Proinflammatory Cytokines and the VEGF in Airway Smooth Muscle

9.1 Summary

Airflow obstruction in chronic airway disease is associated with airway and pulmonary vascular remodeling of which the molecular mechanisms are poorly understood. Paracrine actions of angiogenic factors released by resident or infiltrating inflammatory cells following activation by pro-inflammatory cytokines in diseased airways could play a major role in the airway vascular remodeling process. Here, the pro-inflammatory cytokines, IL-1β and TNF-α were investigated on cell cultures of human airway smooth muscle (ASM) for their effects on mRNA induction and protein release of the angiogenic peptide, vascular endothelial growth factor (VEGF). IL-1β (0.5 ng/ml) and TNF-α (10 ng/ml) each increased VEGF mRNAs (3.9 and 1.7 kb) expression in human ASM cells, reaching maximal levels between 16-24h and 4-8 h, respectively. Both cytokines also induced time dependent release of VEGF, which was not associated with increased ASM growth. Pre-incubation of cells with 1 μM dexamethasone abolished enhanced release of VEGF by TNF-α. Data suggest that human ASM cells express and secrete VEGF in response to pro-inflammatory cytokines and may participate in paracrine inflammatory mechanisms of vascular remodeling in chronic airways disease.

9.2 Introduction

Airway remodeling is a common feature of both asthma and chronic obstructive pulmonary disease (COPD) and is characterized by structural changes including increased airway smooth muscle (ASM) mass, subepithelial fibrosis, glandular hypertrophy and peribronchial fibrosis, widely believed to culminate in poorly reversible airway narrowing. Recently, it has been demonstrated that vascular changes also occur during airway remodeling [7-6]. For instance, in asthma there is hyperemia of the bronchial vasculature and increased number and size of blood vessels. These changes correlate with the severity of disease in asthma [7,8] and the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF) from infiltrating inflammatory or resident structural cells has been implicated in their induction [9].

It is well established that ASM cells exhibit functions in addition to their structural and contractile properties, rather they proliferate and can express a wide range of adhesion molecules and pro-inflammatory or mitogenic factors, including, but not limited to tumor necrosis factor-α (TNF-α), interleukin (IL)-1β [10-12]. TNF-α is an important cytokine in chronic inflammation, its presence may further perpetuate airway inflammation by inducing the secretion of other pro-inflammatory cytokines from multiple cells types in the airways during chronic lung diseases [13,14]. Elevated
levels of TNF-α are found in bronchial tissues during chronic airway diseases. Similarly, IL-1β, is found in high levels in bronchoalveolar fluid and in the airway epithelium of asthmatic patients [13]. Many of the actions of IL-1β are similar to those of TNF-α and the signal transduction pathways of these cytokines may interact closely often resulting in synergism [15].

In many tissues, angiogenesis and increased vascular permeability are characteristic features of the wound healing process and of inflammation [16]. VEGF is a potent endothelial cell mitogen, which is reported to regulate vasculogenesis and postnatal vascular remodelling [17]. Its expression in the lung is increased in COPD and asthma [5], as well as during hypoxia [18] and levels of VEGF are reported to correlate with the increased size and number of blood vessels found in these conditions[8]. Moreover, the degree of VEGF expression correlates with levels of airway hyperresponsiveness in subjects with asthma[19]. VEGF is highly mitogenic for endothelial cells and induces their survival leading to nearby angiogenesis or bronchial vascular remodeling. It is also associated with increased endothelial permeability and induction of endothelial cell expression of chemokines (IL-8), adhesion molecules (ICAM-1) and proteolytic enzymes (matrix metalloproteinases) that promote changes in tissue ECM composition required for endothelial and inflammatory cell migration. Thus, VEGF may play an important role in chronic airway diseases like asthma by contributing to airway and vascular remodelling [2]. Consistent with this possibility, in an animal model, VEGF receptor inhibition prevents both airway inflammation and hyperresponsiveness induced by toluene diisocyanate (25).

The molecular mechanisms underlying the expression and release of VEGF during airway inflammation are largely unknown. The aim of the present study was to investigate whether ASM cells release VEGF when stimulated with the proinflammatory cytokines TNF-α or IL-1β and might therefore contribute to paracrine mechanisms of bronchial vascular remodeling during inflammation in chronic airways disease.

9.3 Materials and Methods

Materials
All cell culture reagents were obtained from Invitrogen (Life Technologies BV, Breda, The Netherlands). Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Recombinant human (rh) TNF-α was purchased from Knoll AG (Ludwigshaven, Germany). Foetal bovine serum (FBS) was obtained from Bio-Whitaker BV (Verviers, Belgium). [Methyl-3H]thymidine and [Methyl-3H]leucine were from Amersham Nederland BV (‘s-Hertogenbosch, The Netherlands). Human specific antibodies and the enzyme-linked immunosorbent assay (ELISA) kits were from
Human ASM cell culture

Human ASM cells were obtained in accordance with procedures approved by the Erasmus University Hospital Research Ethics Committee from the lobar or main bronchus of non-asthmatic patients undergoing lung resection for carcinoma of the bronchus using cell isolation and culture methods described previously [20]. Fluorescent immunocytochemical labelling confirmed that near confluent, FBS-deprived human ASM cells stained >95% for smooth muscle-α-actin. Under these conditions approximately 87% of propidium-iodide labelled cells remained in the G0/G1 phase of the cell cycle. Confluent cells in the 4th - 6th passage were used for all experiments.

Cytokine stimulation of human ASM cells in vitro

The ASM 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 KH2PO4, 8.1 mM Na2HPO4,2H2O, pH 7.4) and then replacing the medium with serum free DMEM supplemented with 1 μM insulin, 5 μg/ml transferrin and 100 μM ascorbate for 60 h. Growth-arrested cell monolayers were treated with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) in fresh FBS-free DMEM for 1, 2, 4, 8, 16, 24h. In additional sets of experiments, ASM cells were pretreated with 1 μM dexamethasone for 1 hr and then TNF-α was added and the incubation continued for 24 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.


Effects of pro-inflammatory cytokines on DNA biosynthesis and total protein biosynthesis were evaluated by incorporation of [methyl-^3]H thymidine and [methyl-^4]H leucine, respectively. Sub-confluent cell monolayers were growth arrested as described above. Cells were incubated with [methyl-^3]H thymidine or [methyl-^4]H leucine (1 μCi/well) in either fresh FBS-free DMEM or similar containing TNF-α, IL-1β or 10% FBS for 8, 24 or 48 h. Following stimulation 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 determined in a Packard 1500 Tri-carb liquid-scintillation counter (Packard-Becker BV, Delft, The Netherlands). Data are expressed as counts per minute of[^3]H thymidine or[^4]H leucine incorporation.
Isolation of total cellular RNA and Northern blot analysis

Treated and untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. Genomic DNA was sheared by passing lysates repeatedly through 23-gauge needles. Total cellular RNA was then isolated using as described previously [20]. Total RNA (10 μg) was 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 onto hybond-N membrane (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) by the alkaline downward capillary transfer method [20]. Filters were air-dried and UV cross-linked (Biorad Laboratories B.V., Veenendaal, The Netherlands) and blots hybridized at 42°C. cDNA insert (950 bp DNA fragment encoding human VEGF) was labeled using 32P-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 BV, 's-Hertogenbosch, The Netherlands) at -80°C. Hybridisation signals were quantified by scanning laser densitometry using the Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden) and normalised against GAPDH mRNA values and expressed as relative optical density (OD) in stimulated cells versus controls.

Measurement of VEGF protein by ELISA

Conditioned media were collected from TNF-α- or IL-1β-treated human ASM cells after 1,2,4,8, or 24h and VEGF levels assessed using a human VEGF specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit. Cell-conditioned medium samples were diluted until VEGF levels were within the linearity limits of the assay standard curve. The concentration of VEGF was expressed in pg/ml. The detection limit of the ELISA assay was 20 pg of VEGF/ml.

Statistical Analysis

All data in the figures are given as mean ± SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at p<0.05.

9.4 Results

Effect of pro-inflammatory cytokines on human ASM cell growth

There are reports that TNF-α and IL-1β can act as mitogens for ASM cells in culture [14,21]. Moreover, VEGF is a potent mitogen, particularly for endothelial cells but also indirectly for ASM cells [22,23]. Thus, we examined whether TNF-α or IL-1β, acting either directly or possibly
indirectly via VEGF production, induced significant changes in DNA synthesis ($[^3]H$/thymidine incorporation) or total protein synthesis ($[^3]H$/leucine) of human ASM. However, treatment of human ASM cells with TNF-α (10ng/ml) or IL-1β (0.5 ng/ml) for 8, 24 or 48 hours did not induce significant changes in $[^3]H$/thymidine or $[^3]H$/leucine uptake when compared with unstimulated cells (Figure 1).

![Graphs showing Thymidine and Leucine incorporation](image)

**Fig 1**

**Fig. 1.** Growth of ASM cells treated with TNF-α or IL-1β

Thymidine (Panel A and C) and Leucine (Panel B and D) incorporation measured after 8, 24 and 48 hours in semi-confluent, growth arrested, human ASM cells stimulated with 10 ng/ml of TNF-α (upper panels) or 0.5 ng/ml IL-1β (lower panels). Values are calculated from quadruplicate experiments and expressed as mean counts per minute (CPM) ±SEM. No significant differences were found between stimulated and unstimulated control cells.

**VEGF mRNA expression in relation to TNF-α or IL-1β**

To examine VEGF mRNA expression human ASM cells were treated with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) for 1, 2, 4, 8, 16 or 24 hours. Using Northern blot hybridization, two mRNA species of 3.9 and 1.7 kb encoding VEGF were detected in cultured human ASM cells treated with TNF-α (Figure 2, Panel A) or IL-1β (Figure 2, Panel B), which were absent in unstimulated
cells. TNF-α-induced VEGF mRNA levels were maximal during 4-8 h; whereas IL-1β-dependent VEGF mRNA content peaked at 16 h and remained elevated after 24 h, when compared with unstimulated cells. 10% FBS also induced the mRNA expression of VEGF in human ASM cells in vitro (not shown).

![Diagram of TNF-α and IL-1β induced expression of VEGF](image)

**Fig. 2**

**Fig. 2.** Pro-inflammatory cytokines induce expression of VEGF mRNAs in human airway smooth muscle cells
Representative Northern blots showing a major 3.9 and a minor 1.7 kb mRNA band for VEGF. Total RNA samples from control (C) and TNF-α (Panel A) or IL-1β (Panel B) treated human ASM cells were hybridized with a cDNA insert encoding human VEGF. Human ASM cells were incubated with TNF-α or with IL-1β or left unstimulated for the time points in hours as indicated on top of the blot. Panel C and D show the densitometric analysis for the blots represented as fold change in VEGF expression relative to GAPDH (as compared to control). Data represent the mean ± SEM of triplicate values from independent blots. * represents significant values (P ≤ 0.05) compared with controls.
Conditioned medium from human ASM cells treated with varying concentrations of TNF-α (0.2, 1, 2, 5, 10, 20 and 100 ng/ml) for 24 hours revealed a concentration-dependent release of VEGF (Figure 3). TNF-α induced VEGF secretion, as measured by ELISA, was 20±3 pg/ml, 110±0.5 pg/ml and 78.5±12.5 pg/ml after 0, 20 and 100ng/ml stimulation, respectively. VEGF release reached maximal levels (166 ±4 pg/ml) after stimulation with 10 ng/ml of TNF-α.

![Dose dependant release of VEGF](chart)

**Fig. 3**

**Fig. 3. Concentration-dependent production of VEGF protein by TNF-α**

Growth arrested human ASM cells were stimulated with 0.2, 1, 2, 5, 10 and 20 ng/ml of TNF-α for 24 hours. Data represent the mean ± SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from three patients. #, * represent significant values (P ≤ 0.05) compared with respective controls (# Vs stimulated cells, * Vs unstimulated cells).

Likewise, the release of VEGF protein from human ASM cells treated either with TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml) was time-dependent. TNF-α-induced VEGF secretion (Figure 4, panel A) was 40.67±6.8 pg/ml, 88.67±11.7 pg/ml and 162.3±59 pg/ml at 1, 4 or 8h, respectively. TNF-α-dependent VEGF release was maximal at 24 hours (173.3 ± 33.9 pg/ml), compared with unstimulated cells (37.67±7.2 pg/ml). Incubation with IL-1β resulted in 66 ±18 pg/ml, 267.3 ±15.5 pg/ml and 356.7 ±31.9 pg/ml at 1, 4 and 8 hours and was maximal after 24 hours where
levels reached $668\pm 116.4$ pg/ml (Figure 4, panel B). Additionally, FBS induced a modest release of VEGF at 24 hours ($290.67\pm 53.9$ pg/ml).

![Diagram A: TNF-α induced VEGF release](image)

![Diagram B: IL-1β induced VEGF release](image)

**Fig. 4**

**Fig. 4. Time-dependent production of VEGF protein by pro-inflammatory cytokines**

Growth arrested human ASM cells were stimulated with TNF-α or with IL-1β for varying times (1, 2, 4, 8, 24h). Control cells (Con) received only serum free medium. Data represent the mean ± SEM of triplicate values from independent experiments using conditioned media from ASM cells cultured from three patients. *$P \leq 0.05$ as compared with controls.

**Effect of dexamethasone on TNF–α induced expression of VEGF**

To assess whether anti-inflammatory glucocorticoids could inhibit release of VEGF by TNF-α (10ng/ml), ASM cells were pretreated with dexamethasone (1μM). Under these conditions, VEGF release was abolished by dexamethasone (Figure 5).

9.5 Discussion

Changes in airway microvasculature and the presence of angiogenesis in inflammatory respiratory diseases are now being documented [3]. Our study provides insight into possible mechanisms through which the angiogenic events may take place in chronic airway disease. We confirmed that human ASM cells express mRNA encoding VEGF and secrete VEGF protein and found that VEGF expression was increased by the proinflammatory cytokines, TNF-α and IL-1β, important modulators of airway function in asthma, and suppressed by anti-inflammatory glucocorticoids.
Inhibition of TNF-α induced VEGF release by Dexamethasone

**Fig. 5**

**Effect of Dexamethasone on TNF-α induced VEGF release**

Growth arrested human ASM cells were stimulated with 10 ng/ml of TNF-α for 24h. Human ASM cells were pretreated with 1 μM dexamethasone for 1 hr prior to stimulation with TNF-α for 24 h. Control cells received only serum free medium. Data represent the mean ± SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from three patients. *P ≤ 0.05 as compared with controls.

We speculate that paracrine actions of VEGF derived from ASM cells could perpetuate the chronic inflammatory process in asthma or COPD in several ways. For example, VEGF is highly mitogenic for endothelial cells and induces their survival leading to nearby angiogenesis or bronchial vascular remodeling. It is also associated with increased endothelial permeability and induction of endothelial cell expression of chemokines (IL-8), adhesion molecules (ICAM-1) and proteolytic enzymes (matrix metalloproteinases) that promote changes in tissue ECM composition required for endothelial and inflammatory cell migration.[24-26].

Recent reports describe the secretion of VEGF by human ASM cells in response to bradykinin and prostanoids (9,) as well as by Th2 cytokines, such as IL-4, IL-5 and IL-13 (20). Other reports reveal only a modest effect of these cytokines on VEGF secretion by bronchial fibroblasts [27]. Cytokines including IL-1 and IL-6 have also been shown to upregulate VEGF protein expression, but their effects appear variable and species or tissue specific. In the present study, up regulation of VEGF by human ASM cells occurred both at the mRNA and protein level, corroborating recent similar findings with IL-1β [23,28]. We also report that TNF-α, another major proinflammatory
cytokine, increased VEGF mRNA and protein secretion in human ASM cells. Induction of VEGF secretion by these cytokines was time dependent, peaking at 24 hours and was concentration-dependent in case of TNF-α with optimum concentration being 10 ng/ml. Our findings with IL-1β showing a relatively slow induction of mRNA and release of protein are in broad agreement with those of others [23] where IL-1β-dependent VEGF secretion was increased at 16 hours after stimulation. However, accumulation of VEGF mRNA after stimulation by TNFα appeared more rapid occurring within 2 to 4 hours and contrasts with Kazi and colleagues [23], who reported no increase in VEGF by TNFα. Reasons for this discrepancy are unclear, but our observation that neither TNFα nor IL-1β induced proliferation of ASM cells under these conditions suggests the increased VEGF levels we observed were not artificially increased due to increased cell numbers.

Increasing experimental evidence suggests inhibition of VEGF activity via blockade of its receptors has potential therapeutic value as an intervention method for decreasing angiogenesis and vascular remodeling [29]. This approach reverses pathophysiologial symptoms including airway hyper-responsiveness and inflammation in a mouse model of asthma [30]. One likely mechanism for the effectiveness of this approach could be prevention of VEGF-induced vascular permeability, thereby suppressing inflammation by reducing vascular leakage and migration of cells and mediators into the airways. However, the VEGF inhibition could be both beneficial and detrimental in different forms of chronic airway diseases. For example, some reports suggest increased levels of VEGF are associated with airflow limitation in bronchitis, whilst decreased levels are also associated with airflow limitation and alveolar destruction in patients with emphysema [31,32]. Likewise, recent evidence demonstrates that long-term glucocorticoids treatment in patients with asthma significantly affects airway remodeling by reducing basement membrane thickness, and reducing indices of submucosal vascularity including blood vessel number and total vascular area [33,34]. Our finding that dexamethasone completely inhibits the release of the angiogenic factor VEGF would support this notion. The inhibitory action of dexamethasone on TNF-α induced VEGF release may be mediated by several mechanisms, including the reduction of cAMP levels or inhibition of p38 MAPK phosphorylation [35]. Dexamethasone suppresses TNF-α-induced AP-1 DNA binding suggesting that glucocorticoids may exert their inhibitory effect on TNF-α by trans-repression of AP-1[36]. The effect of glucocorticoids on airway remodeling, however, remains controversial. Inhibition of VEGF production by ASM cells suggests one mechanism by which glucocorticoids might affect tissue remodeling.

Localization studies in the airways of asthmatics suggest VEGF is expressed by both infiltrating inflammatory and resident cell types including submucosal glandular and alveolar type I and II
epithelial cells, myofibroblasts, eosinophils, macrophages and CD34+ cells [19]. The relative importance of each cell type for release of VEGF under pathophysiological conditions is unknown. However, consistent with ASM also participating in these processes we and others have demonstrated that human ASM cells express VEGF both in vitro and in vivo in the intact myobundles of patients with COPD [5,37,38]. These latter findings suggest that VEGF release by cultured human ASM cells is not a simple culture artifact rather ASM represents a possible pathophysiological important pool of VEGF available during airways inflammation that may be amenable to therapeutic intervention.

In summary, VEGF is secreted by ASM cells in response to the proinflammatory mediators, IL-1β and TNF-α acting via pathways that are sensitive to inhibition by anti-inflammatory glucocorticoids. The current study supports the hypothesis that ASM cells are active sources of mediators relevant to the pathogenesis of airway disease. Proinflammatory cytokines such as IL-1β and TNF-α that associated with chronic inflammation of the airways may drive production of VEGF by ASM cells, which via paracrine mechanisms in the vicinity of bronchial endothelial cells may perpetuate the bronchial vascular remodeling that characterizes asthma and COPD.

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9.6 References


(flk-1 and flt-1) in Patients with Chronic Obstructive Pulmonary Disease. Thorax -in press.


