probe. Antibodies anti-Sp1 and anti-AP-1 were added to binding reactions for 30 min and left on ice before the addition of labelled probe. Samples were resolved on 4% non-denaturing polyacrylamide gels and exposed to an X-ray film overnight for autoradiography. All EMSA experiments were repeated three times.

Statistical analysis

All experiments in this study were performed at least three times. The data shown are representative results. Experimental values are given as means ± SEM. To compare groups and conditions, the data were analysed using a crossed-nested design. When interactions between groups and conditions were significant, each group was analysed using a randomized block design. *A posteriori* multiple comparisons were performed using Tukey's procedure. The mean values ± SEM of quantitative variables were used as representative measures. All reported *P*-values were two-sided and were declared significant at 0.05 levels.

Results

Effect of IL-4 on procollagen I (α$_1$) mRNA

We performed RT-PCR and real-time PCR studies with RNA from the bronchial fibroblasts of both asthmatic and healthy subjects stimulated with (or not) various concentrations of IL-4. IL-4 has a dose–response effect on mRNA, with a maximal effect at 5 ng/mL, as determined by RT-PCR (Fig. 1a) and confirmed by real-time PCR (Fig. 1b). Figure 2 shows that fibroblasts from asthmatics (BAF) have a greater increase in the procollagen I (α_1)/β_2 microglobulin ratio after 6 h of stimulation with 5 ng/mL of IL-4 ($4.1 \times 10^{-2} \pm 0.03$ to $20.8 \times 10^{-2} \pm 0.1$) compared with fibroblasts from normal controls (BNF) ($2.9 \times 10^{-2} \pm 0.006$ to $9.2 \times 10^{-2} \pm 0.08$) ($P = 0.001$).

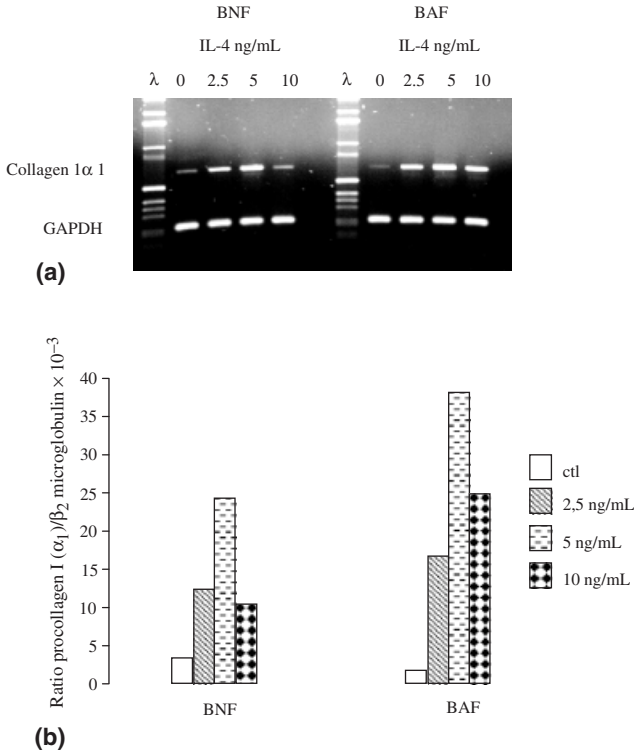

Fig. 1. Effect of IL-4 on procollagen I (α_1) mRNA synthesis by human bronchial fibroblasts. (a) A representative RT-PCR gel of IL-4 dose–response effect on procollagen I (α_1) mRNA levels in BNF and BAF. (b) Quantification by real-time PCR of procollagen I (α_1) mRNA following IL-4 (2.5, 5, and 10 ng/mL) stimulation. Total RNA was extracted after stimulation and PCR analysis was performed for procollagen I (α_1) mRNA and GAPDH or β_2 microglobulin as housekeeping genes. All experiments were repeated at least three times.

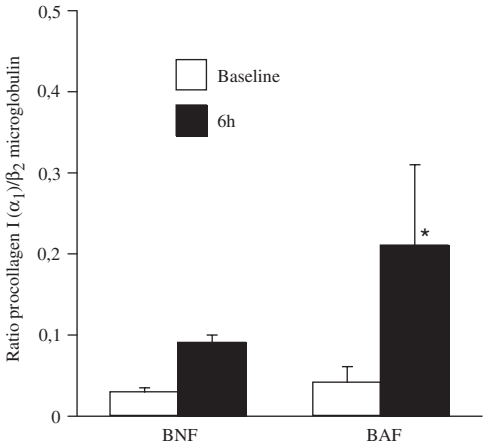

Fig. 2. Quantification of procollagen I (α_1) mRNA by real-time PCR. BAF ($n = 3$) have a greater increase than BNF ($n = 3$) in the procollagen I (α_1)/β_2 microglobulin ratio after 6 h of stimulation with 5 ng/mL of IL-4 (*$P = 0.001$).

Effect of IL-4 on collagen I protein production in culture supernatants

The effect of IL-4 on collagen production by bronchial fibroblasts from asthmatic and healthy subjects was measured

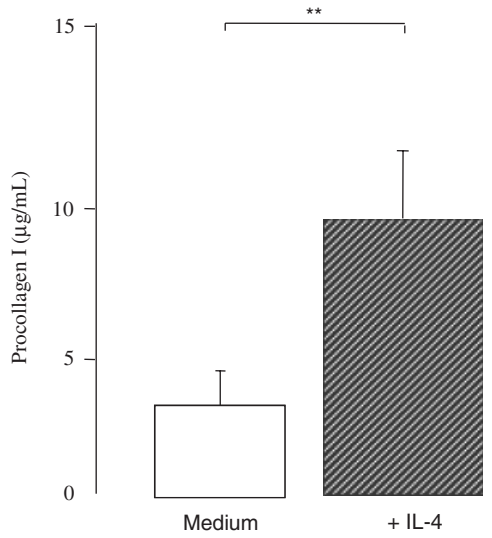

Fig. 3. Effect of IL-4 on procollagen I (α1) production by human bronchial fibroblasts. Cultured human bronchial fibroblasts derived from asthmatic individuals (BAF, $n = 5$) were incubated in the presence (hatched bar) or not (empty bar) of 5 ng/mL of IL-4 for 24 h. Collagen I protein production was measured by RIA in the supernatant culture. Each value represents the average ± SEM (**$P < 0.05$).

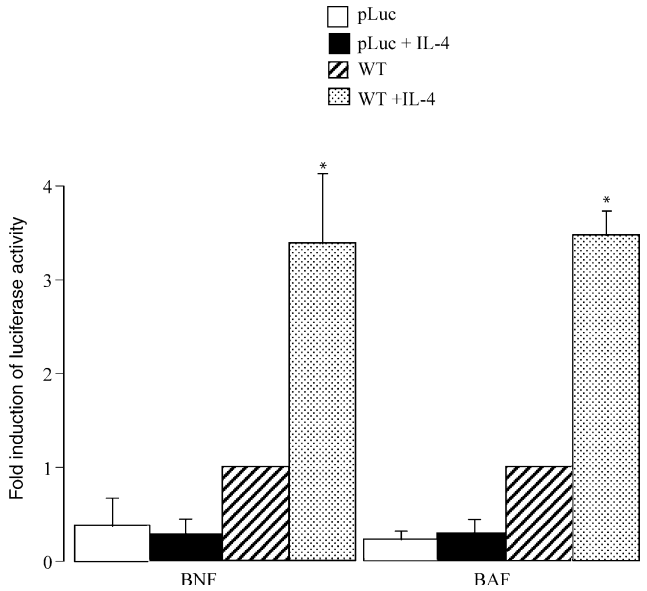

Fig. 4. Effect of IL-4 on luciferase activity measured in human bronchial fibroblasts transfected with procollagen I (α_1) promoter (p204-Luc). Human bronchial fibroblasts were transiently transfected with the p204-Luc wild-type promoter and incubated in the presence or not of IL-4 (5 ng/mL) for 24 h. Luciferase activity from the cell extracts was then measured and is represented as the relative luciferase activity of untreated fibroblasts (1 ×). Values from three stimulated samples in both asthmatic (BAF) and control groups (BNF) are shown. Each value represents the average ± SEM (* differences between conditions $P < 0.01$).

by radioimmunoassay (RIA). Bronchial fibroblasts were either left untreated or stimulated with various concentrations of human recombinant IL-4. The supernatant of bronchial fibroblasts from asthmatic and normal subjects showed a threefold increase in type I collagen, at 5 ng/mL of IL-4. Figure 3 shows data obtained with fibroblasts from asthmatics. The dose effect was also assessed and showed an increase in dose–response with a maximal effect at either 5 or 10 ng/mL. Thus, we demonstrated that the increase in type I collagen mRNA was paralleled by an increase in protein levels.

Bronchial fibroblast transfections with procollagen I (α_1) promoter in the presence of IL-4

To evaluate whether IL-4 positively modulated procollagen I (α_1) transcription, we performed transient transfections of bronchial fibroblasts with a p204-Luc vector containing the procollagen I (α_1) promoter region ($-174/+42$) positioned upstream of the luciferase reporter gene. Previous studies have shown that maximum procollagen I (α_1) promoter activity was contained in the region from 174 to 84 bp upstream to the transcription starting site [33]. Transient transfections of asthmatic and healthy bronchial fibroblasts with p204-Luc demonstrated a threefold increase in promoter activity when stimulated with IL-4 (Fig. 4). Transient transfections of the promoter-less vector pBluescript-Luc showed no significant increase in luciferase activity in the presence of IL-4 (Fig. 4). The transfection efficiency was assessed through co-transfection of the β-galactosidase plasmid and revealed a very constant efficiency of transfect in between conditions. Thus, IL-4 seemed to stimulate procollagen I (α_1) promoter activity and transcription directly.

Regulation of transcription factor by IL-4 in bronchial fibroblasts

To identify which transcription factors are involved in procollagen I (α_1) promoter activation by IL-4, we carried out EMSA studies with nuclear extracts from healthy and asthmatic bronchial fibroblasts, with and without stimulation. The wild-type minimal promoter is known to contain one AP-1-binding site and two NF-1/Sp1 tandem-binding elements. Incubation of the Sp1 probe with bronchial fibroblast nuclear extracts resulted in an early increase in the Sp1-specific signal 30 min after IL-4 stimulation (lane 2) (Fig. 5a). We identified the Sp1 signal as the first band by supershift and competition experiments (data not shown). The lower band represents a non-specific signal not modified by competition or supershift experiments. Similar patterns were observed in both healthy controls and asthmatic patients.

Luciferase activity from fibroblasts transfected with the p204-Luc plasmid in the presence of mithramycin, an Sp1 inhibitor, after IL-4 stimulation was significantly decreased in cells from asthmatic subjects (Fig. 5b) ($P = 0.05$). This result correlates those obtained by RT-PCR, where the mRNA signal decreased with increasing concentrations of mithramycin (Fig. 5c). This transcriptional modulation by IL-4 seems to result, at least in part, from an up-regulation of the Sp1-positive transcription factor as demonstrated by EMSA studies and the use of chemical inhibitors of Sp1.

The implication of the AP-1 transcription factor was also studied. As shown by EMSA, the AP-1 oligonucleotides incubated with bronchial fibroblast nuclear extracts produced one specific band that was less intense in IL-4-treated cells as

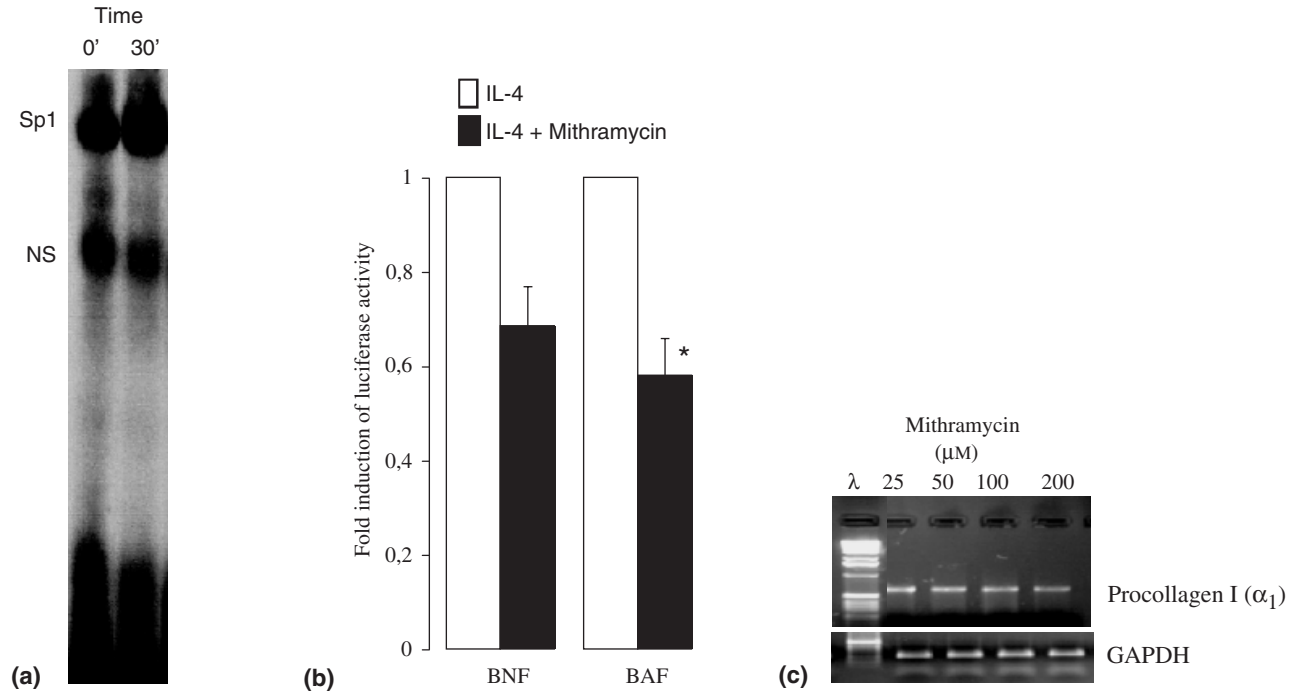

Fig. 5. Sp1 regulation by IL-4. (a) Electrophoretic mobility shift assays of nuclear extracts binding to the Sp1 consensus sequence from human bronchial fibroblast nuclear extracts prepared after IL-4 stimulation (5 ng/mL). Lane 1: time 0; lane 2: 30 min after IL-4 stimulation. (b) Effect of IL-4 in the presence of mithramycin (100 μM) on transient transfections of human bronchial fibroblasts with the p204-Luc wild-type promoter and (c) decreased mRNA expression in stimulated fibroblasts after the addition of increasing concentrations of mithramycin. Values from three stimulated samples in both asthmatic (BAF) and control groups (BNF) are shown. Each value represents the average ± SEM.

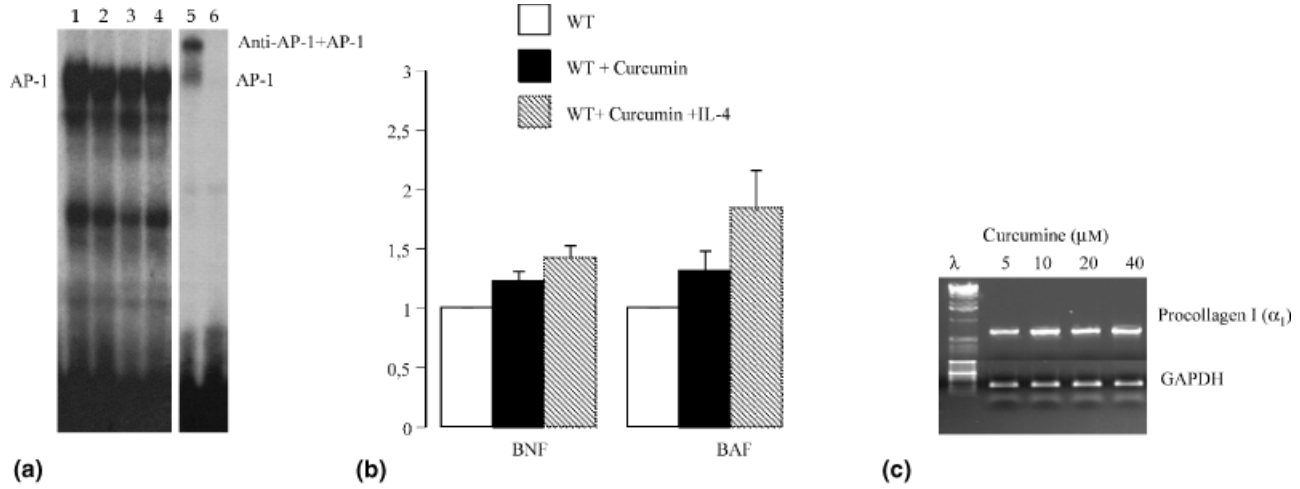

Fig. 6. AP-1 regulation by IL-4. (a) Electrophoretic mobility shift assays of nuclear extracts binding to the AP-1 consensus sequence from human bronchial fibroblast nuclear extracts prepared after IL-4 stimulation (5 ng/mL). Lane 1: time 0; lane 2: 30 min; lane 3: 60 min and lane 4: 360 min after IL-4 stimulation. Lane 5 shows supershift assay (pre-incubation with anti-AP-1) and lane 6 shows positive competition (100-fold molar excess of AP-1 unlabelled probe). (b) Effect of IL-4 in the presence of curcumin (40 μM) on transient transfections of human bronchial fibroblasts with the p204-Luc wild-type promoter and (c) increased mRNA expression in stimulated fibroblasts after the addition of increasing concentrations of curcumin. Values from three stimulated samples in both asthmatic (BAF) and control groups (BNF) are shown. Each value represents the average ± SEM.

early as 30 min (lanes 1 and 2, Fig. 6a). Similar patterns were observed in both groups (data not shown). AP-1 supershift and competition experiments identified the AP-1 signal as the first band (lanes 5 and 6). Transient transfection in the presence of curcumin led to a slight increase in promoter activity (Fig. 6b). This result correlates with those obtained by RT-PCR, where curcumin alone increases the mRNA signal (Fig. 6c). Those results suggest that AP-1 negatively regulates the procollagen I (α_1) promoter and that stimulation by IL-4 results in a decrease in AP-1.

Effect of IL-4 on MMP-2 and TIMP-2 protein production in culture supernatants

ECM components such as collagen I are regulated by the MMP family. We look at a possible modulation of MMP and

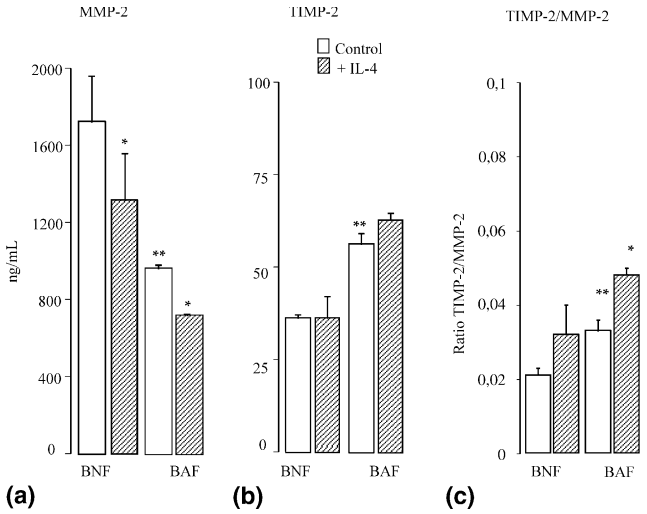

Fig. 7. Effect of IL-4 on MMP-2 (a) and TIMP-2 (b) production by human bronchial fibroblasts. Cultured human bronchial fibroblasts derived from asthmatic (BAF, $n = 5$) and normal individuals (BNF, $n = 7$) were incubated in the presence (hatched bars) or not (empty bars) of 5 ng/mL of IL-4 for 24 h. MMP-2 and TIMP-2 were next quantified by ELISA in supernatant cultures. (c) Ratio of TIMP-2/MMP-2 calculated from ELISA quantification at each condition. Each value represents the average ± SEM (* differences between conditions $P < 0.01$, ** differences between groups $P < 0.05$).

TIMP proteins by IL-4. Figures 7a and b show that at baseline, BAF produce less MMP-2 (956 ± 20 vs. 1719 ± 239 ng/mL, $P = 0.01$) and more TIMP-2 (59 ± 3 vs. 36 ± 1, $P = 0.05$) than BNF. This result confirmed what we had previously found [11]. IL-4 decreased MMP-2 production by bronchial fibroblasts from asthmatic patients and did not alter TIMP-2 production. There is thus a higher TIMP-2/MMP-2 ratio in asthmatics (0.05 ± 0.002) than in normal controls (0.02 ± 0.009) (Fig. 7c). The number of cells and their viability did not change after IL-4 stimulation.

Effect of IL-4 on MMP-2 and TIMP-2 mRNA

The MMP-2 and TIMP-2 mRNA levels were similarly assessed by RT-PCR with mRNA from IL-4-treated healthy and asthmatic bronchial fibroblasts. As shown by Figs 8a and b, the presence of IL-4 triggers a decrease in MMP-2 expression in BAF and maintains the *statu quo* in the amount of TIMP-2 mRNA in both BNF and BAF, thereby leading to a balance that promotes fibrosis through less collagen degradation.

Discussion

In the present study, we demonstrated that IL-4 exerts a direct effect on procollagen I (α_1) synthesis at the transcriptional level in bronchial fibroblasts derived from bronchial biopsies of healthy and mild asthmatic subjects. Our transient transfection studies have shown that procollagen I (α_1) promoter activity increases in the presence of IL-4 in bronchial fibroblasts from healthy and asthmatic subjects. This transcriptional modulation by IL-4 might result in part from an up-regulation of the Sp1 positive transcription factor and a down-regulation of the AP-1 as demonstrated by EMSA studies and inhibitor experiments. This promoter activation leads to an increase in mRNA transcripts and

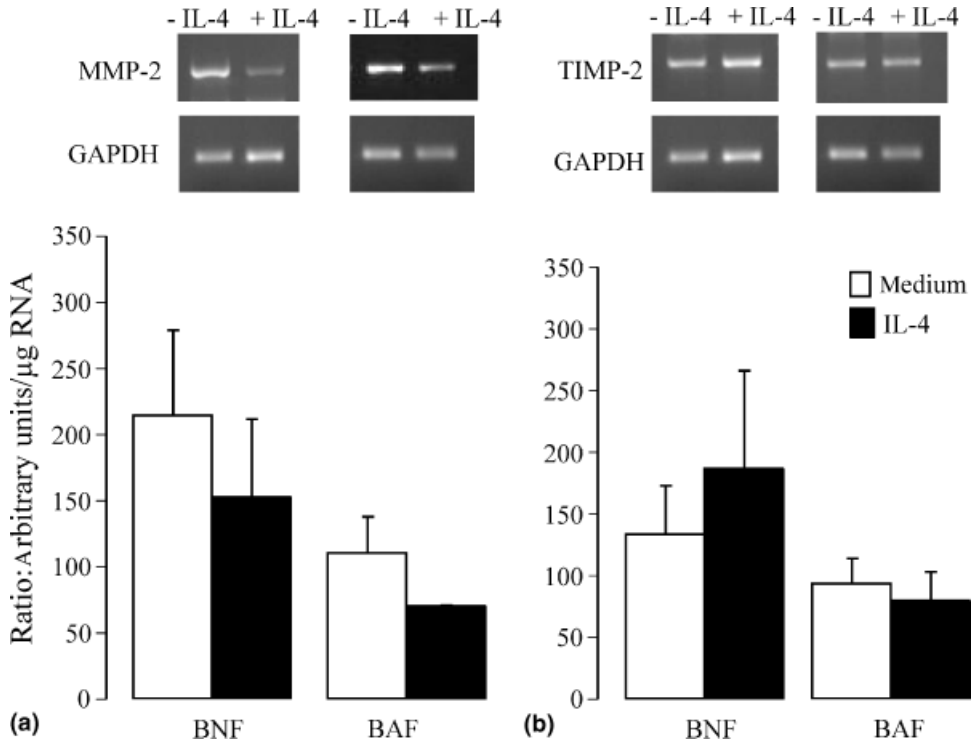

Fig. 8. Effect of IL-4 on MMP-2 (a) and TIMP-2 (b) mRNA synthesis by human bronchial fibroblasts. Cultured human bronchial fibroblasts from normal (BNF, $n = 3$) and asthmatic (BAF, $n = 3$) individuals at baseline (empty bars) and after stimulation with IL-4 (5 ng/mL) for 24 h (black bars). The upper portion of the figure represents the picture of one representative RT-PCR experiment and the bottom shows the relative intensities of the MMP-2 mRNA and TIMP-2 mRNA bands corrected for the mRNA amount. GAPDH mRNA showed constant relative intensities.

subsequently to a rise in protein synthesis. Postlethwaite et al. [19] obtained similar results *in vitro* using human dermal fibroblasts and fibroblastic synovial cell lines from patients with rheumatoid arthritis and osteoarthritis. They reported a twofold increase in collagen production when stimulated with IL-4 as determined by hydroxyproline incorporation assay and SDS-PAGE analysis. They also reported an increase in mRNA synthesis for procollagen I (α_1) and fibronectin. Sempowski et al. [14] showed a proliferating effect and an up-regulation of collagen production in response to IL-4 in a murine lung fibroblast model. These data support our results, where IL-4 has the ability to up-regulate procollagen I (α_1) promoter on human bronchial fibroblasts.

The human collagen promoter has been fully characterized in a systemic sclerosis model [33, 34]. Most of the transcriptional activity in skin fibroblasts occurs in a short region of the promoter (-174 bp to -84 bp) upstream of the transcription-starting site. This region contains two tandem NF-1/Sp1 regulatory elements, one CT-rich region, and one AP-1 consensus sequence. In cardiac fibroblasts, the induction of the AP-1 transcription factor by thyroid hormones inhibits procollagen I (α_1) promoter activity [35]. Mutated Sp1 and NF-1 sites on the minimal procollagen I (α_1) promoter exhibit less CAT activity in transfected human dermal fibroblasts [36, 37]. Li et al. [31] have shown positive regulatory transcriptional activity of Sp1 in expression studies using procollagen I (α_1) promoter construct and an Sp1 expression plasmid in *Drosophila melanogaster* cells [38]. In the Schneider *Drosophila* line 2, which lacks Sp1, transfection with the procollagen I (α_1) promoter construct revealed no detectable expression [39]. All these studies suggest that Sp1 and NF-1 have a positive regulatory role and AP-1 has a negative regulatory role in procollagen I (α_1) expression. The role of AP-1 as a negative transcription factor for the procollagen I (α_1) promoter in our model was demonstrated by inhibitor studies on transient transfection and RT-PCR signal. Sp1 and AP-1 chemical inhibitors, mithramycin and curcumin, respectively, have been previously used by others to study Sp1 and AP-1 involvement [40, 41]. This transcriptional modulation by IL-4 results, at least in part, from an up-regulation of the Sp1 positive transcription factor and a down-regulation of the AP-1 transcription factor as demonstrated by EMSA studies and inhibitor experiments. These results are consistent with previous observations in other models as described earlier. The increase in procollagen I (α_1) promoter activity results in a rise in mRNA transcripts and in collagen I protein synthesis, leading ultimately to a higher collagen content in the bronchial ECM, which is a feature of the pathologic asthma airway remodelling. Our results demonstrate that both control and asthmatic bronchial fibroblasts have the same increase in luciferase activity after IL-4 treatment with respect to the minimal promoter sequence used ($-174/+42$ bp). The higher level of procollagen 1 (α_1) mRNA observed in asthmatic fibroblasts suggests that this group might be more responsive through other promoter regions or possibly the consensus sequences encountered in the first intron.

Homeostasis of the ECM is a dynamic process that involves producing and degrading ECM components. An equilibrate combined effect of these processes results in the preservation of tissue structure. The amount of collagen within the ECM is regulated by a balance between the MMP and the TIMP families. We have shown that MMP-2 protein and mRNA are decreased in IL-4-stimulated bronchial fibroblasts. On the other hand, TIMP-2 protein and mRNA remained constant in the presence of IL-4. These two results together lead to a pro-fibrogenic situation where less collagen is degraded. There are very few studies regarding the effect of IL-4 on the MMP/TIMP degradation pathway. Among them, in a human prostatic tumour model, IL-4 was shown to down-regulate MMP-2 mRNA and protein levels, and has no effect on TIMP-2 mRNA and protein levels [42]. These results correlate with our observation in bronchial fibroblasts.

We thus suggest that, alone, the presence of IL-4 triggers the pro-fibrogenic activation of bronchial fibroblasts, and that the amount of IL-4 in respiratory tissue is the main difference between both studied groups. Inflammation in allergic asthma disease is characterized by a predominant Th2 response differentiation, resulting in an increase in IL-4 expression, which may stimulate bronchial fibroblasts to increase collagen production and to down-regulate degradation. Our results indicate that IL-4 is an important mediator that can not only drive the inflammatory process but can also play a significant role in collagen deposition. This finding has therapeutic implications and blocking the effect of IL-4 may help reduce collagen deposition underneath the basement membrane.

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