

## Transforming growth factor- $\beta_1$ induces angiogenesis *in vitro* via VEGF production in human airway smooth muscle cells

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Increase in size and number of bronchial blood vessels as well as hyperaemia are factors that contribute to airway wall remodelling in patients with chronic airway diseases, such as asthma and chronic obstructive pulmonary diseases (COPD). Expression of transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ), a multifunctional cytokine as well as vascular endothelial growth factor (VEGF), a key angiogenic molecule, has been shown in the inflamed airways in patients with chronic airway diseases. TGF- $\beta_1$  has been implicated in the regulation of extracellular matrix, leading to airway remodelling in patients with chronic airway diseases. However, the role of TGF- $\beta_1$  in regulating VEGF expression in patients with chronic airway diseases, as well as the underlying mechanisms are not yet well established. We investigated whether TGF- $\beta_1$  stimulates VEGF expression *in vitro* and hence could influence vascular remodelling. Cultured human airway smooth muscle cells (HASM) were serum deprived for 60 h before incubation with 5ng/ml of TGF- $\beta_1$  for different time points. Control cells received serum-free culture medium. TGF- $\beta_1$  treatment resulted in time dependent HASMC cell proliferation with maximal values for DNA biosynthesis at 24 h and cell number at 48 h. Northern blot analysis of VEGF mRNA expression showed increased levels in cells treated with TGF- $\beta_1$  for 4 to 8 h. TGF- $\beta_1$  also induced a time-dependent release of VEGF proteins in the conditioned medium after 48 h of treatment. Furthermore, the ability of HASMC-released VEGF proteins to induce human umbilical vein endothelial cells proliferation was inhibited by VEGF receptor antagonist, confirming that TGF- $\beta_1$  induced VEGF was biologically active. We conclude that TGF- $\beta_1$  in addition to an extracellular matrix regulator also could play a key role in bronchial angiogenesis and vascular remodelling via VEGF pathway in asthma.

**Keywords:** Asthma, Chronic obstructive pulmonary diseases (COPD), Human airway smooth muscle cells (HASM), Human umbilical vein endothelial cells (HUVEC), TGF- $\beta_1$ , VEGF, Vascular remodelling

Chronic obstructive pulmonary disease (COPD) and asthma are still among the major health concerns worldwide causing death and disability. The two inflammatory diseases are associated with cellular and structural changes referred to as remodelling that is inappropriate for normal lung functioning<sup>1-3</sup>. The patterns of infiltrated inflammatory cells and of structural changes in the remodelling process are partly different in COPD as compared to asthma. However, airway limitation is a common functional

consequence in both diseases, which is mostly reversible in asthma and not fully reversible in COPD<sup>3</sup>. Accumulation of airway smooth muscle (ASM) mass occurs in bronchi of severe asthmatics and in small airways of patients with COPD<sup>2,3</sup>. The ASM hypertrophy and the involvement of many cytokines, growth factors and other pro- and anti-inflammatory mediators modulate the airway wall remodelling process that promote airway narrowing leading to airflow obstruction.

Airway and pulmonary vascular remodelling are common features in chronic airway diseases. However, mechanisms that underlie this remodelling are incompletely understood<sup>2</sup>. Growth factors like platelet-derived growth factor (PDGF), transforming growth factor- $\beta$  (TGF- $\beta$ ) and epidermal growth factor (EGF) influence the proliferation

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**Abbreviations:** ASM, airway smooth muscle; COPD, chronic obstructive pulmonary diseases; HASMC, human airway smooth muscle cells; HUVEC, human umbilical vein endothelial cells; TGF- $\beta_1$ , transforming growth factor  $\beta_1$ ; VEGF, vascular endothelial growth factor.

of many structural cells, such as myofibroblasts and hyperplasia of airway smooth muscle<sup>4</sup>. The pulmonary expression of TGF- $\beta_1$  in patients with COPD is found to be increased in the epithelium and submucosal cells<sup>5</sup>. This multifunctional cytokine is a potent modulator of fibrotic responses. It has been implicated in the regulation of extracellular matrix homeostasis, leading to airway remodelling in asthma<sup>6</sup>. Moreover, TGF- $\beta_1$  has also been shown to modulate the expression of various growth factors, such as connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)<sup>7</sup> and is also involved in the remodelling of the airway wall<sup>8</sup>.

One of the important features of airway remodelling is angiogenesis and structural vascular changes, where VEGF could be a key growth factor. As potent endothelial cell mitogen, VEGF is reported to regulate vasculogenesis and postnatal vascular remodelling<sup>6</sup>. Increased VEGF expression has also been reported in the airways of asthmatic patients<sup>9</sup>. Though, studies have shown that TGF- $\beta_1$  plays an important role in airway remodelling, the mitogenic action and underlying molecular mechanisms of TGF- $\beta_1$  in vascular remodeling in airway diseases remain largely unknown.

In the present study, we investigated TGF- $\beta_1$ -induced responses in human airway smooth muscle cells *in vitro* and examined the expression and release of VEGF that may contribute to pulmonary vascular remodelling in chronic lung diseases like asthma and COPD.

## Materials and Methods

### Materials

Sodium pyruvate, non-essential amino acid mixture, gentamicin, penicillin/streptomycin, amphotericin B, Dulbecco's modified Eagle's medium (DMEM) and the solution of 0.5% trypsin in 0.02% EDTA were obtained from Life Technologies (Breda, The Netherlands). Insulin, transferrin, ascorbate, bovine serum albumin (fraction V), collagenase (type XI), elastase,  $\alpha$ -smooth muscle actin antibody, smooth muscle-myosin heavy chain (SM1 and SM2) antibody were purchased from Sigma (Zwijndrecht, The Netherlands). Foetal bovine serum (FBS) was procured from Bio-Whitaker (Verviers, Belgium), TGF- $\beta$  from Knoll (Ludwigshaven, Germany), and [*Methyl*-<sup>3</sup>H]thymidine

and [*Methyl*-<sup>3</sup>H]leucine from Amersham Nederland ('s-Hertogenbosch, The Netherlands). Human specific antibodies and the enzyme-linked immunosorbent assay (ELISA) kits were obtained from R & D Systems Europe (Abingdon, UK).

### Human airway smooth muscle cell isolation and culture

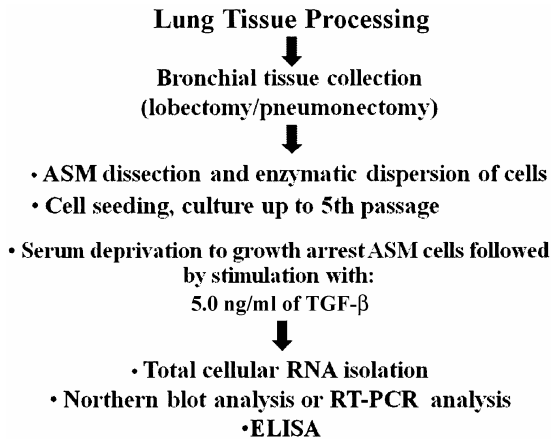
Bronchial smooth muscle was dissected from a fresh, macroscopically normal lobar or main bronchus obtained from patients who underwent surgery for a lung tumour. After removal of the epithelium, parts of smooth muscle was dissected free of adherent connective and parenchymal tissue. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS; Invitrogen, Breda, The Netherlands) containing bovine serum albumin (BSA, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (3.3 U/ml; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) at 37 C in a humidified incubator containing 5% CO<sub>2</sub>/95% air for 30 min.

After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Cambrex, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45  $\mu$ g/ml), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and amphotericin B (1.5  $\mu$ g/ml) (Invitrogen, Breda, The Netherlands). Cells were subsequently seeded at  $2 \times 10^5$  cells per 35 mm dish and maintained in culture by replacing the medium every 48 h. After 10-14 days in culture, ASM cells grew to confluence and were then detached by trypsinization (0.5% trypsin; 0.02% EDTA; Invitrogen, Breda, The Netherlands) and subcultured into 25 cm<sup>2</sup> and 75 cm<sup>2</sup> tissue culture flasks.

### Treatment of airway smooth muscle cells

As shown in the flow diagram below, human airway smooth muscle cell growth was synchronized prior to treatment by washing the cell monolayers twice in phosphate buffered saline (PBS, 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O, pH 7.4) and then replacing the medium with serum-free DMEM supplemented with 1  $\mu$ M insulin, 5  $\mu$ g/ml transferrin and 100  $\mu$ M ascorbate for 60 h. Using flow cytometric analysis of human ASM cells, we found that 60 h of serum deprivation resulted in approximately 87% of the

cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase. Growth-arrested cell monolayers were treated with TGF-β<sub>1</sub> (5.0 ng/ml) in fresh FBS-free DMEM for, 1, 2, 4, 8, 16, 24 and 48 h. Cells were harvested for total RNA isolation and the conditioned media were collected after each time point. Cell-conditioned media were stored at -80°C until assayed for VEGF levels by ELISA.



#### [<sup>3</sup>H]-thymidine incorporation assay

Effects of TGF-β<sub>1</sub> on DNA biosynthesis and protein biosynthesis were evaluated by incorporation of [*methyl*-<sup>3</sup>H] thymidine and [*methyl*-<sup>3</sup>H] leucine, respectively. Confluent cells were washed in PBS, loosened by trypsinisation and transferred into 24-well plates at a seeding density of 3 × 10<sup>4</sup> cells/well. After 24 h in culture, the sub-confluent cell monolayers were growth arrested as described above. Cells were incubated with [*methyl*-<sup>3</sup>H]thymidine (1 μCi/well) in either fresh FBS-free DMEM (control) or DMEM containing TGF-β<sub>1</sub> (0.1, 1.0, 3.0, 5.0 and 10 ng/ml) or 10% serum for 8, 24 and 48 h. After stimulation, the cells were washed in PBS, fixed with ice-cold methanol and exposed to ice-cold trichloroacetic acid (5% w/v). The acid-insoluble fraction was lysed in 0.3 M NaOH and the incorporated radioactivity was determined in a Packard 1500 Tri-carb liquid-scintillation counter (Packard-Becker (Delft, The Netherlands)). Measured radioactivity was expressed as counts/min (CPM) of [<sup>3</sup>H] thymidine incorporation. Data represented were the mean CPM of experiments performed with three different cell isolations.

#### Cell counts

In a parallel series of experiments, cells were incubated with 5.0 ng/ml TGF-β<sub>1</sub> for 24 and 48 h in 24 well plates and processed for cell counting in the Casy<sup>®</sup>1 system (Schärfe system, Reutlingen, Germany). After incubation, the cells were trypsinized for 10 minutes. Cells in suspension were added to 10 ml of Casy<sup>®</sup>1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l boric acid and 0.2 g/l EDTA) and counted and further analysed using Casy<sup>®</sup>1 system software.

#### Isolation of total cellular RNA and Northern blot analysis

Human ASM cells untreated or treated with TGF-β<sub>1</sub> (5.0 ng/ml) for 1, 2, 4, 8, 16, 24 and 48 h were washed in PBS and directly lysed in guanidinium thiocyanate buffer. The lysate was repeatedly passed through a 23-gauge needle in order to shear the genomic DNA. Total cellular RNA was isolated using the method as described earlier<sup>10</sup>. The RNA concentration was estimated by optical density measurements and a DNA/protein ratio of ≥ 1.8 was accepted. Samples of total RNA (10 μg) were denatured at 65°C in formaldehyde containing loading buffer and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide stained gels were photographed and RNA was transferred on to hybond-N membrane (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) by the alkaline downward capillary transfer method<sup>10</sup>. The filters were air-dried and UV cross-linked in a gene linker (Biorad Laboratories, Veenendaal, The Netherlands) and the blots were hybridized at 42°C. A cDNA insert (950 bp DNA fragment encoding human VEGF) was labeled using <sup>32</sup>P-dCTP with a multiprime labeling system. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (American Type Culture Collection, Rockville, USA) was used to rehybridize membranes for reference purposes. Filters were washed under stringent conditions and subsequently exposed to Kodak X-OMAT AR films (Amersham Nederland, 's-Hertogenbosch, The Netherlands) at -80°C. Hybridisation signals were quantified by scanning laser densitometry using the Ultrascan XL enhanced laser densitometer (LKB, Bromma, Sweden). Signals were normalised with respect to GAPDH mRNA values and expressed as relative optical density (OD) in stimulated cells *versus* controls. Values were expressed as mean ± SEM fold induction from three different autoradiographs. Statistical significance was accepted at p ≤ 0.05.

#### Measurement of VEGF proteins by ELISA

Conditioned media were collected from TGF- $\beta_1$  treated human ASM cells after 1, 2, 4, 8, 16, 24 and 48 h. VEGF levels were assessed using human VEGF-specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit. A standard curve using recombinant VEGF protein was first established and subsequently (0.5 ml) of conditioned medium was used to assay for VEGF. Samples of cell-conditioned medium were diluted until the levels of VEGF were within the linearity limits of the standard curve of the assay. Subsequently the samples were pre-incubated with VEGF capture antibody followed by biotinylated VEGF detecting antibody. After addition of streptavidine-peroxidase conjugate, tetramethylbenzidine (TMB) was added and the absorbance of resulting coloured product was measured using an automated spectrophotometer (Biorad Laboratories, Veenendaal, The Netherlands). The concentration of VEGF was expressed in pg/ml. The detection limit of the ELISA method was 20 pg of VEGF/ml.

#### VEGF receptor blockade and endothelial cell growth

Human umbilical vein endothelial cells (HUVEC) were isolated from normal human umbilical cords as described earlier<sup>11</sup>. Briefly, cells were cultured in fibronectin coated tissue culture flasks in culture medium (human endothelial SFM, Invitrogen), with 20% newborn calf serum, 10% human serum (Cambrex, Verviers, Belgium), 20 ng/ml basic fibroblast growth factor and 100 ng/ml epidermal growth factor (Peprotech, London, UK). Passages 5 to 7 were used for the experiments. Cells were plated in fibronectin-coated 96 well plates and allowed to grow for 24 h and then serum deprived and cultured in DMEM for 24 h. Serum-deprived cells were incubated for 48 h with control medium (DMEM alone) or the conditioned medium from TGF- $\beta_1$  treated human ASM cells in 1:1 dilution. Control HUVEC was exposed to medium from untreated ASM cells.

Activity of VEGF present in the conditioned medium on the growth of HUVEC cells was assessed by pre-treating the cells with 10  $\mu$ M VEGF tyrosine kinase receptor blocker 4 [(4'-chloro-2'-fluoro)phenylamino]-6,7-dimethoxyquinazoline (Calbiochem, Darmstadt, Germany). The growth of HUVEC cells was measured using the sulforhodamine B (SRB) assay as described earlier. In short, cells were washed twice with PBS,

incubated with 10% trichloric acetic acid (1 h, 4°C) and washed in distilled water. Cells were stained with 0.4% SRB (Sigma-AldrichBV, Zwijndrecht, The Netherlands) for 15 to 30 min, washed with 1% acetic acid and were allowed to dry. Protein bound SRB was dissolved in TRIS (10 mmol/l, pH 9.4). The absorbance was read at 540 nm. Growth was calculated using the formula: percentage growth = (absorbance test well/absorbance control well) x 100%.

#### Statistical analysis

All data in the figures were given as mean  $\pm$  SEM. Statistical analysis was performed by using two tailed, independent sample "t" test. Significance was accepted at  $p \leq 0.05$ .

#### Results

##### Effect of TGF- $\beta_1$ on human ASM cell proliferation

Figure 1 shows the effect of TGF- $\beta_1$  on human ASM cell growth and proliferation. TGF- $\beta_1$  after 24 and 48 h stimulation significantly increased DNA biosynthesis (assessed by [<sup>3</sup>H] Thymidine incorporation assay) as compared to relevant serum free controls (Fig. 1, Panels A and B). Moreover, comparing with its respective serum free control cells, TGF- $\beta_1$  (5 ng/ml) after 48 h of stimulation significantly proliferated human ASM cells (Fig. 1, Panel C).

##### Expression of VEGF mRNA in relation to TGF- $\beta_1$

In order to examine the VEGF mRNA expression pattern, the human ASM cells were treated with TGF- $\beta_1$  (5 ng/ml) for 1, 2, 4, 8, 16 and 24 h. Using Northern blot hybridisation, two mRNA species of 3.9 and 1.7 kb encoding VEGF were detected in cultured human ASM cells (Fig. 2). In order to compare the expression pattern and to verify the integrity of total RNA samples, GAPDH, a housekeeping gene was used to re-hybridise the membranes. A strong dark band at 1.4 kb, hybridising to a cDNA insert encoding GAPDH in each RNA preparation was observed. TGF- $\beta_1$  induced the VEGF mRNA expression (2-3 fold) which reached maximal levels between 4-8 h of incubation as compared to serum-free controls.

##### Effect of TGF- $\beta_1$ on VEGF release in human ASM cells

The effect of TGF- $\beta_1$  on VEGF protein release in human ASM cells is shown in Fig. 3. Conditioned media obtained from human ASM cells treated with TGF- $\beta_1$  (5 ng/ml) were assessed for VEGF

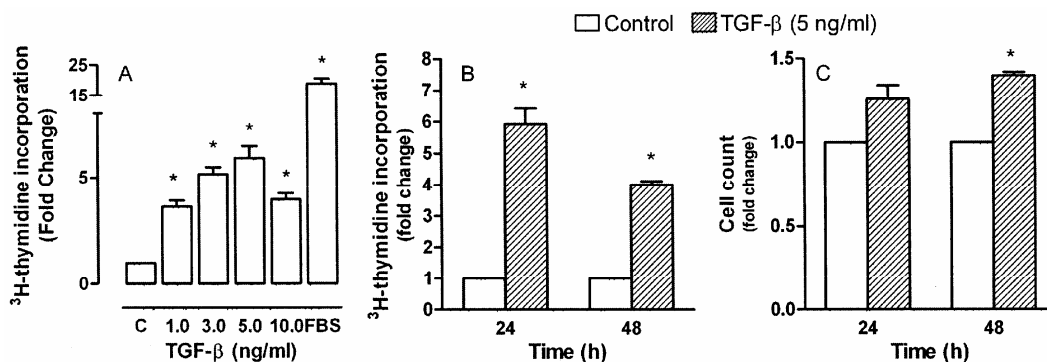


Fig. 1—Effect of TGF- $\beta$  on DNA biosynthesis (Panels A and B) and cell proliferation (Panel C) in human ASM cells [Growth arrested ASM cells were stimulated with TGF- $\beta_1$  and the proliferation was examined by the [ $^3$ H]-thymidine incorporation assay. Panel A shows the fold change increase in DNA biosynthesis due to increasing concentration of TGF- $\beta_1$  (1 -10.0 ng/ml) for up to 48 h, while Panel B depicts the fold change increase in DNA biosynthesis after 24 and 48 h of stimulation with a single concentration of TGF- $\beta_1$  (5 ng/ml). Panel C represents the fold change increase in cell counts after 24 and 48 h of treatment with a single concentration of TGF- $\beta_1$  (5 ng/ml). Values are expressed as mean  $\pm$  SEM. \*: indicates significant differences from control ( $P < 0.05$ )]

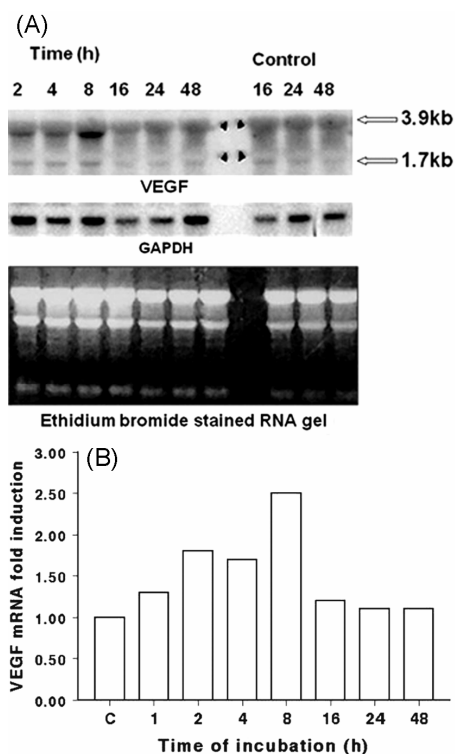


Fig. 2—TGF- $\beta_1$  induced VEGF mRNA expression in cultured human ASM cells [A representative Northern blot showing a major 3.9 and a minor 1.7 kb mRNA band of VEGF (Panel A). Total RNA from control and TGF- $\beta_1$  treated human ASM cells was hybridized with a cDNA insert encoding human VEGF. Panel (B) Graphic representation of the VEGF mRNA expression in ASM cells stimulated with 5 ng/ml of TGF- $\beta_1$  at several time intervals determined by Northern blot analysis. Values represent densitometric measurements (mean  $\pm$  SEM) of three separate isolations relative controls (untreated ASM cells) and normalized to mRNA expression of GAPDH. Values of  $P < 0.05$  between mean from control (untreated ASM cells) and stimulated with TGF- $\beta_1$  were considered as statistically significant as indicated by an asterisk (\*)]

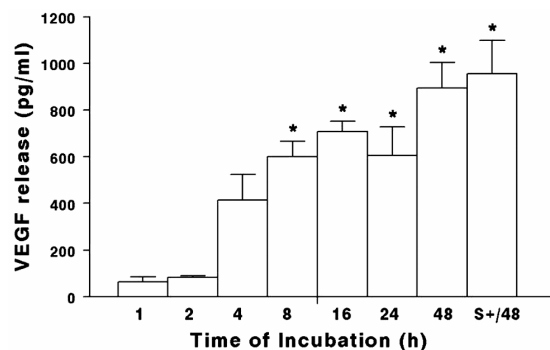


Fig. 3—TGF- $\beta_1$  induced VEGF release in cultured human ASM cells [Graphic representation of the VEGF release (in pg/ml) in culture medium of ASM cells stimulated with 5 ng/ml TGF- $\beta_1$  at several time intervals determined by ELISA method. Values represent (mean  $\pm$  SEM) of three separate isolations. Values of  $P < 0.05$  between mean from control (untreated ASM cells) and stimulated with TGF- $\beta_1$  were considered as statistically significant as indicated by an asterisk (\*)]

protein levels using ELISA method. In our study, we observed a time-dependent significantly increased release of VEGF from human ASM cells treated with TGF- $\beta_1$  ( $40.67 \pm 6.8$  pg/ml,  $88.67 \pm 11.7$  pg/ml,  $88.67 \pm 11.7$  pg/ml and  $162.3 \pm 59$  at 8, 16, 24 and 48 h, respectively). TGF- $\beta_1$  induced release of soluble VEGF protein (8.0-fold) in conditioned medium reached maximal levels at 48 h of incubation ( $173.3 \pm 33.9$  pg/ml) as compared to serum-free control ( $37.67 \pm 7.2$  pg/ml) cells. Serum also induced VEGF release ( $290.67 \pm 53.9$  pg/ml) at 48 h.

#### HUVEC cell proliferation in relation to VEGF tyrosin kinase receptor blocker

To verify the potential mitogenic activity of VEGF secreted from TGF- $\beta_1$  treated human ASM cells, a

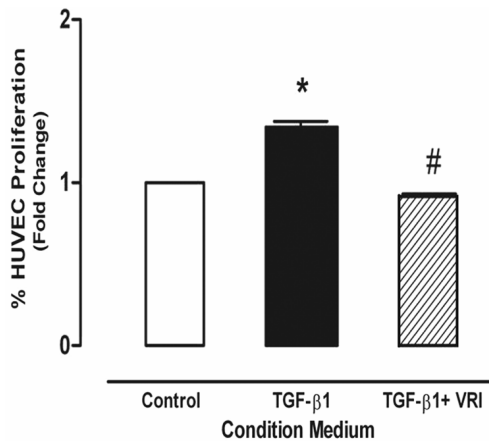


Fig. 4—Angiogenic potential of TGF- $\beta$ <sub>1</sub> treated ASM conditioned medium and effects of VEGF receptor inhibitor on HUVEC proliferation [Graphic representation of fold change in HUVEC proliferation in response to DMEM (control) and DMEM containing conditioned medium from human ASM cells treated with TGF- $\beta$ <sub>1</sub> for 48 h. Proliferation of HUVEC with the TGF- $\beta$ <sub>1</sub> conditioned medium established its angiogenic potential. This stimulating effect was strongly diminished in the presence of 10  $\mu$ M VEGF tyrosine kinase receptor blocker (VRI), 4 [(4'-chloro-2'-fluoro) phenylamino]-6,7-dimethoxyquinazoline pre-treated HUVEC. Values are represented as mean  $\pm$  SEM from experiments performed in quadruplicate. \*compared to medium alone ( $P < 0.05$ ), # in relation to TGF- $\beta$ <sub>1</sub> treated human ASM conditioned medium ( $P < 0.05$ ) at 48 h]

HUVEC based cell proliferation model was exploited. Serum-deprived cultured HUVECs were pre-treated with the VEGF receptor tyrosine kinase inhibitor (VRI) prior to treatment with the conditioned medium of TGF- $\beta$ <sub>1</sub> treated human ASM cells and the data are shown in Fig. 4. We observed a significant increase ( $1.5 \pm 0.2$  fold) in HUVEC proliferation when cells were incubated with conditioned medium as compared to respective control cells ( $P < 0.05$ ). However, pre-treating the HUVEC with VRI showed a significant inhibition in HUVEC proliferation as compared to cells without the VRI pre-treatment ( $P < 0.01$ ) and brought down the proliferation approximate to the untreated control cells (Fig. 4).

## Discussion

In this study, we demonstrated that TGF- $\beta$ <sub>1</sub> induced a time-dependent DNA biosynthesis in cultured human ASM cells. We have previously demonstrated the mitogenic and hypertrophic potential of ANG II in human ASM cells<sup>10</sup>. Furthermore, TGF- $\beta$ <sub>1</sub> induced the mRNA expression and secretion of VEGF from human ASM cells. The conditioned medium derived from TGF- $\beta$ <sub>1</sub> treated ASM cells induced cell

proliferation in porcine pulmonary artery endothelial (data not shown), as well as in human umbilical vein endothelial cells. Moreover, VEGF tyrosine kinase receptor inhibitor blocked the conditioned medium induced mitogenesis in endothelial cells. These results suggested that TGF- $\beta$ <sub>1</sub> contributed to human airway smooth muscle cell growth and up-regulation of VEGF for paracrine action on endothelial cells, which could potentially lead to angiogenesis and vascular remodelling during chronic airway diseases. It is now well established that apart from regulating the bronchial tone and contributing to the hyper responsiveness, airway smooth muscles participate in perpetuation of inflammation by its enhanced secretory nature and thus contributing to the remodelling process in chronic airway diseases. Tissue remodelling is a complex process that involves cell migration, proliferation, angiogenesis and extracellular synthesis that ultimately lead to a modified airways and pulmonary blood vessels.

Besides regulating the bronchomotor tone, recent studies have shown the important roles of airway smooth muscle in the perpetuation of airway inflammation and in the remodelling of the airways<sup>12</sup>. Airway remodelling involves an increase in airway smooth muscle mass through cell proliferation and hypertrophy, and in asthmatic patients, a relationship exists between airway smooth muscle mass and bronchial hyper responsiveness<sup>13</sup>. Though several factors are considered vital to the remodelling process in airways, eosinophils seem to play a significant role in this process. Eosinophil numbers are increased in patients with asthma and in turn eosinophils release several growth factors e.g. TGF- $\beta$ <sub>1</sub> and hence the increase in the density of eosinophils in the airways is associated with an increase in the expression of TGF- $\beta$ <sub>1</sub><sup>14</sup>. Furthermore, TGF- $\beta$ <sub>1</sub> is a potential hypertrophic stimulus and has been shown to positively regulating airway remodelling<sup>8,15</sup>. Therefore, in line with the findings above and consistent with previously published studies<sup>16,17</sup>, our study clearly showed that TGF- $\beta$ <sub>1</sub> significantly increased DNA biosynthesis in and cell proliferation of human ASM cells, indicating its potential involvement in the structural changes patients with chronic airway diseases.

Airway remodelling is a complex healing process, involving cell migration, proliferation, angiogenesis and extracellular matrix deposition,

all ultimately leading to reconstruction of the pulmonary tissue architecture<sup>15</sup>. In our study, we used confluent human ASM cells as a model to study the role of TGF- $\beta_1$  in DNA biosynthesis and cell proliferation, thereby mimicking the remodelling pathways in airways. Our study showed that TGF- $\beta_1$  significantly increased both DNA biosynthesis and cell proliferation in human ASM cells. It is worth to note that the mitogenic effect of TGF- $\beta_1$  depends on the cell density, where the expression of cell surface receptors is abundant such as in our HASC confluent cultures. This would ultimately result in significant binding characteristics of TGF- $\beta_1$  to the cell surface of TGF-beta receptors<sup>16</sup>.

TGF- $\beta_1$  is believed to be associated in angiogenesis process. Gene knockout studies on TGF- $\beta_1$ , its receptors and downstream signaling proteins have demonstrated the essential role of TGF- $\beta$  in the vascular development<sup>18</sup>. TGF- $\beta_1$  exerts its action by binding to type I and type II transmembrane serine/threonine kinase receptors, which in turn trigger activation of various intracellular signaling pathways<sup>19-21</sup>. Smads are intermediate effector proteins that play key roles in biological activities of TGF- $\beta_1$  by transmitting the signals from the cell surface directly into the nucleus and initiating transcription of the targeted genes<sup>19,21</sup>. Therefore, with our findings, we can suggest that TGF- $\beta_1$  by its effects on DNA biosynthesis and cell proliferation could contribute in angiogenesis, a characteristic feature involved in airway remodelling.

Several lines of evidence suggest that VEGF is expressed in variety of cells and its expression is regulated by growth factors and cytokines, including TNF- $\alpha$ , TGF- $\beta$  and interleukin-1 $\beta$ <sup>22-24</sup>. In our study, we found that TGF- $\beta_1$  significantly induced both VEGF mRNA expression and protein. This suggests that TGF- $\beta_1$  is a potent modulator of VEGF release and it supports the available evidence that VEGF levels are up-regulated in patients with asthma<sup>25,26</sup> or COPD<sup>27,28</sup>. Moreover, the increased expression of VEGF in asthmatic airways correlating positively with increased blood vessel numbers and size suggests that VEGF could significantly contribute to angiogenesis<sup>29,30</sup>.

In addition, the TGF- $\beta_1$ -induced expression and release of VEGF clearly supports the notion

that ASM cells are an important source of mediators in the asthmatic airways. The molecular mechanism underlying stimulation of VEGF expression and release by TGF- $\beta_1$  is not entirely clear. However, it has been partly elucidated and seems to involve activation of activator protein-1 transcription factors<sup>31,32</sup>. The angiogenic factors like VEGF produced by ASM cells are present in the vicinity of the endothelial cells and may act via a paracrine mechanism and hence contribute to the pulmonary vascular remodelling. Furthermore, we cultured the HUVEC in conditioned medium of ASM cells that were treated with TGF- $\beta_1$  and with an inhibitor of TGF- $\beta$  and VEGF receptor tyrosine kinase (VRI).

Proliferation of the HUVEC *in vitro* was evident only in response to the conditioned medium obtained from TGF- $\beta_1$  treated HASMC. Whereas, HUVEC proliferation was inhibited in case of the condition medium obtained from HASMC treated with TGF- $\beta_1$  and VEGF receptor inhibitor. These findings clearly demonstrated that the VEGF proteins released from the HASMC treated with TGF- $\beta_1$  were biologically active and depicted the mitogenic properties and hence contributing to the mechanisms of angiogenesis *in vitro*. Undoubtedly, more research focus has been directed towards the molecular events underlying the airway vascular remodelling. Though the precise nature of these events is far from clear, our study provides a possible mechanism of vascular remodelling that could occur in chronic airway diseases.

In conclusion, our results showed that treatment of human ASM cells with TGF- $\beta_1$  significantly increased DNA biosynthesis and cell proliferation, induced the mRNA expression of VEGF, increased the release of VEGF protein in the conditioned medium, and stimulated human umbilical vein endothelial cells proliferation in culture via VEGF protein release. Taken together, our findings suggest that TGF- $\beta_1$  in addition to being an extracellular matrix regulator may play a key role in bronchial vascular remodelling in during chronic airway diseases like asthma and COPD via the VEGF pathway.

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