ORIGINAL ARTICLE

Vasoactive Peptides Upregulate mRNA Expression and Secretion of Vascular Endothelial Growth Factor in Human Airway Smooth Muscle Cells

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Abstract

Airway remodeling and associated angiogenesis are documented features of asthma, of which the molecular mechanisms are not fully understood. Angiotensin (ANG)II and endothelin (ET)-1 are potent vasoconstricting circulatory hormones implicated in asthma. We investigated the effects of ANG II and ET-1 on human airway smooth muscle (ASM) cells proliferation and growth and examined the mRNA expression and release of the angiogenic peptide, vascular endothelial growth factor (VEGF). Serum deprived (48 h) human ASM cells were incubated with ANG II (100 nM) or ET-1 (10 nM) for 30 min, 1, 2, 4, 8, 16, and 24 h and the endogenous synthesis of VEGF was examined in relation to control cells receiving serum free culture medium. ET-1 induced time dependent DNA biosynthesis as determined by [³H]-thymidine incorporation assay. Using northern blot hybridization, we detected two mRNA species of 3.9 and 1.7 kb encoding VEGF in the cultured smooth muscle cells. Both ANG II and ET-1 induced the mRNA expression (two- to threefold) and secretion (1.8- to 2.8-fold) of VEGF reaching maximal levels between 4–8 h of incubation. Induced expression and release of VEGF declined after 8 h of ANG II incubation while levels remained elevated in the case of ET-1. The conditioned medium derived from ET-1-treated ASM cells induced [³H]-thymidine incorporation and cell number in porcine pulmonary artery endothelial as well as human umbilical vein endothelial cells. Moreover, the VEGF tyrosine kinase receptor inhibitor blocked the conditioned medium induced mitogenesis in endothelial cells. Our results suggest a potential role for ANG II and ET-1 in ASM cell growth and upregulation of VEGF that may participate in endothelial cell proliferation via paracrine mechanisms and thus causing pathological angiogenesis and vascular remodelling seen during asthma.

Index Entries: Angiotensin II; endothelin-1; airway smooth muscle; vascular endothelial growth factor; angiogenesis and asthma.

INTRODUCTION

Increased airway smooth muscle (ASM) mass and airway remodelling is a common feature in chronic airway diseases such as asthma (1). Airway remodelling is

accompanied with angiogenesis and microvascular changes wherein the ongoing inflammation seems to be the main contributing factor (2–5). Evidence suggests that the number and size of bronchial vessels is moderately increased in patients with asthma as compared with normal individuals (6–9). Moreover, Salvato and colleagues (10) found that patients with severe asthma had significantly more vessels than those with a mild or

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moderate disease. Previously, we reported that fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) play a pivotal role in the pathogenesis of chronic obstructive pulmonary disease (COPD) (2,3). Similarly, enhanced bronchial localization of various angiogenic growth factors has been observed in patients with asthma and COPD (2,3,11,12). However, the molecular mechanisms underlying bronchial angiogenesis and vascular remodelling remain unclear.

VEGF is a potent endothelial cell mitogen that regulates vasculogenesis and postnatal vascular remodeling (13). Expression of VEGF is upregulated under a variety of pathophysiological conditions, including hypoxia in the lung (14) and during angiogenesis in other biological systems (15,16). The most important role of VEGF in inflammation may be in mediating the angiogenic response (17). Because angiogenesis and increased vascular permeability are characteristic features of wound healing, VEGF may play an important role in airway remodeling in asthma, where wound healing after injury is a common phenomenon (12). VEGF is expressed by human ASM cells in culture and in lungs under pathophysiological conditions (2,9,18,19), and we have shown that COPD is associated with an increased expression of VEGF in both ASM and vascular smooth muscle (VSM) cells in bronchiolar as well as alveolar regions; this increased expression is inversely correlated with the lung function data (2).

Angiotensin (ANG)II and endothelin (ET)-1 are potent vasoconstricting circulatory hormones implicated in asthma (20–24). ANG II has been reported to induce VEGF mRNA expression in rat heart endothelial cells (25) and mitogenesis in human ASM cells (26). Evidence suggests that ET-1 acts as a co-mitogen with EGF and that its action is mediated through activation of ET_A receptors in lungs (27). However, the underlying molecular mechanisms of mitogenic and subsequent signalling elicited by ET-1 in human ASM cells are not yet characterized. We therefore investigated the responses to ANG II and ET-1 in human ASM cells in vitro and examined the expression and release of VEGF that may contribute to pulmonary vascular remodeling in chronic airway diseases. Furthermore, we assessed the conditioned medium obtained from human ASM cells treated with ANG II and ET-1 for in vitro angiogenic potential by examining endothelial cell proliferation and growth.

MATERIALS AND METHODS

Human ASM Cell Isolation and Culture

Human ASM cells were isolated and cultured as described previously (28). Briefly, bronchial smooth

muscle was dissected from fresh macroscopically normal lobar or main bronchi. After removal of the epithelium, parts of smooth muscle was dissected, free of adherent tissue under aseptic conditions. 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% CO₂/95% 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 fetal bovine serum (FBS; Cambrex, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (1.5 µg/mL) (Invitrogen, Breda, The Netherlands). Cells were subsequently seeded at 2×10^5 cells per 35-mm dish and maintained in culture by replacing the medium every 48 h. After 10–14 d 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-cm² and 75-cm² tissue-culture flasks.

Immunocytochemical Characterization of Cultured Human ASM Cells

Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle α -actin and smooth muscle-myosin heavy chain (SM1 and SM2; Sigma-Aldrich BV, Zwijndrecht, The Netherlands) demonstrated that the cultures were essentially free (<5%) of other contaminating cell types (26,28). In order to perform immunocytochemistry, monoclonal antibodies against α -smooth muscle actin were used as markers. In brief, cells were allowed to attach to multi-well slides for 24 h in FBS-containing medium and were subsequently growth-arrested by incubating for 60–72 h in FBS-free DMEM supplemented with apo-transferrin (5 µg/mL), ascorbate (100 µM), and insulin (1 µM) prior to fixation and staining. Following two washes in ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, 8.1 mM Na₂HPO₄ · 2 H₂O, pH 7.4), the cells were fixed in ice-cold methanol and permeabilized in PBS containing 0.1% Tween-20. Nonspecific bindings were blocked by incubating the cells in 1% BSA in PBS, and the cells were then washed and subsequently incubated with anti- α -smooth muscle actin antibodies. The cells were then washed twice in PBS and further incubated with

Fig. 1. Representative examples of human airway smooth muscle (ASM) and pulmonary artery endothelial cells in culture. Photomicrograph depicts the monolayer (upper left panel) of human ASM cells with the characteristic hill and valley appearance. Smooth muscle α -actin (fluorescence) antibody (upper right panel) characterizes the staining in longitudinal actin filaments and assures the purity of the human ASM cells. Photomicrograph depicts the monolayer (lower left panel) of porcine pulmonary artery endothelial cells (PAEC) with the characteristic cobble stone appearance. CD31 (brown) antibody staining (lower right panel) characterizes the purity of the endothelial cells.

affinity purified fluorescein isothiocyanate (FITC)-conjugated antimouse antibody. Unbound antibody was washed away using distilled water and the sections were dehydrated and mounted in glycerol. Specimens were visualized under a fluorescence microscope (Carl Zeiss BV, Weesp, The Netherlands) and photographed. Human ASM cells stained positive for anti- μ -smooth muscle actin (Fig. 1, upper right panel). Under the light microscope, the human ASM cells appeared elongated and spindle-shaped with central oval nuclei containing prominent nucleoli. Confluent human ASM cells in culture showed a specific pattern, aligned in parallel so that the broad nuclear region of one cell lies adjacent to the thin cytoplasmic area of another, giving rise to a typical "hill and valley" appearance (Fig. 1, upper left panel).

Porcine Pulmonary Artery Endothelial Cell Isolation and Culture

The lungs were obtained from Yorkshire $\times$ Landrace female pigs after sacrifice and the major pulmonary artery was identified and dissected free of all connec-

tive tissue and cleaned with HBSS. After incubation with collagen I for 30 min, endothelial cells were isolated by scraping gently the inner surface of the artery with a surgical blade and cultured up to fourth passage in M-199 medium containing 10% fetal calf serum (FCS; Cambrex, Verviers, Belgium) at 37°C in a 5% CO₂-humidified atmosphere. Cells were maintained in culture by replacing the medium every 48 h and when confluent was further passaged using trypsin/EDTA solution into 75-cm² tissue culture flasks. Porcine pulmonary artery endothelial cells had the characteristic cobblestone appearance when cultured in monolayer. Cells were characterized immunocytochemically, using monoclonal antibodies against CD31 and visualized under the light microscope. Porcine pulmonary artery endothelial cells in culture revealed cobblestone appearance (Fig. 1, lower left panel). Immunocytochemical staining of confluent serum-deprived primary cell cultures of using monoclonal antibodies against CD31 (Fig. 1, lower right panel) demonstrated that the cultures were essentially free (<5%) of other contaminating cell types.

Stimulation of Human ASM Cells In Vitro With ANG II or ET-1

Human ASM cells were added in 500 μ L medium to a 24-well multiplate (Corning BV, Schiphol, The Netherlands), at a final concentration of 3×10^4 cells/well. After 72 h, cells were serum deprived for 60 h and subsequently incubated with optimal concentration of ANG II (100 nM) or ET-1 (10 nM), based on previous reports (26,29) and by pilot experiments, for 30 min, 1, 2, 4, 8, 16, and 24 h. Control cells were cultured in serum-free medium (24 h).

Isolation of Total Cellular RNA and Northern Blot Analysis

Total RNA was extracted from the cells incubated with ANG II (100 nM) or ET-1 (10 nM) for 30 min, 1, 2, 4, 8, 16, and 24 h by the guanidinium thiocyanate-phenol-chloroform method. For Northern hybridization, 10 μ g of total RNA was electrophoresed on 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to hybond-N membranes, which were eventually ultraviolet cross-linked and hybridized at 42°C. cDNA insert (950 bp DNA fragment encoding human VEGF) was labeled using (32 P)dCTP with a multiprime labeling system. Filters were washed under stringent conditions and subsequently exposed to Kodak X-OMAT AR films at -80°C . A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to rehybridize membranes for reference purposes. After autoradiography and densitometric measurement of signals, the optical density (OD) of the VEGF signal was divided by OD of the corresponding GAPDH signal and relative mRNA levels were calculated. Values were expressed as fold induction (mean $\pm$ SEM) from four different autoradiograms. Statistical significance was accepted at $p \leq 0.05$.

Measurement of VEGF Protein by Enzyme-Linked Immunosorbent Assay

Conditioned media were collected from human ASM cells treated with ANG II or ET-1 and VEGF levels were assessed using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction (R&D Systems, Abington, UK). As a control, conditioned medium from human ASM cells incubated with only serum-free medium was used. A standard curve using recombinant VEGF protein was first established and subsequently, 100 μ L of conditioned medium was used to assay VEGF. Subsequently, the samples were pre-incubated with VEGF capture antibody followed by biotinylated VEGF-detecting antibody. After addition of the streptavidine-peroxidase conjugate, tetramethylbenzidine (TMB) was added and the absorbance of the resulting colored product was

measured using an automated spectrophotometer (Biorad Laboratories BV, Veenendaal, The Netherlands). The concentration of VEGF was expressed in ng/mL. The detection limit of the VEGF ELISA method was 20 pg of VEGF/mL.

Cell Proliferation Assays

[^3H]THYMIDINE INCORPORATION ASSAY IN HUMAN ASM CELLS TREATED WITH ET-1

Effects of ET-1 on human ASM DNA biosynthesis were evaluated by incorporation of [methyl- ^3H]-thymidine. Confluent cells were washed in PBS, loosened by trypsinization, and transferred into 96-well plates at a seeding density of 1×10^4 cells/well. After 24 h in culture, the subconfluent cell monolayers were growth arrested by 48 h serum deprivation and incubated with different concentrations of ET-1 (1, 10, and 100 nM) for 48 h. Additionally, cells were incubated with ET-1 (10 nM) for time periods varying from 24 to 96 h. Five hours prior to the end of the treatment periods, 10 μ L (1 $\mu\text{Ci}/10 \mu\text{L}$ in HBSS) of [^3H]-thymidine was added to the wells, at a final concentration of 1 MCi/110 μL per well. The plates were frozen overnight at -20°C , after which the cells were harvested on glass fibre filters using a Filtermate 196 cell harvester (Packard, Meridan, USA) and the activity was counted using a Microplate Scintillation β -counter (Topcount, Packard, Meridan, USA). Measured radioactivity was expressed as counts per min (CPM). Data represented is the mean CPM of experiments performed with three different cell isolations.

ENDOTHELIAL CELL PROLIFERATION ASSAY

Porcine pulmonary artery endothelial cells were added in 100 mL medium to a 96-well multiplate (Corning BV, Schiphol, the Netherlands) at a final concentration of 1×10^4 cells/mL. After 48 h, the cells were serum deprived for 24 h and subsequently incubated with the conditioned medium from human ASM cells treated for 8 h with ANG II (100 nM) or ET-1 (10 nM) and positive controls with VEGF (5 ng/mL) or placental growth factor (PIGF) (5 ng/mL) in 100 μ L serum-free M199 medium for 24 h. Control incubations consisted of cells that were incubated with serum-free M199 medium alone. DNA biosynthesis was measured employing [^3H]-thymidine incorporation assay as described above.

ENDOTHELIAL CELL COUNT

In a parallel series of experiments, serum-deprived porcine pulmonary artery endothelial cells were stimulated with the same factors as above for 48 h and processed for cell counting in the Casy[®]1 system

Fig. 2. Graphic representation of human airway smooth muscle (ASM) cell proliferation in relation to endothelin (ET)-1. **A:** Dose-dependent increases in [³H]-thymidine uptake in ASM cells after 48 h stimulation with increasing concentrations (0, 1, 10, 100 nM) of ET-1. **B:** Time course of [³H]-thymidine uptake in ASM cells after stimulation with 10 nM of ET-1. Data represent mean fold increase in relation to control from three independent experiments performed in quadruplicate. Values are shown as mean $\pm$ SEM. * $p \leq 0.05$ vs the control serum-deprived cells.

(Schärfe system GmbH, Reutlingen, Germany). After stimulation, cells were harvested with mild trypsinisation. Cells in suspension were added to 10 mL of Casy1 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), counted and further analyzed using Casy1 system software.

VEGF Receptor Blockade and Endothelial Cell Growth

Human umbilical vein endothelial cells (HUVECs) were isolated from normal human umbilical cords as described earlier (30). 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 FGF, 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 ET-1 (10 nM)-treated (8 h) human ASM cells in 1:1 dilution. Control HUVECs were exposed to medium from untreated ASM cells. Activity of VEGF present in the conditioned medium on the growth of HUVECs was assessed by pretreating the cells with 10 μ M VEGF tyrosine kinase receptor blocker 4-[(4'-Chloro-2'-fluoro)phenylamino]-6,7-chimothoxyquinazoline (Calbiochem, Darmstadt, Germany). The growth of HUVECs was measured using the sulforho-

damine-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-Aldrich BV, 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) $\times$ 100%.

Statistical Analysis

All data in the figures are 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 ET-1 on Human ASM Cell Proliferation

In order to investigate the role of ET-1 on ASM cell proliferation and remodeling, isolated human ASM cells were stimulated in vitro with increasing concentrations of ET-1 (1, 10, and 100 nM). Graphic representations of dose-dependent increase in [³H]-thymidine incorporation are shown in Fig. 2A. ET-1 significantly increased cell numbers at all concentrations after 48 h of incubation. There was no significant difference in the increase in cell proliferation in relation to increase in dose of ET-1. Figure 2B is a graphic representation of time-dependent [³H]-thymidine incorporation at a concentration of

Fig. 3. Angiotensin (ANG) II- and endothelin (ET)-1-induced expression of vascular endothelial growth factor (VEGF) in human airway smooth muscle (ASM) cells. A representative Northern blot showing a major 3.9- and a minor 1.7-kb mRNA band of VEGF. Total RNA from control and treated human ASM cells was hybridised with a cDNA insert encoding human VEGF. Growth arrested cells were incubated with ANG II or ET-1 in serum-free growth medium for the times indicated on the top. Filters were rehybridized with GAPDH cDNA probe for reference purposes.

10 nM of ET-1. After 48 h of stimulation, we found significantly increased cell proliferation as measured by thymidine incorporation as compared to the control cells ($13.6 \pm 1.9 \times 10^3$ CPM vs $8.9 \pm 0.8 \times 10^3$ CPM). Thereafter, cell proliferation induced by ET-1 was significantly higher than the control even up to 96 h of treatment.

VEGF mRNA Expression in Relation to ANG II and ET-1

Human ASM cells were treated for 30 min, 1, 2, 4, 8, 16, and 24 h either with ANG II (100 nM) or ET-1 (10 nM) in order to examine the VEGF mRNA expression. Using Northern blot hybridization, we detected two mRNA species of 3.9 and 1.7 kb encoding VEGF in human ASM cells treated with ANG II (Fig. 3A) and ET-1 (Fig. 3B). In order to compare the expression pattern and to verify the integrity of total RNA samples, GAPDH, a house-keeping gene, was used to re-hybridize the membranes. A strong, dark band at 1.4 kb, hybridizing to a cDNA insert encoding GAPDH in each RNA preparation, is

visible (Fig. 3, lower panels). As shown in the blot and in the graphs expressing the relative mRNA levels, stimulation with ANG II induced the expression of VEGF mRNA reaching maximal levels at 2 h, whereas ET-1 induced VEGF mRNA expression peaked at 4 h and remained elevated as compared to serum-free controls. Serum also induced mRNA expression of VEGF in human ASM cells in vitro (data not shown).

ANG II- and ET-1-Induced Release of VEGF From Human Airway Smooth Muscle Cells

The effects of ANG II and ET-1 on VEGF release in human ASM cells are shown in Fig. 4. Conditioned media obtained from ANG II (100 nM)- and ET-1 (10 nM)-treated human ASM cells were assessed for the VEGF release using the ELISA method. In our study, we observed a time-dependent release of VEGF from human ASM cells treated with ANG II and ET-1. We found a significant increase in VEGF concentration in conditioned medium from ANG II-treated ASM

Fig. 4. Time-dependent production of vascular endothelial growth factor protein in relation to angiotensin (ANG) II or endothelin (ET)-1. Growth-arrested human airway smooth muscle (ASM) cells were stimulated with 100 nM ANG II or 10 nM ET-1 for various time points. Control cells (C) received serum-free medium. Data represent the mean $\pm$ SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from four patients. * $p \leq 0.05$ as compared to respective controls.

cells at 4 h of incubation (391 ± 28 pg/mL) as compared to the control (237 ± 39 pg/mL) (Fig. 4A). In the conditioned medium from ET-1-treated cells, we found significant increase at 4 h (397 ± 60 pg/mL) and 16 h (413 ± 40 pg/mL) and then the levels of secreted VEGF remained elevated at later time points (Fig. 4B). However, ANG II induced the secretion of VEGF protein in a transient manner where the levels declined after 8 h of incubation.

Effects of ASM-Conditioned Medium on DNA Biosynthesis and Cell Number in Porcine Pulmonary Artery Endothelial Cells

To decipher the angiogenic potential of the conditioned medium obtained from vasoactive proteins treated human ASM cells, proliferation assays were conducted on porcine pulmonary artery endothelial cells. Figure 5A shows significant increase in [3 H]-thymidine incorporation after 24 h treatment with conditioned medium from ET-1-treated ASM cells ($2.4 \pm 0.5 \times 10^3$ CPM), 5 ng/mL VEGF ($1.07 \pm 0.3 \times 10^3$ CPM) and 5 ng/mL PIGF ($1.07 \pm 0.2 \times 10^3$ CPM) as compared to serum-free control cells ($0.35 \pm 0.06 \times 10^3$ CPM). However, the conditioned medium obtained from ANG II-treated human ASM cells did not induce significant proliferation ($0.48 \pm 0.6 \times 10^3$ CPM) as compared to the control. Similarly, the cell count experiments revealed enhanced cell numbers (two- to fourfold) after treatment with the conditioned medium from cells treated with ET-1, VEGF, and PIGF at 48 h (Fig. 5B).

Inhibition of Conditioned Medium-Induced Endothelial Cell Proliferation by VEGF Receptor Blocker

To verify the potential mitogenic activity of VEGF secreted from ET-1-treated human ASM cells, a HUVEC-based cell proliferation model was exploited. Serum-deprived cultured HUVECs were pretreated with the VEGF receptor tyrosine kinase inhibitor (VRI) prior to treatment with the conditioned medium of ET-1-treated human ASM cells and the data is shown in Fig. 6. We observed a significant increase (1.45 ± 0.23 -fold) in HUVEC proliferation when cells were incubated with conditioned medium as compared to respective control cells ($p < 0.05$). However, pretreating the HUVECs with VRI showed a drastic inhibition in HUVEC proliferation as compared to cells without the VRI pretreatment ($p < 0.001$) as well as in comparison to control cells ($p < 0.05$).

DISCUSSION

In this study, we have shown that ET-1 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 (26). Furthermore, both ANG II and ET-1 induced the mRNA expression and secretion of VEGF from human ASM cells. The conditioned medium derived from ET-1-treated ASM cells induced cell proliferation in porcine pulmonary artery endothelial as

Fig. 5. Effects of angiogenic factors and conditioned medium obtained from angiotensin (ANG) II or endothelin (ET)-1-treated airway smooth muscle (ASM) cells on the biosynthesis of DNA in porcine pulmonary artery endothelial cells. Conditioned medium (CM; 100 μ L) obtained from human ASM cells treated with ANG II (100 nM) or ET-1 (10 nM)-were added to serum-deprived endothelial cells and [³H]-Thymidine incorporation array (A) and cell number (B) were performed. Values are mean $\pm$ SEM from three experimental determinations. Note that vascular endothelial growth factor (5 ng/mL), placental growth factor (5 ng/mL), and fetal calf serum (FCS) are potent mitogens for the endothelial cells ($p \leq 0.05$). CM from ET-1 (10 nM)-treated ASM cells is mitogenic for the cells at 24 h and 48 h. However, CM from ANG II-treated ASM cells did not elicit the mitogenic effect ($p \leq 0.05$) at 24 h.

Fig. 6. Pretreatment with vascular endothelial growth factor (VEGF) receptor inhibitor and human umbilical vein endothelial cell (HUVEC) proliferation in response to conditioned medium. Graphic representation of fold change in percentage HUVEC proliferation in response to Dulbecco's modified Eagle's medium (Control), and condition medium from human airway smooth muscle (ASM) cells treated with endothelin (ET)-1 for 8 h. Proliferation of HUVECs with the ET-1 conditioned medium increased significantly compared to the control. This stimulating effect was strongly diminished in 10 μ M VEGF receptor blocker (VRI) pretreated HUVECs. Values are represented as mean $\pm$ SEM from experiments performed in quadruplicate and experiments were repeated twice. * Compared to medium alone ($p < 0.05$); # in relation to ET-1-treated human ASM-conditioned medium ($p < 0.05$) at 48 h.

well as human umbilical vein endothelial cells and VEGF tyrosine kinase receptor inhibitor blocked the conditioned medium induced mitogenesis in endothelial cells. These results suggest that ANG II and ET-1 contribute differentially to human ASM cell growth and upregulation of VEGF for paracrine action on endothelial cells, which could potentially lead to angiogenesis and vascular remodelling during chronic airway diseases. Besides regulating the bronchial tone and contributing to the hyper responsiveness (31), studies have clearly shown the important role of ASM in the perpetuation of inflammation by its enhanced secretory nature and thus contributing to the remodelling process in chronic airway diseases. Remodeling is a complex process (32) involving cell migration, proliferation, angiogenesis (2), and extracellular matrix alteration (33), ultimately leading to a modified airway vascular structure.

In our study, we used confluent human ASM cells as a model to study the role of vasoactive proteins in expression and secretion of VEGF, thereby mimicking vascular remodeling pathways in airways. Our data on the increased expression of VEGF in human ASM cells support the notion that this angiogenic molecule is transcribed and translated in these cells for paracrine action on neighbouring endothelial cells. This is in accordance with previous studies where its expression is regulated by growth factors and cytokines, including tumor necrosis factor (TNF)- α , transforming growth factor

(TGF)- β , and interleukin-1 β (18,34). Several lines of evidence suggest that VEGF levels are upregulated in patients with asthma (35,36) and COPD (2,11). Moreover, increased expression of VEGF in asthmatic airways with positive correlation with increase in blood vessel number and size suggests that VEGF significantly contribute to angiogenesis (37) and vascular remodeling and hence to the airway wall remodeling processes. Moreover, the mitogenic effects of the conditioned medium derived from ET-1-treated ASM cells on the pulmonary artery endothelial cells supports the hypothesis that the VEGF synthesized by ASM under influence of these molecules is biologically active. However, the conditioned medium derived from ANG II-treated human ASM cells could not result in endothelial cell proliferation and warrants further investigation. This could also be attributed to the sub-threshold levels of secreted VEGF from ANG II-treated cells as compared to ET-1-treated ASM cells. Both mRNA and protein data on VEGF production in ANG II-treated cells showed transient and rapid increase which declined within 8 h indicating for degradation as compared to stable levels in ET-1-treated cells. Also, it cannot be ruled out that either the released VEGF is an inactive variant or that VEGF, a known survival factor, is being utilized by the cells rapidly. Additionally, the fact that only ET-1 induces significant proliferation of ASM cells, whereas ANG II induces cellular hypertrophy but does not increase cell numbers, as we reported previously (26), may suggest that predominantly proliferating ASM cells secrete VEGF. Inhibition of the conditioned medium-induced HUVEC proliferation with the VEGF receptor blocker clearly demonstrates and confirms the importance of the angiogenic molecule, VEGF in whole milieu.

In conclusion, our study demonstrated that potent vasoactive proteins (ANG II and ET-1), via increased production of VEGF in proliferating human ASM cells, could indirectly contribute to vascular remodelling and angiogenic processes during bronchial pathophysiology. Our previous results (2,18), and the current data clearly demonstrate that ASM cells are an important source of angiogenic mediators in these airways. The mechanism underlying the signaling events leading to the expression of VEGF mRNA in response to vasoactive proteins are not entirely clear. It is now well established that the angiogenic factors, such as VEGF, produced by ASM cells in the vicinity of the endothelial cells appear to be a key paracrine event in the airways that contributes to the pulmonary vascular remodeling. Undoubtedly, more research focus has been recently directed towards the molecular events underlying the airway vascular remodelling. Though the precise nature of these events is far from clear, our study sheds a pos-

sible mechanism of vascular remodeling that could take place in chronic airway diseases.

ACKNOWLEDGMENTS

The authors thank Profs. A. J. J. C. Bogers and Henk C. Hoogsteden for providing fresh human lung tissue for ASM cell cultures. The study was financially supported in part by Netherlands Asthma Funds (Grant # 97.73).

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