TGFβ-induced matrix production by bronchial fibroblasts in asthma: Budesonide and formoterol effects

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Summary
To investigate the mechanisms of enhanced airway deposition of subepithelial collagen in asthma and its sensitivity to drug therapy with combination of an inhaled glucocorticosteroid (GC) and a long-acting β_2-agonist (LABA), a cell model system involving bronchial fibroblasts derived from biopsies from patients with stable mild-to-moderate asthma has been used. To mimic unstable conditions and severe asthma, fibroblasts were stimulated ex vivo with TGFβ1. Primary fibroblasts established from central bronchial biopsies from 8 asthmatic patients were incubated for 24 h with 0.4% serum or TGFβ1 (10 ng/ml) with/without the GC budesonide (BUD; 10 nM) and/or the LABA formoterol (FORM; 0.1 nM). Procollagen peptide I (PICP), metalloproteinase (MMP)-1 and tissue inhibitor of MMPs (TIMP-1) were determined in culture media using ELISA while the activity of MMP-2, -3, -9 by zymography. Metabolically labeled proteoglycans, biglycan and decorin, associated with collagen fibrillation/deposition, were separated using chromatography and SDS-PAGE. The levels of PICP and biglycan were increased 2-fold by TGFβ1 ($p < 0.05$). The BUD and FORM combination reduced the PICP increase by 58% ($p < 0.01$) and the biglycan by 36% ($p < 0.05$) while each drug alone had no effect. Decorin levels were reduced by TGFβ1 in fibroblasts of most patients; BUD alone and BUD and FORM completely counteracted this decrease. MMPs and TIMP-1 were not affected by TGFβ1 or the drugs. These results suggest that BUD and FORM combination therapy, without affecting metalloproteolytic balance, has a potential to counteract enhanced collagen production by bronchial fibroblasts in asthma and to normalize the production of small proteoglycans which may affect collagen fibrillation and deposition.
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Introduction

Subepithelial fibrosis — thickened reticular basement membrane as a result of enhanced deposition of various extracellular matrix (ECM) molecules — is seen in airways of patients with asthma of all severities.[1–5] The extent of subepithelial fibrosis is correlated with the clinical severity of asthma and decrease in lung function.[1–3,5,6] One of the major constituents of the thickened reticular basement membrane in asthma is collagen type I. In the process of collagen type I formation, procollagen type I molecules are released by fibroblasts, and as they are assembled into insoluble collagen fibers, the soluble C-terminal peptide procollagen type I (PICP) is removed into the extracellular space. Hence, the PICP level in serum can be used as a marker of ongoing collagen synthesis.[7] In patients with asthma, mean sputum PICP levels are several-fold higher than those seen in healthy volunteers,[8,9] are increased during asthma exacerbations, and are negatively correlated with patients' forced expiratory volume in 1 s (FEV_1) expressed as a percentage of predicted normal value.[8]

Transforming growth factor β (TGFβ) plays a prominent role in formation of subepithelial fibrosis as it transforms fibroblasts into more active myofibroblasts that are responsible for enhanced production of various ECM components, including collagens and proteoglycans. Frequently, increased TGFβ levels in the airways of asthmatic patients correlate with the severity of asthma[6] and with the thickness of subepithelial basement membrane.[10] According to a new paradigm for persistent asthma, repeated exposures to allergens, respiratory viruses and other environmental insults may lead to damaged epithelium that does not heal completely or appropriately.[11] Subepithelial collagen deposition in the airways of asthmatic patients may be the result of an aberrant repair of this "chronic wound",[12] and both enhanced collagen production by submucosal fibroblasts and repressed collagen turnover would increase collagen deposition. In wound healing, as with organ morphogenesis, collagen deposition and turnover are affected by collagen fibrillation and organization of collagen fibers. Collagen deposition requires formation of fibrillar collagen from soluble procollagen molecules. The process of fiber assembly and spatial organization depends in part on association with small leucine-rich proteoglycans[13] such as decorin[14–16] and biglycan.[16–19] Decorin seems to be a key molecule in regulation of collagen fibrillogenesis while biglycan can fine-tune fibril assembly, especially early in the process, and may provide partial functional compensation for decorin deficiency.[16,20,21] Deposition and turnover of collagen are also affected by the balance between various metalloproteinases (MMPs) and their main tissue inhibitor, TIMP-1. The gelatinases MMP-9 and MMP-2 and collagenase MMP-1 are main regulators of collagens while stromelysin (MMP-3) affects turnover of proteoglycans[22]; all of these MMPs have been detected in the airways of asthmatic patients.[23,24]

Treatment of asthma with a combination therapy comprising an inhaled glucocorticosteroid (GC) and a long-acting β_2-adrenergic agonist (LABA) has been shown to result in improved asthma control.[25–28] However, it remains a matter of debate whether subepithelial fibrosis in asthma may be counteracted by GC and LABA combination therapy.[29] Clinical studies addressing this issue are very few in number[30,31] and their results are fragmentary and equivocal.

To further investigate the mechanisms of airway subepithelial collagen deposition in asthma and its sensitivity to drug therapy with a combination of inhaled GC and LABA, we have used a cell model system involving bronchial fibroblasts derived from biopsy samples taken from stable mild-to-moderate patients with asthma. To mimic unstable conditions, such as exacerbations and severe asthma, bronchial fibroblasts were stimulated *ex vivo* with TGFβ1. We have investigated the TGFβ1-induced synthesis of PICP, biglycan and decorin. We have also analyzed the TGFβ1-induced production of TIMP-1 and MMP-1 as well as the activity of MMP-9, MMP-2 and MMP-3. Concurrently with TGFβ1, patients' bronchial fibroblasts were incubated *ex vivo* with the GC budesonide (BUD) and the LABA formoterol (FORM), either alone or in combination, to evaluate whether combination therapy has the potential to counteract subepithelial fibrosis and whether these drugs exert their effects directly on the production of procollagen, decorin and biglycan or act indirectly on ECM deposition and degradation via effects on metalloproteolytic balance.

Material and methods

Patients

Four female and four male patients, non-smokers, 25–55 years old, with stable mild-to-moderate asthma (according to GINA guidelines: www.ginaasthma.org) and with confirmed airway hyperresponsiveness to methacholine were included in the study. A positive methacholine challenge test was defined as a fall in $FEV_1 \geq 20\%$ (PD_{20}) on a cumulative dose of methacholine < 2000 μg, measured by a tidal volume triggered technique (Automatic Provocation System, Erich Jäger GmbH, Höchberg, Germany). Of the 8 patients, 5 had an $FEV_1\%$ predicted normal of approximately 100% while 3 had values in the range of 62–67%; these 3 patients had also the lowest PD_{20} values (<0.05 μg) (Table 1). Only one of these 3 more severe patients showed reversibility in FEV_1 (defined as $\geq 12\%$ improvement in FEV_1 after inhalation of 400 μg salbutamol) and 2 of 5 patients with mild disease (Table 1). All patients were atopic, with skin prick tests confirming sensitization toward perennial allergens, were free of infections during the six weeks before bronchoscopy and used short-acting β_2-adrenergic agonists as needed. Patients were without inhaled corticosteroids during the last 6 months prior to the start of the study because even in cases inhaled corticosteroids were prescribed, the patients testified that they had not taken any corticosteroid treatment. The study was reviewed and approved by the Swedish Research Ethical Committee (No LU339-00) and informed consent was obtained from all patients. This study is linked to our previous study[32] in which the bronchial biopsy samples from the same patients were used.

Table 1 Baseline characteristics of patients with asthma included in the study.

	Sex	Age (years)	FEV$_1$ (% predicted)	PD$_{20}$ (μg)	FEV$_1$ reversibility
Pt 1	F	28	101	60	N (4%)
Pt 2	F	31	104	15	Y (13%)
Pt 3	M	30	67	<0.05	N (5%)
Pt 4	F	25	95	20	Y (16%)
Pt 5	M	35	62	<0.05	Y (24%)
Pt 6	M	55	103	75	N (4%)
Pt 7	M	26	100	826	N (4%)
Pt 8	F	39	63	<0.05	N (5%)

Abbreviations: F = female; M = male; PD$_{20}$ to methacholine stimulation; Y/N = yes/no FEV$_1$ reversibility was defined as $\geq$ 12% improvement in FEV$_1$ after 400 μg salbutamol.

Bronchoalveolar lavage and sampling of bronchial tissue

Bronchoalveolar lavage (BAL) was performed in right middle lobe with 150 ml buffered saline divided in three aliquots of 50 ml, and used for differential cell analysis. Bronchial biopsy samples were collected from the second and third bronchial generation from right lung as previously described.[33] Cells were collected from BAL fluid by cytospin performed at 1500 rpm for 5 min using a table cytocentrifuge (Shandon Southern Products Ltd., Runcorn, Cheshire, UK). Cells were stained with May-Grünwald and 6% Giemsa solution in buffer (67 mM KH$_2$PO$_4$ and 67 mM Na$_2$HPO$_4$) with separate rounds of buffer between staining. Differential cell counts were performed on 100 cells using light microscopy.

Biopsy-derived bronchial primary fibroblasts

Primary fibroblast-like cells were established, as described previously,[32,33] from biopsy samples that were not different in size, vascular or muscle content. Briefly, the biopsy samples were cut into small pieces and were allowed to adhere to cell culture plastic plates before the addition of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal Clone III serum (Hyclone, Logan, UT, USA), 1% L-glutamine, 0.5% gentamicin and 5 ug/ml amphotericin. Biopsy samples were cultured until there were outgrowths of cells with morphology typical for that of fibroblasts, i.e. cells had a spindle-like shape and several protrusions.[34,35] To ensure that true fibroblasts were cultured, markers for fibroblasts and smooth muscle cells were used as previously described.[32] Isolated primary fibroblasts were split 1:2 at expansions and were used in passages 4—6 for further experiments.

Study design

Biopsy-derived bronchial primary fibroblasts were cultured in 6-well plates containing Earle's minimal essential medium (Sigma—Aldrich, Irvine, UK) supplemented with 10% (v/v) Fetal Clone III serum (Hyclone, Logan, UT, USA), 1% L-glutamine, 0.5% gentamicin and 5 μg/ml amphotericin at 37 °C in a humidified 5% CO$_2$ and 95% atmosphere.

Prior to treatment with TGFβ1 and drugs (or vehicle), cells were starved for 2 h in DMEM supplemented with 1% donor calf serum and 2 mM L-glutamine. Subsequently, cells were incubated for 24 h with TGFβ1 10 ng/ml (R&D Systems, Minneapolis, MN, USA) in 0.4% serum and concurrently with BUD 10^{-8} M or FORM 10^{-10} M or their combination (AstraZeneca, Lund, Sweden) in MgSO$_4$-poor DMEM (Gibco BRL, Paisley, UK). During the last 22 h of the incubation, 50 μCi/ml [^{35}S]-sulfate (PerkinElmer Life Science, Boston, MA, USA) was also present. Fibroblasts incubated with 0.4% serum and drug vehicle (0.1% ethanol) were used as a baseline control. After incubation, the culture media were collected and stored at −80 °C with the exception of the medium aliquots aimed for biglycan and decorin analysis; to these aliquots, 0.1% phenyl-methylsulphon-fluoride (Sigma, St Louis, MO, USA) and 2 mM EDTA together with 0.1 mg/ml chondroitin sulfate-6 and 0.4 mg/ml dextran were added and aliquots were stored at −20 °C. Analyses of PICP, biglycan, decorin, MMP-9, MMP-3, MMP-2, MMP-1 and TIMP-1 were performed in culture media as described below. All experiments with fibroblasts from each patient were performed in homogenous, confluent cell cultures; repeated two or three times, and mean value was calculated for each patient.

The concentration of BUD (10^{-8} M) and FORM (10^{-10} M) used in this study are regarded as clinical relevant; the BUD concentration used reflects the concentration achieved in the airway and lung tissue several hours after inhalation[36—38] while doses of FORM in asthma therapy are typically two orders of magnitude lower than BUD doses.

Radiographic analysis of biglycan and decorin by anion-exchange chromatography

Biglycan and decorin were obtained using radiographic analysis by means of [^{35}S]-sulfate incorporation into the proteoglycan glycosaminoglycan chains and isolated using anion-exchange chromatography as described previously.[39] Briefly, unincorporated radioactive precursors from the collected culture media applied to DEAE cellulose columns were washed out whereon the proteoglycan fraction was eluted and analyzed for [^{35}S] sulfate activity. Eluted fractions were then precipitated, separated, and identified on gradient SDS-PAGE as described previously.[39] The intensity of the bands containing biglycan and decorin were further

analyzed on Fuji FLA 3000 (Seikagaku Kogyo, Tokyo, Japan) and related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Determination of PICP, MMP-1, and TIMP-1 by immunoassay

The concentration of PICP released in the culture media was measured by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Takara Biochemicals Co., Osaka, Japan). Concentrations of MMP-1 and TIMP-1 (TIMP-1 both as the free form as well as complexed with MMPs) were analyzed by ELISA (Amersham, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The obtained concentrations were related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Analysis of MMP-9, MMP-2 and MMP-3 by zymography

The presence and activity of proteolytic enzymes were analyzed using Novex polyacrylamide zymogram gels and a prestained molecular-weight marker BluePlus2 Prestained Standard (Invitrogen, Stockholm, Sweden) as described previously.[43] Briefly, the gelatinase activity of MMP-9 and MMP-2 in the concentrated culture media was determined by preactivating an equal volume of each sample with 1 mM 4-aminophenyl mercuric acetate (APMA) for 24—72 h and then run on 10% zymogram under non-denaturing conditions, all in accordance to the manufacturer's instructions. To determine the caseinolytic activity of MMP-3, samples were preactivated with 5 μg/ml trypsin for 30 min at 37 °C. The activation was terminated by 2 mM phenyl-methyl-sulphon-fluoride and samples were run on 4—16% zymogram (prestained Blue Casein) at 125 V for 2 h at 4 °C. The gels were scanned and gelatinolytic and caseinolytic band areas of activity were quantified by densitometric analysis of an inverted display using a transformation tool program (BIO-RAD's software Quantity One) and expressed as optical density related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Identification of MMP-9, MMP-2 and MMP-3 by Western immunoblotting

The identification of the different MMPs was confirmed by Western blot as described previously.[43] In brief, equal amounts of preactivated samples for MMP-9 and MMP-2 were subjected to 10% Tris-Glycine (Invitrogen, Stockholm, Sweden), or 4—12% for MMP-3, and they were transferred by electro-blotting to a PVDF-P Immobilon membrane (Millipore, Bedford, MA, USA). The following primary antibodies were applied for the various MMPs: MMP-2 mouse anti-human monoclonal IgG/K, MMP-9 rabbit anti-mouse (Gelatinase B) polyclonal full length, and rabbit anti-MMP-3 (Chemicon,Temecula, CA, USA); all were diluted 1:1000 in Tris—HCl, 150 mM NaCl, pH 7.5, 0.05% Tween with 1% BSA.

As polyclonal secondary antibodies, rabbit anti-mouse-HRP for MMP-2, swine anti-rabbit-HRP for MMP-9 and rabbit anti-mouse-HRP for MMP-3 were used at a dilution of 1:1000 (DakoCytomation, Glostrup, Denmark). ECL Western Blotting Detection Reagents (Amersham GE Healthcare) were used for development and band visualization.

Statistical analysis

Data are shown for individual patients either in absolute values or as a percentage of baseline control. For treatment groups, data are presented as arithmetic means ± standard error of the mean (SEM). Data were analyzed by 99% and 95% confidence interval analysis and the results are reported as $p < 0.01$ and $p < 0.05$, respectively. Differences were considered statistically significant at $p < 0.05$. Analysis of each treatment group (TGFβ1 with vehicle or drugs) versus baseline control (0.4% serum with drug vehicle) was performed on data expressed as percentage of baseline control (where baseline control = 100) and checking whether the value of 100 is included within the confidence intervals. The effects of the drugs were analyzed as per cent inhibition of TGFβ1 response and checking whether the zero value is included in the confidence intervals; the per cent inhibition was calculated as:

% inhibition = $100 - 100 \times (D - B)/(T - B)$, where D and T are treatments with TGFβ1 and drugs or TGFβ1 and vehicle, respectively, and B is a baseline control. The correlation analysis was performed by testing the significance of Pearson correlation coefficients (r) with t-test of the regression. All analyses were performed using Astute Software 1.5 (DDU Software, Leeds, UK).

Results

Cellular profile in BAL fluid

To assess the degree of inflammation in the airways of asthmatic patients investigated in this study differential analysis of cells in BAL fluid was performed, counting monocytes/macrophages, lymphocytes, neutrophils eosinophils, basophils and epithelial cells (Table 2). Heterogeneous results were obtained reflecting heterogeneity of the patient population studied. Of the 8 patients investigated, 3 patients (Pt 3, Pt 5, Pt 8) had FEV$_1$% predicted value below 70% and the lowest methacholine PD$_{20}$ dose ($<$0.05 μg) and 5 patients had FEV$_1$% predicted around 100% and methacholine PD$_{20}$ dose between 15 and 826 μg (Table 1). When compared with the 5 patients with mild asthma, the 3 patients with moderate asthma had a somewhat higher percentage of lymphocytes (12—24% vs.1.5—20%) and a slightly lower percentage of monocytes/macrophages (53—70% vs. 60—84%). The percentage of neutrophils and eosinophils was very low in both groups (0—6%); however, unexpectedly, it was higher in the mild asthma group. Interestingly, the highest percentage of eosinophils (2.5—6%) was seen in 3 patients with mild asthma of whom 2 patients (Pt 2 and Pt 4) were the only ones with reversible FEV$_1$ among the 5 patients with mild asthma, which might suggest that these 2 patients were in a more active phase of

Table 2 Cellular profile in bronchoalveolar lavage fluid.

	MO/MC (%)	LYMPH (%)	NEU (%)	EOS (%)	BASO (%)	EPITH (%)
Pt 1	60.0	19.5	6.5	2.5	0.0	9.0
Pt 2	81.0	1.5	4.0	6.0	0.0	4.5
Pt 3	58.0	24.0	0.0	1.0	1.0	5.0
Pt 4	78.0	11.5	0.5	2.5	0.0	0.0
Pt 5	69.5	12.0	2.5	1.0	0.0	13.5
Pt 6	84.0	5.5	0.5	1.5	0.0	6.0
Pt 7	72.5	9.5	0.5	1.0	0.0	4.5
Pt 8	53.0	19.5	1.5	0.0	0.5	19.0

Abbreviations: MO/MC = monocytes/macrophages; LYMPH = lymphocytes; NEU = neutrophils; EOS = eosinophils; BASO = basophils; EPITH = epithelial cells.

the disease. The percentage of epithelial cells in BAL fluid was somewhat higher in the 3 patients with moderate asthma (5—19%) than in those patients with mild asthma (0—9%). An especially high percentage of epithelial cells (13.5% and 19%) was seen in the two patients (Pt 5 and Pt 8, respectively) with the lowest value of FEV_1% predicted (62—63%), which might suggest injured bronchial mucosa with shedding epithelial cells.

Extracellular matrix

Procollagen I

In bronchial fibroblasts from patients with asthma, 2 of the 3 patients with the lowest PD_{20} (<0.05 µg) and the lowest (62—63%) FEV_1% predicted (Pt 5 and Pt 8) showed from 2- to 11-fold higher baseline levels of PICP than the remaining patients (Fig. 1A). TGFβ1 increased PICP levels in 7 (of 8) patients and decreased it in the remaining patient (Pt 8, who in contrast to Pt 5 did not show FEV_1 reversibility). Interestingly, the greatest increase of PICP levels by TGFβ1 (2.5—3.5-fold) was seen in the two patients (Pt 2 and Pt 4) who were the only patients showing FEV_1 reversibility among the 5 patients with normal values of FEV_1% predicted. These two patients had also the highest percentage of eosinophils in BAL (Table 2). These results suggest that PICP production may increase considerably in bronchial fibroblasts from patients with mild asthma during less stable periods of the disease. In contrast, in patients with more severe asthma, PICP levels, although high, may be more stable.

The average increase of PICP by TGFβ1 in fibroblasts from all 8 patients was 1.7-fold ($p < 0.05$) (Fig. 1A). Concomitant incubation of fibroblasts with TGFβ1 and BUD or FORM had no significant effects whereas BUD and FORM in combination decreased considerably variance between PICP levels in fibroblasts from different patients and significantly ($p < 0.01$) decreased PICP levels by an average of 58% (Fig. 1B).

Biglycan

As with PICP levels, baseline levels of biglycan were the highest in bronchial fibroblasts from the two patients with the lowest lung function (Pt 5 and Pt 8) and this was especially striking for Pt 8 with 50-fold higher levels than those for other patients (Fig. 2A). Furthermore, exposure of

fibroblasts from these two patients to TGFβ1 decreased the production of biglycan while the production was increased in fibroblasts from all the other patients. Overall, in fibroblasts from all 8 patients investigated, TGFβ1 increased the production of biglycan 2.2-fold ($p < 0.05$) (Fig. 2A). Concurrent treatment with TGFβ1 and the combination of BUD and FORM decreased the production of biglycan by an average of 36% ($p < 0.05$) whereas neither drug alone had any significant effect (Fig. 2B). Again, the fibroblasts from Pt 5 and Pt 8 deviated from this pattern; their production of biglycan was increased or normalized to baseline levels by all the drug treatments.

Decorin

As with PICP and biglycan, the highest baseline levels of decorin were measured in bronchial fibroblasts from the two patients with the lowest lung function (Pt 5 and Pt 8), displaying from 1.5-fold up to 15-fold higher decorin levels than those for fibroblasts from other patients investigated (Fig. 3A). TGFβ1 exposure decreased the production of decorin by an average of 15% below baseline in bronchial fibroblasts from all 8 patients investigated, however, this decrease was not statistically significant. In fibroblasts from the majority of patients, 5 of 8 (including the 3 patients with more moderate asthma), the production of decorin was decreased by TGFβ1, while it was slightly increased in fibroblasts from 2 patients (Pt 4 and Pt 7) and not affected in one patient (Pt 6) (Fig. 3A). The greatest decrease of decorin production by TGFβ1 was seen in Pt 5 (who had the lowest value of FEV_1% predicted and the highest FEV_1 reversibility among the 8 patients investigated). The decrease of decorin by TGFβ1 in fibroblasts of 5 patients resulted in a level that was on average 40% below baseline ($p < 0.01$). Concurrent treatment with TGFβ1 and BUD alone or in combination with FORM completely counteracted this decrease whereas FORM alone significantly ($p < 0.01$) attenuated it to the levels 15% below baseline (Fig. 3B).

Metalloproteolytic balance

MMP-9 and MMP-2

Gelatin zymography followed by Western blot showed enzymatic activity of MMP-9 in fibroblast medium as an 82-kDa band (Fig. 4A), while the 92-kDa band of the pro-form was often not visible. Activity of MMP-2 was seen as a 62-
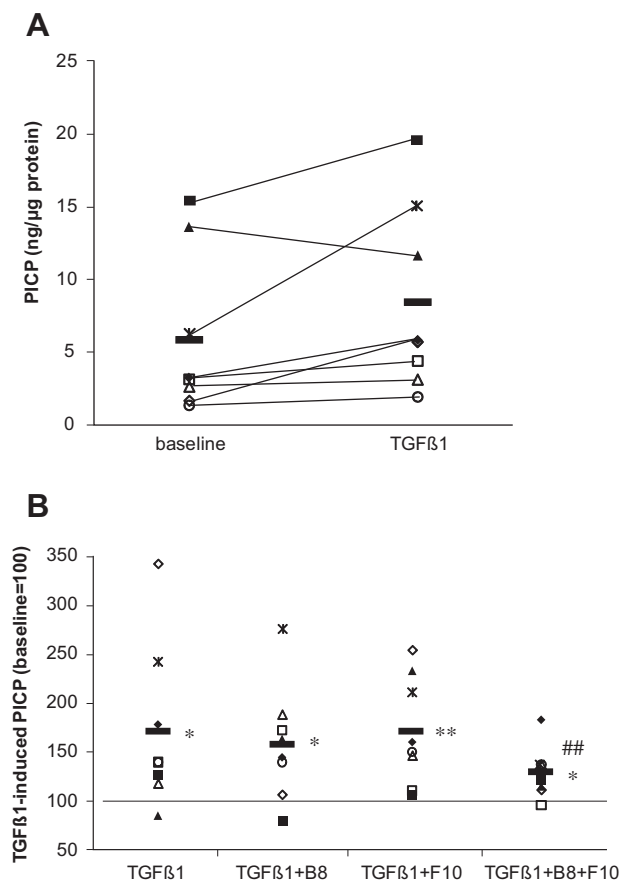
Figure 1 Concentration of procollagen I carboxyterminal propeptide (PICP) in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor β1 (TGFβ1; 10 ng/ml) for 24 h. PICP was measured by ELISA and normalized to the total protein content in the corresponding cell layer. A: PICP concentration (ng/μg protein) at baseline conditions (incubation of fibroblasts with 0.4% serum) and after stimulation of fibroblasts with TGFβ1. B: The effects of treatment with TGFβ1 alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of PICP concentration at baseline (where baseline = 100%). Symbols represent individual patients (Pt): Pt 1 = open triangle, Pt 2 = star, Pt 3 = closed diamond, Pt 4 = open diamond, Pt 5 = closed square, Pt 6 = open circle, Pt 7 = open square, Pt 8 = closed triangle. Means are shown as horizontal bars. ∗$p < 0.05$, ∗∗$p < 0.01$ versus baseline; ##$p < 0.01$ versus TGFβ1.

kDa band and a pro-form 72-kDa band (Fig. 4A). A slight increase of MMP-9 activity (18%) and MMP-2 activity (14%) by TGFβ1 was not statistically significant and the concurrent treatment with BUD and/or FORM had no significant effects on MMP-9 and MMP-2 activity (Fig. 4B and C). The results on both MMP-9 and MMP-2 showed very high heterogeneity. Interestingly, for MMP-9 this was mainly because of the high levels in fibroblasts from the patient with the lowest FEV₁% predicted value and the lowest PD₂₀ dose measured (Pt 5), while for MMP-2 this was to the result of the high levels from the patient (Pt 7) who had normal
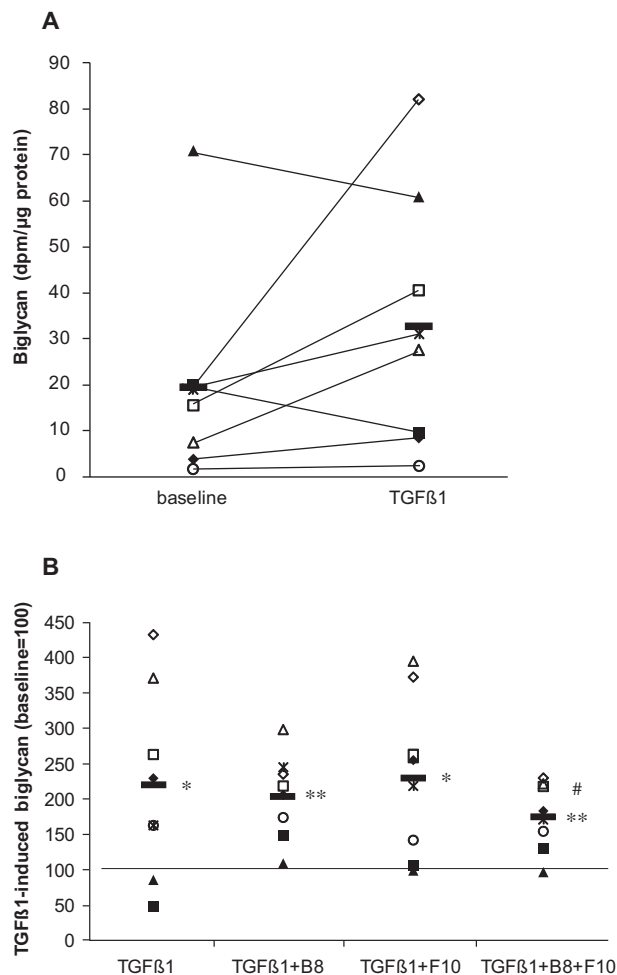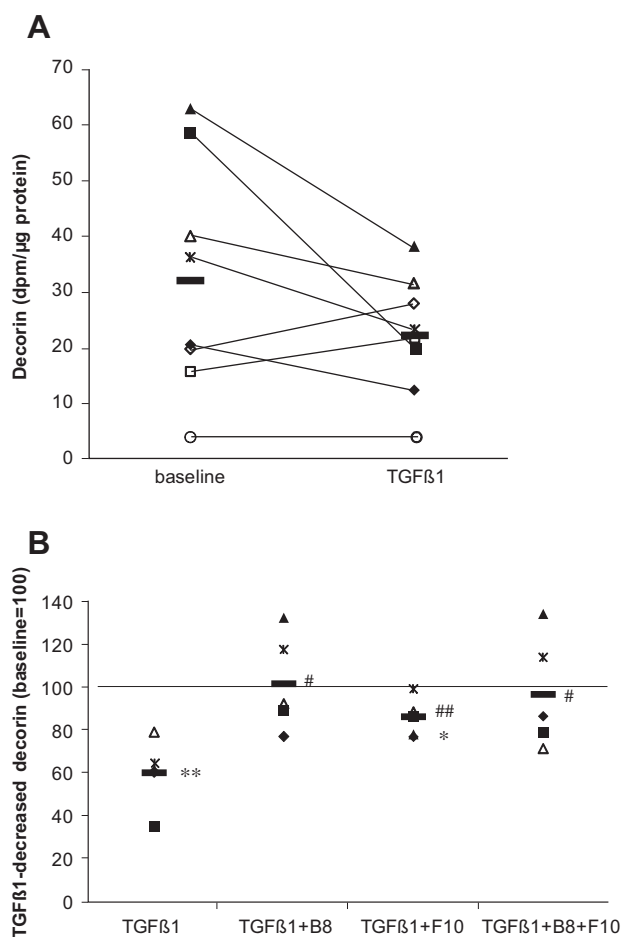

Figure 2 Concentration of the small leucine-rich proteoglycan, biglycan, in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor β1 (TGFβ1; 10 ng/ml) for 24 h. Biglycan was measured by means of [^{35}S]-sulfate incorporation into the proteoglycan glycosaminoglycan chains and using anion-exchange chromatography. Biglycan was identified on gradient SDS-PAGE and the radioactive intensity (expressed as disintegration per minute; dpm) was normalized to the total protein content in the corresponding cell layer. A: Biglycan concentration (dpm/μg protein) at baseline conditions (incubation of fibroblasts with 0.4% serum) and after stimulation of fibroblasts with TGFβ1. B: The effects of treatment with TGFβ1 alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of biglycan concentration at baseline (where baseline = 100%). Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. ∗$p < 0.05$, ∗∗$p < 0.01$ versus baseline; #$p < 0.05$ versus TGFβ1.

FEV₁% predicted value and the highest PD₂₀ dose among all the patients investigated.

MMP-3

Casein zymography followed by Western blot showed enzymatic activity of MMP-3 in fibroblast medium as an active band at 45 kDa, a pro-form double band at 57/59 kDa

A

B

Figure 3 Concentration of the small leucine-rich proteoglycan, decorin, in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor $\beta1$ (TGF$\beta1$; 10 ng/ml) for 24 h. Decorin was measured by means of [^{35}S]-sulfate incorporation into the proteoglycan glycosaminoglycan chains and using anion-exchange chromatography. Decorin was identified on gradient SDS-PAGE and the radioactive intensity (expressed as disintegration per minute; dpm) was normalized to the total protein content in the corresponding cell layer. A: Decorin concentration (dpm/μg protein) at baseline conditions (incubation of fibroblasts with 0.4% serum) and after stimulation of fibroblasts with TGF$\beta1$. B: The effects of treatment with TGF$\beta1$ alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of decorin concentration at baseline (where baseline $=$ 100%) in fibroblasts from 5 (of 8) patients showing decreased decorin production after stimulation with TGF$\beta1$. Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. $*p < 0.05$, $**p < 0.01$ versus baseline; $\#p < 0.05$, $\#\#p < 0.01$ versus TGF$\beta1$.

and a complexed form around 100 kDa (Fig. 5A). After stimulation with TGF$\beta1$, MMP-3 activity slightly decreased (14% below baseline) but this decrease was not statistically significant and concurrent treatment with BUD and/or FORM had no significant effects on MMP-3 activity (Fig. 5B). The high heterogeneity of MMP-3 activity seen after

exposure to TGF$\beta1$ increased further after concurrent drug treatments.

MMP-1

Collagen zymography for analysis of MMP-1 activity was not commercially available and therefore we have measured MMP-1 protein by ELISA. TGF$\beta1$ had no significant effect (an increase of 2.5%) on MMP-1 protein and the concurrent treatment with BUD and/or FORM had no significant effects on MMP-1 levels (Fig. 6). A high heterogeneity in these data was largely to the result of the heterogeneous contribution of fibroblasts from the patients with the lowest FEV$_1$% predicted value; the high levels from Pt 8 and low levels from Pt 5. However, the greatest increase of TIMP-1 protein (2-fold) induced by TGF$\beta1$ was seen in fibroblasts from the patient (Pt 4) who had the greatest FEV$_1$ reversibility (16%) among the 5 patients with normal FEV$_1$% predicted values. In this patient, all the drug treatments concurrent with TGF$\beta1$ lowered MMP-1 protein levels.

TIMP-1

TIMP-1 levels were measured by ELISA. TGF$\beta1$ had no significant effect (an increase of 6.8%) on TIMP-1 protein and there were no significant effects of concurrent treatment with BUD and/or FORM on TIMP-1 levels (Fig. 7). As for MMP-1, the greatest increase of TIMP-1 protein (1.6-fold) induced by TGF$\beta1$ was seen in fibroblasts from the patient (Pt 4) who had the greatest FEV$_1$ reversibility (16%) among the 5 patients with normal FEV$_1$% predicted values. However, in contrast to MMP-1, the drug treatments concurrent with TGF$\beta1$ did not decrease TIMP-1 protein level in this patient. Interestingly, in the fibroblasts from the second patient (Pt 2) with FEV$_1$% reversibility (13%) among the 5 patients with normal FEV$_1$% predicted values, TGF$\beta1$ exposure resulted in a decrease of TIMP-1 levels below baseline but this was counteracted by both FORM alone and the combination of FORM and BUD.

Discussion

We have shown in the present study that following ex vivo exposure to TGF$\beta1$, bronchial fibroblasts derived from biopsy samples from patients with asthma increased their production of PICP, a marker of ongoing collagen I synthesis, and that the combination treatment of BUD and FORM counteracted this increase while neither drug alone had any significant effect. Similarly, the TGF$\beta1$-induced increase of a small proteoglycan, biglycan, was reduced by the BUD and FORM combination but not by either drug alone. Additionally, in fibroblasts from 5 of 8 patients, TGF $\beta1$ decreased production of another small proteoglycan, decorin, and this decrease was completely counteracted by BUD alone and the BUD and FORM combination. Both decorin and biglycan may affect collagen deposition in the ECM since they are involved in fibrillation of collagen fibers and organization of collagen network. In contrast to procollagen and small proteglycans, TGF$\beta1$, BUD and FORM did not affect the production or activity of the various MMPs investigated and their main tissue inhibitor TIMP-1. These results suggest that BUD and FORM combination therapy has a potential to counteract enhanced collagen production

A

B

C
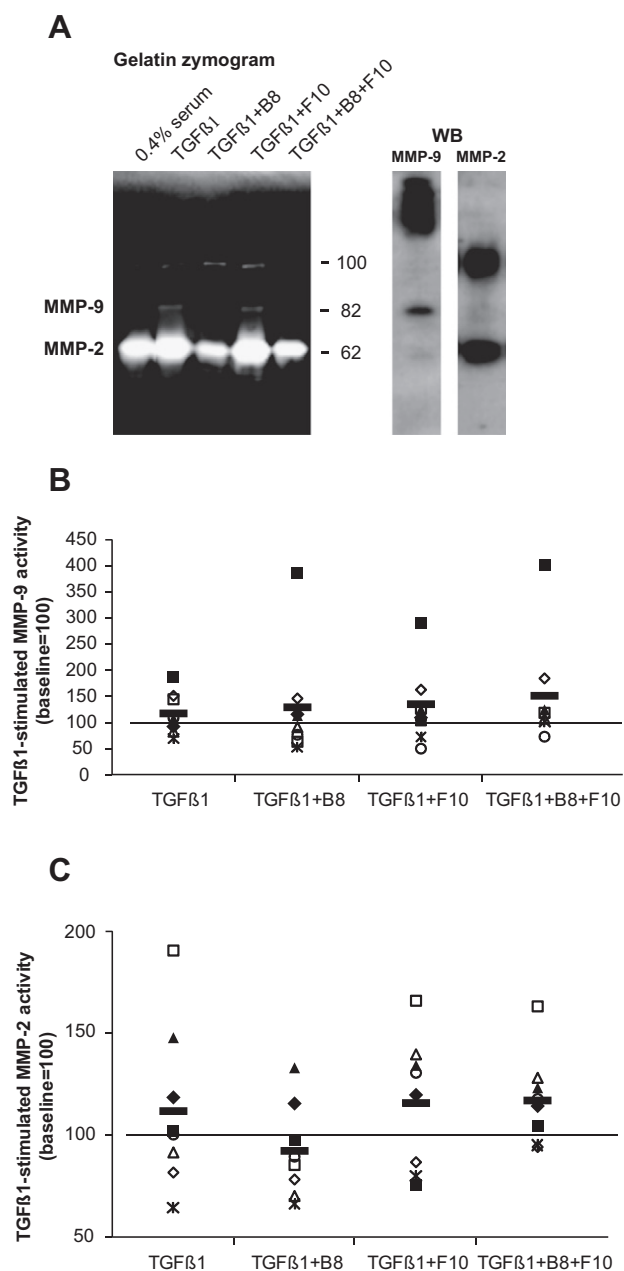
Figure 4 Activity of MMP-9 and MMP-2 in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor $\beta 1$ (TGF$\beta 1$; 10 ng/ml) for 24 h. A: Representative gelatin zymogram and Western blot (WB) revealing both the presence and enzymatic activity of MMP-9 and MMP-2 in culture media at baseline conditions (incubation of fibroblast with 0.4% serum) and after stimulation of fibroblasts with TGF$\beta 1$ alone or with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10). Enzymatic activity for MMP-9 was seen at 82 kDa, slightly visible pro-form enzyme at 92 kDa and high molecular-weight complexes above 100 kDa. For MMP-2, the active enzyme was seen at 62 kDa and the pro-form enzyme at 72 kDa. The activity of the MMP-9 and MMP-2 at 82 kDa and 62 kDa, respectively, was quantified by densitometric analysis of inverted displays and normalized to the total protein content in the corresponding cell layer. B and C: The

and deposition in ECM by bronchial fibroblasts in asthma without affecting metalloproteolytic balance.

The group of 8 patients with mild-to-moderate asthma investigated in the present study was very heterogeneous. They were between 25 and 55 years old, 5 patients had FEV$_1$% predicted value around 100% while this was below 70% for the remaining 3 patients who also had the lowest value of methacholine PD$_{20}$ dose, indicating that these patients had more severe asthma. Two of the 5 patients with normal lung function, and only one patient of the 3 patients with low FEV$_1$% predicted, showed FEV$_1$ reversibility. This clinical heterogeneity was reflected by the cellular profile of BAL fluid. The highest percentage of eosinophils in BAL fluid was seen in 2 patients with normal lung function but who showed FEV$_1$ reversibility, which may suggest that these patients were in a more active phase of the disease. The highest percentage of epithelial cells was seen in the 2 patients with the lowest value of FEV$_1$% predicted, which may suggest ongoing remodeling of injured bronchial epithelium. Interestingly, the same 4 patients with the highest percentage of eosinophils, or epithelial cells in BAL fluid, had the greatest impact on the heterogeneity of the production of PICP, biglycan and decorin and on activities or levels of MMPs and TIMP-1 in their bronchial fibroblasts after stimulation *ex vivo* with TGF$\beta 1$.

In the same 8 patients as investigated in this study, we have recently reported that patients' FEV$_1$% predicted and methacholine log PD$_{20}$ values were negatively correlated with PICP synthesized by patients' bronchial fibroblasts *ex vivo* at baseline conditions while the respective negative associations for proteoglycan production were not statistically significant.[32] In the present study we have investigated these relationships for PICP, biglycan and decorin after stimulation of fibroblasts with TGF$\beta 1$; however, no significant correlations were found (data not shown). TGF$\beta 1$ stimulation was used to mimic unstable conditions and more severe asthma since the collection of biopsies during exacerbations or in severe disease is not feasible. Indeed, in asthma of all severities (mild and moderate-to-severe), TGFβ protein expression in bronchial biopsy tissue is increased and positively correlates with disease severity.[41] The significance of TGFβ in airway remodeling is, in a large part, to the result of its ability to induce a differentiation of fibroblasts into myofibroblasts, which produce a variety of ECM components, including collagen and proteoglycans. Recently, it was shown that an increase of the numbers of submucosal tissue myofibroblasts after allergen inhalation by asthmatic patients was counteracted by inhaled BUD and FORM combination therapy, but not by inhaled BUD alone.[31]

TGFβ is associated with increased expression of collagen type I and III in bronchial tissue.[41] Increased ongoing collagen type I production is reflected by increased PICP
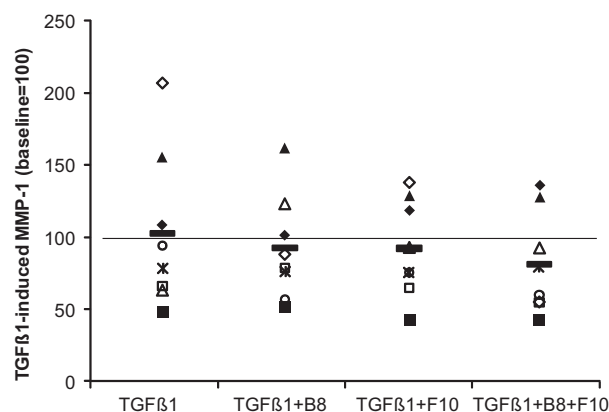
effects of treatment with TGF$\beta 1$ alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) on MMP-9 (B) and MMP-2 activity (C); the effects are expressed as a percentage of the activity at baseline (where baseline = 100%). Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. There were no statistical significant differences between treatment groups.

A

Casein zymogram

B
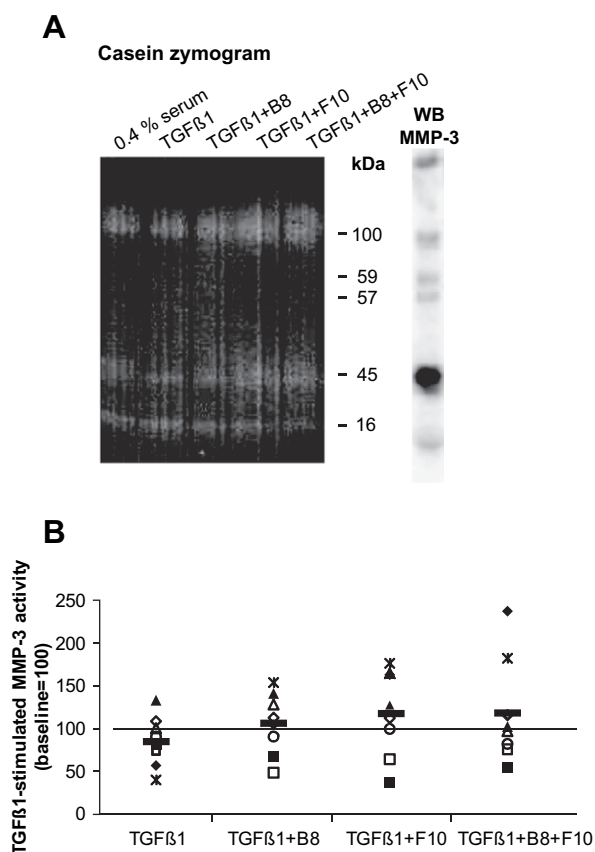
Figure 5 Activity of MMP-3 in culture media of bronchial fibroblasts from asthmatic patients and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor β1 (TGFβ1; 10 ng/ml) for 24 h. A: Representative casein zymogram and Western blot (WB) revealing both the presence and enzymatic activity of MMP-3 at baseline conditions (incubation of fibroblast with 0.4% serum) and after stimulation of fibroblasts with TGFβ1. The active enzyme was seen at 45 kDa, slightly visible pro-form enzyme appeared as a double band at 57/59 kDa, and a complex form around 100 kDa. MMP-3 activity at 45 kDa was quantified by densitometric analysis of the active band inverted display and normalized to the total protein content in the corresponding cell layer. B: The effects of treatment with TGFβ1 alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of the activity at baseline (where baseline = 100%). Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. There were no statistical significant differences between treatment groups.

release, and indeed, PICP sputum levels are enhanced in patients with asthma,[8,9] are further increased during exacerbations[8] and inversely correlate with FEV$_1$% predicted normal.[8] In the study by Kai et al.,[9] PICP levels in sputum from patients with asthma were decreased approximately 30% by inhaled GC, fluticasone propionate (800 μg for 1 month). In the current study, TGFβ1 increased PICP production by bronchial fibroblasts approximately 2-fold and this increase was reduced by almost 60% by BUD and FORM combination treatment while the decrease by

Figure 6 Concentration of MMP-1 in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor β1 (TGFβ1; 10 ng/ml) for 24 h. MMP-1 was measured by ELISA and normalized to the total protein content in the corresponding cell layer. The effects of treatment with TGFβ1 alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of MMP-1 concentration at baseline (where baseline = 100%). Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. There were no statistical significant differences between treatment groups.

BUD alone was not statistically significant. This suggests that inhaled GC and LABA combination therapy may be more efficient than inhaled GC alone in counteracting increased procollagen production and perhaps also collagen deposition in the airways of asthmatic patients.

Collagen deposition requires formation of fibrillar collagen from soluble procollagen molecules and this process depends, in part, on association with small leucine-rich proteoglycans — mainly decorin[14–16] but also the structurally related biglycan.[17–19] Both decorin and biglycan are associated with the deposition of collagen type I[17,42,43] and this may suggest they have a pro-fibrotic function. On the other hand, decorin has been shown to neutralize the biological activity of TGFβ[44,45] and thus act against the development of fibrosis. Indeed, decorin has been shown to prevent scar formation in vivo[46] and to reduce the development of lung fibrosis in a murine model.[45] In the present study, exposure of bronchial fibroblasts to TGFβ1 doubled their production of biglycan while decorin production was not significantly affected when all patients investigated were taken into account. However, in fibroblasts from 5 of 8 patients, decorin production was significantly decreased by 40% below baseline level. The differential regulation of biglycan versus decorin by TGFβ1 was previously shown in human skin fibroblasts,[47] a human lung fibroblast cell line (HFL-1)[49] and in a pulmonary fibrosis model in rats,[49] where TGFβ1 increased biglycan production but either had no effect on decorin production[48] or decreased it.[47,49] This suggests that these small proteoglycans can be independently regulated by TGFβ1. Importantly, increased density of biglycan but decreased density of decorin were also found in the bronchial subepithelial
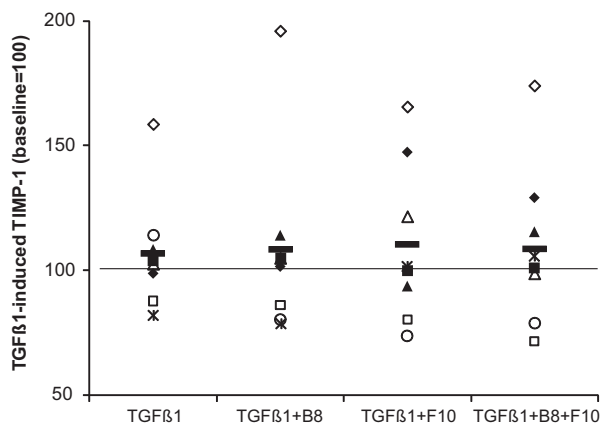
Figure 7 Concentration of TIMP-1 in culture media of bronchial fibroblasts from asthmatic patients, and the effects of budesonide and formoterol in fibroblasts stimulated with transforming growth factor $\beta 1$ (TGF$\beta 1$; 10 ng/ml) for 24 h. TIMP-1 was measured by ELISA and normalized to the total protein content in the corresponding cell layer. The effects of treatment with TGF$\beta 1$ alone or with concurrent budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) expressed as a percentage of TIMP-1 concentration at baseline (where baseline = 100%). Symbols represent individual patients and are shown as in Fig. 1. Means are shown as horizontal bars. There were no statistical significant differences between treatment groups.

layer of patients with mild asthma compared with the normal control group.[50]

In the current study, TGF$\beta 1$-induced decrease of decorin production by bronchial fibroblasts from asthmatic patients was completely reversed by BUD alone and also the combination of BUD and FORM. In contrast, the increased production of PICP and biglycan by TGF$\beta 1$ was significantly inhibited only by the combination of BUD and FORM but not by BUD alone. These results suggest that the combination therapy of BUD and FORM may be more effective at counteracting subepithelial fibrosis in the airways of asthma patients than BUD alone. Indeed, in mild asthma, 4-week treatment with inhaled budesonide did not reduce basal membrane thickening[51] and high doses of inhaled fluticasone propionate for 8 weeks did not affect collagen deposition under the basement membrane.[52] Moreover, a 2-week course of oral prednisolone did not decrease collagen type I in the submucosa of patients with moderate-to-severe asthma.[41] In contrast, therapy with moderate doses of inhaled BUD and FORM for 3 months in patients with mild-to-moderate asthma significantly diminished the thickness of the bronchial reticular basement membrane and normalized the bronchial wall thickness and diameter.[30]

We have previously shown in the human lung fibroblast cell line (HFL-1) that TGF$\beta 1$ increased activity of MMP-2, -3 -9 and TIMP-1 and that BUD and FORM in combination increased the MMP-2/TIMP-1 and MMP-3/TIMP-1 ratios via a decrease of TIMP-1.[40] These results suggest that the combination of BUD and FORM treatment may counteract excessive ECM deposition by increasing fibroblast metalloproteolytic activity. However, in the present study

performed in biopsy-derived bronchial fibroblasts from asthmatic patients, *ex vivo* exposure to TGF$\beta 1$ in the absence or presence of BUD and/or FORM had no effects on MMPs or TIMP-1. Thus, these results suggest direct inhibitory effects of BUD and FORM combination therapy on the TGF$\beta 1$-modified production of procollagen type I, biglycan and decorin.

It was recently shown that a GC, dexamethasone, decreased production of procollagen type I in bronchial fibroblasts isolated from normal donors but not from asthmatic patients and this appeared to be related to increased expression of activator protein-1 (AP-1) transcription factor in bronchial fibroblasts from asthmatic patients.[53] Thus, excessive amounts of AP-1 may reduce the number of activated GR in nuclei and by this mechanism impair GC effects on gene transcription. β_2-Adrenergic agonists are known to increase translocation of GRs from the cytoplasm into the nucleus in various cells including primary human lung fibroblasts.[54] This phenomenon may explain the suppressive effects of BUD and FORM combination treatment on TGF$\beta 1$-induced PICP production in the present study but lack of the effect of BUD alone. We have also previously shown that the inhibitory effects of BUD and FORM combination treatment on the total proteglycan production (induced by serum in HFL-1 cells) were dependent on the presence of functional GRs as well as β-adrenoceptors.[39]

In conclusion, we have shown here that in biopsy-derived bronchial fibroblasts from stable, mild-to-moderate patients with asthma, TGF$\beta 1$-increased synthesis of procollagen type I was directly counteracted by the combination of BUD and FORM but not by either drug alone. The combination of BUD and FORM also normalized production of small proteoglycans which may affect collagen fibrillization, organization and deposition. Importantly, these effects were achieved without affecting metalloproteolytic balance. These results suggest that BUD and FORM combination therapy has a potential to counteract enhanced collagen production and deposition by bronchial fibroblasts in asthma.

Conflict of interest statement

Acknowledgments

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