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Mechanisms of Induction of Airway Smooth Muscle Hyperplasia by Transforming Growth Factor-β

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Abstract

Airway smooth muscle (ASM) hyperplasia is a characteristic feature of the asthmatic airway but the underlying mechanisms that induce ASM hyperplasia remain unknown. Because transforming growth factor (TGF)-β is a potent regulator of ASM cell proliferation, we determined its expression and mitogenic signaling pathways in ASM cells. We obtained ASM cells by laser capture microdissection of bronchial biopsies and found that ASM cells from asthmatic patients expressed TGF-β1 mRNA and protein to a greater extent than non-asthmatic individuals using real-time RT-PCR and immunohistochemistry, respectively. TGF-β1 stimulated the growth of non-confluent and confluent ASM cells either in the presence or absence of serum in a time- and concentration-dependent manner. The mitogenic activity of TGF-β1 on ASM cells was inhibited by selective inhibitors of TGF-β receptor-I kinase (SD-208), of phosphatidylinositol 3-kinase (PI3K, LY294002), ERK (PD98059), JNK (SP600125) and NF-κB (AS602868). On the other hand, p38 MAPK inhibitor (SB203580) augmented TGF-β1-induced proliferation. To study role of the Smads, we transduced ASM cells with an adenovirus vector expressing Smad 4, Smad 7 or negative dominant Smad3 and found no involvement of these Smads in TGF-β1-induced proliferation. Dexamethasone caused a dose-dependent inhibition in TGF-β1-induced proliferation. Our findings suggest that TGF-β1 may act in an autocrine fashion to induce ASM hyperplasia, mediated by its receptor and several kinases including PI3K, ERK and JNK, while p38 MAPK is a negative regulator. NF-κB is also involved in the TGF-β1 mitogenic signaling but Smad pathway does not appear important.

Keywords

Laser capture microdissection; TGF-β1 expression; airway smooth muscle cells; asthma; corticosteroids

Introduction

Increased airway smooth muscle (ASM) mass is one of characteristic structural changes of the asthmatic airway (11) that may contribute to the airflow obstruction of asthma (12). Indeed, both ASM hyperplasia and hypertrophy are observed in the airway wall remodeling present in asthma (12). The mechanisms that mediate these changes in ASM are little known. Transforming growth factor (TGF)-β is a regulator of cell growth and differentiation with pro-fibrotic properties (10;32). Increased expression of TGF-β has been detected in asthmatic bronchial biopsy specimens localised to inflammatory cells in airway mucosa such as eosinophils and to structural cells such as fibroblasts and epithelial cells; increased levels have also been measured in bronchoalveolar lavage fluid samples from patients with asthma (23; 31;35). There has been little focus as to whether ASM cells from asthmatic patients have

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enhanced TGF-β expression (35). Blockade of TGF-β activity prevents airway wall remodeling features including airway smooth muscle hyperplasia in rat and murine models of chronic allergen exposure (19;21), implying that TGF-β may be a potential mediator of ASM hyperplasia in asthma. TGF-β stimulates human ASM cell growth in the presence of 5% FCS and EGF (9), and increases DNA synthesis of human or bovine ASM cells in the presence or absence of serum in a growth factor-independent manner (2;7;25). Paradoxically, TGF-β has been reported to inhibit EGF- and thrombin-induced DNA synthesis of human ASM cells (8) and FBS-induced proliferation of bovine ASM cells (25). Furthermore, TGF-β has also been shown to induce human ASM cell proliferation only in the presence of growth factors such as FGF-2 (3). These conflicting data may result from the use of different culture conditions, such as the presence of serum at varying concentrations, the degree of cell confluence and the addition of different growth inducers.

The intracellular mechanisms that mediate TGF β-induced ASM cell proliferation have been the subject of recent investigation, but there are still areas of uncertainty. Phosphatidylinositol 3-kinase (PI3K) and MAPK may play an important role in the regulation of ASM growth (18;26), since phosphorylation of the D3 position of the inositol ring of membrane phosphoinositides by PI3-K is crucial for control of cell survival, division and migration. PI3K has been implicated both in induction of cell growth and regulation of cyclin-dependent kinase activity. ERK plays an important role in ASM cell proliferation as well as in survival mediated by many growth factors, while JNK and p38 MAPK may regulate ASM cell growth and apoptosis (7). A role for Smads in mediating TGF-β intracellular signaling has been identified in the induction of gene expression, cell proliferation and differentiation (5;15;16;24) but it is uncertain whether Smads mediate TGF-β-induced ASM cell growth. The ubiquitous inflammatory transcription factor, NF-κB, modulates TGF-β expression in airway epithelial cells and can functionally cooperation with TGF-β/Smad signaling pathway (22). However, the role of NF-κB in TGF-β-mediated ASM hyperplasia is not known.

In this study, we determined whether there is an increased TGF-β1 expression in asthmatic ASM cells and its effects on ASM cell growth under different culture conditions, particularly in relation to the state of confluence of ASM cells in the absence or presence of a range of serum concentrations. In order to delineate the intracellular signaling pathways that mediate the growth regulatory effect of TGF-β1, we investigated the role of TGF-β receptor (TβR) kinase, PI3K and MAPKs, and explored whether NF-κB and Smads were involved in the mitogenic signaling.

Materials and Methods

Materials

Recombinant human TGF-β1 was purchased from R&D Systems (Abingdon, UK). PD98059, SB203580 and LY294002 were obtained from Calbiochem (Nottingham, UK). SD-208 was a kind gift from Scios (Fremont, CA) and SP600125 from Celgene (San Diego, CA). AS602868 was from Serono. Primers for TGF-β1 were obtained from Sigma Genosys (Cambridgeshire, UK). RNase-free slides, reagents and other materials for laser capture microdissection (LCM) were purchased from Arcturus (Hertfordshire, UK). Dexamethasone, crystal violet, Dimethylthiazolyldiphenyl-tetrazolium bromide (MTT), FCS, BSA and all other tissue culture reagents and media were form Sigma (Dorset, UK).

Cell culture and treatment

ASM cells were isolated from fresh lobar or main bronchus obtained from lung resection donors and cultured in DMEM supplemented with 10% FCS as described previously (39). ASM cell characteristics were identified by light microscopy with typical ‘hill and valley’ appearance

and by positive immunostaining of smooth muscle (SM) α-actin, SM myosin heavy chain, calponin and SM-22. The cells were maintained in T175 culture flasks at 37°C in a humidified atmosphere of 5% CO_2. ASM cells were studied from passages two to six.

Cells were trypsinized and subcultured in 24-well plates for cell proliferation assay. ASM cells were grown in 10% FCS/DMEM to reach 30% confluence, then FCS concentration was changed to 0.1∼10% in the presence or absence of TGF-β1 or the appropriate test reagents. To test the effect of TGF-β1 on confluent ASM cells, cells were grown in 10% FCS/DMEM to reach confluence before the treatments. For protein analyses by Western blotting, ASM cells were incubated in 6-well plates in 10% FCS/DMEM to reach confluence before the treatments. Control cultures were incubated in the same medium containing vehicle alone. Cells were re-dosed in fresh medium every 2-3 days.

Laser capture microdissection

To examine the expression of TGF-β1 in ASM, bronchial biopsies were obtained from 12 normal volunteers (age=22±3 years, male/female=8/4, FEV_1=101±15% of predicted) and 11 asthmatic patients (age=33±12 years, male/female=5/6, FEV_1=84±18% of predicted), using fiberoptic bronchoscopy (33). The protocols have been approved by the local Ethics Committee and all subjects gave their informed consent. The airway biopsies were embedded in Optimum Cutting Temperature compound (OCT) on dry ice and snap-frozen in liquid nitrogen before storage at −80°C. Frozen sections were cut at 6 μm thickness and mounted on LCM slides (Arcturus). The slides were immediately stored on dry ice and then at −80°C until used. Sections were fixed in 70% ethanol for 30 seconds, and stained and dehydrated in a series of graded ethanol followed by xylene using HistoGene LCM frozen section staining kit (Arcturus) according to the manufacturer's instruction. ASM cells were captured onto the CapSure HS LCM caps (Arcturus) by a Pixcell II Laser Capture Microdissection System (Arcturus, Mountain View, CA) and total RNA was extracted by using a PicoPure RNA isolation kit (Arcturus) according to the manufacturer's instructions.

Real-time PCR

Total RNA extracted from ASM cells collected by LCM was reverse transcribed to cDNA (RoboCycler, Stratagene, USA) using random hexamers and an avian myeloblastosis virus reverse transcriptase (Promega). cDNA was amplified by quantitative real-time PCR (Rotor Gene 3000, Corbett Research, Australia) using SYBR Green PCR Master Mix Reagent (Qiagen). The human TGF-β1 forward and reverse primers were 5'-CCCAGCATCTGCAAAGCTC-3' and 5'-GTCAATGTACAGCTGCCGCA-3'. Each primer was used at a concentration of 0.5 μM in each reaction. Cycling conditions were as follows: step 1, 15 min at 95°C; step 2, 20 sec at 94°C; step3, 20 sec at 60°C; step 4, 20 sec at 72°C, with repeat from step 2 to step 4 for 40 times. Data from the reaction were collected and analysed by the complementary computer software (Corbett Research, Australia). Relative quantitations of gene expression were calculated using standard curve and normalized to GAPDH in each sample (39).

Immunohistochemistry

Immunohistochemistry was performed to detect the expression of TGF-β1 in human bronchial tissue sections. Bronchial biopsies were embedded in OCT and stored at −80°C before use. 6 μm frozen sections were cut and then fixed in cold acetone for 10 min. Sections were incubated in 10% normal horse serum to block non-specific binding, followed by a mouse anti-human TGF-β1 antibody (1 μg/ml, AbCam ab1279) for 1 h at room temperature. Control slides were performed with normal mouse immunoglobulin. Anti-mouse biotinylated secondary antibody (Vector ABC Kit, Vector Laboratories) was applied to the sections for 1 h at room temperature, followed by 1.6% hydrogen peroxide to block endogenous peroxidase activity. Sections were

incubated with the avidin/biotinylated peroxidase complex for 30 min, followed by chromogenic substrate diaminobenzidine for 3 min, and then counterstained in haematoxylin and mounted on aqueous mounting medium. Immunoreactivity for TGF-β1 was expressed as intensity of staining that was graded from 0 to 3 (0: no staining, 1: weak staining, 2: moderate staining, 3: strong staining). The slides were coded and the reader was unaware of the source of the biopsies.

Crystal violet proliferation assay

Crystal violet assay (CVA) was used to determine ASM cell growth (40). On selected days, the cell layer was washed once with PBS. Cell were fixed and stained by 0.5% crystal violet solution in 25% methanol. After 10 min, the excess dye was removed by washing in tap water and the cells were then air-dried for at least 20 h. Image processing was performed using an Axioplan microscope (Zeiss GmbH, Munich, Germany) and images were captured using an Axiocam digital camera (Zeiss GmbH). Then, the incorporated dye was solubilized in 1 ml of 0.1 M sodium citrate solution in 50% ethanol and 100 μl was transferred to a 96-well plate. In order to determine cell number in each sample, the optical density (OD) was measured directly at a wavelength of 550 nm in a Spectramax Plus reader (Molecular Devices). The OD of each sample was then compared with a standard curve, in which the OD was directly proportional to known cell number.

MTT assay

MTT assay was used to determine cell viability and proliferation. On selected days, the culture medium was removed and ASM cells were incubated with 1 mg/ml MTT solution in an incubator at 37°C for 10-30 min. After removing MTT solution, 300 μl DMSO were added to each well of 24-well plate and 100 μl was transferred to a 96-well plate. Absorbency at OD550nm was detected. The OD of each sample was then compared with a standard curve, in which the OD was directly proportional to known cell numbers.

Smad transduction

Smad expressing adenoviruses were a kind gift from Dr Aristidis Moustakas (17). Adenoviruses carrying the vector expressing Flag-tagged Smad4, Smad7, dominant-negative Smad3 (DNS3) or β-galactosidase (the null) were titered by endpoint dilution and plaque assay to determine plaque forming units. Viruses were diluted in 10% FBS/DMEM to a multiplicity of infection of 30 (30 viruses/per cell) before infection of ASM cells. This dose of virus had no effect on cell viability and ~95% transduction efficiencies in ASM cells was obtained as detected by green fluorescent protein expressing adenoviruses (6). The adenovirus-mediated expression of Smads in the infected ASM cells was confirmed by Western blots using an anti-Flag monoclonal antibody (Sigma). In cell growth assays, the adenovirus-containing medium was removed 24 h after infection and cells were stimulated with TGF-β1 in the presence of 2.5% FCS/DMEM. For induction of the expression of connective tissue growth factor (CTGF), which was used as a positive control in this study as it is a target gene of TGF-β, the adenovirus-containing medium was removed 24 h after infection and cells were stimulated with TGF-β1 under serum-free condition for a further 3 days (39).

Western blotting

As described previously (39), total cell protein was extracted and fractionated by SDS-PAGE on a 10% tris-glycine precast gel (Invitrogen), followed by transferring to a nitrocellulose membrane (Amersham). The membrane was incubated overnight at 4°C with an antibody for CTGF (0.5 μg/ml, Abcam). The next day, the membrane was incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody raised against rabbit IgG (1:2000, Cell Signaling Technology) at room temperature. Antibody-bound proteins were visualised by

enhanced chemiluminescence. The membranes were stripped and then reprobed with a mouse anti-GAPDH monoclonal antibody (1:5000, Biogenesis, Poole, UK) in order to control for the loading. Relevant band intensities were quantified by scanning densitometric analysis using software from Ultra-Violet Products (Cambridge, UK). Densitometric data were normalized for GAPDH values.

Data analysis

Data were analysed by ANOVA or t-test (Mann-Whitney U test for immunohistochemistry). Results are expressed as mean ± SD and are representative of at least three separate experiments from three ASM cell donors. $P<0.05$ was taken as statistically significant.

Results

TGF-β1 mRNA and protein expression in ASM cells of asthmatic patients

To determine whether human ASM cells express TGF-β1 mRNA *in-situ*, LCM was performed on sections of human bronchial biopsies obtained from four normal volunteers and three asthmatic patients. Quantitative real-time RT-PCR revealed that *in-situ* ASM cells from asthmatics expressed higher levels of TGF-β1 mRNA than those from non-asthmatic volunteers ($P= 0.029$) (Figure 1A).

Immunohistochemistry of human bronchial biopsy samples (obtained from 10 normal and 8 asthmatic donors) showed weak intensity of immunostaining for TGF-β1 in ASM cells of control samples (Figure 1B). In comparison with the controls, TGF-β1 expression of ASM cells was significant increased in asthmatic patients ($P=0.002$) (Figure 1B & C). There was no staining in the negative control sections in which the mouse anti-TGF-β1 antibody was replaced by normal mouse immunoglobulin (data not shown).

TGF-β1 and ASM cell growth

1) TGF-β1 stimulates non-confluent ASM cell growth in serum-containing media—To investigate the effect of TGF-β1 on growth of non-confluent ASM cells undergoing an exponential growth, cells were incubated in 24-well plates with 10% FCS to 30% confluence, and then exposed to TGF-β1 (10 ng/ml) in the presence of 0.1% to 10% FCS. Cell growth (Figure 2A & B) was detected after 6 days of treatment by CVA. TGF-β1 increased ASM cell growth by 2- to 5-fold in the presence of 0.1-5% FCS with no effect at 10% FCS in comparison with the controls (Figure 2B). Similar results were obtained using MTT assay (data not shown). The mitogenic effect of TGF-β1 was time-dependent, was evident after 3 days of the treatment and maintained until day 7 (Figure 2C). In the absence of TGF-β1, cells were reduced with 0.1% to 0.5% FCS after 3-5 days and cell growth was almost stopped with 1% FCS over 3-7 days (Figure 2C). However, in the presence of 2.5% FCS, ASM cells not only had a marked growth-stimulatory response to TGF-β1 but also kept an autonomous growth. Therefore, 2.5% FCS was chosen for subsequent studies. The effect of TGF-β1 on ASM cell growth was concentration-dependent over the range 0.1-10 ng/ml with 2.5% FCS after 5 days of treatment (Figure 2D).

2) TGF-β1 stimulates non-confluent ASM cell growth in serum-free medium—ASM cells were incubated in 24-well plates with 10% FCS to 30% confluence, and then treated with 10 ng/ml TGF-β1 in serum-free medium with 0.5% BSA. Cell growth was detected after 3-7 days of the treatment. TGF-β1 in the absence of serum induced a 30-40% growth stimulation after 5 days of treatment and this was maintained until day 7 (Figure 3A).

3) TGF-β1 stimulates confluent ASM cell growth in the presence or absence of serum—We next examined the effect of TGF-β1 on confluent ASM cells as most of the

previous studies have been reported on confluent cells. ASM cells were grown in 24-well plates with 10% FCS to confluence, and then exposed to 10 ng/ml TGF-β1 in the presence of 0.5% or 2.5% FCS or 0.5% BSA. Cell number was detected after 2-6 days of the treatment. There was 35-70% growth stimulation with 0.5% FCS, 35-50% with 0.5% BSA and up to 25% with 2.5% FCS following 2-6 days of treatment (Figure 3B). The stimulatory effect of TGF-β1 was almost negligible when ASM cells were too confluent such as after 6 days in 2.5% FCS/DMEM.

Mediation of TGF-β1-induced ASM cell growth by TGF-β receptor-I kinase and PI3K

ASM cells were grown in 24-well plates with 10% FCS to 30% confluence and were pre-treated for 1 h with either a selective inhibitor for TβRI kinase, SD-208 (0.1-1 μM) (34), or for PI3K, LY294002 (1-10 μM) in 2.5% FCS/DMEM, and then co-treated with 5 ng/ml TGF-β1 for 6 days before detecting cell growth. Both SD-208 (Figure 4A) and LY294002 (Figure 4B) induced a concentration-dependent inhibition in TGF-β1-stimulated cell growth. However, LY294002 also inhibited autonomous growth at 10 μM, the highest concentration used.

Regulation TGF-β1-induced ASM cell growth by MAPKs, NF-κB and GR agonist

ASM cells were pre-treated for 1 h with specific inhibitors for MAPKs (PD98059 for ERK, SP600125 for JNK, SB203580 for p38 MAPK) and for NF-κB (AS602868 for IKK2) or with the glucocorticosteroid receptor (GR) agonist, dexamethasone, and then co-treated with 5 ng/ml TGF-β1 for 6 days before assessing cell growth. PD98059 (1-50 μm) inhibited TGF-β1-induced growth in a dose-dependent manner with a significant effect at ≥ 10 μM (Figure 5A). SP600125 also inhibited the mitogenic activity of TGF-β1 but a significant effect was achieved at ≥ 25 μM (Figure 5B). In contrast, SB203580 induced a concentration-dependent increase in TGF-β1-stimulated cell growth with a 45% maximal enhancement (Figure 5C). The IKK2 inhibitor, AS602869, significantly inhibited the ASM cell growth by TGF-β1 at 2.5 μM (Figure 6A). Dexamethasone (0.01-1 μM) down-regulated TGF-β1-stimulated ASM cell growth down to 40% (Fig 6B).

TGF-β1-induced ASM cell growth is Smad-independent

ASM cells were grown to 30-40% confluence with 10% FCS, and were infected with the Smad-expressing adenoviruses for 24 h in 2.5% FCS/DMEM. Cell growth was assessed after 4-6 days of the treatment with TGF-β1 (5 ng/ml) in fresh 2.5% FCS/DMEM (Figure 7A). Transfection of Smad4, Smad7 or DNS3 did not affect the TGF-β1-stimulated ASM cell growth after 4 days of treatment (Figure 7A). Similar results were seen after 6 day treatment with the growth factor (data not shown). To determine the efficacy of the transfection, we show that inhibition of Smad signaling by infection of cells with DNS3 or Smad7 expressing virus down-regulated TGF-β1-induced CTGF protein expression, and enhancement of Smad signaling by introducing increasing amounts of adenovirus-mediated Smad4 into cells up-regulated the CTGF expression as analysed by Western blotting (Figure 7B).

Discussion

In this study, we analyzed TGF-β1 mRNA expression of *in-situ* bronchial ASM cells collected by laser capture microdissection and found that the levels of expression were increased in patients with asthma compared to non-asthma controls. This is also accompanied by an increase in TGF-β1 immunoreactive protein expression in asthmatic ASM. In order to elucidate the effect of TGF-β1 on ASM hyperplasia, we examined its activity on ASM cell proliferation. TGF-β1 stimulated ASM cell growth that is mediated through TβR-I kinase and by the activation of a series of other kinases including PI3K, ERK and JNK. Activation of p38 MAPK was associated with an inhibition of TGF-β1-induced growth. The mitogenic signaling of TGF-β1 also involved the inflammatory transcription factor, NF-κB, but is not dependent on the elements of Smad pathway.

Previous studies have reported conflicting effects of TGF-β1 on ASM cell proliferation (2;3; 7-9), which may be related to different culture conditions used. Our study shows that TGF-β1 retains its proliferative effects under different culture conditions, such as the degree of confluence or the presence of varying concentrations of FCS (0.1-5% FCS) or absence of serum without addition of any other growth factor or stimulator. Treatment of non-confluent ASM cells with TGF-β1 stimulated a 2 to 5-fold increase in cell growth in the presence of 0.1-5% FCS, and the effect was time-and concentration-dependent. Our data support the study by Black *et al*. who showed that TGF-β increased non-confluent bovine ASM cell number in the presence of 2% FBS (2). TGF-β has also been reported to increase cell growth and DNA synthesis of confluent ASM cells at lower serum concentrations (7). We have shown that TGF-β1 stimulated up to 70% increase in confluent ASM cell growth with lower serum concentrations. The mitogenic effect of TGF-β1 on non-confluent cells was greater than on confluent cells, which may be due to the higher growth-rate of non-confluent cells undergoing exponential growth; in addition, TGF-β released by non-confluent ASM cells is much higher than confluent cells (10). TGF-β1 also stimulated non-confluent and confluent ASM cell growth in serum-free medium as has been previously reported (7;25) but the activity is lower than that in serum-containing medium. Although the focus of this work is on hyperplasia, we also observed ASM hypertrophy when ASM cells were treated after 2-3 days in the absence of serum, as has been recently reported (14).

TGF-β signal transduction is first initiated by binding to two cell membrane serine-threonine kinase receptors, termed TβRI and TβRII, followed by their phosphorylation. Two TβRII subunits phosphorylate or activate two TβRI. TGF-β1-stimulated ASM cell growth is directly mediated by its receptor, as inhibition of TβR1 kinase by SD-208 blocked the mitogenic effect of TGF-β1, indicating that the phosphorylation of TβR1 by TGF-β1 is necessary for the mitogenic signalling. The Smad family of proteins are the primary substrates of the phosphorylated TβR1. Phosphorylation of Smad2 and Smad3 leads to the formation of heteromeric complexes with Smad4. These complexes then translocate to the nucleus and regulate gene transcription by binding DNA directly or in association with other transcriptional factors, while Smad7 acts as negatively regulator of the Smad signaling (24). The Smad pathway is involved in TGF-β-stimulated vascular smooth muscle cell growth (16) and mediates TGF-β-enhanced serum response factor-dependent transcription in ASM cells (4). We have previously shown that TGF-β1 induces Smad2/3 phosphorylation and CTGF expression in ASM cells (39), but, in the present study, inhibition of Smad signaling by the virus-mediated expression of DNS3 or Smad7, or activation through the expression of a constitutively active Smad4 did not affect TGF-β1-induced cell growth but regulated TGF-β1-induced CTGF expression. This indicates that TGF-β1-induced ASM cell growth is mediated through Smad-independent pathways. TGF-β has also been shown to activate PAK2 (p21-activated kinase-2) through Smad-independent signaling pathway in the growth-stimulated fibroblastic cells (37).

Evidence from a previous report showed that TGF-β1 activates PI3K via its type I receptor (41), and that PI3K up-regulates cyclin D1 expression in ASM cells (28) and mediates mitogen-induced ASM cell proliferation (18). We also found that PI3K is essential for both TGF-β1-induced and autonomous ASM cell growth because TGF-β1-stimulated ASM cell growth was completely inhibited by the PI3K blocker, LY294002, which also decreased the autonomous cell growth. MAPK pathways have been implicated in both positive and negative regulation of TGF-β signaling (7;38). TGF-β induces the activation of ERK, JNK and p38 MAPK pathways in ASM cells (7) possibly through the upstream mediators RhoA, Ras and via TGF-β activated kinase (1). We showed that ERK and JNK pathways positively regulated TGF-β1-induced ASM cell growth as inhibition of these kinase activation by the specific inhibitors PD98059 and SP25600 down-regulated the mitogenic activity of TGF-β1. ERK is a mitogenic signaling pathway for thrombin and basic FGF as well as TGF-β1 in ASM cells (7;30), and

JNK has also been shown to mediate TGF-β1-induced expression of the target gene, CTGF, in ASM cells (39). Interestingly, inhibition of p38 MAPK activity by the specific inhibitor, SB203580, enhanced TGF-β1-induced ASM cell growth, which may be associated with the observation that p38 MAPK negatively regulates cyclin D1 expression in ASM cells (27). On the other hand, ERK increased cyclin D1 expression in ASM cells (29), illustrating the differential involvement of the different MAPK pathways in TGF-β-induced growth regulation in ASM cells.

TGF-β may activate the inflammatory transcription factor NF-κB (20) and TGF-β signaling has functional co-operation with NF-κB signaling (22). For the first time, we found that inhibition of NF-κB activation by the IKK2 inhibitor, AS602869, significantly attenuated TGF-β1-stimulated cell growth, indicating that NF-κB signaling is required for the TGF-β1 mitogenic activity.

Corticosteroids are anti-inflammatory drugs used for the treatment of asthma, and inhibit mitogen-stimulated ASM cell proliferation (13;36). We observed marked down-regulation of TGF-β1-stimulated ASM cell growth by the glucocorticoid, dexamethasone, an effect that could occur through reduction of cyclin D1 levels and inhibition of pRb phosphorylation (13). However, one of the mechanisms could be through the inhibition of NF-κB activation. Thus, corticosteroid treatment may lead to inhibition of ASM hyperplasia in asthma, an effect that has yet to be confirmed *in vivo* in asthmatic patients treated with inhaled corticosteroids.

In conclusion, increased TGF-β1 expression is observed in ASM of patients with asthma. TGF-β1 induces ASM hyperplasia that is initially mediated by membrane TβR via TβR1 phosphorylation and regulated positively by downstream kinases, PI3K, ERK and JNK, and negatively by p38 MAPK. We also provide evidence that TGF-β1 mitogenic signaling is through NF-κB-dependent but Smad-independent pathways. We speculate that blockage of TGF-β1 activity or signaling may be a therapeutic strategy in asthma to inhibit ASM hyperplasia.

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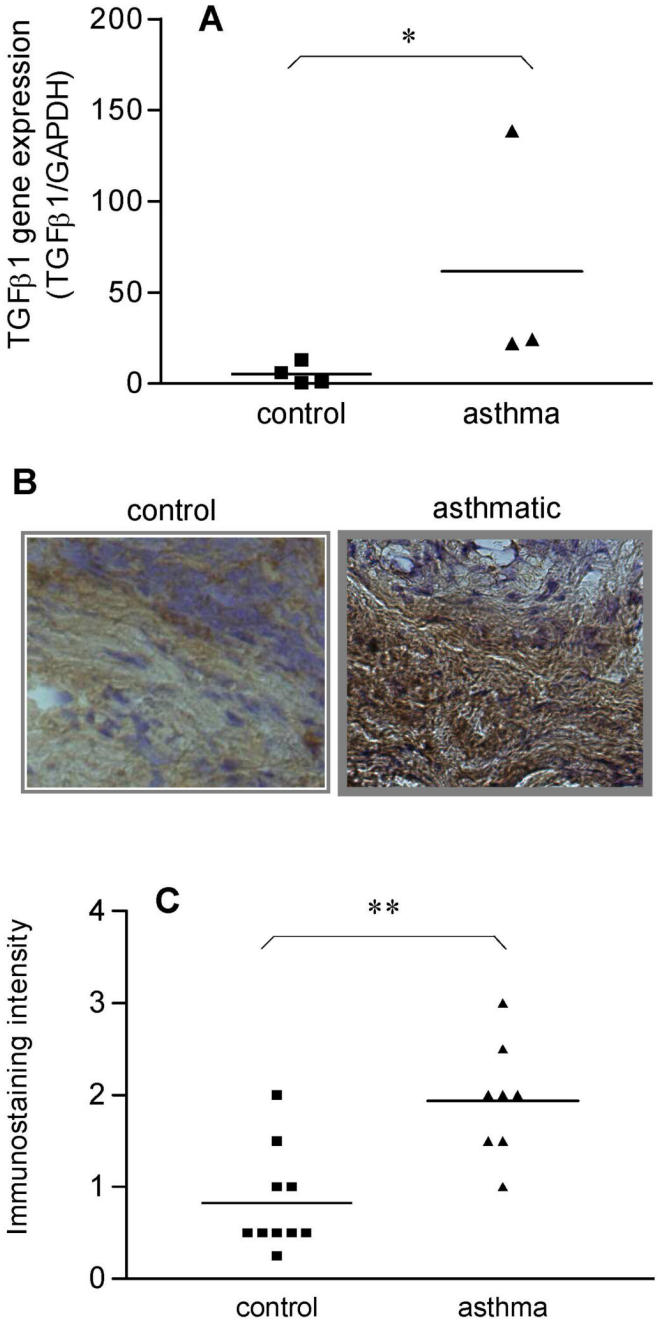

Fig 1.

Increased expression of TGF-β1 mRNA and protein in asthmatic ASM cells. Sections from human bronchial biopsies were prepared. (A) LCM was performed to collect ASM cells. TGF-β1 and GAPDH mRNA expression was analysed by real-time RT-PCR from 4 normal controls and 3 asthmatics. Data were expressed as a ratio of target gene to GAPDH mRNA control. (B) TGF-β1 protein was detected by Immunohistochemistry. (C) Immunostaining intensity was detected from 10 normal controls and 8 asthmatics. *P<0.05, **P<0.01 compared with control.

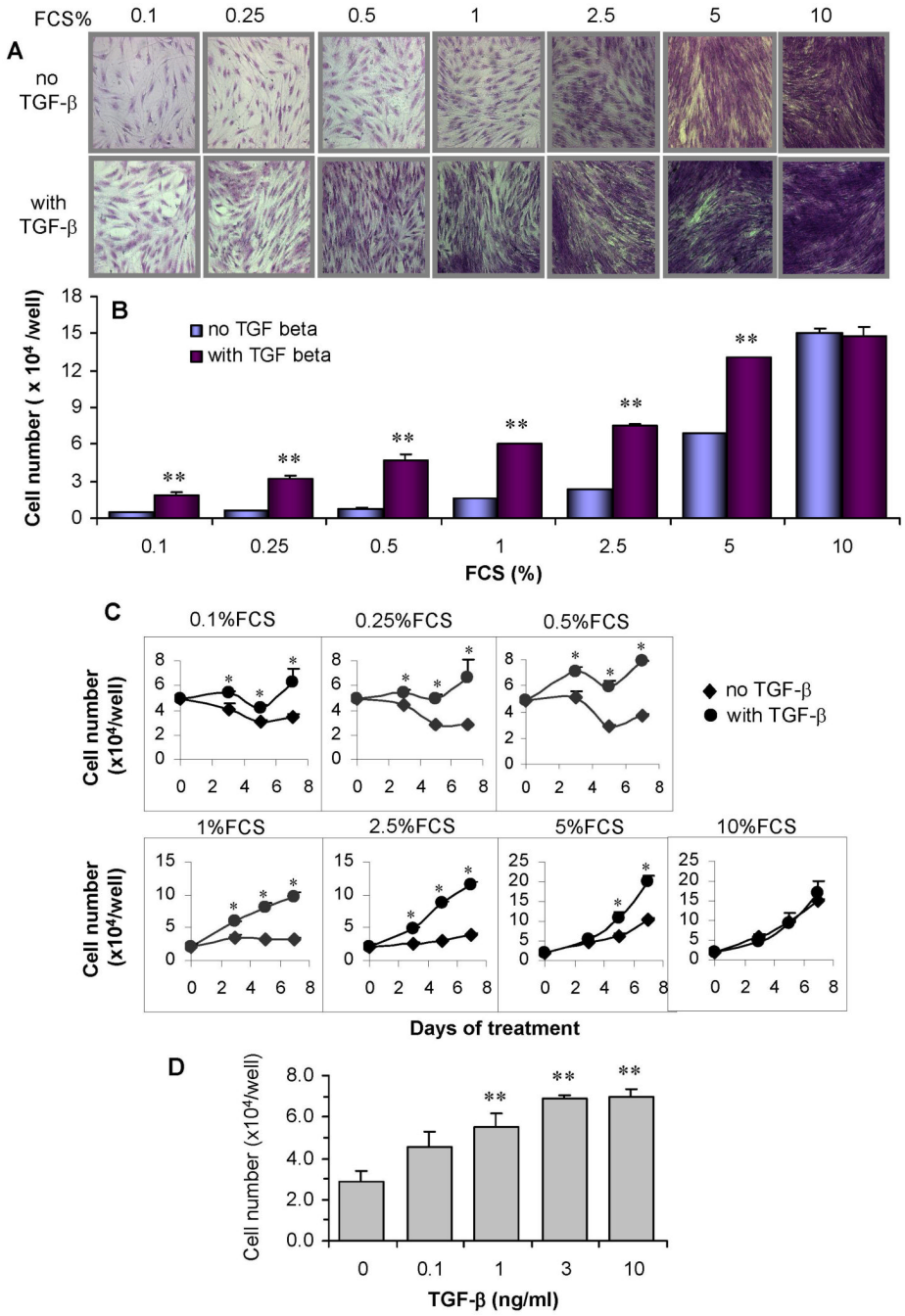

Fig 2.

Stimulation of non-confluent ASM cell growth by TGF-β1 in the presence of serum. (A) Image of ASM cells stained by crystal violet and (B) data from CVA after non-confluent ASM cells were incubated with 0.1 to 10% FCS in the presence or absence of TGF-β1 (10 ng/ml) for 6 days. (C) Time-dependent stimulation by 10 ng/ml TGF-β1 with 0.1-10% FCS for 3-7 days. (D) Concentration-dependent stimulation by TGF-β1 (0.1-10 ng/ml) with 2.5% FCS for 5 days. Cell growth was assessed by CVA. Results are the mean ± SD of triplicate measurements and representative from 3-5 ASM cell donors. *P<0.05, **P<0.01 compared with no TGF-β.

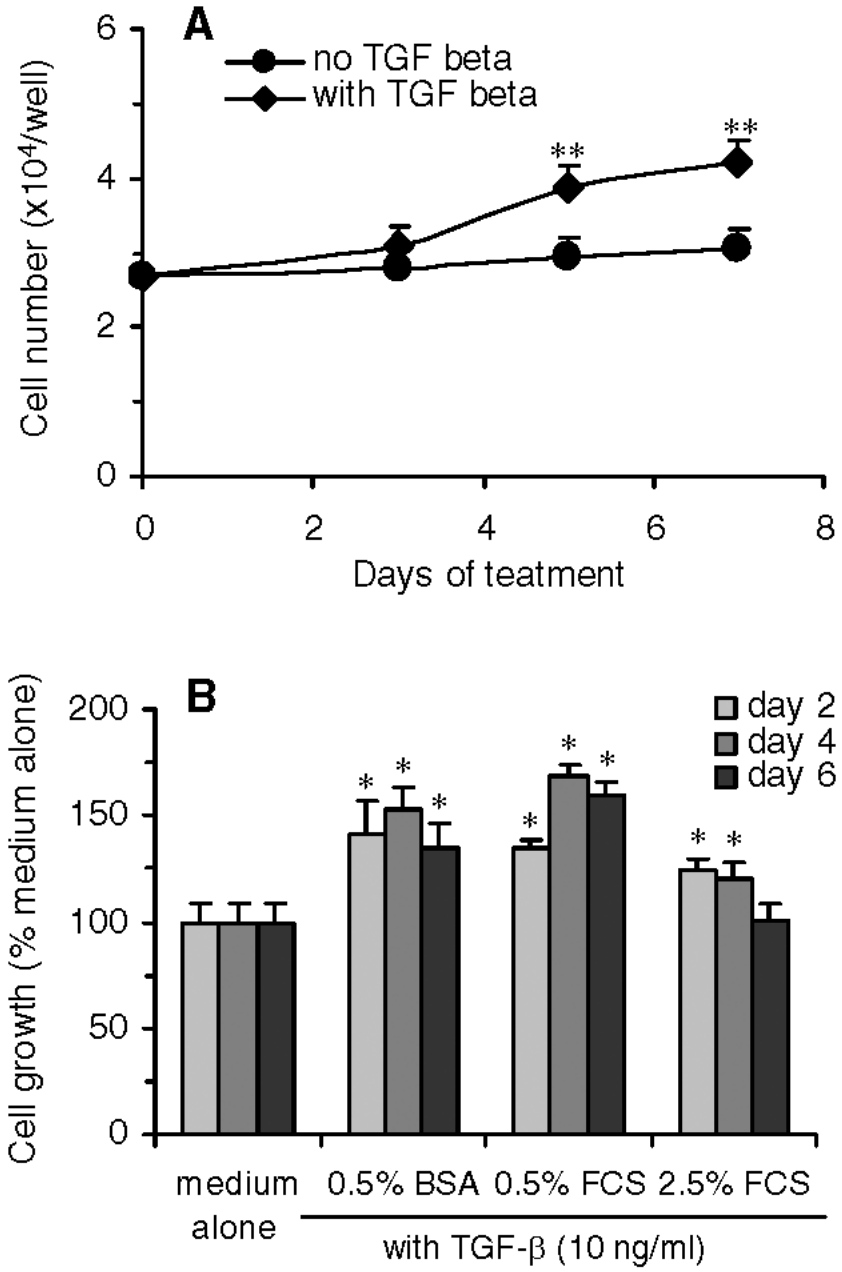

Fig 3.

Stimulation of non-confluent and confluent ASM cell growth by TGF-β1 in the presence or absence of serum. (A) Non-confluent ASM cells were treated with 10 ng/ml TGF-β1 for 3-7 days in serum-free medium containing 0.5% BSA. (B) ASM cells were grown in 10% FCS/DMEM to confluence and then incubated in 0.5% BSA, 0.5% FCS or 2.5% FCS/DMEM with 10 ng/ml TGF-β1 for 2-6 days. Cell growth was assessed by CVA. Results are the mean ± SD of triplicate measurements and representative from three ASM cell donors. The data are expressed as the percentage of the medium alone for (B). *P<0.05, **P<0.01 compared with no TGF-β or medium alone.

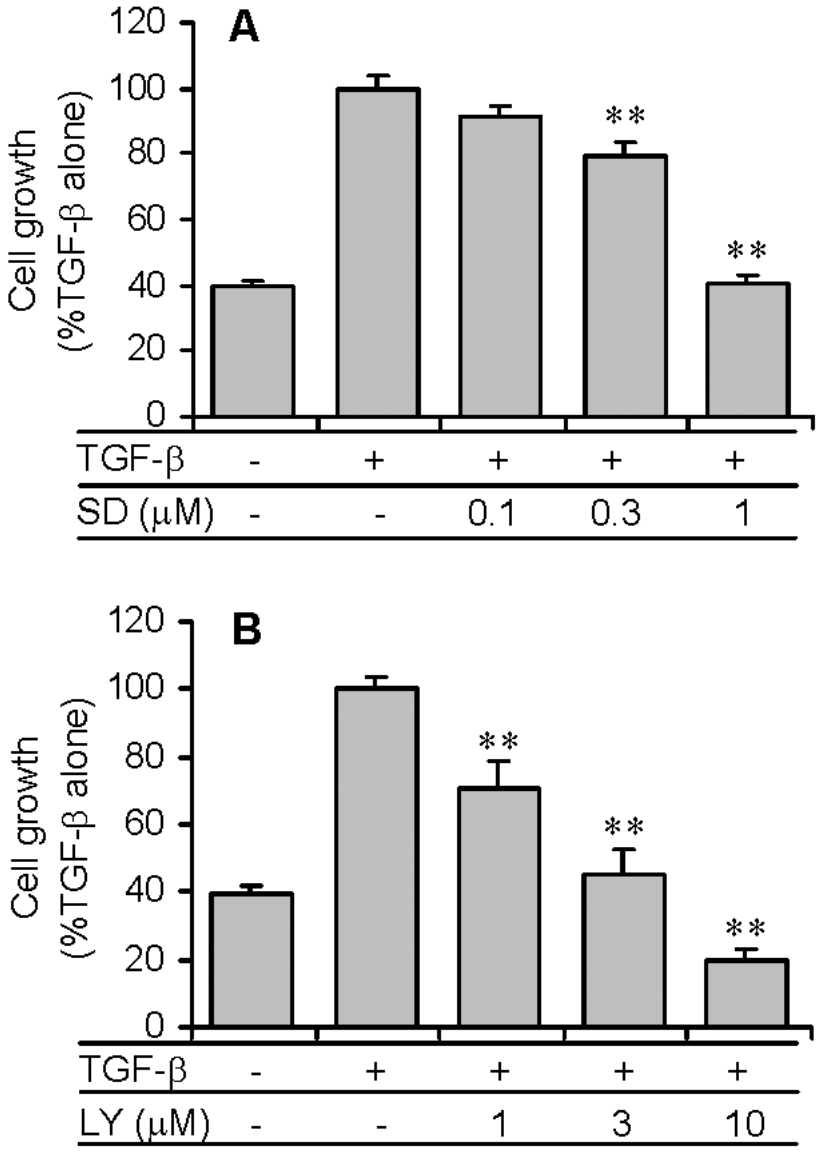

Fig 4.

Inhibition of TGF-β1-stimulated ASM cell growth by TβRI kinase blocker SD-208 and PI3K blocker LY294002. ASM cells were pre-treated for 1 h with SD-208 (A) or LY294002 (B) at the indicated concentrations and then were co-treated with 5 ng/ml TGF-β1 for 6 days in 2.5% FCS medium. Cell growth was assessed by CVA. The data are expressed as the percentage of TGF-β1 alone and are the mean ± SD of triplicate measurements and representative from three ASM cell donors. **P<0.01 compared with TGF-β1 alone.

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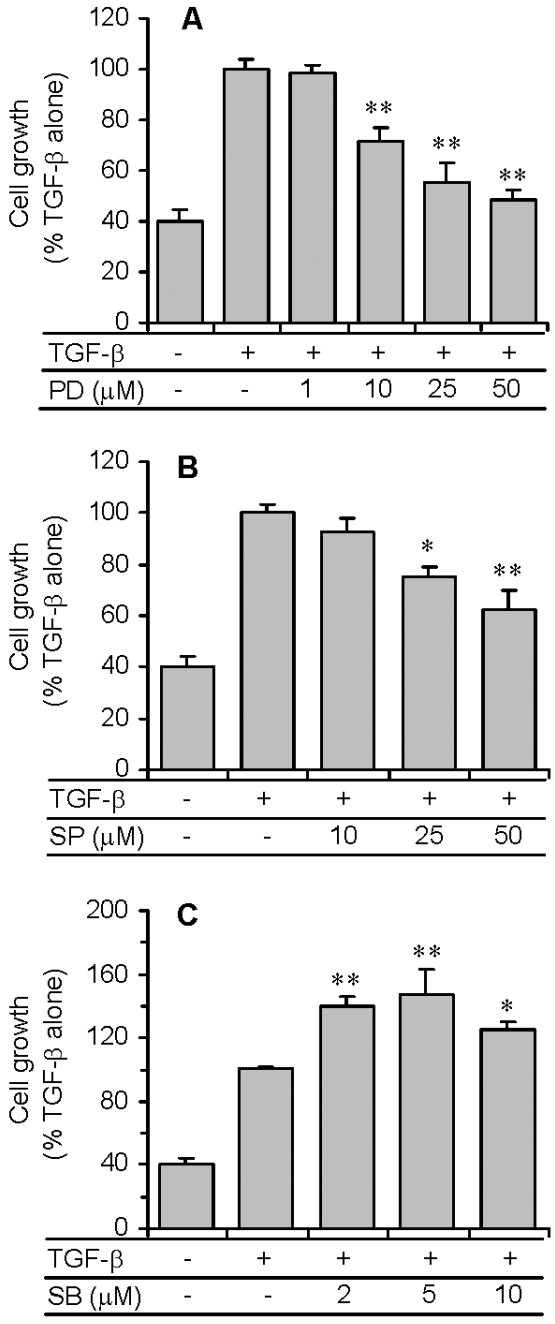

Fig 5.

Regulation of TGF-β1-stimulated ASM cell growth by MAPK inhibitors. ASM cells were pre-treated for 1 h with the inhibitor for ERK, PD98059 (A), for JNK, SP600125 (B) or for p38 MAPK, SB203580 (C) at the indicated concentrations and then were co-treated with 5 ng/ml TGF-β1 for 6 days in 2.5% FCS medium. Cell growth was assessed by CVA. The data are expressed as the percentage of TGF-β1 alone and are the mean ± SD of triplicate measurements and representative from three ASM cell donors. *P<0.05, **P<0.01 compared with TGF-βalone.

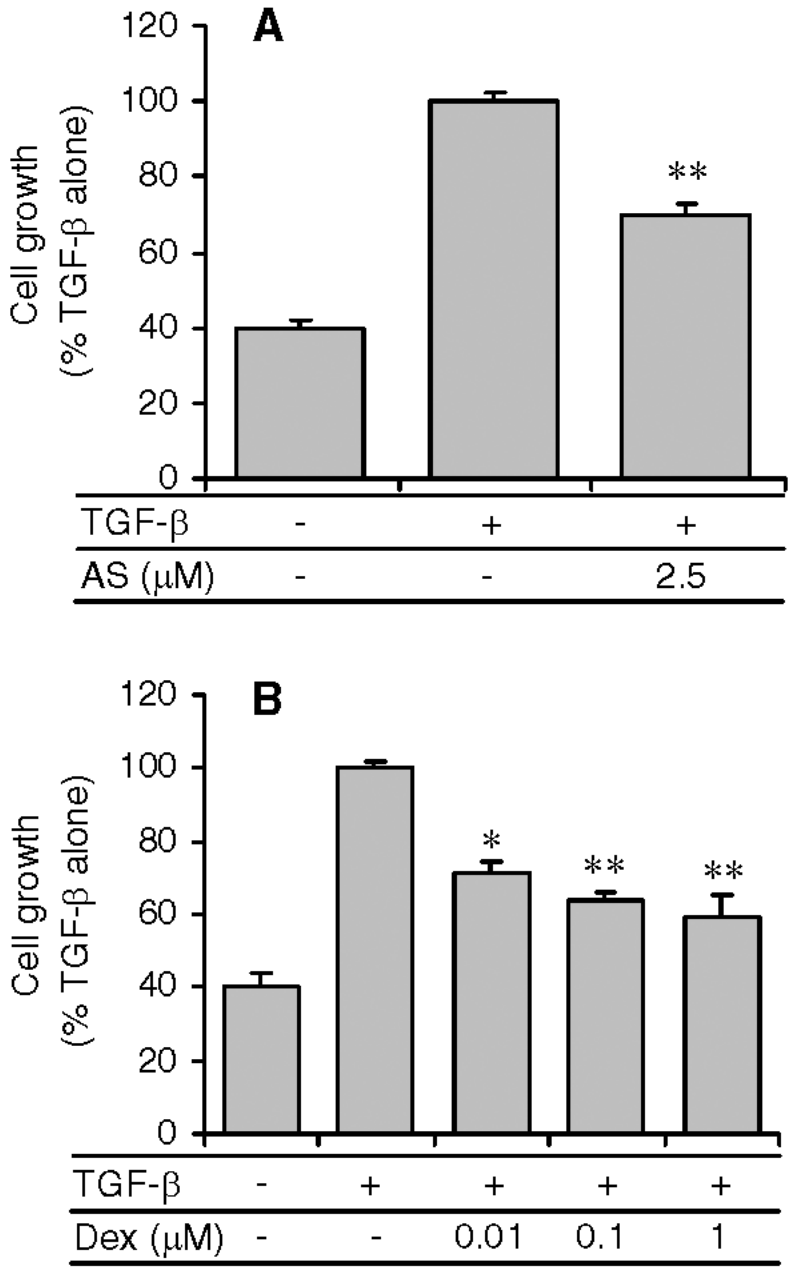

Fig 6.

Down-regulation of TGF-β1-stimulated ASM cell growth by NF-κB signaling inhibition and corticosteroid. ASM cells were pre-treated for 1 h with IKK2 inhibitor, AS602869, or with the corticosteroid, dexamethasone, at the indicated concentrations and then were co-treated with 5 ng/ml TGF-β1 for 6 days in 2.5% FCS medium. Cell growth was assessed by CVA. The data are expressed as the percentage of TGF-β1 alone and are the mean ± SD of triplicate measurements and representative from three ASM cell donors. *P<0.05, **P<0.01 compared with TGF-β1 alone.

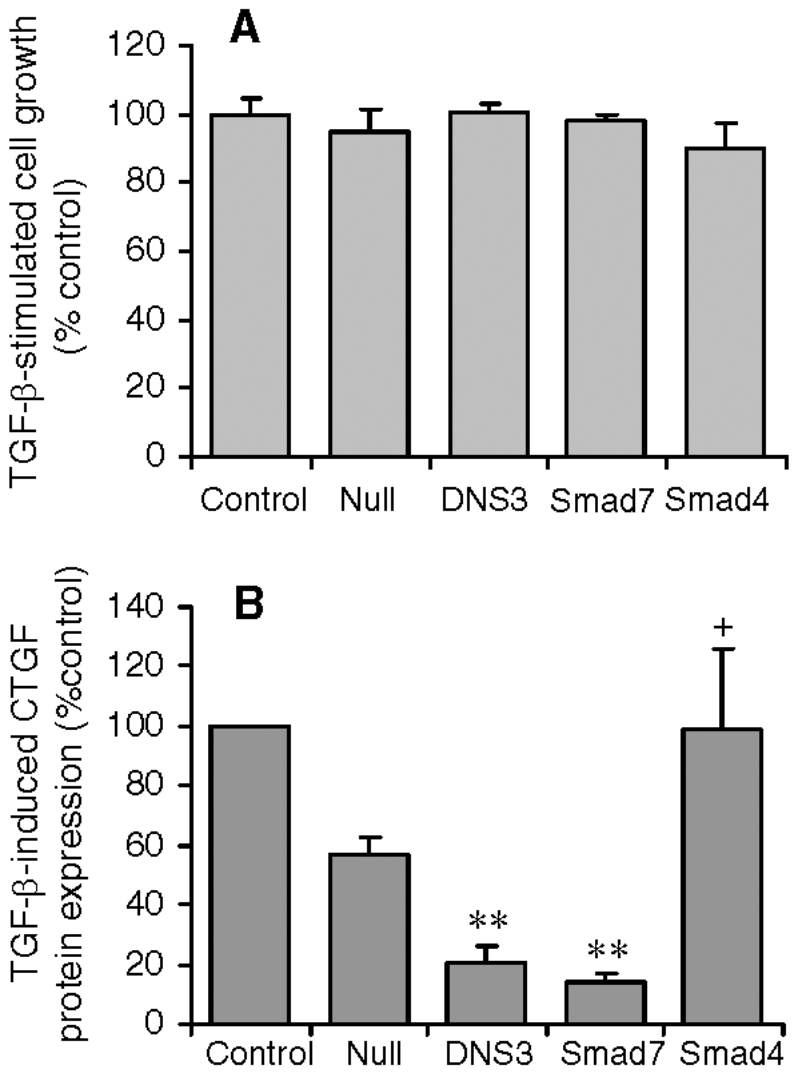

Fig 7.

Role of Smad pathway in TGF-β1-induced ASM cell growth and CTGF expression. ASM cells were infected with the adenoviruses expressing DNS3, Smad7 or Smad4 prior to treatment with TGF-β1 (5 ng/ml) for 4 days to assess cell growth by CVA (A), or for 3 days to analyze CTGF protein expression by Western blotting (B). Control cells were non-infected cells treated with TGF-β1. The data are expressed as the percentage of control and are the mean ± SD from 3 ASM cell donors. **P<0.01, compared with control and Null; +P<0.05, compared with Null.