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Budesonide/formoterol effects on metalloproteolytic balance in TGFβ-activated human lung fibroblasts☆

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Summary
In the airways of asthmatic patients, activated fibroblasts account for an excessive matrix production including proteoglycans (PGs). Transforming growth factor-β (TGFβ), metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play key roles in matrix turnover. It is unclear whether asthma therapy with combination of inhaled glucocorticoids and long-acting β_2-agonists affects metalloproteolytic equilibrium and by that counteracts airway fibrosis.

The effects of the glucocorticoid, budesonide, and the long-acting β_2-agonist, formoterol, on the PG production and the activity of PGs' main regulators: MMP-3, MMP-9, MMP-2 and TIMP-1 were investigated in human lung fibroblasts (HFL-1) treated for 24 h with TGFβ1 (10 ng/ml) without/with budesonide (10^{-9} to 10^{-6} M) and/or formoterol (10^{-11} to 10^{-6} M).

TGFβ1 significantly increased production of PGs and TIMP-1, and the activity of MMP-3, MMP-9 and MMP-2. Concurrent budesonide/formoterol combination counteracted the enhanced: PG and TIMP-1 production, MMP-9 activity and MMP-9/TIMP-1 ratio, whereas MMP-2 and MMP-3 were not affected and so their ratios to TIMP-1 were significantly increased. Budesonide or formoterol alone achieved equal effects as budesonide/formoterol on MMP-9 and MMP-9/TIMP-1 ratio but had no effects on TIMP-1, MMP-2 or MMP-3. In the formoterol absence, higher budesonide concentrations were required to reduce the PG production, whereas formoterol alone had no effects.

These results suggest that the budesonide/formoterol combination enhanced metalloproteolytic activity of human lung fibroblasts via a synergistic decrease of TIMP-1, and that this

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mechanism may be involved in the synergistic inhibition of the TGFβ1-induced PG production. This implies that budesonide/formoterol combination therapy can counteract excessive matrix production and thus pathological airway fibrotic remodeling in asthma.

Introduction

In asthma, extensive structural reorganization in airway and lung tissue occurs, and this is known as airway remodeling. A prominent characteristic of this process is the formation of subepithelial fibrosis due to an enhanced deposition of extracellular matrix (ECM) molecules, particularly collagen, fibronectin and proteoglycans (PGs). Subepithelial fibrosis, described as a thickening of the airway reticular basement membrane, is found in asthma of all severities and correlates with decline in lung function, attack score and airway hyperresponsiveness (reviewed in Ref. [1]). A key factor in the ECM remodeling in asthma is transforming growth factor-β (TGFβ), known to induce the differentiation of fibroblasts into myofibroblasts and increasing their ECM production. TGFβ reactivity and the number of submucosal fibroblasts were both shown to correlate with the increased thickness of the reticular basement membrane in airways of asthmatic patients (reviewed in Ref. [2]).

Subepithelial fibrosis in asthma may occur as a result of an imbalance between metalloproteinases (MMPs), which degrade ECM, and tissue inhibitors of MMPs (TIMPs), particularly TIMP-1 which is the most widely distributed and acts on all active MMPs. Increased levels and/or activity of various MMPs are found in the airways of asthmatic patients, the most prominent being the gelatinases MMP-9[3,4] and also MMP-2[5,6] and the stromelysin MMP-3.[7] These are further enhanced after allergen challenge, during asthma exacerbations, and in severe asthma. Importantly, TIMP levels are also elevated in the airways of patients with asthma[4,5,8,9] and high levels of TIMP-1 are associated with increased airway fibrosis.[8–11] This suggests that pathological airway remodeling in asthma, resulting in airway fibrosis, may be a consequence of over-repair mechanisms.

The reversibility of subepithelial fibrosis in the airways of asthmatic patients and its sensitivity to drug therapy is an issue of ongoing debate.[1] In particular, it is unclear whether the currently most successful asthma therapy — combination therapy with inhaled glucocorticoids (GCs) and long-acting β_2-adrenoceptor agonists — may affect development of subepithelial fibrosis and whether this effect may be greater than with inhaled GC monotherapy. Whereas the beneficial effects of combination therapy on airway myofibroblast[12] and bronchial wall thickness[13] in asthma patients have been recently reported, according to our knowledge data are scarce on the effects of the combination therapy on metalloproteolytic balance in the airways of asthmatics and its effects on ECM deposition.

Here we investigated the effects of the GC, budesonide (BUD), and the long-acting β_2-agonist, formoterol (FORM), alone and in combination, on the proteolytic activity of lung fibroblasts which were stimulated with TGFβ. We studied the activity of MMP-9, MMP-2 and MMP-3 and the production of their main inhibitor TIMP-1. In parallel, we investigated the effects of BUD and FORM on the TGFβ-induced PG production which is a target of these MMPs; predominantly stromelysins (such as MMP-3) but also gelatinases (such as MMP-9 and MMP-2).[14] The contribution of PGs to subepithelial fibrosis in asthma has not received much attention although PG levels are increased in the airways of asthmatic patients[15,16] and positively correlate with patient airway hyperresponsiveness.[16,17]

Material and methods

Study design

Confluent human fetal lung fibroblast cells, HFL-1 (obtained in passage 12 from American Type Culture Collection, Rockville, MD, USA) and used in passages 14—22, were grown in 6- or 24- or 96-well plates in Earle's Minimal Essential Medium (Gibco Brl, Paisley, UK) with 10% bovine donor calf serum, 1% L-glutamine and 1% penicillin—streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Prior to treatment with TGFβ1 and drugs (or vehicle), fibroblasts were starved for 2 h in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% donor calf serum and 1% L-glutamine. Subsequently, cells were incubated for 24 h with TGFβ1 (R&D Systems, Minneapolis, MN, USA) at 10 ng/ml in 0.4% serum concurrently with budesonide (BUD) and/or formoterol (FORM) (AstraZeneca, Lund, Sweden) across a concentration range of 10^{-11} to 10^{-6} M, or with drug vehicle (0.1% ethanol). For the PG analysis, fibroblasts were incubated in $MgSO_4$-poor DMEM (Gibco BRL, Paisley, UK) and for the last 22 h in the presence of 50 μCi/ml [^{35}S]-sulfate (PerkinElmer Life Science, Boston, MA, USA). Fibroblasts incubated in 0.4% serum with drug vehicle were used as a baseline control.

Immunocytochemical and morphological characterization of fibroblasts

Fibroblasts were grown to confluence in 4-well chambers, fixed in 4% paraformaldehyde and permeabilized with 1% Triton in PBS, washed and incubated with monoclonal mouse anti-human antibodies for α-smooth muscle actin (α-SMA). Negative controls were incubated without primary antibody. Secondary antibody used was goat anti-mouse Alexa Flour 594 (Molecular Probes, Eugene, OR) and nuclei were stained with Hoechst (H33342 Sigma Chemical Co., St. Louis, MO, USA). Slides were mounted with Cytomation Fluorescent Mounting Medium (Dako, Glostrup, Denmark) and analyzed with fluorescence microscopy (Nikon Eclipse TE 2000-U with Nikon digital camera DXM 1200, Japan). For stress fibre analysis, Alexa Fluor™488 phalloidin probe diluted in blocking buffer was used and fluorescence microscope was used to examine the cells.

Proteoglycan analysis

PGs produced and released into the cell culture medium were isolated by anion-exchange chromatography and the total PG production was measured as [^{35}S]-sulfate incorporation into the glycosaminoglycan chains as previously described.[18] The amount of radioactivity for the purified total PG was quantified and related to the total protein amount of the respective cell layer.

Protein determination

The cell layer was extracted with 50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 10% glycerol and 1% NP-40, pH 7.4 with pepstatin A, aprotenin and leupeptin (1 μg/ml). Total protein amount for the cell layers was determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

αSMA mRNA analysis

Total RNA was extracted from the cell layers using RNeasy kit (Qiagen GmbH, Hilden, Germany) and first strand cDNA was synthesized using first strand cDNA synthesis kit for Reverse Transcriptase-Polymerase Chain Reaction (Roche Applied Science, Indianapolis, USA) followed by real time PCR measurements using SYBR Green I PCR Master Mix (Roche Applied Science) as described previously.[18] As a negative control, mRNA samples were used that had not undergone reverse transcription. A melting curve was performed to determine the melting temperature of the amplicons, and consequently, the specificity of the PCRs. Results, related to the internal control (18S), were analyzed using the Lightcycler (Bromma, Sweden) software. Real time-PCRs were performed in duplicates or triplicates. Primers used were generated using the oligonucleotide design program Primer3 (MIT) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and ordered from A/S DNA Technology, Denmark; for α-SMA: forward 5′-gaa gga ata gcc acg ctc ag-3′ and reverse 5′-ttc aat gtc cca gcc atg ta-3′; for 18S: forward 5′-cga acg tct gcc cta tca ac-3′ and reverse 5′-tgc ctt cct tgg atg tgg ta-3′.

α-SMA and TIMP-1 by ELISA

α-SMA protein expression was measured by ELISA. 96-Well Nunc-Immuno Plate with Maxisorb surface was coated with polyclonal rabbit anti-α-SMA (Abcam, UK) and blocked with 3% BSA. Cell lysate containing 10 μg of total protein was added to each well, and bound α-SMA was detected using a monoclonal mouse anti-human α-SMA (DakoCytomation, Denmark) and an alkaline phosphatase (AP)-conjugated secondary antibody (mouse immunoglobulin AP, DakoCytomation, Denmark). 2 mg/ml *p*-Nitrophenyl Phosphate (pNPP) dissolved in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8, was added for development and absorbance was measured at 405 nm. The concentration of TIMP-1 in the conditioned media was analyzed by ELISA according to manufacturer instructions (Amersham, GE Healthcare, Buckinghamshire, UK) and normalized to the total protein amount in the corresponding cell layers.

Cell proliferation assay

Fibroblast proliferation was assessed using the method of crystal violet dye as described previously.[19] After serum starvation overnight, cells were incubated with TGFβ1 and the drugs or vehicle for 24 h and 48 h. As a positive control 10% serum was used and 0.4% serum as a negative control. Proliferation rate was determined spectrophotometrically at 595 nm.

Gelatin and casein zymography

Conditioned media from treated fibroblasts were concentrated (Centric YM-10 Millipore, Watford, UK) and analyzed on Novex polyacrylamide zymogram gels (Invitrogen, Stockholm, Sweden). For gelatinase activity, equal volume of all samples was activated with 1 mM 4-aminophenyl mercuric acetate (APMA) for 24–72 h and diluted in Tris–Glycine SDS Sample buffer (Invitrogen, Stockholm, Sweden). The samples were run on 10% zymogram under non-denaturing conditions according to the manufacturer instructions (Invitrogen, Stockholm, Sweden). For caseinolytic activity, samples were preactivated with 5 μg/ml trypsin for 30 min at 37 °C and activation was terminated by 2 mM phenyl-methyl-sulphonylfluoride. The molecular weight corresponding to the gelatinolytic and caseinolytic areas was estimated using the prestained molecular weight marker BluePlus2 Prestained Standard (Invitrogen, Stockholm, Sweden). Bands were analyzed and quantified using BIORAD's software Quantity One. All densitometry analyses were related to the total amount protein in each sample.

For identification of the MMPs, Western blot was performed where preactivated samples were subjected to 10% Tris–Glycine gels for MMP-2 and MMP-9, or 4–12% for MMP-3 (Invitrogen, Stockholm, Sweden). Separated samples were transferred to PVDF-membranes using electro-blotting. Primary antibodies used: 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 diluted 1:1000 in Tris–HCl, 150 mM NaCl, pH 7.5, 0.05% Tween with 1% BSA. Polyclonal secondary antibodies used: rabbit anti-mouse-HRP for MMP-2; swine anti-rabbit-HRP for MMP-9, and rabbit anti-mouse-HRP for MMP-3, all diluted 1:1000 (DakoCytomation, Glostrup, Denmark). Bands were visualized after development with ECL Western Blotting Detection Reagents (Amersham GE Healthcare).

Statistical analysis

Data are expressed as a percentage of baseline control where 100% corresponds to baseline conditions (0.4% serum), and displayed as mean ± SEM. Effects of TGFβ1 were analyzed versus baseline control. Effects of drugs versus TGFβ1 were analyzed as per cent inhibition: % inhibition $= 100 - 100 \times (D - B)/(T - B)$, where T and D represent treatment with TGFβ1 with vehicle or with drugs, respectively, and B represents baseline control. Statistical

analysis was performed using Astute software 1.5 (DDU Software, Leeds, UK) by analysis of 99%, 95% and 90% confidence intervals obtained from one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results

Fibroblast activation

Proteoglycan production

To activate fibroblasts and to enhance their ECM production, cells were incubated with TGFβ1 at 10 ng/ml for 24 h. Control fibroblasts (exposed to 0.4% serum) appeared elongated in a spindle-like shape with long cytoplasmic projections, whereas fibroblasts treated with TGFβ1 appeared in a rounded form with a more compact cytoplasm and with more well-defined stress fibres.

The total PG production increased with increasing TGFβ1 concentrations and a 2.5-fold increase was obtained at 10 ng/ml ($p < 0.01$). This increase was reduced in a concentration-dependent manner by BUD 10^{-9} to 10^{-6} M (Fig. 1) with a significant 35% reduction achieved at 10^{-6} M ($p < 0.05$). FORM 10^{-11} to 10^{-6} M had no inhibitory effects, but when applied together with BUD in a 100:1 BUD:FORM ratio (which approximately reflects the clinical dose ratio), it enhanced the effect of BUD. Accordingly, a significant 39% reduction ($p < 0.05$) was achieved by BUD 10^{-8} M with FORM 10^{-10} M and a 54% reduction ($p < 0.01$) by BUD 10^{-6} M with FORM 10^{-8} M.

For subsequent experiments, we chose the lowest drug concentrations that when used in combination exerted significant inhibition of the total PG production, i.e. BUD at 10^{-8} M and FORM at 10^{-10} M. These concentrations are considered clinically relevant. We have found that at these concentrations, the BUD and FORM combination also reduced the total baseline PG production (cells incubated with 0.4% serum; data not shown) and that this accounted for approximately one quarter of the reduction seen in the presence of TGFβ1.

Differentiation and proliferation

The effects of BUD and FORM on TGFβ1-induced fibroblast differentiation and proliferation were analyzed to assess whether they contributed to the effects on PG production. For the assessment of fibroblast differentiation towards active myofibroblasts, the myofibroblast marker α-SMA was analyzed. The fraction of cells stained for α-SMA increased with increasing TGFβ1 concentration and exposure time. With TGFβ1 at 10 ng/ml and 24 h incubation time, the majority of cells (72%) showed a strong α-SMA staining compared with 23% of cells exposed to 0.4% serum ($p < 0.01$). Measurements of α-SMA mRNA and protein levels (ELISA) confirmed increased synthesis of α-SMA in TGFβ1-stimulated cells; α-SMA mRNA increased 7-fold and α-SMA protein 1.8-fold ($p < 0.01$ for both) but this increase was not counteracted by concurrent treatment with BUD 10^{-8} M and/or FORM 10^{-10} M (Fig. 2A).

Neither TGFβ1 alone (10 ng/ml) nor together with BUD 10^{-8} M and/or FORM 10^{-10} M had significant effects on fibroblast proliferation after 24 h or 48 h incubation (Fig. 2B) whereas proliferation was increased significantly (1.3-fold) after 24 h incubation with 10% serum used as a positive control.[18]

Metalloproteinases

MMP-9

Gelatin zymography showed enzymatic activity of both MMP-9 and MMP-2 in the fibroblast-conditioned medium and Western blot analysis confirmed the presence of enzyme pro-forms and high molecular weight complexes for both MMPs (Fig. 3A). For MMP-9, quantification by densitometry showed that TGFβ1 significantly increased the active form (82 kDa) 3.8-fold ($p < 0.05$) (Fig. 3B) as well as the pro-enzyme (92 kDa) and high molecular

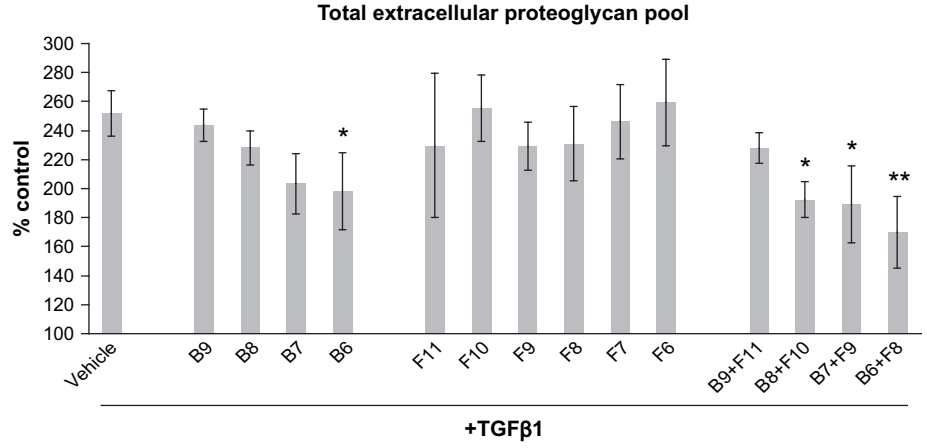

Figure 1 The total production of extracellular proteoglycans in human lung fibroblasts (HFL-1) stimulated with transforming growth factor-β1 (TGFβ1; 10 ng/ml) for 24 h (versus incubation with 0.4% serum), and the effects of concurrent treatment with budesonide 10^{-9} M (B9) — BUD 10^{-6} M (B6), or FORM 10^{-11} M (F11) — FORM 10^{-6} M (F6), or their combination. Data ($n = 3$) were normalized to the total protein amount in the corresponding cell layers and expressed as a percentage of control (0.4% serum = 100%); for all bars $p < 0.01$ versus control (0.4% serum); *$p < 0.05$, **$p < 0.01$ versus TGFβ1+vehicle.

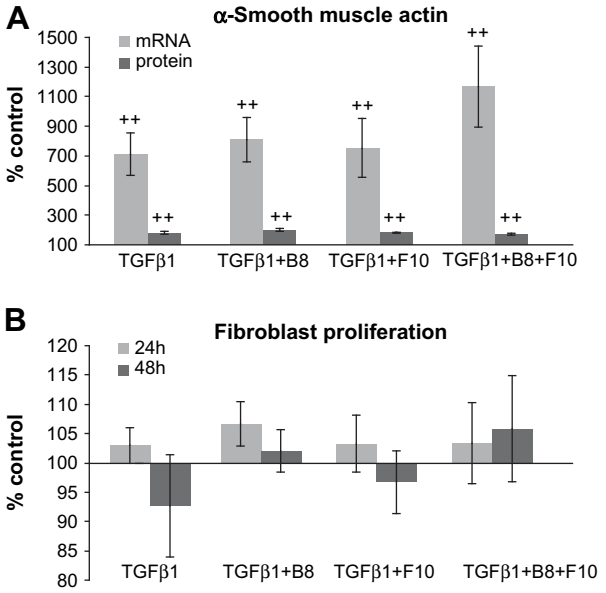

Figure 2 Characterization of human lung fibroblasts (HFL-1) stimulated with transforming growth factor β1 (TGFβ1; 10 ng/ml) for 24 h (versus incubation with 0.4% serum), and the effects of concurrent treatment with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10). (A) α-smooth muscle actin expression at mRNA level ($n = 6–7$; light bars) and protein level (ELISA) normalized to the total protein amount in the corresponding cell layers ($n = 2$; dark bars). (B) fibroblast proliferation after 24 h (light bars) or 48 h (dark bars) incubation ($n = 3$). Data are expressed as a percentage of control (0.4% serum = 100%); $^{++}p < 0.01$ versus 0.4% serum (there are no statistically significant differences versus TGFβ1).

weight complexes (around 200 kDa) 4–6-fold ($p < 0.05$). All forms of MMP-9 detected in the presence of TGFβ1 were reduced by both BUD and FORM alone and in combination to levels that were not significantly different from those at baseline conditions (0.4% serum). Compared with TGFβ1-induced response, the active enzyme was reduced 76% by BUD, 49% by FORM and 84% by the BUD/FORM combination ($p < 0.01$ for all; Fig. 3B), and the pro-form of the enzyme by 70–90% for all drug treatments ($p < 0.01$). BUD alone and in combination with FORM also reduced the baseline activity of MMP-9 (cells incubated with 0.4% serum; data not shown) and this accounted for approximately one half of the reduction seen in the presence of TGFβ1.

MMP-2

The activity of MMP-2 at baseline conditions (0.4% serum) was approximately 10-fold greater than that of MMP-9 (Fig. 3A), which is in agreement with constitutive expression of MMP-2 by lung fibroblasts. At baseline conditions, MMP-2 was detected as a pro-form (72 kDa) and as active enzyme at 62 kDa (Fig. 3A). Both forms were significantly increased by TGFβ1; the pro-form enzyme 4.8-fold ($p < 0.05$) and the active enzyme 3.3-fold ($p < 0.01$; Fig. 3C). Concurrent treatment with BUD and FORM alone and in combination had no effect on either the active form (Fig. 3C) or the pro-form of the MMP-2.

MMP-3

Casein zymography followed by Western blot revealed both the presence and enzymatic activity of MMP-3 in the conditioned medium of fibroblasts at control conditions (in 0.4% serum) and after stimulation with TGFβ1. The pro-enzyme appeared as a double band at 59 kDa and 57 kDa (representing a glycosylated and non-glycosylated form, respectively), the active enzyme as a band at 45 kDa, and a complexed form as a band at around 100 kDa (Fig. 4A). Densitometric analysis revealed that TGFβ1 increased the active band 1.6-fold ($p < 0.05$) (Fig. 4B), and a 1.5–2.0-fold increase was also seen for all the other MMP-3 forms detected. Concurrent treatment with BUD and/or FORM did not significantly affect the active MMP-3 or the other MMP-3 forms, except for 59 kDa pro-enzyme which was further enhanced by BUD/FORM combination treatment [from the 2.0-fold increase by TGFβ1 ($p < 0.05$) to a 2.8-fold increase after addition of BUD/FORM ($p < 0.05$)].

TIMP-1

As evaluated by ELISA, TGFβ1 significantly increased TIMP-1 protein (1.6-fold, $p < 0.01$). Concurrent treatment with BUD had no effect and the further 45% increase by FORM was not statistically significant ($p > 0.1$). However, treatment with the BUD/FORM combination significantly ($p < 0.05$) and completely counteracted the TGFβ1-induced TIMP-1 increase (Fig. 5).

Metalloproteolytic equilibrium

Considering the 3–4-fold increase of active MMP-9 and MMP-2 by TGFβ1 and only 1.6-fold increase of TIMP-1 protein, TGFβ1 exposure led to a pro-proteolytic environment; with a 2-fold increase of the ratios of MMP-9/TIMP-1 ($p < 0.1$) and MMP-2/TIMP-1 ($p < 0.01$) as compared to the baseline conditions (exposure to 0.4% serum). The increase of MMP-9/TIMP-1 ratio was completely abolished by concurrent treatment with BUD and FORM alone and in combination (Fig. 6A). In contrast, the increase of MMP-2/TIMP-1 ratio was further potentiated by BUD/FORM combination treatment (but not by either drug alone), reaching a 3-fold increase when compared to baseline conditions ($p < 0.01$; $p < 0.05$ versus TGFβ1 alone; Fig. 6B).

In contrast to MMP-9 and MMP-2, TGFβ1 increased active MMP-3 to the same extent as it increased TIMP-1 protein (1.6-fold in both cases) and thus did not affect the ratio of MMP-3/TIMP-1. This ratio was not affected by concurrent BUD or FORM treatment, whereas BUD/FORM combination increased it 2.5-fold as compared to baseline conditions ($p < 0.05$; $p < 0.1$ versus TGFβ1 alone; Fig. 6C).

Discussion

In this study, we have shown that stimulation of human lung fibroblasts with TGFβ1 resulted, along with increased α-SMA expression, in increased production of PGs and TIMP-1 protein as well as enhanced activities of gelatinases MMP-9 and MMP-2 and the stromelysin MMP-3. We have further shown that concurrent BUD/FORM combination treatment counteracted the TGFβ1-induced production of PGs and TIMP-1, as well as enhanced MMP-9 activity and MMP-9/TIMP-1

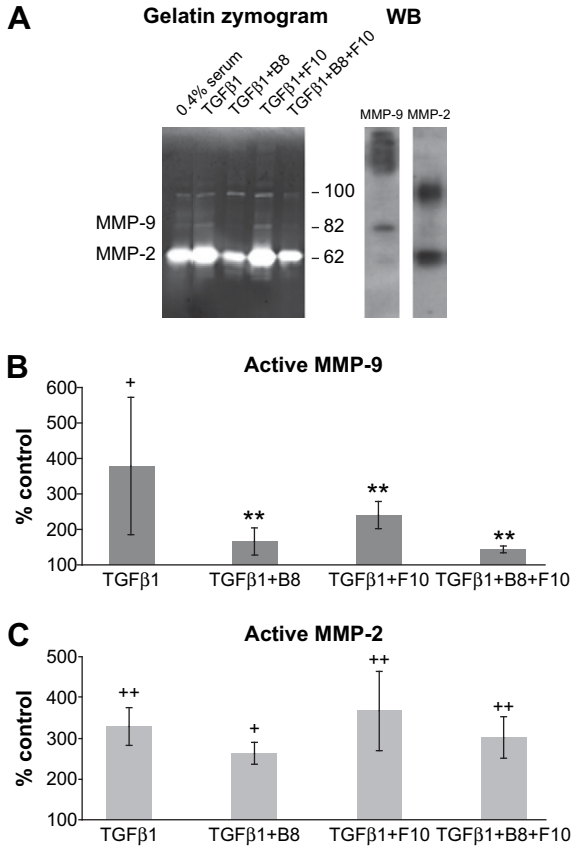

Figure 3 The production and activity of metalloproteinase-9 (MMP-9) and -2 (MMP-2) by human lung fibroblasts (HFL-1) stimulated by transforming growth factor-β1 (TGFβ1; 10 ng/ml) for 24 h (versus 0.4% serum) and the effects of concurrent treatment with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10). (A) Representative gelatin zymogram and Western blot (WB) using equal volume samples, revealing both the presence and the enzymatic activity of MMP-9 and MMP-2 in the cell culture medium. For MMP-9, the pro-form enzyme is visible as a band at 92 kDa, the active enzyme as a band at 82 kDa, and high molecular weight complexes at around 200 kDa. For MMP-2, the pro-form enzyme is visible as a band at 72 kDa and the active enzyme as a band at 62 kDa. In (B) and (C) data show enzyme activity measured by densitometric analysis of active bands and normalized to the total protein concentration in each sample: 82 kDa for MMP-9 ($n = 3$) and 62 kDa for MMP-2 ($n = 4$). In (B) and (C) data are expressed as a percentage of control (0.4% serum = 100%); $^{+}p < 0.05$, $p^{++} < 0.01$ versus 0.4% serum; and $^{**}p < 0.01$ versus TGFβ1.

ratio. In contrast, increased α-SMA expression and activity of MMP-2 and MMP-3 were not affected by BUD/FORM combination while MMP-2/TIMP-1 and MMP-3/TIMP-1 ratios were significantly increased. TGFβ1-induced MMP-9 activity and enhanced MMP-9/TIMP-1 ratio was as effectively counteracted by BUD or FORM alone as by the BUD/FORM combination, whereas neither BUD nor FORM alone affected the activity of MMP-2 or MMP-3 and the production of TIMP-1. Moreover, higher concentrations of BUD were required to reduce the PG production than when BUD was used together with FORM, although FORM had no effect on its own. These results suggest that the inhibitory, synergistic effects of BUD/FORM combination treatment on TGFβ1-induced PG production may involve increased metalloproteolytic turnover of PGs through a synergistic decrease of TIMP-1.

We have chosen the human fetal lung fibroblasts, HFL-1, to address the aims of the present study. These cells have been previously validated as suitable to study the differentiation of lung fibroblast into myofibroblasts and their ECM production under exposure to TGFβ, which is regarded as crucial for the development of subepithelial fibrosis in asthma. These cells show similar morphology and production of connective tissue, including PGs, as primary human bronchial fibroblasts derived from central bronchial lung biopsies from human subjects.[20,21]

We have investigated a broad range of BUD (10^{-9} to 10^{-6} M) and FORM (10^{-11} to 10^{-6} M) concentrations on the total PG production. The drugs were combined in a 100:1 ratio (BUD:FORM), which approximately reflects dose ratio of these drugs in asthma therapy. For further analyses we chose BUD 10^{-8} M, and consequently FORM 10^{-10} M, which reflects the concentration of BUD in airway and lung tissue some hours after inhalation of a moderate dose.[22]

The total PG production by TGFβ1-stimulated lung fibroblasts was decreased by BUD in a concentration-dependent manner, and while FORM had no effect on its own, it potentiated the effect of BUD, suggesting synergistic drug interaction. This synergistic effect regards predominantly the TGFβ1-induced PG production since the reduction of the baseline production by the drug combination was responsible only for approximately one quarter of the reduction in the presence of TGFβ1. The drugs, either alone or in combination, did not affect TGFβ1-induced fibroblast differentiation into more active myofibroblast, or fibroblast proliferation. Altogether, these results resemble very closely our earlier findings on the effects of BUD and FORM on PG production in HFL-1 fibroblasts stimulated with 10% serum.[18]

PGs are a large component of ECM and are involved in many pathophysiological processes that occur in the ECM. They modulate inflammatory response, influence tissue repair and remodeling, maintain fibre network structure and function, and affect fluid balance and consequently tissue resilience and mechanics.[23] Total PG levels are increased in the airways of asthmatic patients.[15,16] An increased production of PGs by bronchial fibroblasts from asthmatic patients was associated with increased patient airway hyperresponsivness,[17] and a positive correlation between the enhanced bronchial subepithelial deposition of some specific PGs and airway hyperresponsiveness was found in patients with mild asthma.[16]

The major endogenous factor leading to airway ECM remodeling in asthma is regarded an imbalance between activity of MMPs and TIMPs, and especially between gelatinase MMP-9 and TIMP-1.[3] The level and/or activity of MMP-9 were shown to be increased in asthma patients' sputum[4,5,9] and bronchoalveolar lavage fluid,[24] and increased further after allergen challenge,[4,25] during asthma exacerbations,[6,26] and in severe asthma.[4] MMP-9 immunoreactivity in subepithelial basement membrane was also associated with asthma severity.[27] A direct effect of MMPs is ECM degradation, which may counteract formation of airway fibrosis. However, an excessive degradation over a longer period of time may also result in a feedback of

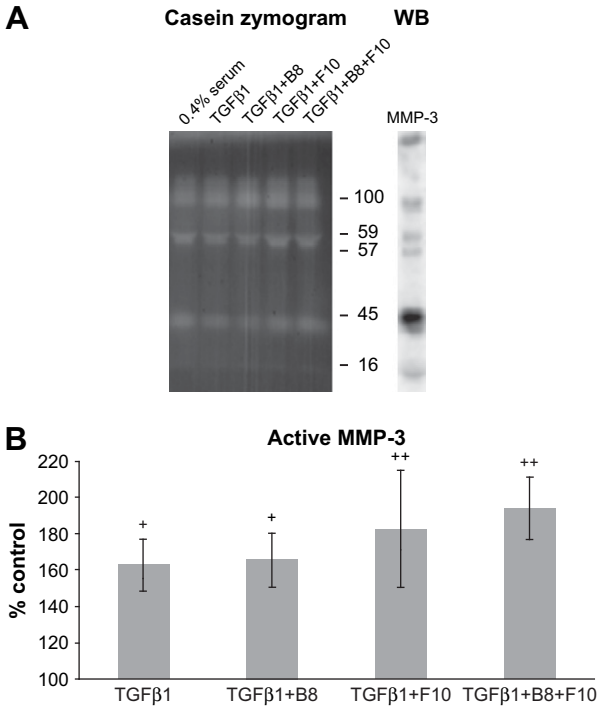

Figure 4 The production and activity of metalloproteinase-3 (MMP-3) by human lung fibroblasts (HFL-1) stimulated by transforming growth factor-β1 (TGFβ1; 10 ng/ml) for 24 h (versus 0.4% serum), and the effects of concurrent treatment with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10). (A) Representative casein zymogram and Western blot (WB) using equal volume samples, revealing both the presence and the enzymatic activity of MMP-3 in the cell culture medium. The pro-form enzyme is visible as a double band at 59 kDa and 57 kDa, the active enzyme as a band at 45 kDa, and a complexed form as a band at around 100 kDa. (B) Activity of MMP-3 measured by densitometric analysis of the 45-kDa band and normalized to the total protein concentration in each sample. In (B) data ($n = 3$) are expressed as a percentage of control (0.4% serum = 100%); $^{+}p < 0.05$, $p^{++} < 0.01$ versus 0.4% serum (there are no significant differences versus TGFβ1).

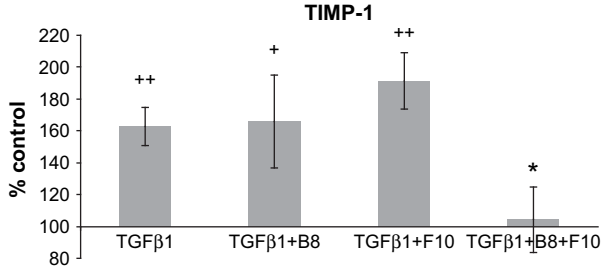

Figure 5 The production (ELISA) of extracellular tissue inhibitor of metalloproteinases-1 (TIMP-1) by human lung fibroblasts (HFL-1) stimulated by transforming growth factor-β1 (TGFβ1; 10 ng/ml) for 24 h (versus 0.4% serum) and the effects of concurrent treatment with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10). Data ($n = 4$) were normalized to the total protein amount in the corresponding cell layers and expressed as a percentage of control (0.4% serum = 100%); $^{+}p < 0.05$, $p^{++} < 0.01$ versus 0.4% serum; and $^{*}p < 0.05$ versus TGFβ1.

over-repair cycles, leading to increased synthesis and deposition of ECM. Indeed, the levels of TIMP-1 are also elevated in the airways of asthmatic patients[4,5,8,9] and TIMP-1 molar concentrations often exceed the concentrations of MMP-9 and other MMPs.[8–10] Furthermore, high levels of TIMP-1 in sputum of asthmatic patients are associated with increased airway obstruction[9] and with airway structural changes in patients with severe asthma.[10,11] These findings suggest that although TIMP-1 protects airway tissue against enhanced MMP activity, its increase may also be pathogenic and lead to enhanced airway fibrosis. It is conceivable that for a proper healing and repair processes in the airways, both a pro- and an anti-proteolytic environment is required at different stages of this process. Importantly, the airway fibrotic remodeling in asthma suggests an over-repair as a consequence of a disturbance of this process.

In the present study, the active gelatinases MMP-9 and MMP-2 were 3–4-fold increased by TGFβ1 and their ratios to TIMP-1 protein were increased 2-fold. For MMP-9, these changes were effectively counteracted by BUD and FORM, both alone and in combination. However, such effects were not observed for MMP-2; in contrary, its ratio to TIMP-1 was further increased by BUD/FORM combination while either drug alone had no effect. Similarly, the ratio of active MMP-3 to TIMP-1 protein was increased by BUD/FORM combination treatment although it was not increased by TGFβ1 alone or together with either BUD or FORM. The increased ratio of these MMPs to TIMP-1 was caused by the fact that BUD/FORM combination treatment completely counteracted the TGFβ1-induced increase of TIMP-1 while it had no effects on MMP-2 or MMP-3 activity. Although we do not know whether TIMP-1 equally inhibits various MMPs, these results suggest that a decrease of TIMP-1 by BUD/FORM combination results in an enhanced activity of MMP-2 and MMP-3, which then may lead to an increased turnover of PGs. Thus, this mechanism may be involved in the inhibition of the TGFβ1-induced PG production by the BUD/FORM combination.

The effects of BUD and FORM on the production of PGs and TIMP-1 at the low/moderate drug concentrations investigated appeared to be synergistic as neither BUD nor FORM alone had any effects at these concentrations. We have previously shown that a synergistic decrease in the total PG production by BUD and FORM in these cells (stimulated by 10% serum) was dependent on the presence of functional GC receptors and β-adrenoceptors.[18] These effects can be explained by the ability of β_2-agonists to enhance activation and nuclear translocation of GC receptors and, as a consequence, affect GC-regulated gene transcription,[28,29] as well as by the ability of GCs to increase β_2-adrenoceptor expression and signalling (reviewed in Ref. [29]). The latter may counteract desensitisation and downregulation of β_2-adrenoceptors by TGFβ1.[30]

In conclusion, these results suggest that the BUD/FORM combination enhanced metalloproteolytic activity of human lung fibroblasts via a synergistic decrease of TIMP-1,

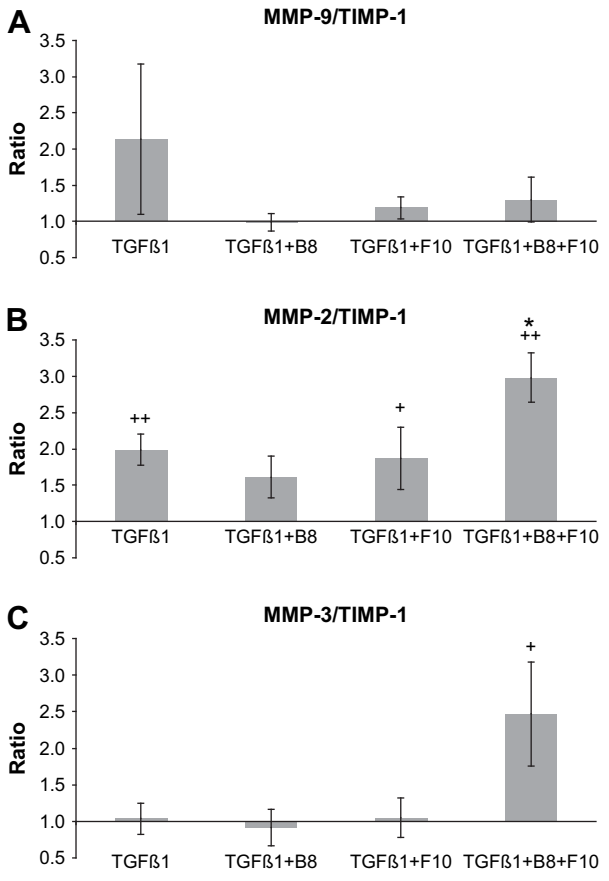

Figure 6 The effects of transforming growth factor-β1 (TGFβ1; 10 ng/ml; 24 h incubation) and the concurrent treatment with budesonide 10^{-8} M (B8) and/or formoterol 10^{-10} M (F10) on the equilibrium (shown as ratio) between active metalloproteinases (MMP-9, MMP-2 and MMP-3) and tissue inhibitor of metalloproteinases-1 (TIMP-1) protein in human lung fibroblasts (HFL-1) incubation medium, as compared to control (0.4% serum) conditions (shown here as the ratio = 1). $^{+}p < 0.05$, $p^{++} < 0.01$ versus 0.4% serum; and $^{*}p < 0.05$ versus TGFβ1.

and that this mechanism may be involved in the synergistic inhibition of the TGFβ1-induced PG production by BUD/FORM treatment. This implies that BUD/FORM combination therapy can counteract excessive ECM production and deposition and thus pathological airway fibrotic remodeling in asthma. Extension of these findings to other ECM molecules, and investigation of lung fibroblasts from patients with asthma are warranted.

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Conflict of interest statement

LT has received a grant from AstraZeneca and financial support to ERS and ATS meetings. EG and AM-L are employees of AstraZeneca.

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