Dissecting the Dualistic Effects of Transforming Growth Factor (TGF)-β on Fibroproliferation and Extracellular Matrix Production in Primary Human Lung Fibroblasts – The Role of p38δ MAP Kinase

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Abstract: Rationale: Inflammation, increased fibroblast proliferation, and increased deposition of extracellular matrix (ECM) are hallmarks of early lung fibrosis and asthma. Transforming growth factor-β (TGF-β) has been suggested as a key regulator of lung tissue homeostasis with several and often opposite effects on fibroblast proliferation and ECM production. In human and animal model systems, it has been shown that TGF-β induced several signaling cascades including Smads, p38 mitogen-activated protein (MAP) kinases, and extracellular signal-regulated kinases 1/2 (ERK). Information on how TGF-β regulates and controls normal primary lung fibroproliferation and ECM production is not present.

Objectives: We sought to dissect the effects of TGF-β on fibroproliferation and ECM production of primary adult human lung fibroblasts and elucidate the involved signaling pathways.

Results: Depending on the presence of fetal bovine serum (FBS; 10%), TGF-β exerted opposite effects on fibroproliferation. In the absence of FBS, low TGF-β concentrations (0.01 and 0.001 ng/ml) significantly *induced* fibroproliferation. In the presence of FBS, TGF-β (1 ng/ml, 10 ng/ml) significantly *reduced* fibroproliferation. TGF-β dose-dependently increased ECM deposition, which was independent of the presence of FBS. The anti-proliferative effect of TGF-β was associated with increased prostaglandin E_2 (PGE_2) production, that was induced *via* p38δ and ERK 1/2 MAP kinases. Indomethacin (2.5 μM) and a small interfering RNA specific for p38 MAP kinase completely reversed the TGF-β-dependent inhibition of fibroblast proliferation.

Conclusions: Both pro- and anti-proliferative cascades can be activated by TGF-β. In a mitogenic or inflammatory environment TGF-β induces PGE_2 synthesis *via* activation of p38δ MAP kinase, which then exerts a strong anti-proliferative effect. This dualistic nature of TGF-β may exist in order to maintain lung tissue integrity.

Keywords: Primary human lung fibroblasts, transforming growth factor-β signaling, p38 MAP kinase isoforms, lung homeostasis, fibrosis.

INTRODUCTION

Remodeling of the lung is a hallmark of both the early phase of pulmonary fibrosis and asthma, and may reflect a situation in which the organ cannot cope with repeated physical injury or infectious insult. The remodeling is characterized by an increase of the bulk of fibroblast tissue and an enhanced deposition of extracellular matrix (ECM), and may underlie the distortion of the normal lung architecture and/or airway wall thickening [1-3]. The idiopathic nature of many fibroproliferative disorders of the lung make them very hard to treat, but careful biomolecular studies have suggested transforming growth factor-β (TGF-β) as a target. TGF-β may be a key regulator of lung tissue homeostasis [4, 5], because it was shown to exert opposite effects on fibroproliferation and ECM synthesis depending on the microenvironment of the lung. Firstly, TGF-β has been reported to stimulate the synthesis of ECM proteins and glycoproteins, while inhibiting the degradation of collagen [1]. TGF-β has also been associated with structural lung tissue remodeling and fibrosis in both humans and animal studies [6-12]. Secondly, TGF-β has been shown to inhibit the proliferation of fibroblasts in both humans and animal models [13-15].

A straightforward understanding of the role of TGF-β in fibroproliferative diseases is hampered, however, because most studies have been performed in distinct cellular systems, either in animal models or primary and transformed human cell lines. Therefore, it is hardly possible to draw compelling conclusions with respect to TGF-β's role in lung tissue remodeling and fibrosis. That is the reason why we recently

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introduced an interactive cell culture model of primary human airway epithelium plus primary human lung fibroblasts. With this model we showed that epithelium-derived TGF-β actively inhibited the proliferation of fibroblasts, and that this inhibitory effect was mediated by prostaglandin E_2 (PGE_2) produced by the lung fibroblasts [16].

In addition, we have demonstrated that the cellular environment – in particular the cell density and exposure to serum – determined the activity, function and intracellular signaling of primary fibroblasts [17, 18]. Considering these data, we argued that the effects exerted by TGF-β may heavily depend on the microenvironment of the lung, i.e. in the presence of additional inflammatory and/or fibrogenic stimuli, because TGF-β signaling may depend on and/or integrate with additional signaling systems that are activated in a proinflammatory environment.

Indeed, TGF-β has the ability to activate several signaling cascades, some of which are specific to the action of TGF-β, whereas others are of a more general character: 1) the Smad pathway, 2) p38 mitogen-activated protein (MAP) kinases, 3) extracellular signal-regulated kinase (ERK) 1/2 MAP kinase, 4) c-Jun NH_2-terminal kinase (JNK), and 5) the phosphoinositol-3 kinase (PI3K) pathway [5, 19]. Dissecting these signaling pathways in primary human lung fibroblasts is crucial for the understanding of the role of TGF-β in tissue homeostasis and of the pathogenesis of lung remodeling associated with fibrosis. Therefore, we examined the effect of TGF-β on fibroproliferation and ECM deposition using resting and mitogen-stimulated primary human lung fibroblasts and elucidated the associated signaling pathways.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Reagents

Material was purchased from: Invitrogen (Heidelberg, Victoria, Australia): Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, amphotericin B. JRH (Melbourne, Victoria, Australia): fetal bovine serum (FBS), phosphate buffered saline (PBS). Sigma-Aldrich (Saint Louis, MO, USA): trypsin, TGF-β_2, TGF-β_3, indomethacin. Calbiochem (EMD Biosciences, San Diego, CA, USA): SB203580, PD98059, primary antibodies p38 MAP kinase, p-p38 MAP kinase. Cell signaling Technology, Inc. (Danvers, MA, USA): primary antibodies p-p38 MAP kinase, Smad2/3, p38α MAP kinase, p38β MAP kinase, p38γ MAP kinase, p38δ MAP kinase. Santa Cruz Biotechnology (Santa Cruz, CA, USA): primary antibody p38 MAP kinase, goat-anti-mouse antibody, goat-anti-rabbit antibody, siRNA p38 MAP kinase, scrambled siRNA, siRNA transfection reagent. Promega (Madison, WI, USA): primary antibodies ERK 1/2, p-ERK 1/2, caspase-3/7 assay kit. R&D Systems (Minneapolis, MN, USA): TGF-β_1, TGF-β_1 ELISA kit (Quantikine).

Primary Cell Culture

Normal human lung tissue was obtained from resected human lungs (Human Ethics Committee, Sydney University, South West Sydney Area Health Service; Ethics Committee, Faculty of Medicine, University Hospital Basel), and cultures of primary human lung fibroblasts and primary human bronchial smooth muscle cells were established as reported previously [16, 20, 21]. All experiments were performed using cells between passage 3 to 6.

TGF-β Analysis

Total TGF-β_1 was measured in the cell culture medium using a commercial ELISA kit.

Thymidine Incorporation into DNA

Cell proliferation assays were performed as described previously [20]. Fibroblasts were incubated with different concentrations of human recombinant TGF-β_1, TGF-β_2, or TGF-β_3, respectively, for 24 hours, and stimulation with 10% FBS was used as a positive control [20]. To exclude apoptosis as a cause of reduced cell proliferation, caspase-3/7 activity was assessed in the cell culture medium using a commercial kit for caspase-3/7 activity and was found negative at all concentrations tested, indicating that TGF-β did not induce apoptosis (data not shown).

Total ECM and Collagen Deposition

Serum-deprived fibroblasts in 24-well plates were treated with 10% FBS, and/or with TGF-β_1 in the presence of 1 μCi/ml [^{3}H]-proline (Amersham, Little Chalfont, UK). [^{3}H]-proline incorporation was measured by liquid scintillation counting. Total ECM and collagen deposition were calculated as described previously [18].

Prostaglandin E_2 Analysis

PGE_2 levels were quantified in the cell culture medium using a commercial ELISA kit, following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

Nuclear and Cytosolic Extracts and Western Blot Analysis

Total, nuclear and cytosolic extracts were prepared from 80% sub-confluent cell cultures, as described previously [21]. To determine Smad2/3, ERK 1/2 MAP kinase, phosphorylated (p-)-ERK 1/2 MAP kinase, p38 MAP kinase, p-p38 MAP kinase, p38α MAP kinase, p38β MAP kinase, p38γ MAP kinase, and p38δ MAP kinase expression, equal amounts of protein (5μg) were size-fractionated in a 10% SDS-polyacrylamide gel, transferred onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) (25 mA/gel, 100 V, 120 minutes, 4°C), and equal loading was confirmed by Ponceau's red. Membranes were blocked for 1 hour (10 mM Tris, 150 mM NaCl, 0.05 % Tween 20, 5% FBS) followed by incubation with one of the primary antibodies: rabbit-anti-human (Smad2/3, ERK 1/2 MAP kinase, p-ERK 1/2 MAP kinase, p-p38 MAP kinase, p38α MAP kinase, p38β MAP kinase, p38γ MAP kinase, p38δ MAP kinase), or mouse-anti-human (p38 MAP kinase) and were used at 1: 500 to 1: 3´000 dilution in blocking-buffer (4°C overnight). The membranes were then washed (3 x 5 minutes) with blocking buffer, and incubated with a goat-anti-rabbit, or a goat-anti-mouse secondary antibody, conjugated to horseradish peroxidase (diluted 1: 5´000, 1 hour at room temperature). Protein detection was performed by chemiluminescence (Pierce, Rockord, IL, USA).

Inhibition of Cyclooxygenase, ERK 1/2 and p38 MAP Kinase

Cyclooxygenase (COX) activity was blocked by indomethacin (2.5 μM). ERK 1/2 MAP kinase was inhibited by pre-incubation (30 minutes) of fibroblasts with PD98059

(10μM), and p38 MAP kinases were inhibited by pre-incubation (30 minutes) with SB203580 (10 μM) [22].

Transfection with Small Interfering RNA

Fibroblasts were seeded in 12-well plates (1 x 10^5 cells/ml) and grown to 60 - 70% sub-confluence in DMEM (10% FBS), without antibiotics. Cells were washed with FBS-free DMEM, before transfection with 3.6 μl/well of 10 μM p38 MAP kinase small interfering RNA (siRNA) together with 2.4 μl of transfection reagent. Cells incubated with scrambled siRNA or transfection reagent alone served as controls. After 6 hours growth medium (DMEM + 20% FBS + 1x antibiotics) was added for 20 hours. Cells were then washed once with PBS and subsequently stimulated with 10% FBS plus 10 ng/ml TGF-β_1 or with 10% FBS alone. After 24 hours, cells were lysed (1 M Tris-HCl [pH 6.8], 2% SDS, 2% mercaptoethanol, 10% glycerol) and total protein was collected.

For proliferation assays in 96-well plates the quantity of siRNA and transfection reagent were scaled in proportion to the surface area of the well size. Nineteen hours after TGF-β stimulation [^{3}H]-thymidine (Perkin Elmer, Wellesley, MA, USA) was added to the cells for 5 hours.

Statistical Analysis

For analysis of repeated measurements ANOVA was used. Paired observations were analyzed by Student's paired t-test. Correlations between parameters were assessed using Pearson's correlation test with logarithmic regression analysis (StatView), and p-values ≤ 0.05 were considered significant. Where applicable, data are shown as mean ± standard error of the mean (SEM).

RESULTS

Dissecting the Effects of TGF-β on Fibroblast Proliferation and ECM Production

Over a period of 24 hours 10% FBS significantly increased fibroblast proliferation by five times compared to cells in serum-free conditions (n=3, Student's paired t-test, p<0.0001; Fig. **1A**). Likewise, total ECM-deposition was

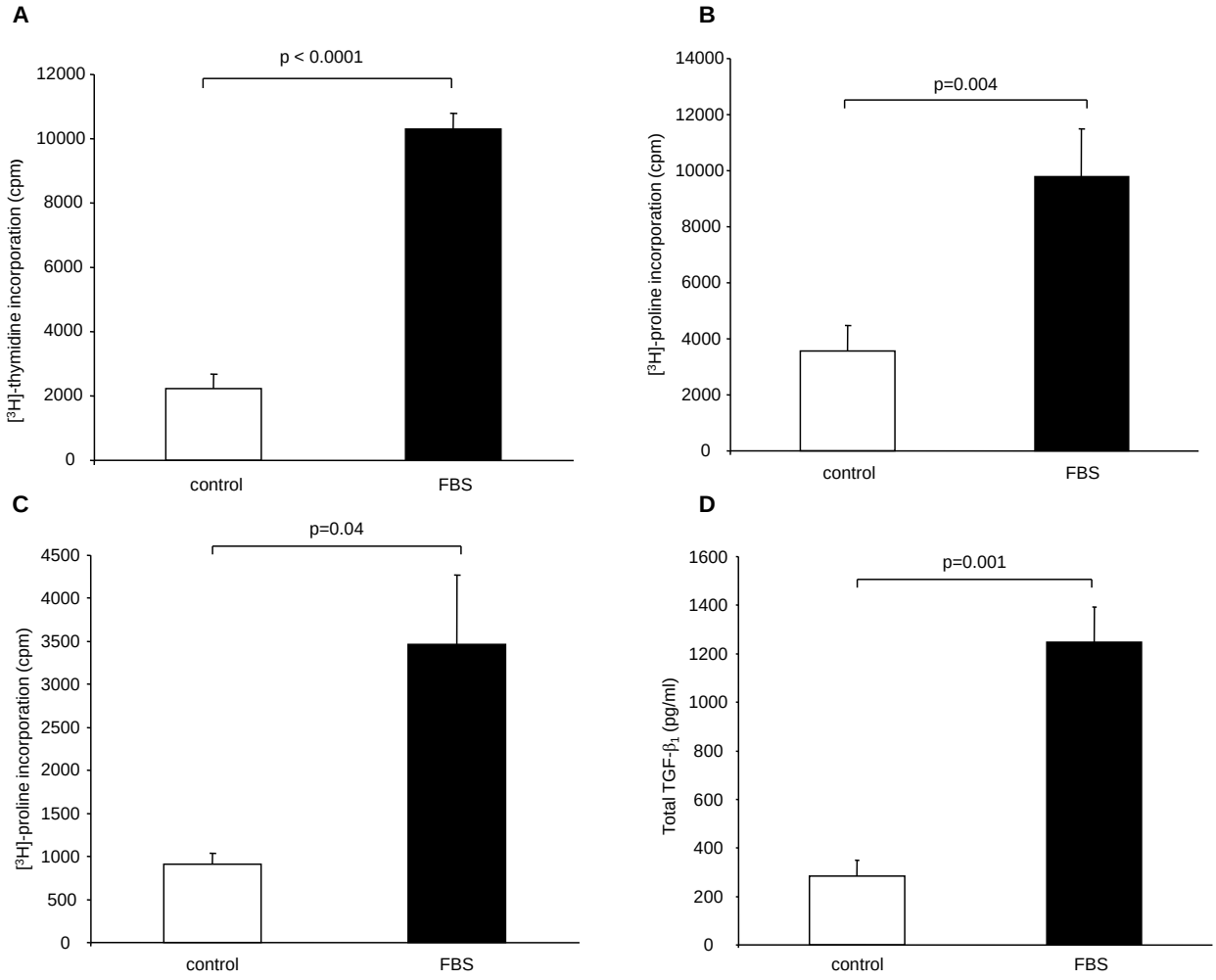

Fig. (1). FBS induces a pro-proliferative and pro-fibrogenic environment. Effect of 10% FBS (black bars) on fibroblast proliferation (**A**), ECM deposition (**B**), collagen deposition (**C**), and TGF-β_1 secretion (**D**) of primary human lung fibroblasts compared with serum-free DMEM (=control, open bars). Serum starved primary human lung fibroblasts were incubated with DMEM plus 10% FBS or with serum-free DMEM. Cell proliferation was assessed by [^{3}H]-thymidine incorporation, and total ECM/collagen deposition were determined by [^{3}H]-proline incorporation. Levels of total TGF-β_1 were measured in the cell culture medium by specific ELISA. Values are presented as mean ± SEM of independent experiments performed in at least three different fibroblast cell lines.

increased by three times by 10% FBS compared to serum-free conditions (n=5, Student's paired t-test, p=0.004; Fig. **1B**), which was confirmed by an increased collagen-deposition (n=4, Student's paired t-test, p=0.04; Fig. **1C**). In the absence of FBS lung fibroblasts spontaneously secreted low levels of TGF-β_1 (282 ± 67 pg/ml), and this was significantly increased by 24 hours of incubation in the presence of 10% FBS (1248 ± 144 pg/ml) (n=4, Student's paired t-test, p=0.001; Fig. **1D**).

Then, we determined the effect of TGF-β_1, -β_2, and β_3, in the absence or presence of 10% FBS on fibroblast proliferation. In the absence of FBS only TGF-β_1 at concentrations 0.001 ng/ml and 0.01 ng/ml significantly enhanced fibroblast proliferation by maximal 20% (n=3, ANOVA, $p \leq 0.03$; Fig. **2A**). In contrast, the addition of any of the three TGF-β-isoforms at concentrations ≥ 0.1 ng/ml had no effect on fibroblast proliferation (n=3, ANOVA; Fig. **2A**). In the presence of FBS TGF-β_1, -β_2, and β_3 significantly and dose-dependently decreased fibroblast proliferation (n=3, ANOVA, $p < 0.02$; Fig. **2B**). The inhibitory effect of TGF-β_3 was most pronounced and already significant at 0.001 ng/ml (n=3, ANOVA, $p < 0.02$; Fig. **2B**).

Since tissue remodeling consists of fibroblast hyperplasia and increased ECM deposition, we next evaluated the effect of TGF-β_1 on the deposition of total ECM and collagen. As TGF-β_1 is the isoform that is most consistently linked to

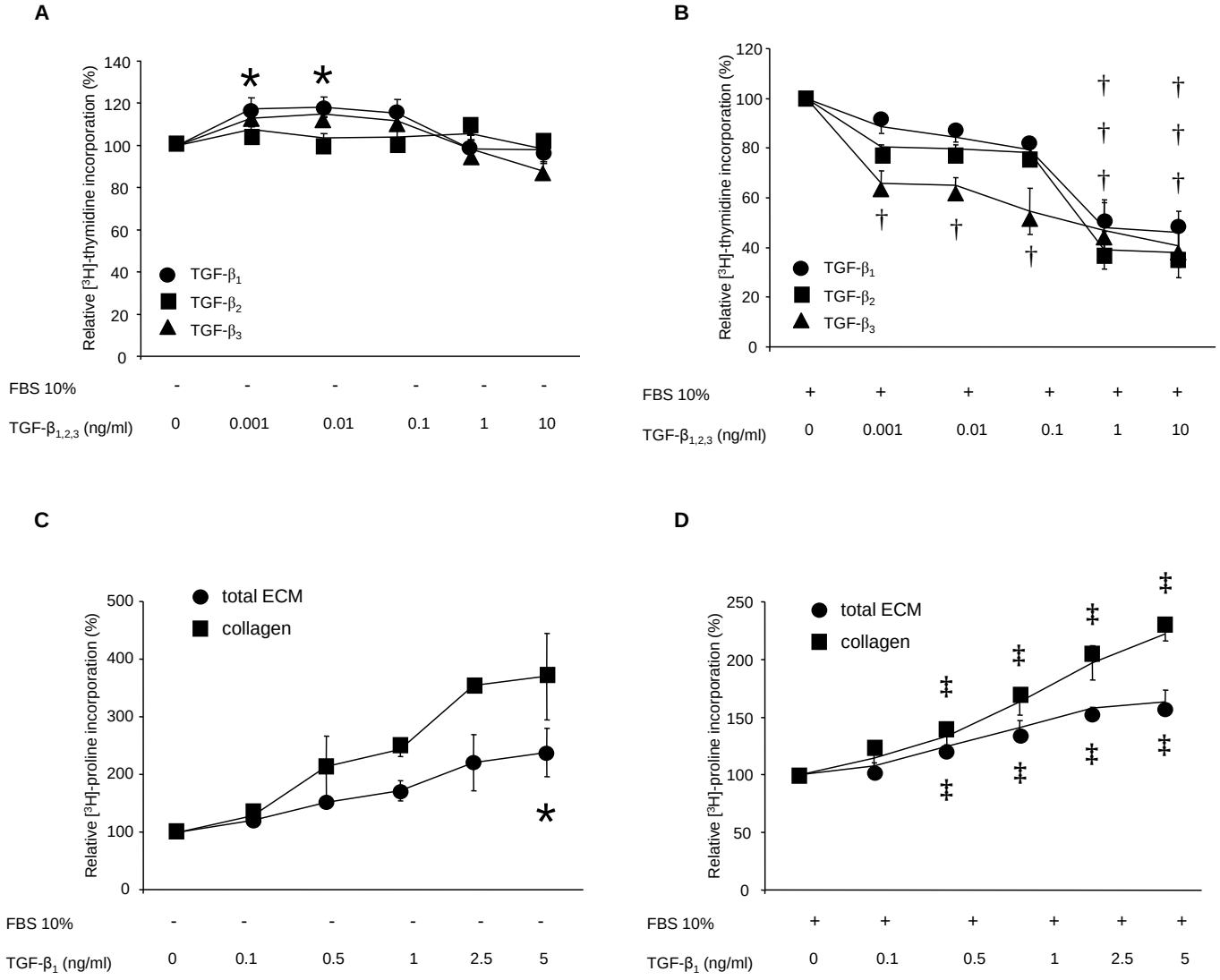

Fig. (2). The modulatory effect of TGF-β on fibroblast proliferation and matrix production. Effect of TGF-β_1 (●), TGF-β_2 (■), and TGF-β_3 (▲) on fibroblast proliferation in the absence (**A**) or presence (**B**) of 10% FBS. Serum starved primary human lung fibroblasts were incubated for 24 hours with serum-free DMEM (**A**) or with DMEM plus 10% FBS (**B**) containing increasing concentrations of TGF-β_1, TGF-β_2, or TGF-β_3, respectively. Cell proliferation was assessed by [^{3}H]-thymidine incorporation, and expressed as relative proliferation compared with the respective control (serum-free DMEM or DMEM plus 10% FBS). Values are presented as mean ± SEM of independent experiments performed in three different fibroblast cell lines. *$p \leq 0.03$, †$p \leq 0.02$. Effect of TGF-β_1 on total ECM (●) and collagen (■) deposition in the absence (**C**) or presence (**D**) of 10% FBS. Serum starved primary human lung fibroblasts were stimulated with increasing concentrations of TGF-β_1 in the absence (**C**) or presence (**D**) of 10% FBS. Total ECM and collagen deposition were determined by [^{3}H]-proline incorporation. Values are presented as mean ± SEM of independent experiments performed in at least three different fibroblast cell lines. *$p \leq 0.03$, ‡ $p \leq 0.009$.

tissue fibrosis [23] and is the most abundant isoform in the human lung [24], we focused on this isoform in all further experiments.

Under serum-free conditions TGF-β_1 increased the deposition of both total ECM and collagen in a dose dependent pattern (n=3, ANOVA, p=0.03; Fig. **2C**). When TGF-β_1 was added to fibroblasts in the presence of 10% FBS the relative effect of increase was unchanged, indicated by the slope in Fig. **2C**, **D** (n=3, ANOVA, $p \leq 0.009$; Fig. **2D**).

Next, we assessed the role of TGF-β_1-induced PGE_2 synthesis by lung fibroblasts. Indomethacin, a non-selective inhibitor of COX, significantly reversed the anti-proliferative effect of TGF-β_1 (1 ng/ml or 10 ng/ml) (n=3, ANOVA, $p \leq 0.003$; Fig. **3A**), whereas indomethacin alone did not affect proliferation. In Fig. (**3B**) the correlation between PGE_2 levels secreted by TGF-β_1-stimulated fibroblasts and the growth-inhibitory effect of TGF-β_1 is presented as it was observed in 12 fibroblast cell lines (n=12, Pearson's correlation test, r = 0.78, p = 0.0003, $R^2 = 0.63$; Fig. **3B**).

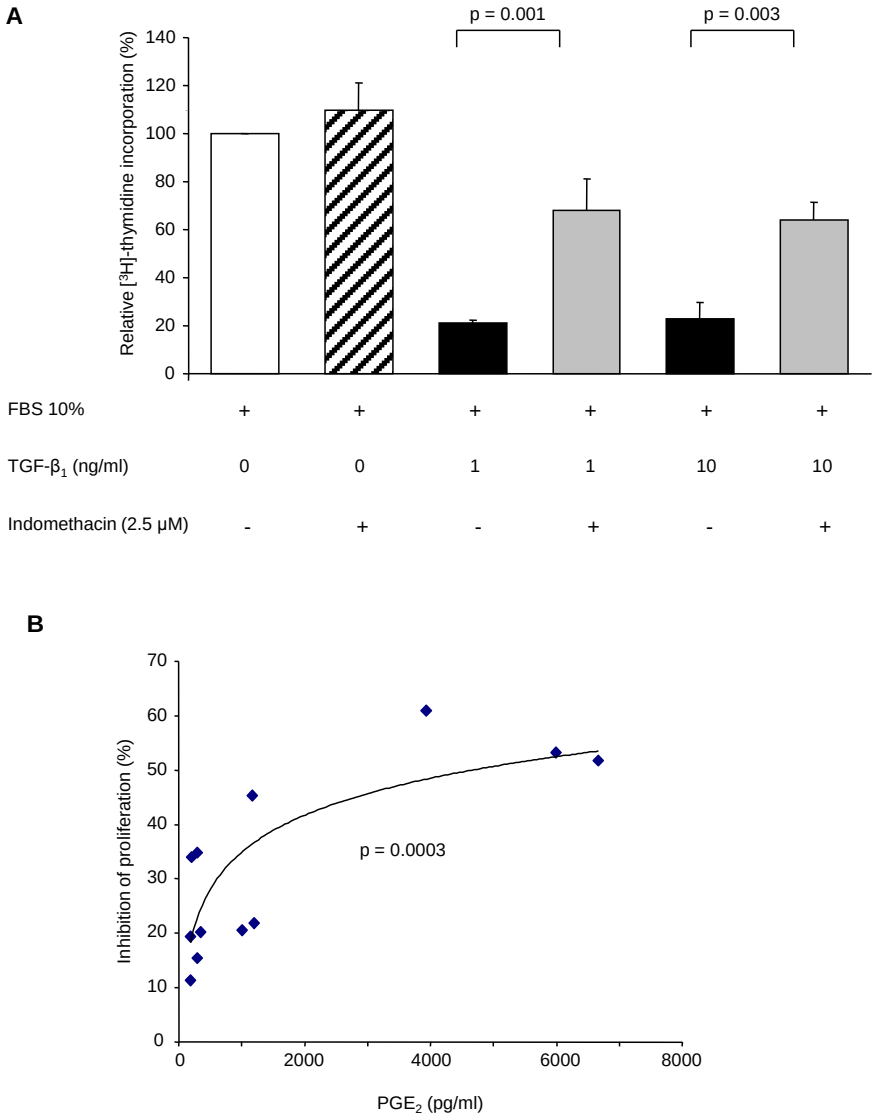

Fig. (3). Indomethacin reverses the anti-proliferative effect of TGF-β. (**A**) Effect of TGF-β_1 on fibroblast proliferation in the presence (grey bars) or absence (black bars) of 2.5 µM indomethacin compared with 10% FBS (open bar). DMEM plus 10% FBS containing 2.5 µM indomethacin served as control to demonstrate indomethacin's neutral effect on fibroblast proliferation (striped bar). Serum starved primary human lung fibroblasts were incubated for 24 hours with DMEM plus 10% FBS containing 1 ng/ml or 10 ng/ml TGF-β_1 with and without 2.5 µM indomethacin. Cell proliferation was assessed by [^{3}H]-thymidine incorporation, and expressed as relative proliferation compared with the control (DMEM plus 10% FBS). Values are presented as mean ± SEM of independent experiments performed in three different fibroblast cell lines. (**B**) Correlation between PGE_2 content in the cell culture medium of fibroblasts treated with various concentrations of TGF-β_1 and the inhibitory effect of TGF-β_1 on fibroblast proliferation. Serum starved primary human lung fibroblasts were incubated for 24 hours with DMEM plus 10% FBS containing various concentrations of TGF-β_1. PGE_2 levels were measured in the cell culture medium of fibroblasts after 24 hours TGF-β-treatment by specific ELISA. Cell growth was then assessed by [^{3}H]-thymidine incorporation and expressed as relative growth inhibition compared to the control (DMEM plus 10% FBS). Each value represents the PGE_2 content in the cell culture medium and the corresponding inhibitory effect of the cell culture medium on fibroblast proliferation (n=12, r=0.78, p=0.0003, R^2=0.63).

The p38δ MAP Kinase Isoform Mediates the TGF-β-Induced Anti-Proliferative Effect

Next, we analyzed the signaling pathways involved in the TGF-β-induced growth inhibition. As shown in Fig. (**4A**), TGF-β_1 (10 ng/ml) significantly increased ERK 1/2 phosphorylation in both the cytosolic and the nuclear fractions at 15 to 60 minutes (n=3; Fig. **4A**). Similarly, upon TGF-β_1 stimulation phosphorylated p38 MAP kinases appear in both the cytosol and the nucleus (n=3; Fig. **4B**).

To our surprise FBS-stimulated primary adult human lung fibroblasts expressed the third TGF-β signal transducer, Smad2/3, only at relative low levels when compared to other resident lung cells, such as bronchial smooth muscle cells (BSMC) (Fig. **4C**). Upon TGF-β_1 treatment we did not observe any phosphorylation or nuclear translocation of Smad2/3 in primary human lung fibroblasts.

The central role of MAP kinases in the mediation of the TGF-β-induced anti-proliferative effects is further demonstrated in Fig. (**4D**). SB203580, a specific inhibitor of p38 MAP kinase [22], completely blocked the TGF-β_1-induced increase in PGE_2 secretion by fibroblasts (n=3, ANOVA, p = 0.01; Fig. **4D**), whereas PD98059, a specific inhibitor of ERK 1/2, had no effect on PGE_2 production. Neither SB203580 nor PD98059 alone had any effect on PGE_2 synthesis (data not shown).

To further confirm the role of p38 MAP kinase in the TGF-β_1-induced inhibition of proliferation, lung fibroblasts were incubated with siRNA targeting the p38 MAP kinase. As demonstrated in Fig. (**4E**), the TGF-β_1-induced anti-proliferative effect was completely and specifically reversed in p38 MAP kinase siRNA-transfected fibroblasts (n=3, Student's paired t-test, p = 0.02; Fig. (**4E**), fifth bar).

Finally, we analyzed which of the p38 MAP kinase isoforms (p38α, p38β, p38γ, and p38δ) are involved in the TGF-β-induced growth-inhibition. In Fig. (**5**) it is shown that upon stimulation with TGF-β_1 the cytosolic fraction of p38 MAP kinase is comprised predominantly of the p38β and –δ MAP kinase isoforms, whereas only the p38δ isoform is translocated into the nucleus.

DISCUSSION

In the present report, we dissected two pivotal functions of TGF-β which are crucial in maintaining a healthy lung homeostasis. It was observed that TGF-β is able to activate both pro- and anti-fibrogenic cascades. In resting fibroblasts TGF-β, which may reflect normal non-pathological conditions, TGF-β had a moderately fibrogenic effect. This was manifest as a slightly increased fibroproliferation and ECM deposition. In the presence of a mitogenic stimulus, TGF-β markedly increased the deposition of ECM, which may reflect the situation found in early lung fibrosis and asthma [1, 3, 25-28]. In contrast to this strong pro-fibrotic condition, exogenous TGF-β had a strong anti-proliferative effect which was mediated *via* the secretion of PGE_2. These findings are in line with our previously published data demonstrating that airway-epithelium-derived TGF-β actively inhibits the proliferation of both normal and fibrotic fibroblasts [16]. Our current findings show that TGF-β can have opposite effects, which depend on the microenvironment, and demonstrate TGF-β's pivotal role in tissue homeostasis. The anti-proliferative effect of TGF-β observed under mitogenic (or inflammatory) conditions might represent a control mechanism that maintains tissue homeostasis in the lung by preventing an overshoot of the fibroproliferative response, hence counteracting tissue remodeling. The observation that normal primary human airway epithelial cells produce TGF-β [17] indicates a participation of TGF-β in the regulation of physiologic processes. Indeed, basal concentrations of TGF-β have been shown to maintain subepithelial collagen matrix homeostasis [29]. Our finding that the ultimate effect of TGF-β is dependent on the microenvironment, and that under inflammatory/fibrogenic conditions TGF-β controls proliferation and ECM deposition by two distinct signaling pathways may have major implications for the understanding of the role of TGF-β in early lung fibrosis and asthma.

TGF-β had been reported to stimulate the proliferation of many different cell types in various species [30-32], and its association with tissue fibrosis has been demonstrated both *in vitro* and *in vivo* in human and animal studies [9-11, 33-38]. In our *in vitro* model only very low concentrations of TGF-β_1 (0.001 and 0.01 ng/ml) exerted a weak, but significant mitogenic effect in a serum-free, non-inflammatory, non-fibrotic cell culture condition, but not in a pro-inflammatory condition. Compared to this weak mitogenic effect, the inhibitory effect of all TGF-β isoforms at higher concentrations on FBS-induced fibroblast proliferation was more prominent. These findings are in accordance with other reports demonstrating that TGF-β isoforms caused both proliferative and anti-proliferative effects depending on its concentration and on the presence of other mitogenic stimuli [13-15].

In our experiments the anti-proliferative effect of TGF-β was associated with increased secretion of PGE_2 *via* a COX-dependent mechanism. These findings are supported by the observation that impaired production of PGE_2 correlated with enhanced fibrogenesis in the lung, and that COX was protective against pulmonary fibrosis [39-44]. Furthermore, the proliferation of fibroblasts isolated from fibrotic human lungs could not be inhibited by TGF-β, and correlated with an impaired ability to synthesize PGE_2 [15, 45, 46]. In addition, in idiopathic pulmonary fibrosis COX-2 expression was down-regulated [47], and the respiratory epithelium from patients with aspirin-sensitive asthma have a diminished capacity to synthesize PGE_2 [48]. Considering these facts, it may be of clinical importance to boost the anti-proliferative signals initiated by TGF-β in early lung fibrosis, in particular the signaling pathway leading to PGE_2 synthesis.

The TGF-β signal transduction network involves receptor serine/threonine kinases and their substrates, the Smad proteins [19]. Smads mediate the growth-inhibitory effect of TGF-β in immortalized human cell lines and in rodent fibroblasts [13]. In contrast to these findings, the primary human lung fibroblasts used in this study expressed only very low levels of Smad2/3, indicating that in this cell type Smad proteins are not be the primary anti-proliferative signaling pathway of TGF-β.

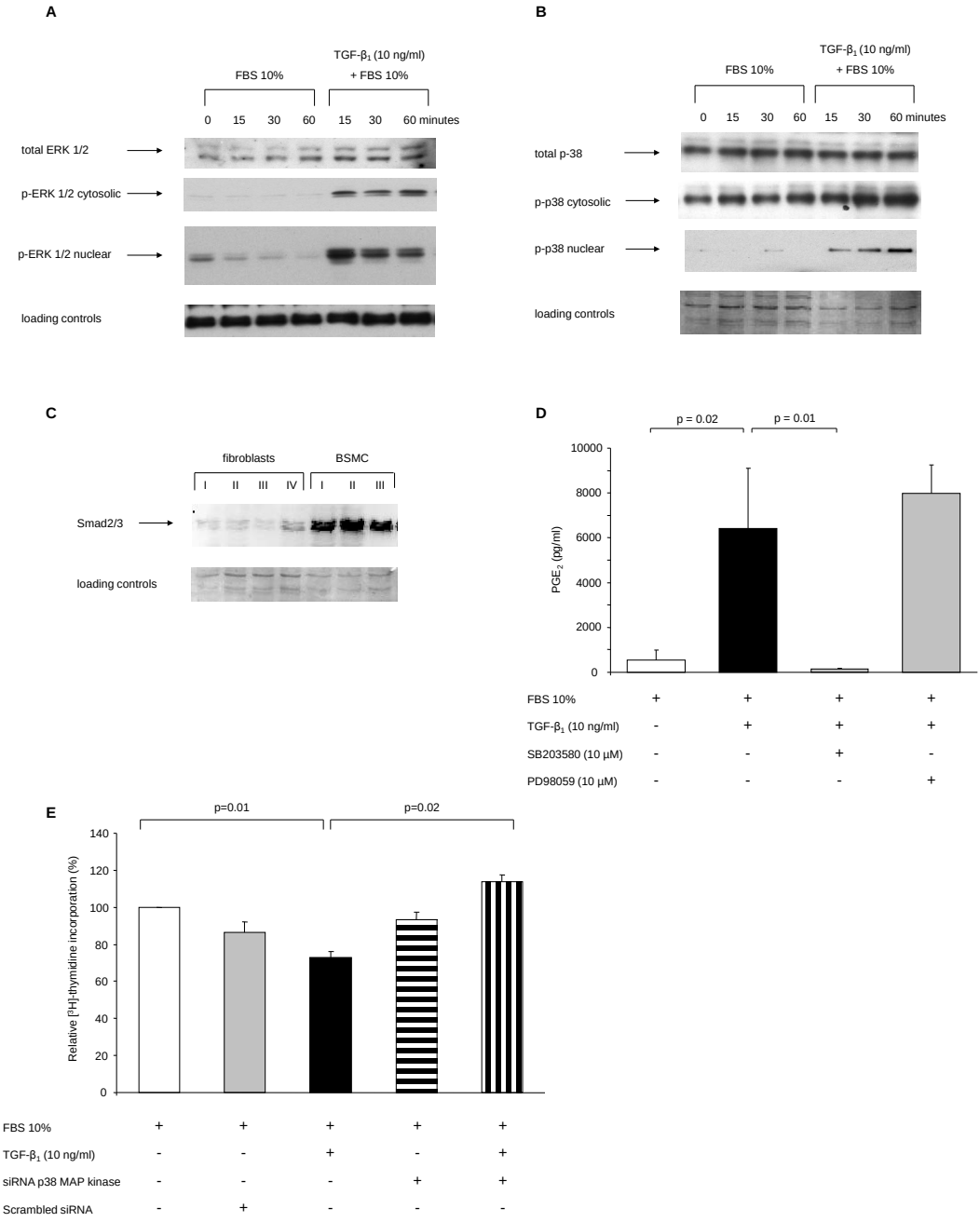

Fig. (4). TGF-β_1 activates ERK 1/2 and p38 MAP kinases. Western blots showing the effects of TGF-β_1 (10 ng/ml) on phosphorylation and localization of ERK 1/2 (**A**) and p38 MAP kinase (**B**) in FBS-stimulated primary human lung fibroblasts. Primary human lung fibroblasts (n=3) were incubated with serum-free DMEM for 24 hours and then stimulated with 10 ng/ml TGF-β_1 plus 10% FBS for 15, 30, or 60 minutes, respectively. Expression patterns of (phosphorylated) ERK 1/2 (**A**) and (phosphorylated) p38 MAP kinase (**B**) were examined in total protein extracts, cytosolic and nuclear fractions. (**C**) Western blots showing the expression of Smad2/3 in four different lung fibroblast cell lines and three different bronchial smooth muscle cell (BSMC) lines. Primary human lung fibroblasts and primary bronchial smooth muscle cells were incubated with serum-free DMEM for 24 hours, stimulated with DMEM plus 10% FBS for 24 hours and were then lysed at 4°C. (**D**) Effect of TGF-β_1 on PGE_2 release by fibroblasts in the absence (black bar) or presence (grey bars) of the p38 MAP kinase inhibitor SB203580 or the ERK 1/2 inhibitor PD98059, respectively, compared with control (open bar). Serum starved primary human lung fibroblasts were incubated for 24 hours with DMEM (plus FBS) containing 10 ng/ml TGF-β_1 in the presence or absence of 10 µM SB203580 or 10 µM PD98059, respectively. PGE_2 levels were measured in the cell culture medium by specific ELISA. Results are expressed as mean ± SEM of independent experiments with three different fibroblast cell lines. (**E**) Effect of TGF-β_1 on fibroblast proliferation in non-transfected (black bar) and transfected cells (vertically striped bar) compared with 10% FBS (first bar). Primary human lung fibroblasts (n=3) were transfected with p38 MAP kinase specific siRNA. After 26 hours fibroblasts were stimulated for another 24 hours with DMEM, 10% FBS containing 10 ng/ml TGF-β_1, or with DMEM, 10% FBS alone. Non-transfected fibroblasts served as control. Cell proliferation was assessed by [^{3}H]-thymidine incorporation, and expressed as relative proliferation compared with the control (DMEM plus 10% FBS). Values are presented as mean ± SEM of independent experiments performed in three different fibroblast cell lines.

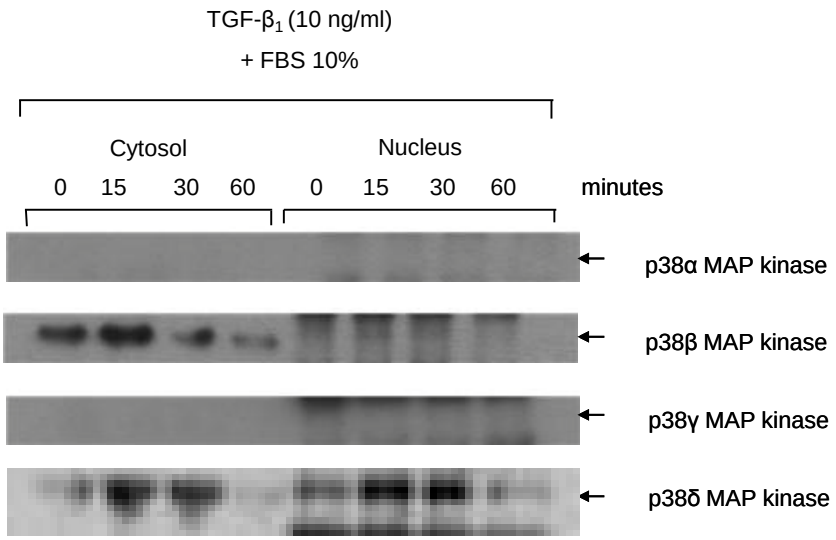

Fig. (5). TGF-β_1 activates the p38δ MAP kinase isoform. Effects of TGF-β_1 (10 ng/ml) on the localization of the p38 MAP kinase isoforms p38α, p38β, p38γ, and p38δ in FBS-stimulated primary human lung fibroblasts. Primary human lung fibroblasts (n=3) were incubated with serum-free DMEM for 24 hours and then stimulated with 10 ng/ml TGF-β_1 plus 10% FBS for 15, 30, or 60 minutes, respectively. Expression patterns of the four p38 MAP kinase isoforms were examined in cytosolic and nuclear fractions.

TGF-β activated ERK 1/2, JNK, and p38 MAP kinases [49]. JNK and p38 MAP kinase are activated by stress and link TGF-β to apoptosis [50, 51], whereas the ERK 1/2 pathway was associated with cell proliferation and protected from apoptosis [52]. We observed that TGF-β induced the secretion of PGE_2, which was associated with a rapid phosphorylation and nuclear translocation of both p38 and ERK 1/2 MAP kinases. Only the p38 MAP kinase pathway was involved in the PGE_2 release, because the effect was reversed by SB203580. Our data are in accordance with those found in mouse vascular smooth muscle cells [53] and in a human breast cancer epithelial cell line [54], which demonstrated that p38 MAP kinase mediated the anti-proliferative effect of TGF-β.

The expression pattern of the four different p38 MAP kinase isoforms (α, β, γ, and δ) is cell type and tissue specific, and each of the isoforms may have distinct functions [55]. Importantly, elevated expression of the p38δ MAP kinase isoform was found in lung tissue [56]. In murine mesangial cells p38δ MAP kinase mediated TGF-β_1-induced vascular endothelial growth factor expression [57], and there is evidence for a p38δ-ERK 1/2 complex formation [58]. In this study, we showed that TGF-β_1 only activated the p38δ MAP kinase, but not the p38α, -β, and –γ isoforms. A selective activation of the p38δ MAP kinase pathway to block proliferation of fibroblasts may therefore be a novel strategy to control fibroproliferative lung diseases.

In conclusion, TGF-β may elicit both pro- and anti-proliferative effects on fibroblast. The ultimate effect of TGF-β depended on additional inflammatory, pro-proliferative and pro-fibrotic signals in the microenvironment. The anti-proliferative effect was mediated by PGE_2 and required the activation of p38δ MAP kinase. Based on these findings and on our previous studies [16], we propose that the anti-proliferative effect of TGF-β represents a control feedback loop to counteract an overshooting fibrogenic stimulation, and prevents further remodeling and/or tissue fibrosis. Failure of such intrinsic regulatory mechanism operating in the epithelial-mesenchymal trophic unit could lead to uncontrolled fibroblast proliferation and may result in ongoing tissue remodeling and/or tissue fibrosis. Therefore, boosting the anti-proliferative TGF-β/p38δ MAP kinase signaling cascade might be a powerful way to intervene in early lung tissue remodeling and fibrosis.

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