

Airway smooth muscle cell responses  
in relation to mediators of asthma

Sue McKay

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AIRWAY SMOOTH MUSCLE CELL RESPONSES  
IN RELATION TO MEDIATORS OF ASTHMA

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relatie tot mediators van astma

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## **General introduction and aims of the studies**

## 1.1 Asthma

“Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough. These symptoms are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli” [1]. These stimuli include tobacco smoke, fog, nitrogen dioxide [2], sulphur dioxide, ozone, viral infections [3], exposure to cold air and smooth muscle agonists such as methacholine and histamine [2, 4-8]. Exposure to specific stimuli such as allergens (e.g. house dust mite and pollen) can also lead to an increase in airway hyperresponsiveness [9-11].

Asthma severity can be classified as mild intermittent, mild persistent, moderate persistent, and severe persistent to accurately reflect the clinical manifestations of the disease. Patients at any level of severity can have mild, moderate, or severe exacerbations [1]. Asthma pharmacotherapy should be instituted in conjunction with environmental control measures that reduce exposure to factors known to increase the patient’s asthma symptoms. A stepwise approach to therapy is recommended, with the type and amount of medication dictated by asthma severity. Because inflammation is considered an early and persistent component of asthma, therapy for persistent asthma must be directed toward long-term suppression of the inflammation. Medications are categorised into two general classes: *long-term-control medications* (controllers) to achieve and maintain control of persistent asthma and *quick-relief medications*

(relievers) to treat symptoms and exacerbations [12]. Currently available anti-asthma therapies include glucocorticosteroids, sodium cromoglycate, nedocromil sodium, sustained release theophylline, long acting  $\beta_2$  agonists, anti-allergic agents and leukotriene antagonists (controllers) to reduce inflammation and short acting inhaled or oral  $\beta_2$  agonists, glucocorticosteroids, anticholinergics and short acting theophylline to dilate the bronchi [13].

Asthma is one of the most common chronic airway disorders with 150 million sufferers worldwide, leading to 1 million unnecessary deaths each decade [1, 12]. The prevalence of asthma is increasing in most countries (20-50% every 10 years) especially in children and adolescents. Important pathological features of asthmatic airways include inflammatory cell infiltration: neutrophils especially in sudden-onset, fatal asthma exacerbations, eosinophils and  $Th_2$ -lymphocytes. Denudation of airway epithelium, goblet cell hyperplasia, basement membrane thickening, oedema, mast cell activation and increased mass of airway smooth muscle (ASM) [14-19].

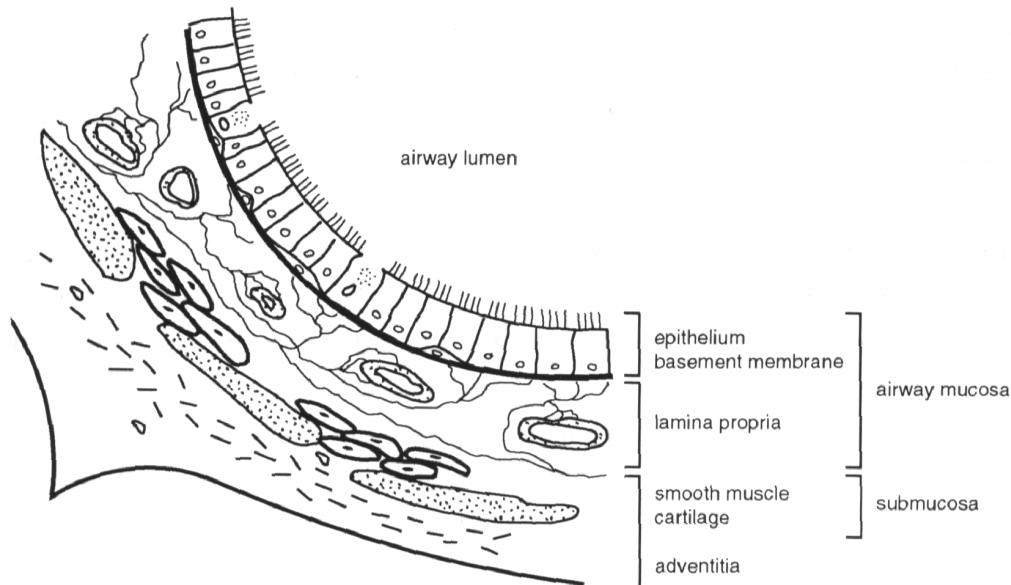
## **1.2 Airway structure and function**

The airway mucosa consists of an epithelial layer, the basement membrane and the lamina propria (figure 1.1).

### ***1.2.1 Epithelium***

The epithelium is a multifunctional tissue built up from a number of different cell types with varying functions, these include ciliated cells, secretory cells and basal cells. The ciliated cells primarily promote the flow of mucus in a cranial direction. The secretory cells, including goblet cells and Clara cells, play a key role in the generation and

maintenance of the mucociliary system. The basal cells, closely attached to the basement membrane, are considered to be progenitor cells for the airway epithelium.



**Figure 1.1** Schematic diagram of an airway wall from a medium sized bronchus

The epithelium serves as a barrier between the respiratory system and the external environment. It forms not only a physical barrier but also an immunologic and metabolic barrier [20]. Epithelial damage, observed in asthma, may result in increased permeability and easier access of allergens, pollutants, and inflammatory and contractile agents to subepithelial layers [16, 21].

### **1.2.2 Basement membrane**

The basement membrane consists of two layers, the basal lamina comprising type IV collagen, laminin, actin, proteoglycans and fibronectin, and the lamina reticularis comprising collagen fibrils [22-25]. The basement membrane separates the epithelium

from the lamina propria and provides an elastic support for epithelial cells. Extensive thickening of the basement membrane has been reported in asthma [16, 21, 26].

### ***1.2.3 Lamina propria***

The lamina propria consists of arterioles, capillaries and postcapillary venuoles embedded in fibrous tissue. An increase in the permeability of this microvasculature and plasma exudation are considered to be important features of asthmatic airways [27-32]. New vessel formation (angiogenesis) can contribute to the thickening of the airway wall.

### ***1.2.4 Airway smooth muscle***

The submucosa contains smooth muscle, glands and cartilage (figure 1.1). The smooth muscle in the trachea is inserted dorsally between the ends of the horseshoe-shaped cartilage rings and runs transversely to form a strip in the membranous part of the trachea. In the bronchi the smooth muscle is configured in helical strands around the airway circumference and is located internally with respect to the cartilage plates. Smooth muscle tone is important in determining airway diameter and thus affects airway resistance. In asthma an increase in ASM mass has been reported by a number of authors [18, 33-35]. Studies using detailed computer models of human airways indicate that increased ASM mass is one of the most important abnormalities responsible for excessive airway narrowing of the airways in asthma [33]. The increased amount of ASM in asthmatics is an indication of abnormal cell proliferation and growth. Both hyperplastic (i.e. increase in cell number) and hypertrophic (i.e. increase in cell size) changes are thought to contribute to the increased ASM content of the airway wall [17, 18, 36].

ASM cells have been shown to express either protein or mRNA encoding various receptors and cell surface molecules, these are summarised briefly in table 1.1.

**Table 1.1** Receptors/surface molecules expressed by ASM cells.

Cytokines	Adhesion molecules	Growth factors	Broncho-constrictors	Broncho-dilators	Others
GM-CSF [37]	ICAM-1 [41]	PDGF- $\alpha/\beta$ [42]	H <sub>1</sub> [44, 45]	$\beta_2$ [56]	MHC II [58]
IL-2R [37]	VCAM-1	FGFR-1/2 [43]	M <sub>3</sub> [46]	EP <sub>2/4</sub> [57]	CD44 [41]
IL-5R [37]	[41]		Cys-LT <sub>1</sub> [47]		Fc $\epsilon$ RII [59]
IFN- $\gamma$ R [37]			ET-A/B [48,		LTB <sub>4</sub> [47]
IL-12 [37]			49]		M <sub>2</sub> [60]
TNF			TP [50, 51]		H <sub>2</sub> [61]
- p75 [38]			NK <sub>2</sub> [52, 53]		
- p55 [38]			5-HT <sub>2</sub> [54]		
- CD40 [39]			5-HT <sub>3</sub> [55]		
TGF- $\beta_1$ [40]			B <sub>2</sub> [51]		
IL-1 $\beta$ [62, 63]		EGF [65]	Ang II [67]		
IL-6 [64]		IGF [66]			

Shaded area includes receptors that have not yet been directly identified on ASM cells, but are assumed to be present due to responses that have been measured.

### 1.3 Airway wall inflammation in asthma

Airway wall inflammation is an important pathological feature of asthmatic airways, with infiltration and activation of eosinophils, T lymphocytes, mast cells and macrophages [14-16, 68, 69]. These cells can influence airway function through secretion of preformed and newly synthesised mediators. Furthermore, subpopulations of T lymphocytes (Th<sub>2</sub>) have been identified as important cells that may regulate allergic inflammation in the airway through the release of selective cytokines [70]. In addition, constituent cells of the airway, including fibroblasts, endothelial cells, epithelial cells and ASM cells, also contribute to this process by releasing cytokines and chemokines (table 1.2).

Allergic airway inflammation develops following the uptake and processing of inhaled allergens by antigen-presenting cells such as dendritic cells and macrophages. It is apparent that asthma is not caused by either a single cell or single inflammatory mediator but rather results from complex interactions among inflammatory cells, mediators, and other cells and tissues resident in the airways. It is hypothesised that the initial release of inflammatory mediators from bronchial mast cells, macrophages, T lymphocytes and epithelial cells leads to the subsequent migration and activation of other inflammatory cells, such as eosinophils and neutrophils, to the airway. The release of inflammatory mediators and cytokines, by these infiltrating cells, results in epithelial damage, smooth muscle contraction, mucus hypersecretion and increased ASM responsiveness.

#### **1.4 Airway wall remodelling**

Airway wall remodelling can be defined as structural changes in the airway, such as sub-epithelial fibrosis, mucous gland and goblet cell hyperplasia, epithelial disruption, increased ASM mass (hypertrophy and hyperplasia) and new vessel formation (angiogenesis). The continuous process of healing and repair, due to chronic airway inflammation, together with the release of cytokines, chemokines and growth factors by infiltrating inflammatory cells and resident airway cells, can lead to airway wall remodelling [16, 21, 71-75]. The cellular and molecular events underlying the remodelling process are still poorly understood, and the ability of currently available anti-asthma therapies to prevent or reverse airway wall remodelling is uncertain [76-78].

## **1.5 Airway hyperresponsiveness**

Airway hyperresponsiveness (AHR) is an exaggerated bronchoconstrictor response to a wide variety of stimuli. AHR leads to clinical symptoms of wheezing and dyspnea after exposure to allergens, environmental irritants, viral infections, cold air or exercise [1]. Airway inflammation is not the only factor that contributes to airway hyperresponsiveness. An increase in airway wall thickening, due to increased ASM mass, sub-epithelial fibrosis, matrix deposition and mucus gland hyperplasia, can increase bronchial hyperresponsiveness and profoundly affect airway narrowing due to smooth muscle contraction [33, 36, 79-82]. Small changes in airway wall thickness, that have little effect on baseline airway resistance, can markedly increase airway responsiveness to inhaled agonists and a subsequent increase in resistance to airflow caused by smooth muscle shortening [82].

## **1.6 Mediators of asthma**

Infiltration of inflammatory cells into the airways and the subsequent release of pro-inflammatory mediators, cytokines, chemokines and growth factors can induce airway hyperreactivity and airway wall remodelling [13, 68, 72-75, 83]. The potential sources of inflammatory mediators in asthmatic airways are summarised in table 1.2.



**Table 1.2** Sources of cytokines, growth factors and inflammatory mediators in inflamed airways

Source	Inflammatory mediators	Ref
Airway smooth muscle cell	IL-1, IL-2, IL-5, IL-6, IL-8, IL-11, IL-12, IFN- $\gamma$ , LIF, eotaxin, RANTES, GM-CSF, MCP, MIP, FGF-2, PDGF, IGF-2, TGF- $\beta$ <sub>1</sub> , VEGF, prostaglandins, TX,	[37, 63, 66, 84-100]
Fibroblast	MCP's, MIP, RANTES, IL-6, IL-8, GM-CSF, SCF	[101-104]
Eosinophil	MBP, EDN, ECP, EPO LTC <sub>4</sub> , PAF, O <sub>2</sub> <sup>-</sup> , MMP-9 IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TGF- $\beta$ , GM-CSF, TNF- $\alpha$ , MIP-1 $\alpha$ , TIMP-1, PDGF	[105-108]
Epithelium	IL-1 $\beta$ , IL-6, IL-8, GM-CSF, ET-1, FGF, RANTES, MCP, MIP-1 $\alpha$ , TIMP-1, TNF- $\alpha$ , TGF- $\beta$ , PDGF, PGE <sub>2</sub> , EGF, SubsP, LTD <sub>4</sub>	[84, 105]
Macrophage	IL-1, IL-6, IL-10, GM-CSF, TNF- $\alpha$ , prostaglandins, TX's, LT's, PAF, FGF, ET-1, TGF- $\beta$ , PDGF, MCP, IGF, EGF	[84, 105]
Mast cell	IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, GM-CSF, TNF- $\alpha$ , TGF- $\beta$ , histamine, tryptase, chymase, bradykinin, prostaglandins, PAF, LT's, heparin	[105, 106]
Neutrophil	myeloperoxidase, lysozyme, LTA <sub>4</sub> , LTB <sub>4</sub> , IL-1 $\beta$ , IL-6, IL-8, prostaglandins, PAF, TXA <sub>2</sub> , TNF- $\alpha$ , TGF- $\beta$ , elastase, collagenase, MMP-9	[106]
Th <sub>1</sub> -lymphocyte	IL-2, IL-3, GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$	[105]
Th <sub>2</sub> -lymphocyte	IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF	[105]
Plasma/platelet	5-HT, Ang II, EGF, PDGF, IGF-1, TGF- $\beta$ <sub>1</sub> , ET-1, thrombin, LPA	[109, 110]

## 1.7 Transcription factors

Transcription factors are DNA binding proteins that regulate the expression of genes, including genes encoding inflammatory molecules and enzymes involved in the synthesis of inflammatory mediators (both chemical and peptide). They can bind to regulatory sequences, often found in the promoter region of the gene of interest, and

their function is to increase or decrease the rate of transcription of that gene. Transcription factors include activator protein-1 (AP-1), nuclear factor- $\kappa$ B (NF- $\kappa$ B) and early growth response gene-1 (*egr-1*), which are universal transcription factors that are involved in the expression of many inflammatory and immune genes and may play an important role in regulating the inflammatory response in the airway [111-118].

Immediate early response genes, such as *c-fos*, *c-jun* and *egr-1* are genes whose induction can occur without intervening protein synthesis; they require only the modification of pre-existing transcriptional modulators. They are induced by many different stimuli (e.g. growth factors, cytokines, ionising radiation) and are associated with cellular responses such as mitogenesis, cell differentiation, cell survival and apoptosis [112-114, 119-125].

The proteins FOS and JUN form a heterodimer, held together by a “leucine zipper” – leucine rich areas on both FOS and JUN allowing the two proteins to dimerise. JUN can also form homodimers but FOS cannot. FOS-JUN and JUN-JUN dimers can bind to the AP-1 DNA consensus sequence, 5'-TGA(C/G)TCA-3'. There are also three other FOS-related proteins, FRA-1, FRA-2 and FOS-B and two other JUN-related proteins JUN-B and JUN-D that can form dimers, these can also bind to AP-1 DNA sequences. However, not all of the heterodimers can transcriptionally activate AP-1 specific sequences. Additionally JUN and FOS proteins can form dimers with cAMP response element binding protein (CREB) that bind to CRE sequences rather than AP-1 sequences. The EGR-1 protein contains three zinc finger motifs that bind and regulate transcription via the DNA consensus-binding site 5'-GCG(G/T)GGGCG-3'.

In the context of asthma, transcription factors activated by inflammatory stimuli and growth factors regulate the transcription rate of inflammatory proteins and proteins necessary for cell mitogenesis. Transcription factors may therefore be considered as potential targets for the development of drugs influencing airway wall inflammation or airway wall remodelling [126]. Many transcription factors have been identified, but we have concentrated our studies on the expression of the immediate early response genes encoding *c-fos* and *c-jun* (constituents of the AP-1 transcription factor) and *egr-1* because they are implicated in the regulation of many inflammatory and immune genes that may play an important role in regulating the inflammatory response in asthmatic airways [111-118].

Many genes contain binding sites for the AP-1 and *egr-1* transcription factors. The variability in the biological response to a particular ligand can occur as a consequence of:

(i) Differential quantitative induction of early response genes, e.g. *c-fos* and *egr-1* upregulation, but no *c-jun*, and as a result no formation of the AP-1 transcription factor but upregulation of *egr-1* responsive genes.

(ii) Differences in induction kinetics of the response genes and their proteins, e.g. if *c-fos* is rapidly induced but *c-jun* induction follows 30 minutes later, regulation of AP-1 responsive genes will not be immediate.

(iii) Formation of changing patterns of heterodimeric transcription factors with differing transcriptional capacities, e.g. junD-JUN heterodimers do not activate AP-1 binding sites in the promoter region of inflammatory genes as well as FOS-JUN heterodimers. Fra-1-JUN heterodimers bind efficiently to DNA but do not transcriptionally activate AP-1 sequences.

(iv) Cell-specific restriction of the expression of subsets of early response genes, e.g. a cell that does not express *c-fos* at all, will not be able to express FOS-JUN heterodimers.

(v) Ligand- and cell-specific posttranslational modification of early response gene products and consequent alterations in the biological properties of these proteins, e.g. the phosphorylation of JUN protein so that it cannot form a heterodimer with FOS protein, and not activate the AP-1 site.

(iv) Differential production of autocrine and paracrine factors that modulate initial inductive responses, e.g. repression of *c-fos* transcription by FOS protein

## **1.8 Aims of the studies**

The increase in ASM mass in asthmatic airways could contribute to the exaggerated airway narrowing observed in this disease and may result from the action of cytokines, growth factors and other chemical mediators released during the chronic inflammatory process. In addition, ASM cells may modulate the inflammatory processes in the airway through release of cytokines, chemokines or other pro-inflammatory mediators. Therefore, the ASM could potentially exhibit different phenotypes: a “contractile phenotype” responsible for the regulation of airway calibre, a “proliferative phenotype” allowing an increase in ASM cell numbers and/or size, as well as a “synthetic phenotype” resulting in the synthesis and release of mediators.

ASM cells in culture provide a convenient model for studying a wide variety of airway responses at a cellular and/or molecular level. The aim of the studies described in this thesis were (i) to identify specific factors involved in the stimulation of ASM mitogenesis, (ii) to investigate the expression of the immediate early response genes *c-fos*, *c-jun* and *egr-1*, and (iii) to examine ASM cell secretory capabilities and the

underlying mechanisms.

ASM muscle is considered to be a target for mediators released during airway wall inflammation. A number of studies suggest that the induction of the immediate early response genes (e.g. c-fos, c-jun, egr-1) can be considered as markers for proliferation and/or differentiation. Additionally, ASM cells are reported to synthesise and release cytokines and chemokines that are potentially important for the perpetuation, development or modulation of airway wall inflammation. We hypothesised that the pro-inflammatory cytokines IL-1 $\beta$ , IL-5, IL-6 and TNF- $\alpha$  and the contractile agonists ET-1 and histamine, all upregulated during symptomatic asthma, were involved in the stimulation of ASM cell mitogenesis. The aim of the study described in **Chapter 2** was to investigate the effects of these pro-inflammatory mediators on the expression of mRNA encoding c-fos, and subsequent cell proliferation as well as the expression and secretion of the pleiotropic cytokine IL-6.

Early growth response gene-1 (egr-1) is a sequence specific, zinc finger transcription factor that plays a regulatory role in the expression of various genes, such as five-lipoxygenase, TGF- $\beta_1$  and fibronectin, implicated in asthma. We hypothesised that human ASM cells would express egr-1 in the presence of pro-inflammatory cytokines and growth factors and that this would be followed by the expression of other genes implicated in asthma. The aim of the study described in **Chapter 3** was to investigate the expression of egr-1 in human ASM cells treated with the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  or treated with the growth factors PDGF-AB and FGF-2. Fibronectin mRNA expression and cell proliferation were also measured.

Angiotensin II (Ang II) is a potent vasoconstrictor and mitogen for a wide variety of cells and has been implicated in bronchoconstriction in asthmatics. We hypothesised that this octapeptide would induce expression of immediate early response genes as well as proliferation and/or hypertrophy of ASM cells. Expression of *egr-1*, *c-fos* and *c-jun* transcription factors and TGF- $\beta_1$  mRNA and protein were measured in Ang II treated human ASM cells. This study is described in **Chapter 4**.

The AP-1 transcription factor can bind to the AP-1 binding site in the promoter region of many inflammatory genes, including the IL-6 gene, leading to the increased synthesis of inflammatory proteins. AP-1 is a heterodimer of FOS and JUN oncoproteins. We hypothesised that TNF- $\alpha$ -induced IL-6 protein secretion was regulated through the AP-1 transcription factor, and that IL-6 protein secretion would be increased with increasing concentrations of TNF- $\alpha$ . The aim of the study described in **Chapter 5** was to investigate expression of mRNA for *c-fos* and *c-jun* (genes encoding the proteins FOS and JUN) and to determine whether these genes were involved in the regulation of IL-6 expression.

PDGF-AB is a mitogen for human ASM cells. We hypothesised that IL-6 gene expression is also induced by the growth factor PDGF-AB and regulated through the transcription factors AP-1, C/EBP $\beta$  and NF- $\kappa$ B. The aim of this study was to determine part of the transcriptional regulatory pathway of IL-6 gene expression in human ASM cells. This is described in **Chapter 6**.

**Chapter 7** reviews the role of ASM in the autocrine regulation of airway inflammation.

In **Chapter 8** we discuss the studies described in this thesis and the implications of our findings for future research.

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**Pro-inflammatory stimuli induce c-fos expression  
followed by IL-6 release in human  
airway smooth muscle cells**

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## 2.1 Summary

Airway smooth muscle (ASM) is considered to be a target for mediators released during airway inflammation. We investigated the ability of various pro-inflammatory cytokines (tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin(IL)-1 $\beta$ , IL-5, IL-6), and other mediators (histamine, endothelin-1 (ET-1)) to induce the expression of c-fos, a constituent of the transcription factor activator protein-1 (AP-1). In addition, we measured the release of IL-6 into the conditioned medium of stimulated ASM cells as well as DNA biosynthesis and changes in cell number. All of the mediators investigated induced a rapid (within 1 h) and transient increase in the expression of mRNA encoding c-fos followed by the expression and enhanced release of IL-6. Cell proliferation, assessed by thymidine incorporation assays and cell counting, remained unchanged in cytokine- and mediator-stimulated cells. Our results suggest that cytokine and mediator induced c-fos expression in ASM cells could be described as a marker of cell 'activation', during airway inflammation, rather than as a marker of proliferation.

## 2.2 Introduction

Asthma is a chronic disease of the airways characterised by reversible airway obstruction and airway hyper-responsiveness. Important pathological features of asthmatic airways include inflammatory cell infiltration, epithelial shedding, basement membrane thickening and increased mass of ASM. Both hyperplasia (an increase in cell number) and hypertrophy (an increase in cell size) of ASM cells contribute to the increased smooth muscle content of the airway wall [1-3]. Human ASM cells in culture provide a useful model for investigating physiological responses, and studies of human ASM cell mitogenesis and the identification of the specific factors involved in regulating ASM

growth are important for understanding the mechanisms of airway remodelling.

Bronchoalveolar lavage fluid and serum from symptomatic asthma patients contain significantly increased levels of pro-inflammatory cytokines, growth factors and other inflammatory mediators, suggesting that these mediators may also be in the vicinity of the airway mucosa [4-11]. Moreover, pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 have been reported to stimulate DNA synthesis in cultured ASM cells derived from different species [12-14]. ET-1 and histamine have also been implicated in ASM cell mitogenesis [15-20], providing a potential link to the observed hyperplasia and hypertrophy of smooth muscle in asthmatic airways [2, 3, 21].

The purpose of the present study was to investigate the effects of a range of pro-inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , IL-5, IL-6, histamine, ET-1) on the expression of the immediate early response gene, c-fos, as well as the release of the pleiotropic cytokine IL-6 by cultured human ASM cells. The nuclear proto-oncogene c-fos encodes a protein that dimerises with c-jun to form the transcription factor AP-1. Induction of the c-fos gene, and subsequent cell proliferation and/or differentiation suggests that c-fos can be considered as a proliferation marker [19, 22]. IL-6 has pro-inflammatory effects that may be relevant to airway wall inflammation, including mucus hypersecretion, the terminal differentiation of B cells into antibody producing cells, upregulation of IL-4-dependent IgE production as well as differentiation of immature mast cells [23-26]. Possible anti-inflammatory properties of IL-6 include the inhibition of macrophage cytokine production, stimulation of the production of anti-inflammatory molecules such as IL-1 receptor

antagonist and soluble TNF receptor p55 and reduced airway responsiveness [27-32]. Our results demonstrate that all of the pro-inflammatory mediators investigated induced a rapid and transient expression of mRNA encoding c-fos. Moreover, they induced the release of IL-6 protein into the conditioned medium. However, none of the mediators stimulated DNA biosynthesis or ASM cell mitogenesis. These results suggest that it may be preferable to describe c-fos as a marker of 'activation' in ASM cells – leading to the expression of cytokines, such as IL-6, rather than as a marker of proliferation.

### **2.3 Materials and Methods**

#### *Human airway smooth muscle cell isolation and culture*

Human airway smooth muscle cells were isolated and cultured as described earlier [33]. Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained immediately following surgery of patients with lung carcinoma. After removal of the epithelium, pieces of smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle fragments were incubated in HBSS containing BSA (fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) at 37°C in a humidified incubator (ASSAB, Clean Air Techniek BV, Woerden, The Netherlands, model T154) containing 5% CO<sub>2</sub> in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in DMEM containing 10% (v/v) FBS 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). Cells were subsequently seeded in 35 mm dish and maintained in culture by replacing the medium every 72 h. After 10-14 days in culture, ASM cells grew to confluence and were then removed from the plastic base



of each dish using 0.5% trypsin/0.02% EDTA, and sub-cultured into 25cm<sup>2</sup> tissue culture flasks. At confluency, cells were further passaged using trypsin/EDTA solution into 75cm<sup>2</sup> tissue culture flasks. Confluent cells in the 4<sup>th</sup> - 6<sup>th</sup> passage were used for experiments.

#### *Stimulation of human airway smooth muscle cells*

The ASM cell growth was synchronised 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 µM insulin, 5 µg/ml transferrin and 100 µM ascorbate for 72 h. Using flow cytometric analysis of human ASM cells, we previously found that 72 h of serum deprivation resulted in approximately 85% of the cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase. Growth-arrested cell monolayers were treated with either TNF-α (10 ng/ml), IL-1β (0.5 ng/ml), IL-5 (2 ng/ml) or IL-6 (5 ng/ml); ET-1 (1 nM), histamine (1 µM) or serum (10%) in fresh FBS-free DMEM in a time dependent manner. Stimulation of human ASM cells with cytokines can induce the release of prostaglandins, which may then inhibit the proliferation of ASM cells [34]. Therefore, we also examined the effects of TNF-α, IL-1β, IL-5 or IL-6 on human ASM cell proliferation in the presence of indomethacin (1 µM), which is a non-specific cyclo-oxygenase inhibitor.

#### *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. 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 [33]. 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 a 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 hybrid-N membrane (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) by the alkaline downward capillary transfer method also described earlier [33]. The filters were air-dried and UV cross-linked in a gene linker (Biorad Laboratories B.V., Veenendaal, The Netherlands). Blots were hybridised with radiolabelled mouse c-fos (2.1 kb fragment) or a reference house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (American Type Culture Collection, Rockville, USA. [33]). Hybridisation signals were quantified by scanning laser densitometry using the Ultrosan 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.

#### *Measurement of IL-6 protein levels by ELISA*

IL-6 protein levels in ASM cell-conditioned medium, after 24 h stimulation, were determined using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) as described earlier [35]. The increase in IL-6 secretion was expressed as ng/ml, and data are shown as fold increase in IL-6 secretion relative to respective controls. The detection limit of the IL-6 ELISA method was 50 pg of IL-6/ml. No correction for cell number variation was made since the cells were serum deprived 16-24 h after plating out, allowing insufficient time for proliferation.

### *[<sup>3</sup>H]Thymidine incorporation assay*

Effects of cytokines, growth factors and inflammatory peptides on DNA biosynthesis was evaluated by incorporation of [*methyl*-<sup>3</sup>H]thymidine. Cells were transferred into 24-well plates at a seeding density of  $3 \times 10^4$  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  $\mu$ Ci/well) in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ 10% serum) or DMEM containing the various stimuli for 1, 2, 3, 4 or 5 days. 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 liquid-scintillation counter (Packard 1500 Tri-carb liquid scintillation analyser, Packard-Becker BV, Delft, The Netherlands). The data are expressed as fold increase in thymidine incorporation relative to controls.

### *Cell counting*

In order to assess changes in cell number in relation to pro-inflammatory stimuli we also performed direct cell counting. Cells were incubated with the above mentioned mediators for 7 days. Following stimulation, the cell-conditioned medium was removed and the cells were washed twice in PBS and then detached in 50  $\mu$ l of trypsin-EDTA by incubating at room temperature for 10 min. We then added 50  $\mu$ l PBS to each well and the cells were dispersed by gently pipetting. The resulting cell suspension was added to 10 ml isotonic counting solution (sodium chloride 6.38 g/l, sodium tetraborate 0.2 g/l, boric acid 1.0 g/l, EDTA 0.2 g/l) and the cells were counted in a CASY-1 Coulter counter (Schärfe system, Reutlingen, Germany).

### *Materials*

The following solutions and reagents were obtained from Life Technologies BV (Breda, The Netherlands): Hank's balanced salt solution (HBSS), sodium pyruvate, non-essential amino acid mixture, gentamicin, penicillin/streptomycin and amphotericin B, Dulbecco's modified Eagle's medium (DMEM), 0.5% trypsin;0.02% EDTA. Bovine serum albumin (BSA, fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml), insulin, transferrin and ascorbate, IL-1 $\beta$ , IL-5, ET-1, 5-HT, histamine were purchased from Sigma BV (Zwijndrecht, The Netherlands). We procured foetal bovine serum (FBS) from Bio-Whitaker BV (Verviers, Belgium), TNF- $\alpha$  from Knoll AG (Ludwigshaven, Germany), [*Methyl*-<sup>3</sup>H]thymidine from Amersham Nederland BV ('s-Hertogenbosch, The Netherlands) and IL-6 from Promega (Leiden, The Netherlands). Antibodies used in the IL-6 ELISA were procured from Medgenix (Breda, The Netherlands).

### *Statistical Analysis*

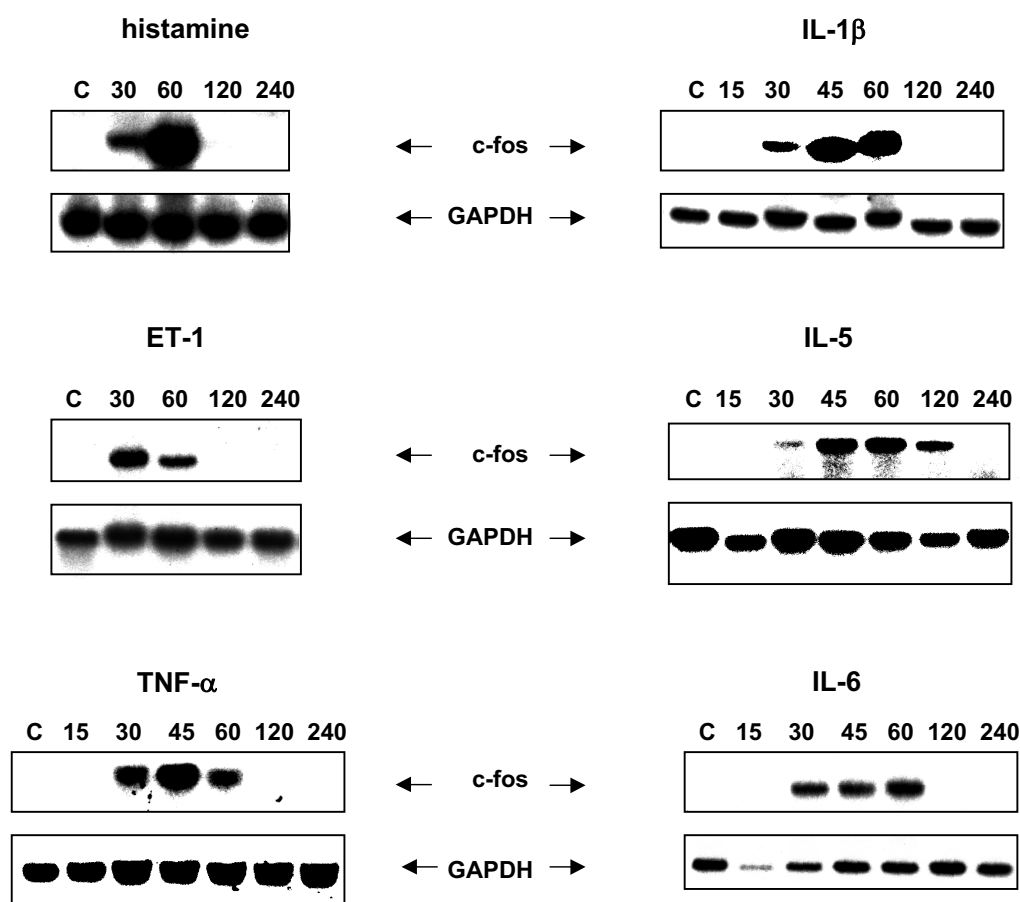
All data in the figures and tables are given as mean  $\pm$  SEM. Statistical analysis was performed by using two-tailed, independent sample "t"-test. Significance was accepted at  $p < 0.05$ .

## **2.4 Results**

### *Expression of the c-fos proto-oncogene*

Expression of the proto-oncogene c-fos, was examined in cultured human ASM cells treated with different cytokines and inflammatory mediators. Representative Northern blots showing the expression pattern of the proto-oncogene c-fos in relation to TNF- $\alpha$  (10 ng/ml), IL-1 $\beta$  (0.5 ng/ml), IL-5 (2 ng/ml), IL-6 (5 ng/ml), histamine (1  $\mu$ M) or ET-1 (1 nM)

are shown in figure 2.1. Densitometric analysis of these Northern blots revealed that all of the mediators investigated induced the rapid (within 1 h) and transient expression of mRNA encoding c-fos. The induction of c-fos mRNA reached a maximum between 30-60 min followed by an abrupt decline. 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 was expressed in each RNA preparation (figure 2.1 lower panels).

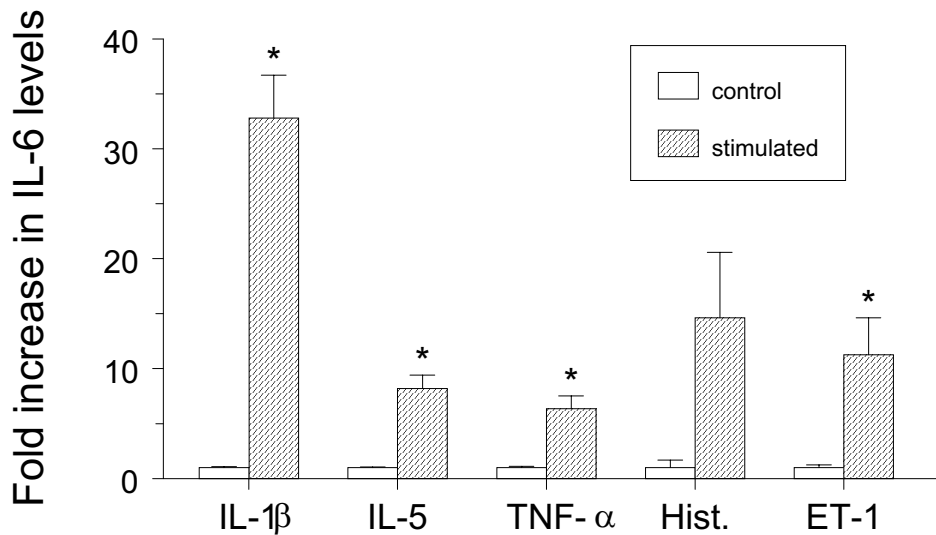


**Figure 2.1.** Northern blot analysis of c-fos expression in human ASM cells

Human ASM cells were treated with histamine (1  $\mu$ M), ET-1 (1 nM), TNF- $\alpha$  (10 ng/ml), IL-1 $\beta$  (0.5 ng/ml), IL-5 (2 ng/ml) or IL-6 (5 ng/ml) for the times indicated at the top of each lane. Total RNA from control=C (cultured in serum-free DMEM) and treated cells was subjected to Northern hybridisation with a radiolabelled c-fos cDNA probe. Rehybridisation of each filter with a GAPDH cDNA probe was performed for reference purposes. These Northern blots are representative of blots from three experiments.

*Release of IL-6 protein into the conditioned medium*

The concentration of IL-6 in the conditioned medium of cultured human ASM cells following 24 h of stimulation with IL-1 $\beta$ , IL-5, TNF- $\alpha$ , histamine and ET-1 was  $6.56 \pm 0.78$  ng/ml,  $1.17 \pm 0.22$  ng/ml,  $3.17 \pm 0.64$  ng/ml,  $1.16 \pm 0.92$  ng/ml and  $1.92 \pm 0.55$  ng/ml respectively. Conditioned medium derived from control cells showed only a negligible amount ( $0.14 \pm 0.02$  ng/ml) of IL-6. The fold increase in IL-6 concentration in relation to the different cytokines and mediators after normalising with their respective controls is shown in figure 2.2.



**Figure 2.2.** Production and release of IL-6 by human ASM cells.

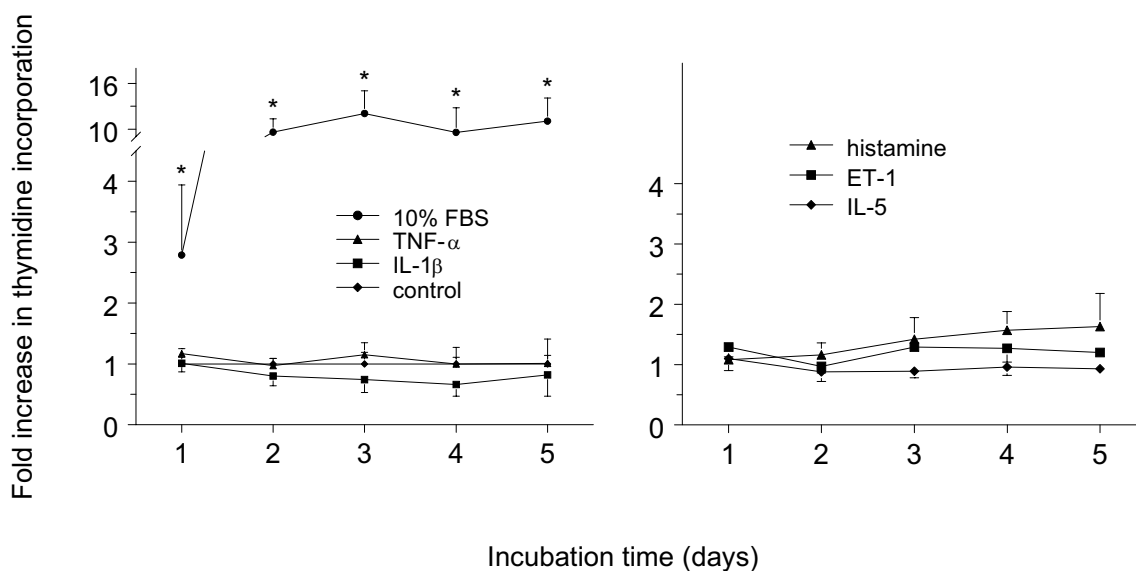
Growth arrested ASM cells were stimulated in the absence (control) or presence of different cytokines or mediators for 24 h. Data represent the mean  $\pm$  SEM of triplicate values from 3-5 independent experiments using conditioned medium from ASM cells cultured from different patients. Data are expressed as fold increase in IL-6 secretion compared to respective controls.

IL-1 $\beta$  was the most potent cytokine for cultured human ASM cells in producing IL-6 protein where levels rose to over 30 fold as compared to the respective control. In order to verify that the measured amounts of IL-6 in the conditioned medium were due to the release of *de novo* synthesised IL-6, we also assessed the concentration of IL-6 in the cell

lysates of unstimulated human ASM cells. These IL-6 levels were found to be negligible ( $0.1 \pm 0.01$  ng/ml).

### *Thymidine incorporation*

Figure 2.3 shows the DNA biosynthesis data assessed by  $^3\text{H}$ -thymidine incorporation into DNA during a period of five days. We found that 10% FBS increased thymidine incorporation up to 10 fold higher than in cells cultured in medium deprived of FBS (controls). Surprisingly, none of the cytokines or other mediators used in this study stimulated DNA biosynthesis in this cultured human ASM cell model. Even in the presence of the cyclo-oxygenase inhibitor indomethacin, the cytokines studied failed to elicit a proliferative response in these cells after 3 days of stimulation (table 2.1).



**Figure 2.3.**

Thymidine incorporation measured after 5 days in semi-confluent, growth arrested, human ASM cells stimulated with 10% FBS (circles), control (diamonds), 10 ng/ml TNF- $\alpha$  (triangles), 0.5 ng/ml IL-1 $\beta$  (squares), 2 ng/ml IL-5 (diamonds), 1  $\mu\text{M}$  histamine (triangles) or 1 nM ET-1 (squares). Values are calculated from 3-4 independent experiments of four determinations each and expressed as fold increase in thymidine incorporation relative to controls.

*Cell counting*

After 7 days incubation, 10% FBS potentiated cell proliferation by almost 3.5 fold (table 2.2). The cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-5 and IL-6 did not increase cell numbers after 7 days of incubation, as also predicted by the thymidine incorporation assays. Also histamine and ET-1 did not induce increases in cell numbers after 7 days of incubation, in agreement with our thymidine incorporation results.

**Table 2.1** Thymidine incorporation into human ASM cells in the presence or absence of indomethacin.

Treatment	TNF- $\alpha$	IL-1 $\beta$	IL-5	IL-6
- 1 $\mu$ M indomethacin	1.00 $\pm$ 0.11	1.00 $\pm$ 0.11	1.00 $\pm$ 0.11	1.08 $\pm$ 0.16
+ 1 $\mu$ M indomethacin	0.88 $\pm$ 0.12	1.08 $\pm$ 0.20	0.97 $\pm$ 0.20	0.88 $\pm$ 0.12

Thymidine incorporation into DNA, measured after 3 days in semi-confluent, growth arrested human ASM cells stimulated with TNF- $\alpha$  (10 ng/ml), IL-1 $\beta$  (0.5 ng/ml), IL-5 (2 ng/ml) and IL-6 (5 ng/ml) in the presence or absence of 1  $\mu$ M indomethacin. Values are calculated from three or four independent experiments of four determinations each and expressed as fold increase in thymidine incorporation relative to controls.



**Table 2.2** Human ASM cell numbers after 7 days of stimulation with different cytokines

Treatment	Cell number	
FBS	3.24 ± 0.75*	
TNF- $\alpha$	0.97 ± 0.10	Human ASM cell numbers were determined after 7 days of stimulation with various cytokines. Values are calculated from 3-4 independent experiments of four determinations each and expressed as fold increase in cell number relative to controls. * $P$ ≤ 0.05 as compared with respective controls.
IL-1 $\beta$	0.77 ± 0.12	
IL-5	1.30 ± 0.39	
IL-6	1.06 ± 0.13	
Histamine	1.21 ± 0.12	
ET-1	0.96 ± 0.10	

## 2.5 Discussion

The purpose of the present study was to investigate the relative contributions of a number of pro-inflammatory stimuli by evaluating their individual ability to induce the expression of the immediate early response gene, c-fos, as well as the release of the pleiotropic cytokine IL-6. Also, the ability of these stimuli to induce cell mitogenesis, under identical experimental conditions, was evaluated. Our results demonstrate that a rapid and transient expression of mRNA encoding c-fos was induced by all of the stimuli investigated. However, none of the stimuli induced DNA biosynthesis or an increase in cell numbers in this human ASM cell model. Only serum stimulation resulted in mitogenesis in these cells, supporting existing evidence that serum-derived growth factors are potentially important mediators in airway remodelling in asthma. Moreover, all of the stimuli investigated induced the release of *de novo* synthesised IL-6 protein into the cell-conditioned medium.

The rapid and transient induction of the immediate early response gene c-fos, and subsequent cell proliferation and/or differentiation has previously suggested that c-fos can be considered as a proliferation marker in ASM cells [15, 19, 22]. However, it has also been shown that c-fos induction alone is insufficient for cell proliferation in a number of cells of mesenchymal origin [36, 37]. Histamine and ET-1 have previously been shown to increase levels of c-fos mRNA, peaking at 30 minutes of stimulation, in canine and ovine ASM cells respectively [15, 19]. Both of these studies associated c-fos expression with ASM cell mitogenesis. However, we could not demonstrate that c-fos expression was directly associated with a proliferative response in our human ASM cells. These findings are also supported by one of our previous studies where we showed that angiotensin II induced the expression of c-fos without cell proliferation [33].

Stimulation of human ASM cells with either TNF- $\alpha$  (10 ng/ml) or IL-1 $\beta$  (0.5 ng/ml), in serum-free medium, induced the expression of c-fos, but did not result in an increase in thymidine incorporation or cells numbers in our study. Although work from other groups has previous shown a mitogenic response, it is important to emphasise that the culture media, TNF- $\alpha$  (10 ng/ml) in the study from Amrani *et al.*, and IL-1 $\beta$  (0.1 ng/ml) in the study from De *et al.* contained serum [12, 14]. TNF- $\alpha$ , IL-1 $\beta$  and growth factors (present in serum) activate mitogen-activated protein (MAP) kinases in smooth muscle cells [38-40]. MAP kinases are key transducers of extracellular signals in pathways leading to cell proliferation and differentiation. Signals activated by TNF- $\alpha$  or IL-1 $\beta$  together with serum may synergistically induce proliferation, possibly explaining the previously reported proliferative response. Stewart *et al.* found an increase in thymidine incorporation using TNF- $\alpha$  at very low concentrations (5.0 pg/ml to 0.5 ng/ml) in human

ASM cells, but at higher concentrations (5.0 ng/ml) this effect was abolished [12]. De *et al.* also found that IL-1 $\beta$  in the absence of indomethacin inhibited proliferation of ASM cells [13]. We did not observe proliferative effects in either the presence or in the absence of indomethacin, again suggesting that considerably low concentrations of serum derived factors may synergistically contribute to ASM cell mitogenesis.

IL-5 stimulation of human ASM cell proliferation has not previously been reported, although ASM cells have been reported to express and secrete IL-5 in response to the serum obtained from allergic asthma patients [41]. We found that IL-5 (2 ng/ml) is not mitogenic for human ASM cells, even in the presence of indomethacin. The cytokine IL-6 has previously been reported to induce a concentration dependent (1–4 ng/ml) increase in guinea pig tracheal smooth muscle cell proliferation [13]. We found, however, that 5ng/ml IL-6 did not induce human ASM cell proliferation in the presence or in the absence of the cyclo-oxygenase inhibitor indomethacin [35]. The upregulation of c-fos expression, in human ASM cells, by the pro-inflammatory stimuli investigated in our study, was thus not sufficient for the induction of cell proliferation. Radiolabelled thymidine incorporation demonstrated that 10% serum induced human ASM cell DNA biosynthesis which is a well established phenomenon in ASM cell culture.

The c-fos protein, which is able to translocate to the nucleus and bind to c-jun, forms a heterodimeric complex known as activator protein-1 (AP-1). This transcription factor can activate gene transcription by binding to the AP-1 site in the promoter region of target genes. Several recent studies have shown that ASM cells stimulation by cytokines leads to the expression and subsequent release of a number of inflammatory cytokines and chemokines [35, 42-44]. The majority of these pro-inflammatory factors contain

AP-1 binding sites in the promoter region of their genes. We have recently shown that c-fos upregulation precedes IL-6 protein secretion by human ASM cells [35]. The transient upregulation of c-fos that we measured in this study may contribute to the induction and release of cytokines, such as IL-6, thereby playing a role in the regulation of inflammatory responses in asthmatic airways. IL-6 is upregulated in the bronchoalveolar lavage fluid from symptomatic asthma patients and has pro-inflammatory effects that may be relevant to airway wall inflammation. These include mucus hypersecretion, the terminal differentiation of B cells into antibody producing cells, upregulation of IL-4-dependent IgE production, and differentiation of immature mast cells [23-26].

Our data suggest that ASM cells may play a role in the regulation of airway inflammation, through the production of cytokines such as IL-6. This may occur via upregulation of different genes, including cytokines and chemokines such as TGF- $\beta_1$ , IL-8, GM-CSF or RANTES [33, 42-44]. Although the individual pro-inflammatory mediators and cytokines investigated in our study did not induce ASM cell proliferation, we cannot exclude that their concerted actions in asthmatic airways may contribute to ASM remodelling. Evidence for the importance of combinations of mediators has recently been published [45]. Whether the endogenous generation of cytokines by ASM cells, and especially the release of IL-6, contributes to the pathogenesis of airway inflammation and remodelling is unclear, but the capacity of ASM cells to produce IL-6 suggests that these cells could participate in local inflammatory events in the airways.

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**Cytokines and growth factors induce expression of early  
growth response gene-1, a zinc finger transcription  
factor, in human airway smooth muscle cells**

### 3.1 Summary

Chronic inflammation and airway remodelling are important features of asthmatic airways contributing to the pathogenesis of bronchial hyperresponsiveness. Early growth response gene-1 (*egr-1*) is a sequence specific transcription factor that plays a regulatory role in the expression of various genes, such as five-lipoxygenase (5-LO), transforming growth factor (TGF)- $\beta$  and fibronectin, implicated in asthma. We investigated the expression pattern of *egr-1* in cultured human airway smooth muscle (ASM) cells treated with tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , platelet-derived growth factor (PDGF)-AB and fibroblast growth factor (FGF)-2. In addition, cell proliferation and expression of fibronectin mRNA were examined in human ASM cells. Growth arrested ASM cells were incubated with 10 ng/ml of TNF- $\alpha$ , 0.5 ng/ml of IL-1 $\beta$ , 50 ng/ml of PDGF-AB or 0.5 ng/ml of FGF-2 for various time points. Control cells were incubated in serum free culture medium. Total cellular RNA was isolated after each incubation and analysed by Northern blot hybridisation for the expression of *egr-1* and fibronectin. *Egr-1* mRNA levels were rapidly (within 15 min) elevated and declined after 2-4 h of stimulation with all of the above mentioned stimuli. PDGF-AB and FGF-2 induced a 3.5- and 2.5-fold increase in thymidine incorporation, respectively as compared to controls, whereas the cytokines did not. Fibronectin mRNA levels were upregulated by IL-1 $\beta$ , PDGF-AB and FGF-2 after 8 h of stimulation. Our results show upregulation of *egr-1*, followed by fibronectin, in human ASM cells in response to inflammatory stimuli. This early signalling event may influence long term changes in gene expression, thereby contributing to airway wall remodelling, as observed in asthma.

## 3.2 Introduction

Asthma, a chronic inflammatory disease of the airways, is characterised by reversible airway obstruction, airway hyperresponsiveness and airway wall inflammation. Important pathological features of asthmatic airways include inflammatory cell infiltration, epithelial shedding, basement membrane thickening and increased mass of ASM. Hyperplasia and hypertrophy of ASM cells contribute to the increase in smooth muscle content of the airway wall, the resulting changes in airway structure form the basis for airway wall remodelling [1-4].

The immediate early response gene *egr-1* is a sequence specific, zinc finger transcription factor that plays a regulatory role in the expression of various disease-associated effector genes *in vitro*. *Egr-1* could be of particular importance in asthmatic airways through the regulation of TNF- $\alpha$  [5], 5-LO [6], IL-2 [7], FGF-2 [8], CD44 and intercellular adhesion molecule-1 (ICAM-1) [9, 10] expression. Furthermore, TGF- $\beta$  [11] and fibronectin [12, 13], molecules involved in airway remodelling, are potential targets of regulation by this nuclear transcription factor. Smooth muscle cells also express a number of these potential targets [14-16].

Bronchoalveolar lavage fluid and serum from symptomatic asthma patients contain significantly increased levels of pro-inflammatory cytokines and growth factors, suggesting that these mediators may also be in the vicinity of the airway mucosa [17, 18]. PDGF and FGF-2 are known ASM mitogens [19, 20] derived in large quantities from alveolar macrophages and eosinophils, but also produced by epithelial cells and mesenchymal cells of the airways [21-24]. Interestingly, a number of cytokines, chemokines and growth factors are also produced by ASM cells [25-30].

We have recently demonstrated that stimulation of human ASM cells with the potent vasoconstrictor angiotensin II (Ang II), an octapeptide implicated in bronchoconstriction in mild asthmatics [31], results in the rapid expression of *egr-1* followed by the expression and secretion of TGF- $\beta$  [29]. TGF- $\beta$  is a multifunctional protein and a potent regulator for the synthesis of extracellular matrix proteins such as fibronectin. Also, VSM cells can be stimulated to release fibronectin [12]. Fibronectin is an important component of the subepithelial connective tissue and may contribute to the increased thickness of the basal lamina, which is a characteristic feature of asthmatic airways.

Evidence for a correlation between induction of *egr-1* and a proliferative response has been documented for several cell types [32-34]. However, induction of *egr-1* has also been described to play a role in events other than growth control, such as cell differentiation and apoptosis, where non-proliferating cells express high levels of *egr-1* suggesting that the cells are in an activated state [7-10, 35, 36]. Therefore, in this study, we investigated *egr-1* expression in cultured human ASM cells following stimulation with the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  as well as the growth factors PDGF-AB and FGF-2. Furthermore, we compared the expression of *egr-1* with subsequent proliferative responses as well as with the expression of mRNA encoding fibronectin in human ASM cells.

### **3.3 Materials and Methods**

#### *Human airway smooth muscle cell isolation and culture*

Human airway smooth muscle cells were isolated and cultured as described previously [29, 37]. Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained from patients who underwent surgery for lung carcinoma. After removal of the epithelium, pieces of smooth muscle were dissected free of adherent

connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (Life Technologies BV, Breda, The Netherlands) containing bovine serum albumin (BSA, fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) (Sigma BV, Zwijndrecht, The Netherlands) at 37°C in a humidified incubator (ASSAB, Clean Air Techniek BV, Woerden, The Netherlands, model T154) containing 5% CO<sub>2</sub> in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Life Technologies BV) containing 10% (vol/vol) foetal bovine serum (FBS; Bio-Whitaker BV, 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) (Life Technologies BV). Cells were subsequently seeded at approximately 2x10<sup>5</sup> cells per 35 mm dish and maintained in culture by replacing the medium every 72 h. After 10-14 days in culture, ASM cells grew to confluence and they were then removed by trypsinisation (0.5% trypsin; 0.02% ethylenediaminetetraacetic acid [EDTA]; Life Technologies BV) and subcultured into 25cm<sup>2</sup> tissue culture flasks. Cells were further passaged into 75cm<sup>2</sup> tissue culture flasks. Confluent cells in the fourth to sixth passages were used for experiments.

Immunocytochemical staining of confluent serum-deprived human ASM cells, during passage 4-6, using monoclonal antibodies to smooth muscle  $\alpha$ -actin and smooth muscle-myosin heavy chain (SM1 and SM2) (Sigma BV) [29, 38, 39], demonstrated that the cultures were essentially free (>95%) of other contaminating cell types.

*Stimulation of airway smooth muscle cells*

Human ASM cells were harvested from 75 cm<sup>2</sup> flasks by treatment with trypsin during passages 4 to 6. Cells were either seeded into 24-well plastic tissue culture plates at a density of  $3 \times 10^4$  cells/well and allowed to adhere for 24 h, or seeded into 25 cm<sup>2</sup> flasks and allowed to grow to confluence. To synchronise cellular growth the cells were washed 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 cultured in serum-free DMEM containing 1 μM insulin, 5 μg/ml transferrin and 100 μM ascorbate (Sigma BV) for 72 h. Using flow cytometric analysis of human ASM cells stained with propidium iodide, we previously found that 72 h of serum deprivation resulted in approximately 85% of human ASM cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase. Growth-arrested cell monolayers were stimulated with TNF-α (10 ng/ml), IL-1β (0.5 ng/ml), PDGF-AB (50 ng/ml) or FGF-2 (0.5 ng/ml) in fresh FBS-free DMEM in a time-dependent manner.

*Isolation of total cellular RNA and Northern blot analysis*

Human ASM cells were stimulated for either 15, 30, 45 min, 1, 2, 4, 8, 16 or 24 h. Treated and untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. The lysate was repeatedly passed through a 23G needle in order to shear the genomic DNA. Total cellular RNA was isolated using the method described previously [29, 40]. The RNA concentration was estimated by optical density (OD) measurements and a DNA/protein ratio of  $\geq 1.8$  was accepted. For Northern hybridisation, samples of total RNA (10 μg) were denatured at 65°C in a 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 [41]. The filters were air-dried and UV cross-linked in a gene linker (Biorad Laboratories BV, Veenendaal, The Netherlands). Blots were hybridised as described previously [29]. The cDNA probes used for hybridisation were: mouse *egr-1* (300 bp DNA fragment of zinc finger region) and human fibronectin (531 bp DNA fragment). A GAPDH cDNA probe (American Type Culture Collection, Rockville, MD) was used to rehybridise membranes for reference purposes. Hybridisation signals were quantified by scanning laser densitometry using the Ultrosan XL enhanced laser densitometer (LKB, Bromma, Sweden). Signals were normalised with respect to GAPDH mRNA values and expressed as arbitrary densitometric units (ADU).

#### *[<sup>3</sup>H]Thymidine incorporation assay*

Effects of the different cytokines and growth factors on DNA biosynthesis was evaluated by incorporation of [*methyl*-<sup>3</sup>H]thymidine. Cells were incubated with [*methyl*-<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) in either fresh FBS-free DMEM (control) or DMEM containing the various stimuli for 1, 2, 3, 4 or 5 days. 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 liquid-scintillation counter (Packard 1500 Tri-carb liquid scintillation analyser, Packard-Becker BV, Delft, The Netherlands). The data are expressed as fold increase in thymidine incorporation relative to controls.

### *Statistical Analysis*

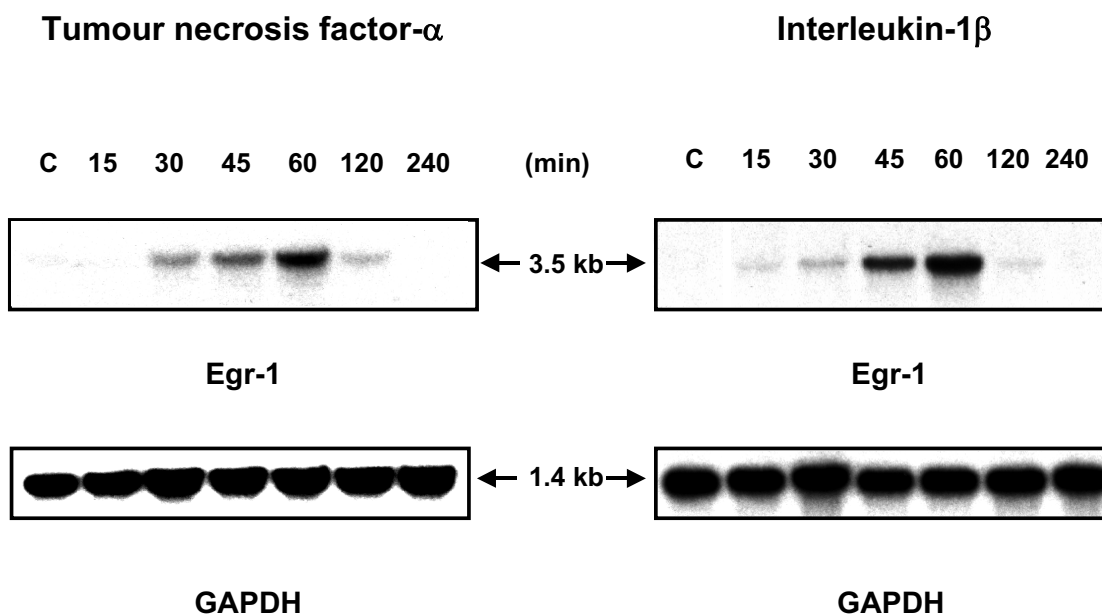
Data in the text and figure legends are expressed as mean  $\pm$  standard error of the mean (SEM) of observations from cells isolated from at least three different patients. Statistical analysis was performed by using the two-tailed, independent samples *t*-test. Significance was accepted at  $p < 0.05$ .

## **3.4 Results**

### *Cytokines induced egr-1 expression*

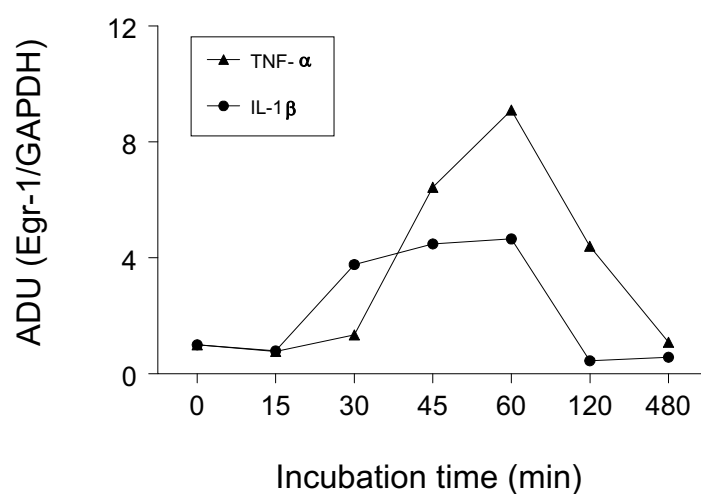
Expression of the *egr-1* gene was examined in human ASM cells treated with the pro-inflammatory cytokines TNF- $\alpha$  (10 ng/ml) and IL-1 $\beta$  (0.5 ng/ml) for increasing periods of time. Representative Northern blots showing the expression pattern of *egr-1* in relation to TNF- $\alpha$  and IL-1 $\beta$  are depicted in figure 3.1. Densitometric analyses of the Northern blots (fig 3.2) revealed that both cytokines rapidly induced the expression of mRNA encoding *egr-1* in human ASM cells as early as 30 min and the expression levels reached a maximum at 60 min (fig 3.1, upper panels). The transiently induced *egr-1* mRNA levels declined abruptly after 60 min, and were undetectable after 120 min with IL-1 $\beta$  stimulation and after 240 min with TNF- $\alpha$  stimulation. In order to compare the expression pattern and to verify the integrity of total RNA samples we used a housekeeping gene, GAPDH, to rehybridise membranes (fig 3.1, lower panels).





**Figure. 3.1** Northern blot analysis of egr-1 mRNA expression.

Human ASM cells were treated with TNF- $\alpha$  (10 ng/ml) or IL-1 $\beta$  (0.5 ng/ml) for the times indicated at the top of each lane, and total cellular RNA was extracted and subjected to Northern blot hybridisation using a mouse egr-1 cDNA probe. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes.

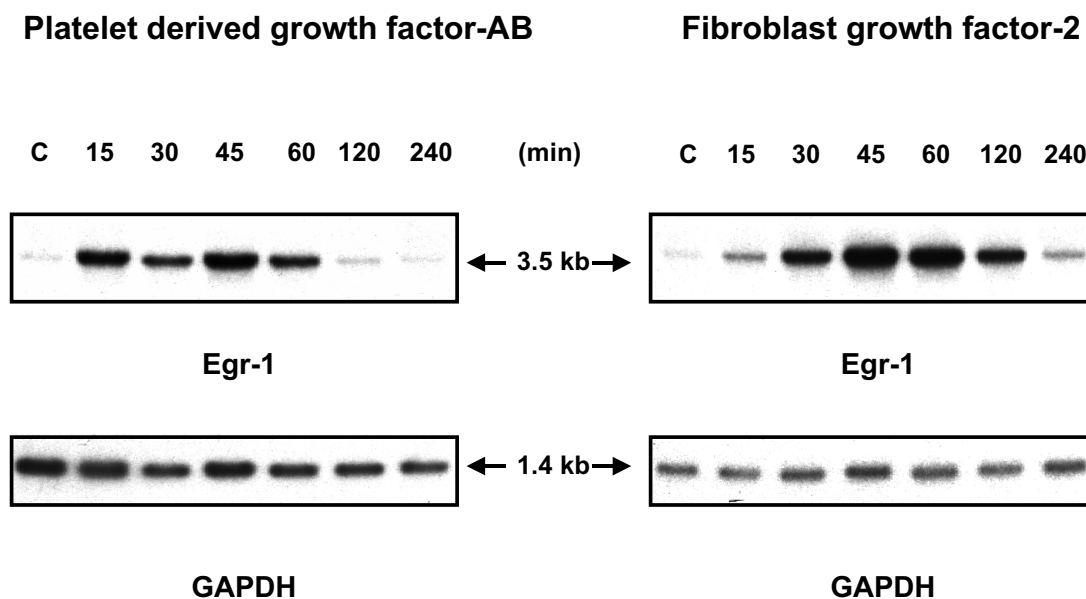


**Figure. 3.2** Densitometric analysis of egr-1 mRNA expression.

Autoradiographs were scanned and densitometric values were normalised with GAPDH. Values are presented as arbitrary densitometry units (ADU). Results depict the values from the representative blots.

*Egr-1 expression in relation to growth factors*

Expression of the *egr-1* gene was also examined in quiescent human ASM cells treated with PDGF-AB (50 ng/ml) and FGF-2 (0.5 ng/ml) for increasing periods of time. Representative Northern blots showing the expression pattern of *egr-1* in relation to PDGF-AB and FGF-2 are depicted in figure 3.3. Densitometric analyses of the Northern blots (figure 3.4) revealed that both growth factors rapidly induced the expression of mRNA encoding *egr-1* in human ASM cells as early as 15 min and the expression levels reached a maximum around 60 min (fig 3.3, upper panels). The transiently induced mRNA levels declined after stimulation for 120 min with PDGF-AB and after 480 min with FGF-2. In order to compare the expression pattern and to verify the integrity of total RNA samples, GAPDH was to rehybridise membranes (fig 3.3, lower panels).



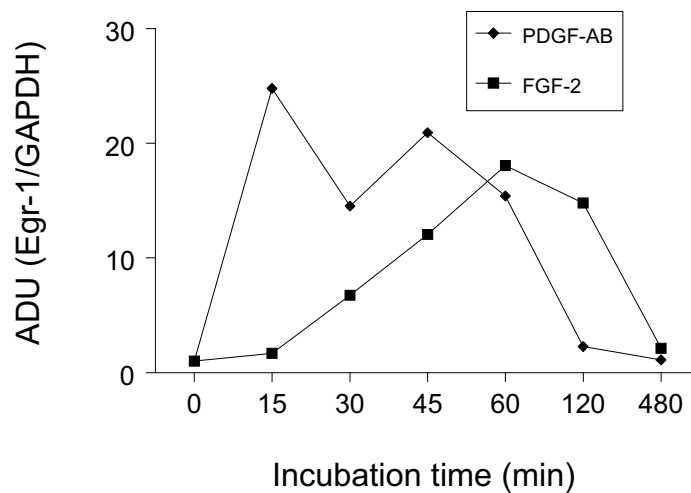
**Figure 3.3** Northern blot analysis of *egr-1* mRNA expression.

Human ASM cells were treated with PDGF-AB (50 ng/ml) or FGF-2 (0.5 ng/ml) for the times indicated at the top of each lane, and total cellular RNA was extracted and subjected to Northern blot hybridisation using a mouse *egr-1* cDNA probe. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from different individuals, and each experiment showed a similar pattern.

**Figure 3.4**

Densitometric analysis of egr-1 mRNA expression.

Autoradiographs were scanned and densitometric values were normalised with GAPDH. Values are presented as arbitrary densitometry units (ADU). Results depict the values from the representative blots.



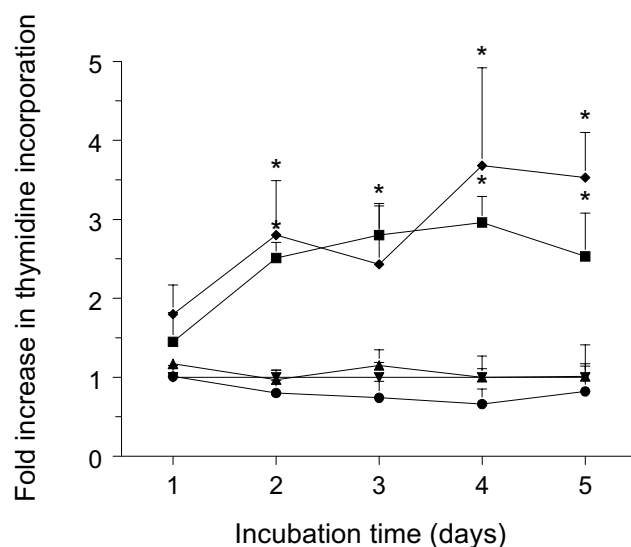
### *Proliferative responses*

Figure 3.5 shows the DNA biosynthesis data assessed by  $^3\text{H}$ -thymidine incorporation during a period of five days. We found that PDGF-AB and FGF-2 increased thymidine incorporation up to 3.5- and 2.5-fold, compared to controls, respectively. Neither TNF- $\alpha$  nor IL-1 $\beta$  resulted in the induction of DNA biosynthesis in these cultured human ASM cells.

**Figure 3.5** DNA biosynthesis

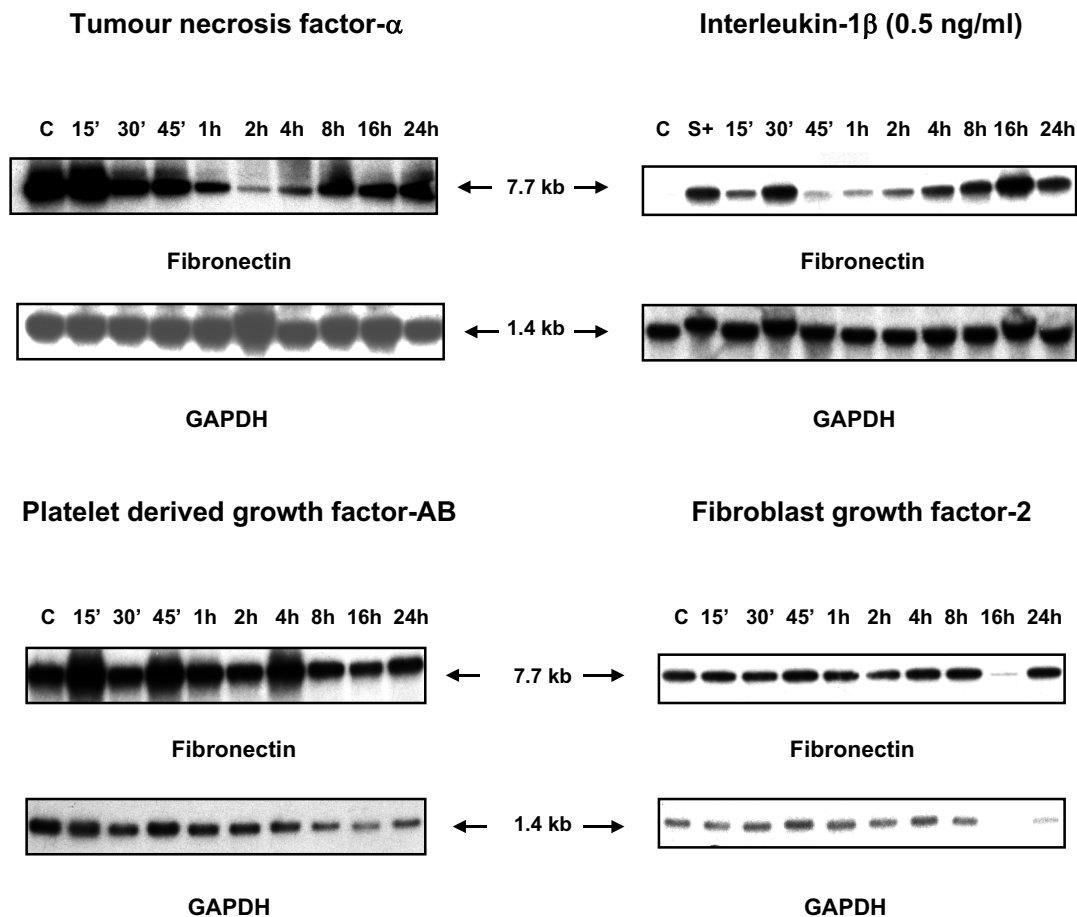
data assessed by  $^3\text{H}$ -thymidine incorporation.

During a period of five days in semi-confluent, growth arrested human ASM cells were stimulated with 50 ng/ml PDGF-AB (diamonds), 0.5 ng/ml FGF-2 (squares), 10 ng/ml TNF- $\alpha$  (triangles) or 0.5 ng/ml IL-1 $\beta$  (circles). Values are calculated from 3-4 independent experiments of four determinations each and expressed as fold increase in thymidine incorporation relative to controls (inverted triangles).



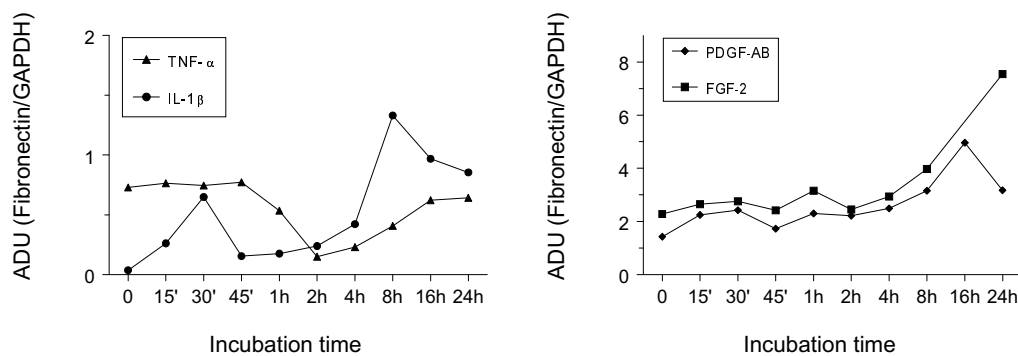
**Expression of mRNA encoding fibronectin**

To evaluate the effects of cytokine or growth factor stimulation on the expression of RNA encoding fibronectin we analysed total cellular RNA from quiescent human ASM cells treated with TNF- $\alpha$ , IL-1 $\beta$ , PDGF-AB and FGF-2 for various time intervals and compared the expression pattern with unstimulated cells. Using a human-specific cDNA probe encoding fibronectin, we detected mRNA species of 7.7 kb in human ASM cells (Figure 3.6). Fibronectin expression increased after 8 h of stimulation in IL-1 $\beta$ , PDGF-AB and FGF-2-treated human ASM cells. Densitometric analysis (figure 3.7) showed that mRNA levels became apparent after 8 h and did not decline even after 24 h.



**Figure 3.6** Northern blot analysis of fibronectin mRNA expression.

Human ASM cells were treated with TNF- $\alpha$  (10 ng/ml), IL-1 $\beta$  (0.5 ng/ml), PDGF-AB (50 ng/ml) or FGF-2 (0.5 ng/ml) for the times indicated at the top of each lane, and total cellular RNA was extracted and subjected to Northern blot hybridisation using a human fibronectin cDNA probe. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from different individuals, and each experiment showed a similar pattern.



**Figure 3.7** Northern blot analysis of fibronectin mRNA expression.

Autoradiographs were scanned and densitometric values were normalised with GAPDH. Values are presented as arbitrary densitometry units (ADU). Results depict the values from the representative blots.

### 3.5 Discussion

The immediate early response gene *egr-1* has been shown to encode a zinc finger transcription factor that has been proposed to regulate the expression of various genes, including a number of genes implicated in asthma. Important pathological features of asthmatic airways include increased ASM mass, a result of ASM cell hyperplasia and hypertrophy, as well as a thickened basement membrane. The purpose of the present study was, therefore, to investigate the expression of mRNA encoding *egr-1* in cultured human ASM cells following stimulation with the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  as well as the growth factors PDGF-AB and FGF-2. In addition, subsequent ASM cell proliferative responses were examined, as well as expression of mRNA encoding fibronectin, an *egr-1* responsive gene and an important component of the basement membrane.

Our findings demonstrate the induction and expression of *egr-1* in human ASM cells exposed to these pro-inflammatory stimuli. Both cytokines and growth factors induced a rapid and transient increase in mRNA encoding *egr-1*. Furthermore, PDGF-AB- and FGF-2-induced *egr-1* expression, but not TNF- $\alpha$ - or IL-1 $\beta$ -induced *egr-1* expression, was followed by an increase in <sup>3</sup>H-thymidine incorporation in ASM cells, demonstrating that induction of *egr-1* in human ASM cells is not necessarily accompanied with cell proliferation. Considering that the ASM cells also expressed *egr-1* in the absence of an increase in thymidine incorporation, it would seem likely that *egr-1* is expressed by cells that are in an activated state. Of particular relevance to airway wall remodelling is the observation that IL-1 $\beta$ , PDGF-AB and FGF-2 stimulated the induction and expression of mRNA encoding fibronectin in human ASM cells. Fibronectin is an important component of the subepithelial connective tissue and may contribute to the increased thickness of the basement membrane, which is a characteristic pathological feature of asthmatic airways.

It has been proposed that chronic asthmatic airway inflammation leads to airway wall remodelling. Potentially important inflammatory target genes under regulation of the *egr-1* nuclear, zinc finger transcription factor include TNF- $\alpha$  [5], 5-LO [6], IL-2 [7, 42], CD44 and intercellular adhesion molecule-1 (ICAM-1) [9, 10]. Other molecules important for airway remodelling include TGF- $\beta$  [11] and fibronectin [12, 13], they are involved in the synthesis of extracellular matrix proteins and are reported to be regulated by the *egr-1* transcription factor [11-13]. *Egr-1* binding motifs are also present in the promoters of insulin-like growth factor (IGF)-II, FGF-2 [8] and PDGF-A and the PDGF-B chain [43, 44], all molecules capable of inducing ASM cell mitogenesis [19, 20, 45]. Recent data show that smooth muscle cells can express and secrete many of these pro-inflammatory molecules, thereby supporting the hypothesis that *egr-1* could be an important transient

transcription factor that is may regulate a number of nuclear events in ASM cells [14-16]. Also, a number of researchers have demonstrated that inhibition of egr-1 transcription factor binding to DNA results in inhibition of cellular proliferation [33]. It remains to be shown that this mechanism could result in prevention of ASM cell proliferation and airway wall remodelling [32-34].

Summarising, we have established that the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  as well as the growth factors PDGF-AB and FGF-2 transiently induce the expression of mRNA encoding egr-1. Our results also demonstrate that egr-1 expression is followed by the elevated expression of fibronectin mRNA in human ASM cells. Furthermore, growth factor stimulation resulted in the proliferation of human ASM cells. We believe that egr-1 could be an important target gene for the development of novel therapeutic strategies against ASM proliferation and eventually for airway wall remodelling in asthma.

### **Acknowledgements**

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**Angiotensin II induces expression of transcription factors,  
cellular hypertrophy and cytokine production**

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## 4.1 Summary

Increased smooth muscle mass due to hyperplasia and hypertrophy of airway smooth muscle (ASM) cells is a common feature in asthma. Angiotensin II (Ang II), a potent vasoconstrictor and mitogen for a wide variety of cells, has recently been implicated in bronchoconstriction in asthmatics. However, a possible mitogenic role as well as underlying molecular mechanisms of this octapeptide in human ASM cells are not yet known. We studied the effects of Ang II on ASM cell proliferation and growth and on the expression of three transcription factors, *egr-1*, *c-fos* and *c-jun*, as well as a cytokine, transforming growth factor- $\beta$ 1 (TGF- $\beta$ <sub>1</sub>). Human ASM cells were isolated by enzymatic digestion of bronchial smooth muscle obtained from lung resection tissue. Confluent cells were growth arrested and subsequently incubated with Ang II (100nM) for different time periods and processed for the measurement of cell growth and gene expression. Ang II significantly induced DNA and protein synthesis in human ASM cells at 8 h resulting in a net increase in the accumulation of protein over DNA (i.e. cellular hypertrophy) at 16 h of incubation. Cell counts and MTT-reduction assay, however, showed no increase in cell number as a result of Ang II stimulation. Ang II stimulated the expression of *egr-1* and *c-fos* as early as 15 min, reaching maximum levels at 45 min, whereas the expression of *c-jun* peaked at 2 h of Ang II exposure. Furthermore, steady-state mRNA levels of TGF- $\beta$ <sub>1</sub> were upregulated by Ang II after 4 h and reached peak levels at 16 h of incubation. Secretion of biologically active TGF- $\beta$ <sub>1</sub> from human ASM cells was significant ( $P \leq 0.02$ ) enhanced by Ang II incubation after 8 h, which remained elevated until 24 h. Our results suggest that the Ang II-induced transient early expression of transcription factors may regulate autocrine genes like TGF- $\beta$ <sub>1</sub>, of which the subsequent late upregulation could contribute to cellular hypertrophy during, for example, airway remodelling in asthma.

## 4.2 Introduction

Airway remodelling with inflammatory cell infiltration, epithelial shedding, basement membrane thickening and increased mass of airway smooth muscle (ASM) is an important determinant of bronchial obstruction and hyperresponsiveness in asthma [1-4]. Studies using detailed computer models of human airways indicate that increased ASM mass is by far the most important abnormality responsible for excessive airway narrowing and compliance of the airway wall in asthma [1,5]. In analogy to vascular smooth muscle accumulation in hypertension and atherosclerosis, ASM growth in asthma is a complex phenomenon of which the underlying mechanisms are difficult to investigate *in vivo*. The increased amount of ASM in asthmatics is an indication of abnormal cell proliferation and growth. Both hyperplastic (i.e. increase in cell number) and hypertrophic (i.e. increase in cell size) changes contribute to the increased smooth muscle content of the airway wall [1,2,6,7], but little is known regarding the molecular mechanisms and factors that regulate ASM cell proliferation and growth in asthma.

Recently, a number of growth factors and cytokines derived from inflammatory cells have been implicated in ASM cell division and growth [8-12]. The potent circulating hormone of the renin-angiotensin system, angiotensin II (Ang II) has been implicated in bronchoconstriction in mild asthmatics [13]. Plasma levels of Ang II have been shown to be elevated in patients with acute severe asthma [14]. At plasma levels similar to those observed in acute asthma, Ang II was found to enhance methacholine-evoked bronchoconstriction, both in human bronchi *in vitro* and in mild asthmatics *in vivo*, thus suggesting a novel role for Ang II as a putative mediator in asthma [13]. Although Ang II has been shown to stimulate proliferation and/or hypertrophy in a wide variety of cells, such as cardiac myocytes, cardiac fibroblasts and vascular smooth muscle cells [15-17], it

remains to be established whether this octapeptide stimulates proliferation and growth of ASM cells.

It is known that growth and differentiation factors stimulate signal transduction pathways in the cell leading to the expression of nuclear proto-oncogenes that include the FOS, JUN and EGR family [18]. Fos and Jun proteins constitute the heterodimer AP-1 [19]. The AP-1 and egr-1 transcription factors can regulate a number of target genes during growth factor stimulation and thereby influence cellular growth and differentiation [16,20,21]. Ang II has been shown to induce a rapid increase in the expression of c-fos and c-jun (members of the leucine zipper family) and egr-1 (a member of the zinc finger family) in vascular smooth muscle cells as well as in cardiac fibroblasts prior to hypertrophy and/or hyperplasia [16,20,22]. Furthermore, an important cytokine, transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), has synergistically been associated with Ang II. TGF- $\beta_1$  is synthesised as a biologically inactive propeptide which must be cleaved to form the active peptide, by proteases like plasmin [23], before it can exert its function in tissue repair after injury or act as a regulatory peptide in remodelling processes in various tissues including the heart and the lung [16,24,25]. Also, TGF- $\beta_1$  stimulates the synthesis of extracellular matrix components such as collagens and fibronectin in response to Ang II in vascular smooth muscle cells [17,25,26].

To elucidate the potential role of Ang II in ASM growth in asthma, we investigated hyperplasia and hypertrophy in cultured human ASM cells and examined the expression pattern of three proto-oncogenes, namely egr-1, c-fos and c-jun. Additionally, we studied the expression of the extracellular matrix regulator TGF- $\beta_1$  in human ASM cells in relation to Ang II.

### 4.3 Materials and Methods

#### *Cell Culture*

Human airway smooth muscle cells were isolated and cultured according to the methods described earlier [10,27]. Briefly, bronchial smooth muscle was dissected from the lobar or main bronchus from lung resection specimens obtained from patients undergoing surgery for lung cancer. After removal of the epithelium, pieces of macroscopically normal smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS) containing 10 mg/ml of bovine serum albumin (BSA, fraction V), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) at 37°C in a humidified ASSAB model T154 CO<sub>2</sub> incubator (Clean Air Techniek BV, Woerden, The Netherlands). After enzymatic digestion, the tissue was centrifuged and the resultant pellet was washed in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100µg/ml) and amphotericin B (1.5 µg/ml) and subsequently seeded at  $2 \cdot 10^5$  cells per 35 mm dish. Fresh medium was replaced every 72 h. After 10-14 days in culture, ASM cells grew to confluence and were then removed from the plastic base of each dish using non-enzymatic cell dissociation buffer and subcultured in to tissue culture flasks. At confluency, cells were further passaged by means of trypsinization. Confluent cells in the 5<sup>th</sup> passage were used for experiments.

#### *Flow cytometer analysis of ASM cell growth arrest*

To establish the optimal time necessary for maximal growth arrest human ASM cells were incubating in FBS-free DMEM supplemented with apo-transferrin (5 µg/ml), ascorbate (100 µM) and insulin (1 µM) for 2-7 days. Following serum deprivation the medium was

removed and the cell monolayers were washed twice in PBS+BSA (0.5%). 900  $\mu$ l of trypsin solution (30 mg/ml, in stock solution) was added at room temp (RT). After 10 min the cells were gently loosened using a pipet and transferred to a 15 ml tube. 750  $\mu$ l trypsin inhibitor/Ribonuclease A solution was added (500mg/ml; 100 mg/ml respectively, in stock solution) and the solutions were mixed by inversion at RT. After 10 min 750  $\mu$ l ice-cold propidium iodide (PI)/spermine tetrahydrochloride solution (416 mg/ml; 1.16 g/ml respectively in stock solution) was added and the samples were filtered through a 30  $\mu$ m nylon mesh. Stock solution: trisodium citrate 1 g/ml, NP-40 0.1%, spermine tetrahydrochloride 1.5 mM, Tris buffer 0.5 mM: pH 7.6. The number of cells in the G<sub>0</sub>/G<sub>1</sub> phase or S/M phase of the cell cycle were measured using a flow cytometer (Becton Dickinson FACScaliber, San Jose, CA, USA).

#### *Immunocytochemical characterisation of ASM cells*

Monoclonal antibodies against  $\alpha$ -smooth muscle actin and smooth muscle cell specific myosin (SM-1 and SM-2) were used as markers to characterise human ASM cells in culture [28]. Cells were allowed to attach to multiwell slides for 24 h in FBS-containing medium and were subsequently growth arrested, as described above, 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 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), the cells were fixed in ice-cold methanol and permeabilised in PBS containing 0.1% Tween-20. Non-specific binding was blocked by incubating the cells in 1% BSA in PBS, the cells were then washed and subsequently incubated with anti- $\alpha$  smooth muscle actin or anti-smooth muscle myosin antibodies. After incubation, the cells were washed twice in PBS and further incubated with affinity purified FITC-conjugated anti-mouse antibody. Unbound antibody was washed away using distilled water and the sections were dehydrated and mounted in



glycerol. Specimens were visualised under a fluorescence microscope (Carl Zeiss BV, Weesp, The Netherlands) and photographed. Human ASM cells stained positive for anti- $\alpha$  smooth muscle actin and anti-smooth muscle myosin. Additionally the cultures were stained for endothelial and epithelial cell contamination using anti-CD31 and anti-cytokeratin antibodies respectively. The staining showed that human ASM cell cultures were essentially free (>95%) of other contaminating cell types. 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 as described earlier by Twort and Breeman [27].

#### *RNA isolation and Northern blot analysis*

Total RNA was extracted from the ASM cells by the guanidinium thiocyanate-phenol-chloroform method described earlier [16]. The RNA concentration was measured by spectrophotometry. For Northern hybridisation, samples of total RNA were denatured at 65°C and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide stained gels were photographed and RNA was transferred to hybond-N membrane by alkaline downward capillary transfer [29]. The filters were air dried and UV cross linked in a gene linker (Biorad B.V., Veenendaal, The Netherlands). Blots were hybridised at 42°C in a buffer containing 50% deionized formamide, 1.0 M sodium chloride, 1% sodium dodecylsulfate (SDS), 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 0.2% bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 2% dextran sulphate and denatured herring sperm DNA (2 mg/ml). cDNA probes used for hybridisation were: mouse c-fos (2.1 kb fragment), mouse c-jun (2.6 kb fragment), mouse egr-1 (300 bp

fragment of zinc finger region) and human TGF- $\beta_1$  (1050 bp fragment). CDNA inserts were labelled employing the redi-prime labelling system to a specific activity of approximately  $10^9$ cpm/ $\mu$ g DNA. Filters were washed under stringent conditions, wrapped in household plastic film and exposed to Kodak X-OMAT AR films at  $-80^\circ\text{C}$  for 1-3 days. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to rehybridise membranes for reference purposes. Hybridisation signals were quantified by scanning laser densitometry using the Ultrascan XL enhanced laser densitometer (LKB, Bromma, Sweden). Densitometric values for each gene were normalised with respective GAPDH mRNA values and expressed as relative O.D. in Ang II stimulated cells versus control. An optimal concentration of Ang II (100 nM) for the early response of human ASM cells was determined by incubating growth arrested cells for 1 h in  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  M [Sar<sup>1</sup>]-Ang II, and subsequently examining the expression pattern of the proto-oncogene egr-1.

#### *[<sup>3</sup>H]Thymidine and [<sup>3</sup>H]Leucine incorporation assays*

Effects of Ang II on DNA and protein biosynthesis were assessed by incorporation of [*methyl*-<sup>3</sup>H]thymidine and [*methyl*-<sup>3</sup>H]leucine, respectively. An optimum concentration of Ang II (100 nM) was determined by incubating growth arrested ASM cells for 24 h in  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M [Sar<sup>1</sup>]-Ang II, and subsequently examining [<sup>3</sup>H]thymidine and [<sup>3</sup>H]leucine incorporation. Confluent cells were washed in PBS, de-attached by trypsinisation and transferred into 24-well plates at a seeding density of  $2 \times 10^4$  cells/well. After 5 days in culture the cells were growth arrested as described above. Cells were incubated with [*methyl*-<sup>3</sup>H]thymidine or [*methyl*-<sup>3</sup>H]leucine (1  $\mu$ Ci/well) in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum) or DMEM containing 100 nM [Sar<sup>1</sup>]-Ang II for 4, 8, 16, 24 or 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 analyser (Packard-Becker BV, Delft, The Netherlands) [9].

#### *Assessment of cellular hypertrophy and hyperplasia*

*Protein/DNA ratio.* ASM cell hypertrophy in relation to Ang II was assessed by calculating the ratio of total protein to DNA content. Cells were plated at a seeding density of  $2 \times 10^4$  cells/well in 24-well plates. After 5 days the cells were growth arrested as described above, and then incubated in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum) or DMEM containing 100 nM [Sar<sup>1</sup>]-Ang II for 4, 8, 16, 24 or 48 h. After stimulation, the cells were washed in ice-cold PBS and lysed in 0.3 M NaOH. The total DNA content was determined fluorimetrically using the method described by Kapuscinski and Skoczylas [30]. The total protein content was estimated colorimetrically using the method described by Bradford [31]. Serial concentrations of calf thymus DNA and BSA were used for the calibration curves.

*MTT Assay.* In order to assess cellular hyperplasia in response to Ang II, MTT assays were performed. Cells in the 5<sup>th</sup> - 6<sup>th</sup> passage were transferred to 24-well plates at a seeding density of  $2 \times 10^4$  cells/well. After 24 h, the cells were growth arrested as described above, and subsequently incubated in either fresh FBS-free DMEM or DMEM containing 100 nM [Sar<sup>1</sup>]-Ang II for 4, 8, 16, 24 or 48 h. Proliferation was assessed using the MTT dye technique, which relies on the specific metabolic reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by living cells as described by Hirst [14,32]. Following incubations, the cells were washed twice in PBS then 200  $\mu$ l of

MTT in DMEM (final concentration 0.5 µg/ml) was added to each well, and the cells were incubated for 5 h at 37°C. After incubation, the blue formazan product is solubilized by the addition of 200 µl of solubilisation solution (10% SDS in 0.01 M HCl) to each well for 16 h at 37°C. An aliquot of 200 µl from each duplicate well was then transferred to a 96-well microtiter plate, and the optical density was determined by automated dual wavelength spectrophotometry (Bio-Rad Labs BV, Veenendaal, The Netherlands) at a test wavelength of 595 nm and a reference of 690 nm. The change in optical density ( $\Delta_{OD}$ ), which correlates directly with changes in cell numbers, was plotted against time as described previously by Hirst *et al.* [9].

*Haemocytometry.* Cells were seeded into 24 well plates and treated as described above. Following stimulation, the medium was removed and the cell monolayers were resuspended in 100 µl trypsin-EDTA at room temperature for 10 min. An equal volume of trypan blue was added to the cell suspension before counting in a Bürker haemocytometer (Marienfeld, Germany). At least 120 cells were counted from each well to minimise counting error.

#### *Detection of TGF- $\beta_1$ protein in conditioned media*

Confluent cells in the fifth passage were growth arrested as described above, and then incubated in either fresh FBS-free DMEM (control), FBS-containing DMEM (+ serum) or DMEM containing 100 nM [Sar<sup>1</sup>]-Ang II for 4, 8, 16 or 24 h. Media were removed and stored at -20°C until assay. Biologically active TGF- $\beta_1$  protein was quantified by enzyme-linked immunosorbent assay (ELISA) using a TGF- $\beta_1$  E<sub>max</sub> ImmunoAssay System (Promega Corp. BNL, Leiden, The Netherlands) following the suppliers' instructions.

*Statistical Analysis*

Data are given as mean  $\pm$  SEM. Statistical analysis was performed by using the Students "t"-test. Significance was accepted at  $P \leq 0.05$ .

*Materials*

Bovine serum albumin, collagenase, elastase, apo-transferrin, ascorbate, insulin, non-enzymatic cell dissociation solution, mouse monoclonal anti- $\alpha$  smooth muscle actin and anti-smooth muscle myosin, anti-mouse IgG FITC conjugate, 4',6-diamidino-2-phenylindole-2HCl (DAPI), calf thymus DNA, herring sperm DNA and trypan blue were purchased from Sigma-Aldrich B.V. (Zwijndrecht, The Netherlands). HBSS, DMEM, sodium pyruvate, MEM non-essential amino acids, gentamicin, penicillin:streptomycin, Fungizone/amphotericin B and trypsin-EDTA were purchased from Life Technologies BV (Breda, The Netherlands). Foetal bovine serum (FBS) was purchased from Bio-Whitaker BV (Verviers, Belgium). [*Methyl*- $^3\text{H}$ ]-thymidine, [*methyl*- $^3\text{H}$ ]-leucine, ( $\alpha$ - $\text{P}^{32}$ )-dCTP, hybond-N and redi-prime labelling kit were obtained from Amersham Nederland BV ('s-Hertogenbosch, The Netherlands). The MTT-reduction assay kit was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Whereas, the TGF- $\beta_1$  E<sub>max</sub> ImmunoAssay System was procured from Promega Corp. BNL (Leiden, The Netherlands). Anti-cytokeratin and anti- $\alpha$ -CD31 antibodies were obtained from Dako A/S (Glostrup, Denmark). The cDNA probe for human specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from American Type Culture Collection (Rockville, USA). Tissue culture plasticware was obtained from Life technologies B.V. (Breda, The Netherlands). All other chemicals were of molecular biology and/or tissue culture grade and were procured from local suppliers.

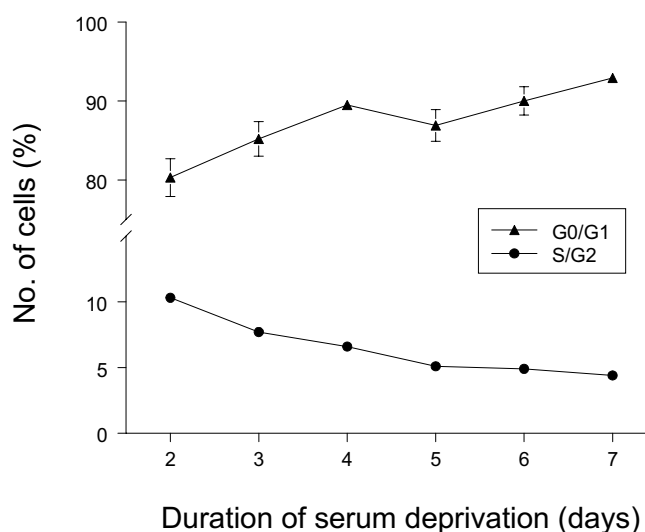
### 4.3 Results

#### *Flow cytometric analysis of ASM cells in serum-deprived culture medium*

Figure 4.1 shows percentages of ASM cells in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle after increasing incubation time in serum-deprived medium. After 3 days more than 85% of the cells were growth arrested.

**Figure 4.1** The percentage of cells in the G<sub>0</sub>/G<sub>1</sub> or S/G<sub>2</sub> phase of the cell cycle.

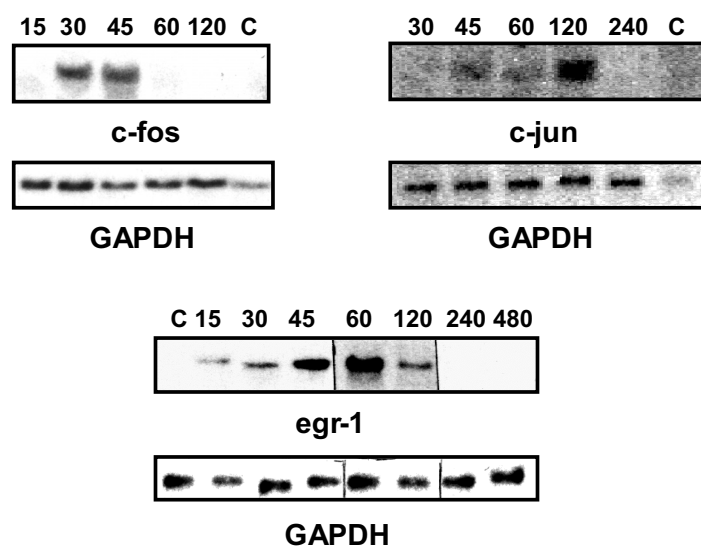
ASM cell nuclei were isolated and stained with PI according to the method of Vindelov [47]. The percentage of cells measured in the G<sub>0</sub>/G<sub>1</sub> or S/G<sub>2</sub> phase of the cell cycle are shown.



#### *Expression of transcription factors in relation to angiotensin II*

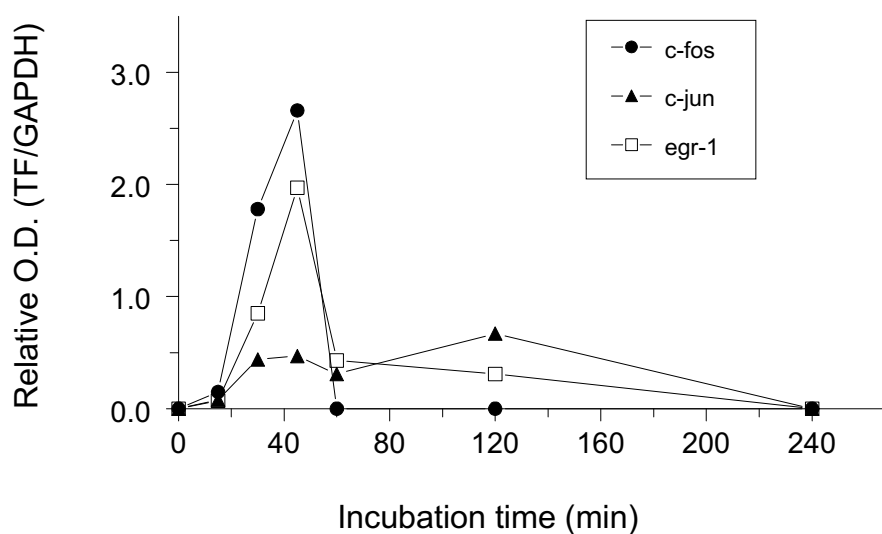
Representative Northern blots showing the expression pattern of three proto-oncogenes, following Ang II stimulation of human ASM cells, are shown in figure 4.2. Ang II induced the expression of mRNAs encoding egr-1 and c-fos as early as 15 min, while c-jun expression was increased after 45 min of Ang II stimulation. Figure 4.3 in shows the quantitative analysis, by scanning laser densitometry, of the Northern blots for each proto-oncogene. Densitometric analysis revealed that the induction of c-fos and egr-1 was transient and reached a maximum at 45 min followed by an abrupt decline; whereas the expression of c-jun was also transient but reached a maximum at 120 min, and gradually

returned to basal levels after 4 h. The relative O.D. values for the levels of mRNA encoding *egr-1*, *c-fos* and *c-jun* increased from 0 to their maximum values of 1.97, 2.66 and 0.67, respectively, after Ang II stimulation of human ASM cells.



**Figure 4.2** Northern blot analysis of *egr-1*, *c-fos* and *c-jun* expression in human airway smooth muscle cells treated with Ang II.

Representative Northern blots for *egr-1* (3.5 kb mRNA band), *c-fos* (2.2 kb mRNA band), and *c-jun* (2.3 kb mRNA band) are shown. ASM cells were incubated with Ang II (100 nM) in serum free medium for the times indicated at the top of each panel. Control=C (untreated). Rehybridisation of each filter with a GAPDH cDNA probe (*lower part of panel*, 1.4 kb mRNA band) was performed for reference purposes.



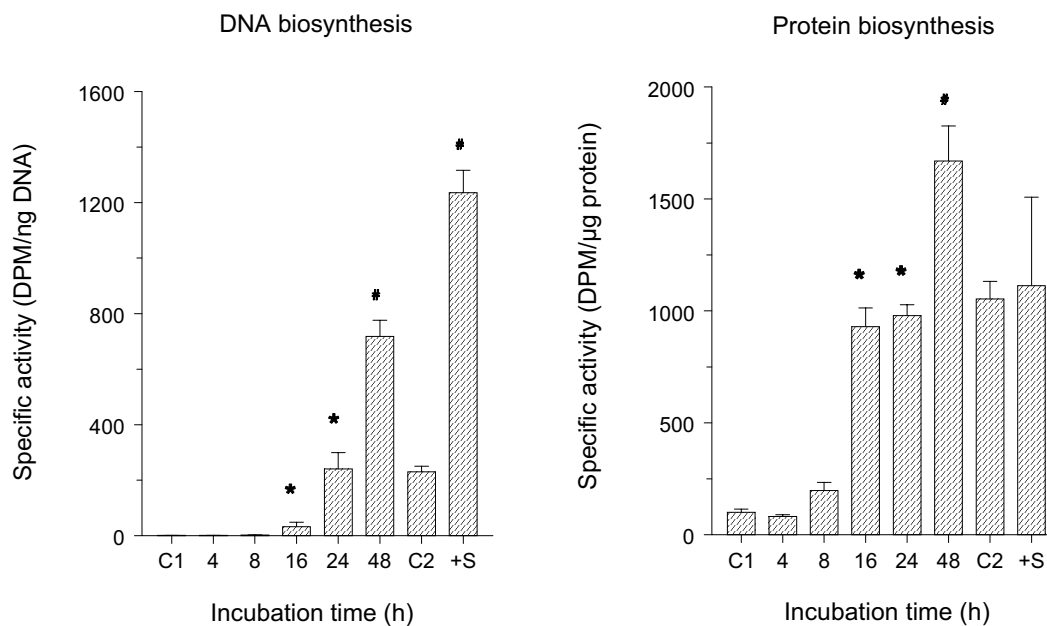
**Figure 4.3** Line diagram showing quantification of mRNA expression pattern for different transcription factors (TF).

Scanning densitometric values for each proto-oncogene were normalised with respective GAPDH mRNA values and expressed as relative optical density (OD). Results depict the values from the representative blots. Each experiment was repeated at least three times using human ASM cells originating from different individuals, and each experiment showed a similar pattern.

#### *Effects of Angiotensin II on ASM proliferation and growth*

The initial aim of this study was to determine whether Ang II induces an increase in DNA and protein synthesis in ASM cells made quiescent in defined serum-free medium. Biosynthesis of DNA and protein from their respective labelled precursors (thymidine and leucine) was calculated as specific activity and represented as dpm/ng DNA or dpm/ $\mu$ g protein (figure 4.4). Data show that the biosynthesis of both DNA and protein was minimal until 8 h of Ang II incubation. However, a significant increase was observed in both DNA and protein biosynthesis at 16 h of Ang II stimulation and these levels remained elevated until 48 h as compared to controls.



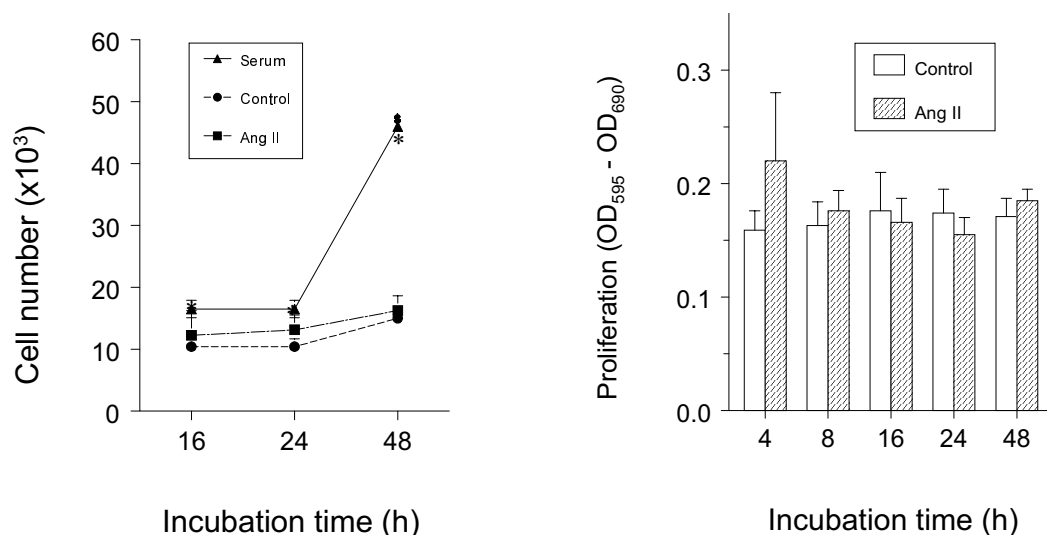


**Figure 4.4** Ang II induced DNA and protein biosynthesis in human airway smooth muscle cells.

[<sup>3</sup>H]Thymidine and [<sup>3</sup>H]leucine incorporation were measured in confluent, growth arrested ASM cells that were stimulated with 100 nM Ang II and compared with untreated cells (C1=4 h; C2=48 h) or cells treated with medium containing 10% FBS (+S). Values are calculated from three independent experiments of four determinations each and expressed as specific activity (dpm/ng DNA or dpm/μg protein). \* $P \leq 0.05$  compared to C1, # $P \leq 0.05$  compared to C2.

In order to assess whether the DNA biosynthesis in human ASM cells, treated with Ang II, was accompanied with an increase in cell number, we performed haemocytometry and MTT assays. Data on haemocytometry showed that Ang II incubation did not significantly increase the cell number with time as compared to respective controls. However, serum induced a significant ( $P \leq 0.05$ ) increase in cell numbers already at 16 h as compared to controls, and at 48 h cell numbers were considerably higher ( $P \leq 0.001$ ) as compared to respective control and Ang II treated cells (figure 4.5). MTT-reduction assay data showed no significant change in O.D. values at 4, 8, 16, 24 or 48 h of Ang II incubation as

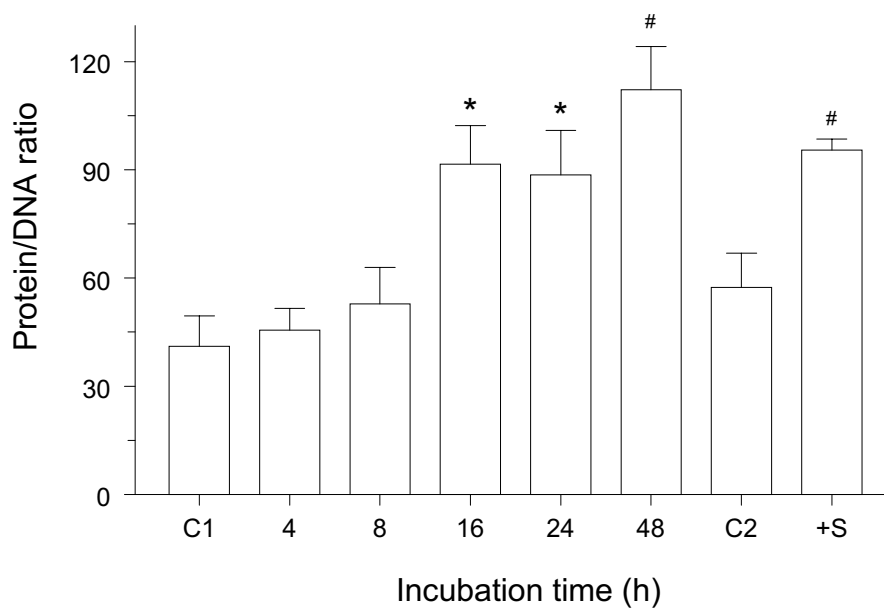
compared to respective controls, indicating that Ang II did not cause ASM cell proliferation (figure 4.5).



**Figure 4.5** Proliferative response of human ASM cells exposed to Ang II.

Proliferation was assessed by haemocytometry (left) and by using the MTT dye technique (right). Values are represented as mean  $\pm$  SEM of four measurements from 3 separate experiments. \* $P \leq 0.05$  compared with control; <sup>s</sup> $P \leq 0.05$  compared with Ang II treated cells.

In a separate set of experiments, the total DNA and protein content of the ASM cells were determined in relation to Ang II stimulation with time. Figure 4.6 shows the effect of Ang II on the protein/DNA ratio in human ASM cells. In order to assess Ang II induced hypertrophy in human ASM cells, we calculated the protein-to-DNA ratio at different time points. After 16 h of Ang II incubation, the protein/DNA ratio of the ASM cells increased significantly ( $P \leq 0.001$ ) compared to controls, indicating that the cells were synthesising more protein than DNA, suggesting cellular hypertrophy.



**Figure 4.6** Ang II induced cellular hypertrophy in human ASM cells.

Total DNA and protein contents of airway smooth muscle cells in the presence and absence of Ang II (100 nM) were measured. DNA and protein values were calculated from three independent experiments of four determinations each, and the Protein/DNA ratio was calculated. Values are expressed as mean  $\pm$  SEM. C1 = control (untreated) cells at 4 h; C2 = control cells at 48 h; +S = cells incubated with medium containing 10% FBS. \* $P \leq 0.05$  compared to C1; # $P \leq 0.05$  compared to C2.

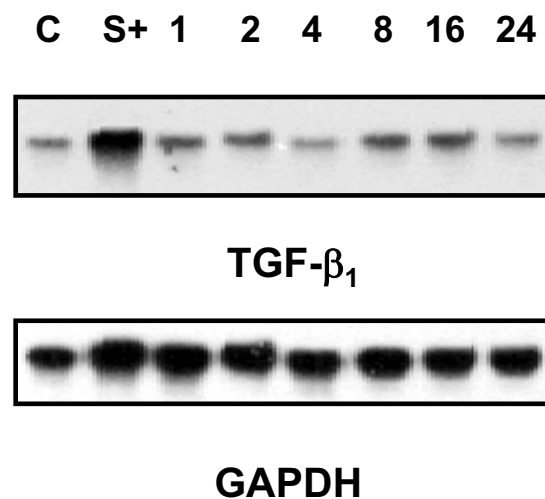
#### *Effects of angiotensin II on TGF- $\beta_1$ expression*

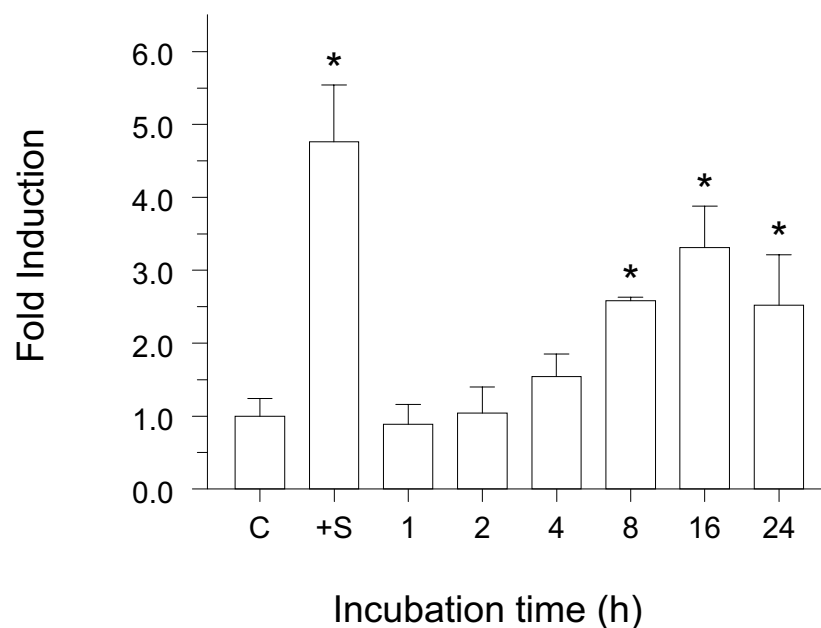
To evaluate the effects of Ang II on TGF- $\beta_1$  expression, we analysed total cellular RNA from serum-fed ASM cells and cells stimulated with 100 nM Ang II for various time intervals and compared the expression pattern with respective controls. Using a human specific cDNA probe encoding TGF- $\beta_1$ , we detected a mRNA species of 2.4 kb in human ASM cells (figure 4.7). TGF- $\beta_1$  expression was drastically increased ( $P \leq 0.001$ ) in serum-fed cells as compared to the control cells. Scanning laser densitometric analysis of TGF- $\beta_1$  expression showed that Ang II significantly induced ( $P \leq 0.001$ ) the steady-state mRNA levels which became apparent after 8 h and reached a maximum level at 16 h ( $P \leq 0.001$ ) as

compared with controls (figure 4.8). To evaluate whether the increase in mRNA levels for TGF- $\beta_1$  were accompanied with an increase in secreted protein, biologically active TGF- $\beta_1$  was measured in the conditioned medium after stimulation of the ASM cells with Ang II for various time intervals. Indeed, a significant increase in TGF- $\beta_1$  protein was observed after 8 h, which remained elevated at 24 h (Table 4.1).

**Figure 4.7** Northern blot showing TGF- $\beta_1$  mRNA expression in human ASM cells in relation to Ang II.

A representative Northern blot showing the major 2.5 kb mRNA band encoding TGF- $\beta_1$ . RNA from control and Ang II treated human ASM cells was hybridised with a radiolabelled human TGF- $\beta_1$  probe. Human ASM cells were incubated with Ang II (100 nM) in serum free medium for the times indicated at the top of the panel. Filters were reprobbed with a radiolabelled GAPDH cDNA for reference purposes (*lower part of panel*).





**Figure 4.8** Bar graph showing quantitative analysis of the Ang II induced TGF- $\beta_1$  expression.

Scanning densitometric values for TGF- $\beta_1$  were normalised with respective GAPDH mRNA values. Values are means of the normalised signal  $\pm$  SEM (n = 4) and expressed as fold induction versus control (control value set at 1.0). # $P \leq 0.05$  versus control (control cells at 24 h).

**Table 4.1.** Effects of Ang II on secretion of biologically active TGF- $\beta_1$

Incubation time with Ang II (h)	Active TGF- $\beta_1$ (pg/ml)
Control	161.6 $\pm$ 12.0
8	446.0 $\pm$ 3.0
24	393.3 $\pm$ 11.9

Biologically active TGF- $\beta_1$  was measured in the conditioned medium of human ASM cells treated with Ang II for 8 and 24 h. Values are mean  $\pm$  SEM.

## 4.5 Discussion

This is the first study showing that Ang II, in the absence of serum, induces hypertrophy in human airway smooth muscle cells *in vitro*. In an attempt to discern whether Ang II could induce cell hypertrophy, cell hyperplasia or both, we used several biochemical markers. On the basis of [<sup>3</sup>H]leucine incorporation, protein over DNA accumulation in the cell [33], MTT-reduction assays and cell number determinations our results clearly indicate a hypertrophic response in human ASM cells. Our data also show that Ang II induced an increase in [<sup>3</sup>H]thymidine incorporation into DNA without significantly altering the cell number in human ASM cells. Furthermore, the magnitude of increase in [<sup>3</sup>H]thymidine incorporation was much lower (55%) than that seen with foetal bovine serum, suggesting that Ang II may act as a weak mitogen for human ASM cells.

Studying vascular smooth muscle cells *in vitro*, several groups have demonstrated cell proliferation [34], whereas others have shown only cellular hypertrophy after Ang II stimulation [15,17,35]. Ang II-induced hypertrophy in vascular smooth muscle cells has been shown to be associated with an increase in expression and secretion of the autocrine growth factor platelet derived growth factor-AA (PDGF-AA) [15,36]. PDGF is a potent mitogen for vascular smooth muscle cells and it is quite possible that in analogy to these cells, human ASM cells express and secrete PDGF in response to Ang II resulting in an increase in DNA synthesis. Moreover, Ang II induces the synthesis and release of other autocrine-paracrine mitogenic factors such as endothelin-1 (ET-1) and basic fibroblastic growth factor (bFGF) [8,15]. Therefore, in this study, Ang II-induced DNA synthesis may be attributed to the autocrine induction of such growth factors, although it does not appear that the cells are capable of completing the cell cycle (i.e. no increase in cell number). In this regard, Jahan *et al.* [37] demonstrated that Ang II did not induce the transition of aortic

smooth muscle cells in the  $G_0$  phase to the  $G_1$  phase; rather it acted as a progression factor stimulating cells remaining in the  $G_1$  phase to synthesize protein and DNA. Additionally, they found that PDGF stimulated the entry of cells in the  $G_0$  phase into the  $G_1$  phase without a further progression into the S and M phases, whereas Ang II incubation stimulated the progression of these PDGF-pretreated  $G_1$  cells to the S and M phases. Perhaps, in our study, DNA repair could be partly responsible for the increase in [ $^3\text{H}$ ]-thymidine incorporation into DNA with time in the Ang II treated cells.

In this study, we chose 60 h of serum free culture conditions in order to synchronise and growth arrest human ASM cells. Fluorescent-activated cell sorter analysis verified that more than 85% of cells were in the  $G_0/G_1$  phase of the cell cycle after 60 h of serum deprivation, allowing us to examine the direct effects of Ang II on cell division and growth. Others have reported earlier that 48 h of serum deprivation was successful in growth arresting ASM cells in culture [10-12,38]. The data presented here demonstrate that Ang II is a weak mitogen and a potent hypertrophic stimulus for human ASM cells *in vitro*. However, the cellular mechanisms involved in Ang II-stimulated mitogenesis in ASM cells are undefined and may be distinct from vascular smooth muscle cells. Previous studies have established that Ang II can induce a rapid increase of the growth associated nuclear proto-oncogenes c-myc, c-fos, c-jun and egr-1 in a variety of cell types [20,22,39]. Similarly, in human ASM cells, nuclear events associated with Ang II activation appear to be the induction of immediate early genes (c-fos, c-jun and egr-1). The observed increase in DNA biosynthesis could be attributed to the transient expression of the transcription factors c-fos, c-jun and egr-1, which are previously reported to be involved in cell growth and differentiation [18,20,21,39]. Additionally, the Fos and Jun proteins form a heterodimeric complex, known as the AP-1 transcription factor, which activates gene

transcription by binding to an AP-1 site in target genes that may subsequently contribute to cell proliferation and growth [19,21,40]. Previous studies have demonstrated that Ang II stimulates angiotensin II type-1 (AT<sub>1</sub>) receptor mediated hypertrophy in cardiac myocytes and hyperplasia in cardiac fibroblasts [18,41]. In vascular smooth muscle cells proliferative effects of Ang II are attributed to the AT<sub>1</sub> receptor which is coupled to G-proteins and classic intracellular second messenger systems [42]. In contrast, the function and the signal transduction pathways for the AT<sub>2</sub> receptor, which exhibits only 32% homology to the AT<sub>1</sub> receptor, are not fully understood. However, AT<sub>2</sub> receptor mediated growth inhibition in endothelial cells treated with Ang II has been reported [43]. We might speculate that the Ang II-induced up-regulation of immediate early genes in human ASM cells may be mediated by AT<sub>1</sub> receptors.

In the present study we have demonstrated that Ang II induces TGF- $\beta_1$  mRNA expression and the secretion of biologically active TGF- $\beta_1$  protein in human ASM cells. Our findings are in agreement with previous reports where Ang II has been shown to stimulate TGF- $\beta_1$  both at mRNA and protein level in vascular smooth muscle cells [17,26,34]. One explanation for the induction of TGF- $\beta_1$  seems to be the positive regulation by the AP-1 transcription factor. The TGF- $\beta_1$  promoter region contains an AP-1 site which can modulate TGF- $\beta_1$  gene expression, suggesting that the Ang II-induced expression of c-fos and c-jun may participate in the induction of TGF- $\beta_1$  gene expression [17,44]. Several groups have shown that the incubation of vascular smooth muscle cells with TGF- $\beta_1$  induces cellular hypertrophy and inhibits mitogen-stimulated proliferation [34,45,46]. Apart from the mechanisms involving induction of growth factors like TGF- $\beta_1$  and PDGF, Ang II induced hypertrophy in human ASM cells may be attributed partly to changes in the contractile phenotype of these cells in response to Ang II stimulation. Koibuchi and



colleagues have shown that vascular smooth muscle cell growth is mediated by the autocrine production of active TGF- $\beta_1$  [26]. This may also be the case in ASM cells. The autocrine production of biologically active TGF- $\beta_1$  is a major determinant of whether vascular smooth muscle cells grow by hypertrophy or hyperplasia [17]. It remains to be determined whether the direct incubation of human ASM cells with biologically active TGF- $\beta_1$  can stimulate cellular hypertrophy.

In conclusion, we have established that Ang II induces DNA biosynthesis and protein biosynthesis, with the net effect being the accumulation of protein over DNA indicating hypertrophy in human ASM cells. Caution must be exercised in extrapolating from *in vitro* studies to *in vivo* observations, but our data suggests a potentially important role for Ang II in ASM growth in airway remodelling in asthma. This possibility is supported by the findings that plasma levels of Ang II are elevated in patients with acute severe asthma [13,14]. Our results also demonstrate that the ASM cells are activated by Ang II and that *egr-1*, *c-fos* and *c-jun* appear to be the primary response genes. The transient expression of these transcription factors is evidence in favour of induction of the nuclear transcriptional machinery that can subsequently be involved in cellular hypertrophy, since *egr-1*, *c-fos* and *c-jun* upregulation generally precede the induction of growth-associated genes in a wide variety of cells. Furthermore, the late and sustained induction of TGF- $\beta_1$  in response to Ang II could contribute, in an autocrine manner, to the process of hypertrophy in human ASM cells. Hence, Ang II activates a proliferative pathway (increase in DNA synthesis), as well as a possible antiproliferative/hypertrophic pathway (TGF- $\beta_1$  upregulation) [17], such that the net effect is hypertrophy in human ASM cells.

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**Tumour necrosis factor- $\alpha$  induces mRNA expression  
and secretion of interleukin-6**

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## 5.1 Summary

Airway smooth muscle (ASM) is considered to be an end target cell for the effects of mediators released during airway wall inflammation. Several reports suggest that activated ASM may be capable of generating various pro-inflammatory cytokines. We investigated the effects of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a potent pro-inflammatory cytokine, on cultured human ASM cells by examining the expression and release of the cytokine interleukin-6 (IL-6), cell proliferation and the expression pattern of c-fos and c-jun, two nuclear proto-oncogenes constituting the activator protein-1 (AP-1) transcription factor. Growth-arrested cell monolayers were stimulated with human recombinant TNF- $\alpha$  in a concentration- and time-dependent manner. TNF- $\alpha$  stimulated the expression of IL-6 messenger RNA (mRNA) which was detected after 15 min, reaching a maximum at 1 h. IL-6 protein was readily detected in ASM cell-conditioned medium after 2 h of TNF- $\alpha$  stimulation. Protein levels increased in a time- and concentration-dependent manner. Release of IL-6 elicited by TNF- $\alpha$  was significantly inhibited by dexamethasone, cycloheximide and nordihydroguaiaretic acid (NDGA). TNF- $\alpha$  did not alter DNA biosynthesis up to 48 h or cell numbers up to 120 h. Northern blot analysis of proto-oncogene expression revealed that c-fos and c-jun mRNA levels were elevated after 30 min of TNF- $\alpha$  incubation with maximum levels at 1 h and 45 min respectively. Expression of c-fos mRNA was downregulated by NDGA. Four hours of TNF- $\alpha$  treatment resulted in translocation of c-jun immunofluorescence from the cytoplasm to the nucleus in human ASM cells. Our results suggest that despite the lack of a mitogenic response to TNF- $\alpha$ , upregulation of primary response genes in human ASM cells may account for the induction of pro-inflammatory cytokines, such as IL-6, in human airways.

## 5.2 Introduction

Asthma is a chronic disease of the airways characterised by reversible airway obstruction and airway hyperresponsiveness. Important pathological features of asthmatic airways include inflammatory cell infiltration, epithelial shedding, basement membrane thickening and increased mass of airway smooth muscle (ASM) [1-3]. Infiltration of lymphocytes and granulocytes, especially eosinophils, and their concomitant release of cytokines appears to play a central role in mediating the airway inflammatory response [4]. Several inflammatory cell-derived cytokines have also been implicated in ASM cell division and growth [5-7] and may therefore be linked to the hyperplasia and hypertrophy of smooth muscle observed in asthmatic airways [1, 2]. In addition to their contractile and proliferative properties, ASM cells may modulate airway

inflammation by the synthesis and secretion of proinflammatory secondary mediators, thereby acting as “immune-effector” cells in the perpetuation of the airway inflammatory reaction [8-12]. TNF- $\alpha$  is a potent proinflammatory cytokine and its role as a potential mediator in asthma has been well described [13, 14]. It has been shown in humans that inhaled TNF- $\alpha$  increases bronchial responsiveness [15]. TNF- $\alpha$  can also modulate cultured ASM cells to proliferate [6, 7]. Another important biologic action of TNF- $\alpha$  is its ability to induce an influx of inflammatory cells into tissues through either chemotactic mechanisms or increased expression of adhesion molecules [16, 17].

Interleukin (IL)-6, a pleiotropic cytokine, has proinflammatory effects relevant to airway wall inflammation [18] including mucus hyper-secretion [19] as well as stimulation of hyperplasia and hypertrophy of cultured guinea pig ASM cells [5]. Expression of the IL-6 gene can be induced in many different cell types following stimulation with TNF- $\alpha$  [20] and decreased by treatment with corticosteroids [21, 22]. The gene contains sequences for the serum responsive element (SRE) and the consensus sequences for transcription factors such as AP-1 and nuclear factor kappa-B (NF- $\kappa$ B). These regulatory sequences play an important role in transcriptional activation of the IL-6 gene [23]. TNF- $\alpha$  can induce NF- $\kappa$ B and AP-1 in human lung, which could be of relevance for the expression of IL-6 [24-26].

Several investigators have shown that the levels of both TNF- $\alpha$  and IL-6 are upregulated in patients with acute severe asthma [27-29]. However, the molecular mechanisms involved in the relationship between TNF- $\alpha$  and IL-6 gene expression have not been investigated in airway inflammation. In this study we investigated the effects of TNF- $\alpha$  on the expression and release of IL-6 by human ASM cells. We also examined the mRNA expression pattern of the constituent proteins of the AP-1 transcription factor complex, c-fos and c-jun. ASM cell proliferation was also assessed in relation to TNF- $\alpha$  and IL-6. Furthermore, we determined whether the secretion of IL-6 by human ASM cells was sensitive to inhibition by the glucocorticosteroid dexamethasone, as well as the protein synthesis inhibitor cycloheximide (CHX) and the lipoxygenase pathway inhibitor NDGA.

### **5.3 Materials and Methods**

#### *Human airway smooth muscle cell isolation and culture*

Human airway smooth muscle cells were isolated and cultured as described previously [30, 31]. Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained from patients who underwent surgery for lung carcinoma. After removal



of the epithelium, pieces of smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (Life Technologies BV, Breda, The Netherlands) containing bovine serum albumin (BSA, fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) (Sigma BV, Zwijndrecht, The Netherlands) at 37°C in a humidified incubator (ASSAB, Clean Air Techniek BV, Woerden, The Netherlands, model T154) containing 5% CO<sub>2</sub> in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Life Technologies BV) containing 10% (vol/vol) foetal bovine serum (FBS; Bio-Whitaker BV, 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) (Life Technologies BV). Cells were subsequently seeded at approximately 2x10<sup>5</sup> cells per 35 mm dish and maintained in culture by replacing the medium every 72 h. After 10-14 days in culture, ASM cells grew to confluence and they were then removed by trypsinisation (0.5% trypsin; 0.02% ethylenediaminetetraacetic acid [EDTA]; Life Technologies BV) and subcultured into 25cm<sup>2</sup> tissue culture flasks. Cells were further passaged into 75cm<sup>2</sup> tissue culture flasks. Confluent cells in the fourth to sixth passages were used for experiments.

Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle  $\alpha$ -actin and smooth muscle-myosin heavy chain (SM1 and SM2) (Sigma BV) [30, 32, 33], demonstrated that the cultures were essentially free (>95%) of other contaminating cell types.

#### *Stimulation of airway smooth muscle cells*

Human ASM cells were harvested from 75 cm<sup>2</sup> flasks by treatment with trypsin during passages 4 to 6. Cells were seeded into 24-well plastic tissue culture plates at a density of 3x10<sup>4</sup> cells/well and allowed to adhere for 24 h. To synchronise cellular growth, human ASM cells were washed 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 cultured in serum-free DMEM containing 1µM insulin, 5µg/ml transferrin and 100µM ascorbate (Sigma BV) for 72 h. Using flow cytometric analysis of human ASM cells stained with propidium iodide, we previously found that 72 h of serum deprivation resulted in approximately 85% of human ASM cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase. Growth-arrested cell monolayers were stimulated with TNF- $\alpha$  (Knoll AG, Ludwigshaven, Germany) in fresh FBS-free DMEM in a concentration-dependent (0, 1, 5, 10, 25, 50, 100 and 500 units TNF- $\alpha$ /ml; 24 h) and time-dependent (15, 30, 45 min, 1, 2, 4, 8, 16, 24, 48, 72, 96, 120 h; 50 units/ml

TNF- $\alpha$ ) manner. In a separate set of experiments, serum-deprived cells were stimulated with human recombinant IL-6 (Promega, Leiden, The Netherlands) in fresh FBS-free DMEM in a concentration-dependent (0, 1, 5, 10, 50 ng IL-6/ml; 72 h) manner.

An appropriate concentration of TNF- $\alpha$  (50 units/ml) was selected for further experiments. Where inhibitors were used, cells were preincubated for 1 h with either dexamethasone (1 nM to 1  $\mu$ M) (US Biochemicals and Amersham Nederland BV, 's-Hertogenbosch, The Netherlands), CHX (1 nM to 1000 nM), or NDGA (1  $\mu$ M to 10  $\mu$ M) (Sigma BV) before the addition of TNF- $\alpha$  for 1 h (c-fos mRNA expression), 2 and 4 h (IL-6 mRNA expression), or 16 and 24 h (IL-6 protein assay). Cell-conditioned medium (0.5 ml) was collected and stored at -20°C until assayed for IL-6 levels by enzyme-linked immunosorbent assay (ELISA).

To measure cell-associated IL-6 levels, human ASM cells were lysed in single detergent lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 1  $\mu$ g/ml aprotinin, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride) after 4 h incubation in either serum-deprived medium or with TNF- $\alpha$ . The lysate was stored at -80°C until assayed for IL-6 levels by ELISA.

ASM cell viability was determined immediately by mitochondrial-dependent reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma BV) to formazan as described previously [12, 31, 34]. Cell monolayers were washed in 0.5 ml PBS, MTT (200  $\mu$ l in DMEM; final concentration 0.5  $\mu$ g/ml) was added to each well, and the cells were incubated for 5 h before overnight solubilization in an additional 200  $\mu$ l 10% sodium dodecyl sulphate in 0.1M HCl. A 200  $\mu$ l aliquot from each duplicate well was transferred to a 96-well microplate, and the optical density (OD) was determined using an automated dual wavelength spectrophotometer (3550 microplate reader; Bio-Rad, Veenendaal, The Netherlands) at a test wavelength of 595 nm and a reference of 690 nm. Cell viability was expressed as arbitrary optical density (OD) units.

#### *Isolation of total cellular RNA*

TNF- $\alpha$ -treated and -untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. The lysate was repeatedly passed through a 23G needle in order to shear the genomic DNA. Total cellular RNA was isolated using the method described previously [30, 35]. The RNA concentration was estimated by OD measurements and a DNA/protein ratio of  $\geq 1.8$  was accepted. Furthermore, the quality of RNA was tested on a

denaturing formaldehyde agarose gel. RNA samples were stored at  $-20^{\circ}\text{C}$  until processed for reverse transcriptase/polymerase chain reaction (RT-PCR) and Northern blot analysis.

#### *RT-PCR*

RT-PCR was performed to detect IL-6 mRNA expression in cultured human ASM cells and subsequently to prepare a human specific complementary DNA (cDNA) probe. Reverse transcription of 5  $\mu\text{g}$  of total RNA was performed using AMV reverse transcriptase (20 U) (HT Biotechnologies, Cambridge, UK), 1 mM of dATP, dCTP, dGTP and dTTP, 2  $\mu\text{g}$  oligo(dT)<sub>12-18</sub> primer (Pharmacia Biotechnologies Woerden, The Netherlands), 1 U/ $\mu\text{l}$  RNase inhibitor (Promega, Leiden, The Netherlands), 5.0 mM  $\text{MgCl}_2$ , 50 mM KCl, 25 mM Tris-HCl (pH 8.3) and 2.0 mM DTT in a total volume of 50  $\mu\text{l}$ . Oligo(dT) and dissolved RNA were incubated at  $70^{\circ}\text{C}$  for 10 min and placed on ice. Subsequently the remaining ingredients were added and samples were incubated at  $42^{\circ}\text{C}$  for 40 min.

Five microliters of the cDNA samples were amplified. PCR was performed using a 0.5  $\mu\text{M}$  concentration of forward and reverse primers (Life Technologies BV); dATP, dGTP, dCTP and dTTP at a final concentration of 0.25 mM each; 0.5 U *Taq* polymerase (Perkin Elmer, Gouda, The Netherlands); 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl; 10 mM Tris-HCl (pH 8.3) in a final volume of 20  $\mu\text{l}$ . Oligonucleotide primers for IL-6 were sense primer 5'-TACATCCTCGACGGCATCTCA-3' and anti-sense primer 5'-AGTTGTCATGTCCTGCAGCCA-3', giving rise to a PCR product of 398 bp. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5'-GGCCATCCACAGTCTTCTGGGT-3' and 5'-CCGAGCCACATCGCTCAGAC-AC-3' primers, giving rise to a product of 594 bp, was used as a reference. The PCR was carried out in a PTC-100 programmable thermal controller (MJ Research Inc. Watertown, MA) at  $94^{\circ}\text{C}$  for an initial 10 min followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 min, annealing at  $55^{\circ}\text{C}$  for 30 sec, and extension at  $72^{\circ}\text{C}$  for 90 sec. Final extension was for 10 min at  $72^{\circ}\text{C}$ . Products were resolved by electrophoresis on a 1% agarose ethidium bromide-stained gel and then visualized using ultraviolet luminescence and photographed. The PCR product was purified using a Wizard<sup>®</sup> PCR purification system (Promega) before sequencing in an automated Applied Biosystems Prism 310 genetic analyzer (Perkin Elmer, Nieuwekerk a/d IJssel, The Netherlands). Cycle sequencing reactions using an ABI prism dye terminator kit were performed according to the manufacturers instructions (Perkin Elmer). The IL-6 PCR product was cloned into the pGEM-T easy plasmid vector and transformed into JM109 cells (Promega). The insert positive clones were processed for plasmid DNA isolation using a Wizard<sup>®</sup> DNA purification kit (Promega).

### *Northern blot analysis*

For Northern hybridisation, samples of total RNA (10  $\mu$ g) were denatured at 65°C in a 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) by the alkaline downward capillary transfer method [36]. The filters were air-dried and UV cross-linked in a gene linker (Biorad Laboratories BV). Blots were hybridised as described previously [30]. The cDNA probes used for hybridisation were: human IL-6 (360 bp fragment), mouse c-fos (2.1 kb fragment) and mouse c-jun (2.6 kb fragment). A GAPDH cDNA probe (American Type Culture Collection, Rockville, MD) was used to rehybridise membranes for reference purposes. 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 OD in TNF- $\alpha$ -stimulated cells versus controls.

### *Measurement of IL-6 protein levels by ELISA*

IL-6 protein levels in ASM lysates and cell-conditioned medium were determined using a solid-phase sandwich ELISA (Medgenix, Breda, The Netherlands). Samples of cell-conditioned medium were diluted until the level of IL-6 was within the linearity limits of the standard curve of the assay. Subsequently the samples were preincubated with IL-6 capture antibody followed by biotinylated IL-6 detecting antibody. After addition of the streptavidine-peroxidase conjugate (Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands) tetramethylbenzidine (TMB) (ICN Biomedicals Inc., Costa Mesa, CA) was added and the absorbance of the resulting coloured product was measured at 450 nm using an automated spectrophotometer (Biorad Laboratories BV). The concentration of IL-6 was expressed in nanograms per milliliter. The detection limit of the IL-6 ELISA method was 50 pg of IL-6/ml. No correction for cell number variation was made since the cells were serum deprived 16 to 24 h after plating out, allowing insufficient time for proliferation.

### *Evaluation of cellular proliferation*

*[<sup>3</sup>H]Thymidine incorporation assay.* Effects of recombinant TNF- $\alpha$  or IL-6 on DNA biosynthesis was evaluated by incorporation of [*methyl*-<sup>3</sup>H]thymidine (Amersham Nederland BV). Confluent cells in the fourth passage were washed in PBS, detached by trypsinisation and transferred into 24-well plates at a seeding density of  $6 \times 10^4$  cells/well. After 24 h, the sub-confluent cell monolayers were growth arrested as described previously. Cells were incubated with [*methyl*-<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) in either fresh FBS-free DMEM (control) or DMEM containing

TNF- $\alpha$  (50 units/ml) for 16, 24 or 48 h, or IL-6 (0, 1, 5, 10, 50 ng/ml) for 72 h in order to assess DNA biosynthesis. After stimulation, the cells were washed in PBS, fixed with ice-cold methanol, and exposed to ice-cold trichloroacetic acid (5% wt/vol). The acid-insoluble fraction was lysed in 0.3 M NaOH and the incorporated radioactivity was determined by liquid-scintillation spectrometry in a Packard 1500 Tri-carb liquid scintillation analyzer (Packard-Becker BV, Delft, The Netherlands).

*Cell counting.* Cells were incubated in either fresh FBS-free DMEM or FBS-free DMEM containing TNF- $\alpha$  (50 units/ml) for 24, 48, 72, 96 or 120 h, or FBS-free DMEM containing IL-6 (0, 1, 5, 10 or 50 ng/ml; 7 days). After stimulation, the cell-conditioned medium was removed, and the cells were dispersed in 50  $\mu$ l of trypsin-EDTA by incubating at room temperature for 10 min. Cells were counted in a Bürker hemocytometer (Bürker, Marienfeld, Germany) using the trypan blue dye (Sigma BV) exclusion method.

#### *Immunocytochemistry*

An affinity purified rabbit polyclonal antibody against human c-jun (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to demonstrate the cellular localisation of the AP-1 transcription factor in human ASM cells stimulated with TNF- $\alpha$ . Cells were allowed to attach to multiwell slides for 24 h in FBS-containing medium and were subsequently synchronised before stimulation with TNF- $\alpha$  (50 units/ml) for 2 or 4 h, or FBS-free medium. After two washes in ice-cold PBS the cells were fixed in ice-cold methanol and permeabilised in PBS containing 0.5% Tween-20. Non-specific binding was blocked by incubating the cells in PBS containing 10% serum and 5% BSA, the cells were then washed and subsequently incubated with c-jun antibody in a dilution of 1:100; the negative control cells were not incubated with the primary antibody. After incubation, the cells were washed twice in PBS/Tween and further incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Santa Cruz Biotechnology). Unbound antibody was washed away using PBS and the sections were mounted in glycerol. Specimens were visualised under a microscope equipped with fluorescence ultra violet optics (Carl Zeiss BV, Weesp, The Netherlands) and photographed.

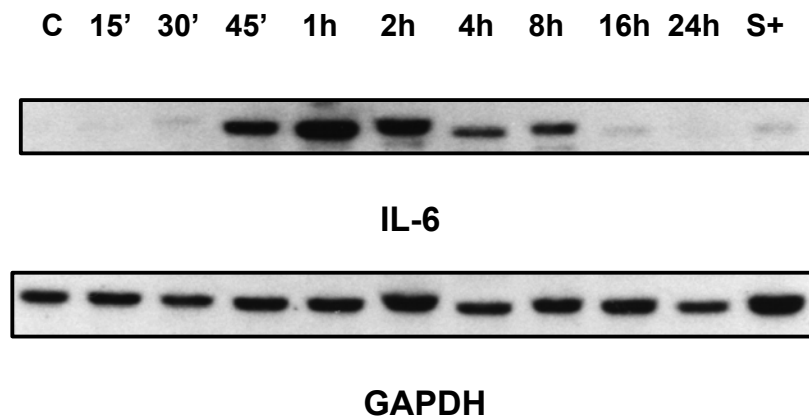
#### *Statistical Analysis*

Data in the text and figure legends are expressed as mean  $\pm$  standard error of the mean (SEM) of observations from  $n$  patients. Statistical analysis was performed by using the two-tailed, independent samples  $t$ -test. Significance was accepted at  $p < 0.05$ .

## 5.4 Results

### *IL-6 mRNA expression in relation to TNF- $\alpha$*

To examine IL-6 mRNA expression in human ASM cells treated with TNF- $\alpha$  (50 units/ml) for 1, 2 or 4 h, we performed RT-PCR employing human specific-IL-6 oligonucleotide primers. Agarose gel electrophoresis revealed PCR products of 398 bp in size. The PCR product was purified and subjected to DNA sequencing which confirmed that the amplified PCR product was indeed IL-6, with a 100% sequence homology to the human IL-6 cDNA, reported previously by Tonouchi *et al.* [37]. The IL-6 PCR product was subsequently cloned into a pGEM-T easy vector and the recombinant plasmid DNA encoding IL-6 was used as a probe to detect and quantify IL-6 mRNA by Northern blot analysis. Densitometric analysis of Northern blots showed that TNF- $\alpha$  induced the expression of mRNA encoding IL-6 after 15 min of stimulation with a maximal induction at 1 h and then the expression decreased gradually (figure 5.1). The gene remained slightly upregulated for up to 16 h.

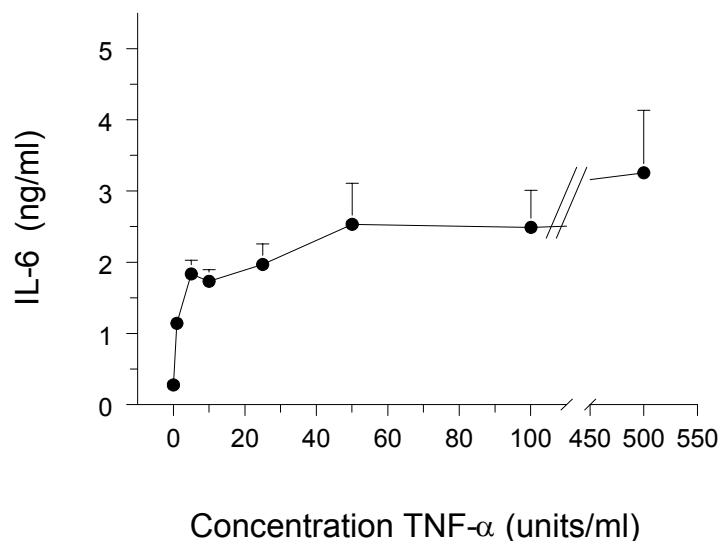


**Figure 5.1** Northern blot analysis of IL-6 mRNA expression.

Human ASM cells were treated with TNF- $\alpha$ , for the times indicated at the top of each lane, and total cellular RNA was extracted and subjected to Northern hybridisation using human IL-6 cDNA probe. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes. C = control.

### Release of IL-6 protein in response to TNF- $\alpha$

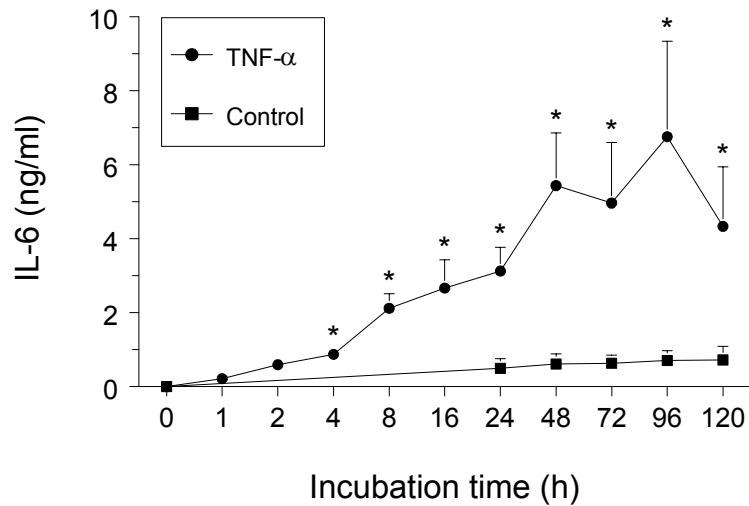
A concentration-dependent increase in the release of IL-6 protein from human ASM cells was found after stimulation by TNF- $\alpha$  for 24 h (figure 5.2). The highest IL-6 production occurred in response to 500 units/ml. Conditioned media derived from serum-deprived untreated ASM cells contained very low ( $\pm 0.25$  ng/ml) levels of IL-6 protein. To verify that TNF- $\alpha$ -stimulated human ASM cells release the *de novo* synthesised IL-6, we also measured IL-6 levels in the cell lysates of stimulated and unstimulated control cells. IL-6 levels in cell lysates of unstimulated ASM cells were negligible ( $0.10 \pm 0.01$  ng/ml). MTT reduction assays showed that ASM cell viability was not affected by TNF- $\alpha$  (up to 500 units/ml; untreated cells,  $0.24 \pm 0.02$  versus TNF- $\alpha$ -treated cells,  $0.26 \pm 0.03$  OD units,  $n=4$ ,  $P<0.05$ ).



**Figure 5.2** TNF- $\alpha$  concentration dependent production of IL-6 by human ASM cells.

Growth arrested ASM cells were stimulated in the absence (control) or presence of varying concentrations of TNF- $\alpha$  for 24 h. Data represent the mean  $\pm$  SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from four patients.

In a second set of experiments we found that TNF- $\alpha$  (50 units/ml) caused a time-dependent increase in IL-6 protein in the ASM cell-conditioned media (figure 5.3). IL-6 could be detected after just 2 h of stimulation with TNF- $\alpha$  and continued to increase before reaching a plateau around 48 h. The levels remained elevated for up to 120 h of stimulation. IL-6 levels were significantly higher in the conditioned media derived from TNF- $\alpha$  treated ASM cells compared with untreated cells at all the time points studied.



**Figure 5.3** Time-dependent production of IL-6 protein by TNF- $\alpha$  stimulated human ASM cells.

Growth arrested human ASM cells were stimulated with 50 units/ml of TNF- $\alpha$  for various time points. Control cells received only 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.

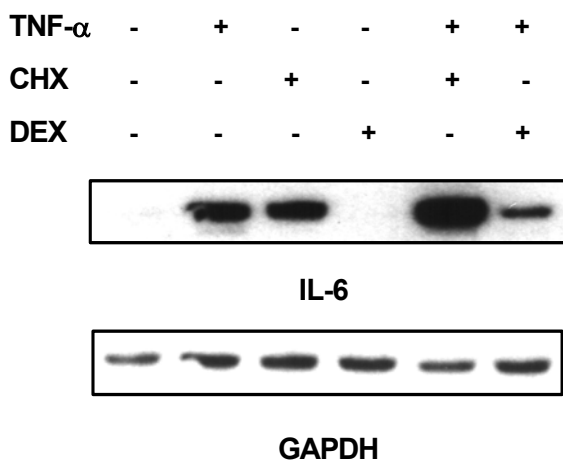
#### *Influence of inhibitors on IL-6 expression and secretion*

In figure 5.4, the effects of dexamethasone and CHX on the expression of IL-6 mRNA from untreated and TNF- $\alpha$ -treated (50 U/ml) cells are shown. Interestingly, treatment with CHX (1  $\mu$ M) resulted in increased IL-6 mRNA levels comparable to those present during TNF- $\alpha$  treatment. When cells were incubated with both TNF- $\alpha$  and CHX, IL-6 mRNA levels were further increased by 2.8 fold. However, dexamethasone significantly inhibited TNF- $\alpha$ -induced expression of IL-6 mRNA by more than 50%.

Addition of TNF- $\alpha$  (50 units/ml) to ASM cell cultures preincubated with dexamethasone (1 nM - 1  $\mu$ M) showed a concentration-dependent inhibition in the production of IL-6 from these cells, measured by ELISA (figure 5.5). Dexamethasone, at a concentration of 100 nM, significantly decreased the TNF- $\alpha$  induced IL-6 protein levels by 83% from  $3.98 \pm 0.81$  to  $1.17 \pm 0.18$  ng/ml. Levels of IL-6 in cell-conditioned medium after treatment with dexamethasone at 1  $\mu$ M were not significantly different to IL-6 levels in conditioned medium from unstimulated cells ( $n = 4$ ,

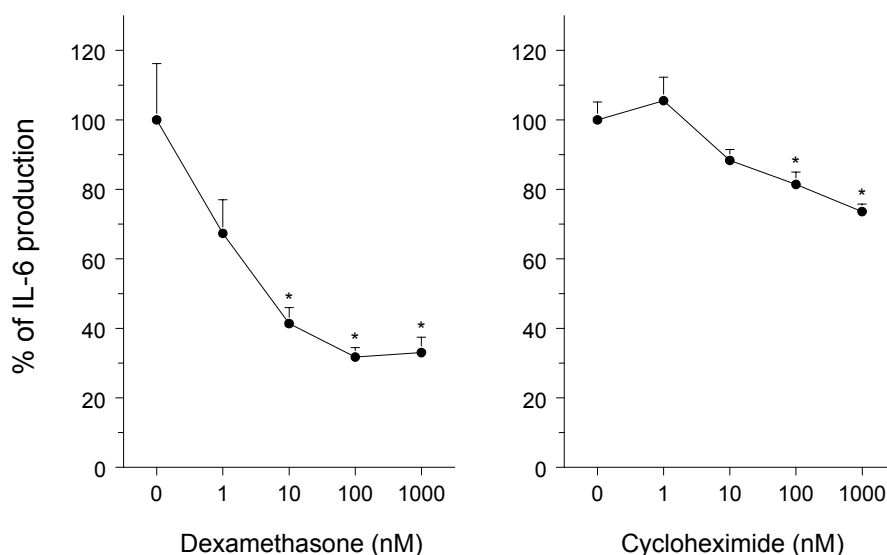


$P < 0.05$ ). Preincubation of human ASM cells with CHX (1 nM - 1  $\mu$ M) also showed a concentration-dependent inhibition in the production of IL-6 protein by cells stimulated with an optimal concentration (50 U/ml) of TNF- $\alpha$  for 24 h (figure 5.5). A total of 1  $\mu$ M of CHX reduced IL-6 release by 25% from  $3.08 \pm 0.86$  ng/ml to  $2.28 \pm 0.62$  ng/ml ( $n = 4$ ,  $P < 0.05$ ).



**Figure 5.4** Effects of CHX and dexamethasone on IL-6 mRNA expression.

Human ASM cells were pretreated with 1  $\mu$ M of either CHX or dexamethasone (DEX) for 1h before incubation with or without 50 units/ml of TNF- $\alpha$  for 2h. Total cellular RNA was extracted and subjected to Northern hybridisation using a human IL-6 cDNA probe. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes.

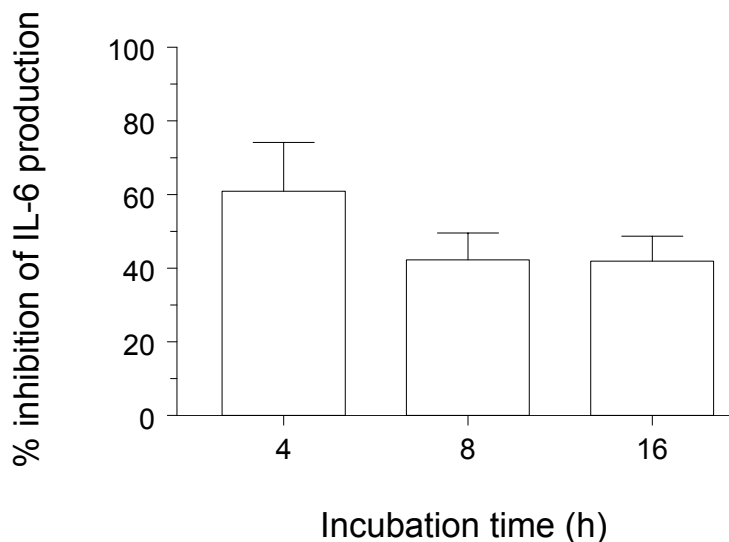


**Figure 5.5** Effects of dexamethasone and CHX on IL-6 release from TNF- $\alpha$ -treated human ASM cells.

Growth arrested human ASM cells were incubated with 50 units/ml TNF- $\alpha$  in the presence of increasing concentrations of dexamethasone or CHX. Data represent the mean  $\pm$  SEM of triplicate values from independent experiments using conditioned medium from ASM cells from four patients. \* $P \leq 0.05$  compared with TNF- $\alpha$ -stimulated cells in the absence of an inhibitor.

The lipoxygenase pathway inhibitor NDGA reduced the secretion of IL-6 protein at a concentration of 1  $\mu$ M. As shown in figure 5.6, NDGA significantly inhibited the TNF- $\alpha$ -induced production of IL-6 at 4, 8 and 16 h by  $60.9 \pm 13.3$ ,  $42.3 \pm 7.3$ , and  $41.9 \pm 6.8\%$ , respectively ( $n = 3$ ,  $P < 0.05$ ), whereas IL-6 levels in cell-conditioned medium after treatment with NDGA alone were not significantly different from unstimulated control cells.

We also examined the effects of dexamethasone and CHX on cell viability by using the MTT reduction assay. Performing treatment with the previously mentioned reagents, cell viability was not significantly affected up to 24 h of stimulation. For instance, in TNF- $\alpha$ +dexamethasone and TNF- $\alpha$ +CHX treated cells, the MTT reduction assay values were  $0.28 \pm 0.01$  and  $0.28 \pm 0.01$  versus  $0.27 \pm 0.01$  ODU for untreated cells ( $n=4$ ,  $P < 0.05$ ).



**Figure 5.6** Effect of NDGA on TNF- $\alpha$ -induced release of IL-6.

Growth-arrested human ASM cells were incubated with 50 units/ml of TNF- $\alpha$  in the presence of 1  $\mu$ M NDGA. Data represent a percent mean  $\pm$  SEM inhibition of TNF- $\alpha$ -induced IL-6 production by NDGA. IL-6 production in NDGA-treated cells was significantly ( $P \leq 0.05$ ) inhibited at 4, 8, and 16 h of incubation. Data were calculated from quadruplicate values from three independent experiments (three different patients).

#### *Effects of TNF- $\alpha$ and IL-6 on ASM proliferation*

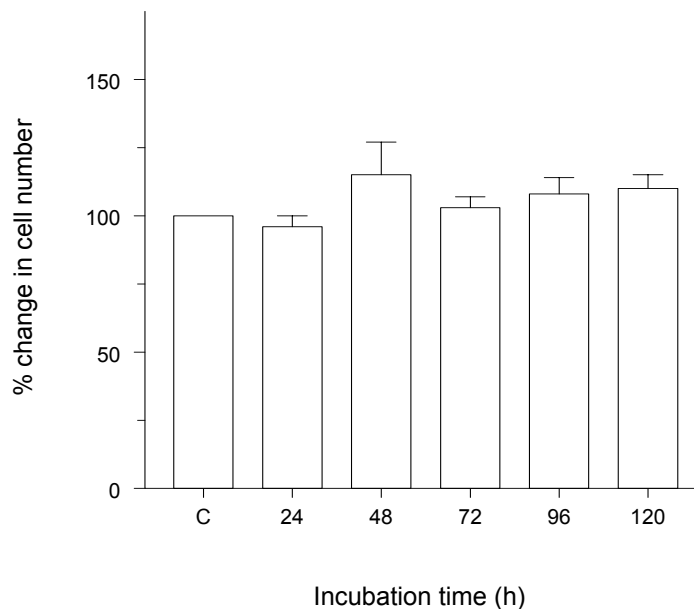
To ascertain whether human recombinant TNF- $\alpha$  induces cell proliferation,  $^3\text{H}$ -thymidine incorporation up to 48 h and changes in cell number during 120 h of incubation were determined. Treatment of human ASM cells with TNF- $\alpha$  (50 units/ml) up to 48 h of incubation did not induce

DNA biosynthesis as compared with untreated control cells (data not shown). Furthermore, TNF- $\alpha$  incubation did not significantly increase ASM cell number as compared to controls (figure 5.7).

**Figure 5.7**

Proliferation of human ASM cells in relation to TNF- $\alpha$ .

Growth arrested human ASM cells were stimulated with 50 units/ml TNF- $\alpha$  for 24, 48, 72, 96 and 120 h, and cellular proliferation was assessed by cell counting in relation to non-stimulated cells using a hemocytometer. Values are represented as mean percent change in cell numbers relative to controls  $\pm$  SEM of four measurements from four separate experiments.

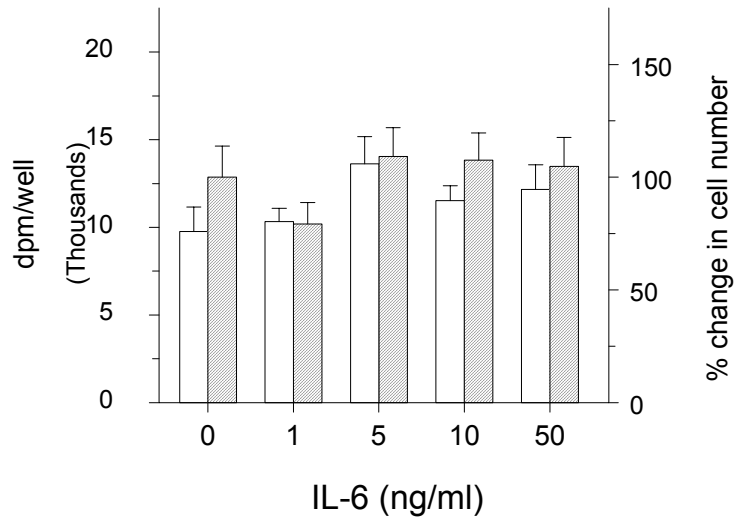


To establish whether human recombinant IL-6 induces human ASM cell proliferation,  $^3\text{H}$ -thymidine incorporation and changes in cell numbers were determined using varying concentrations of IL-6 (including the concentration found to be secreted by TNF- $\alpha$ -stimulated ASM cells). Data shown in figure 5.8 depict that IL-6 did not significantly alter the DNA biosynthesis as well as cell number in human ASM cells.

**Figure 5.8**

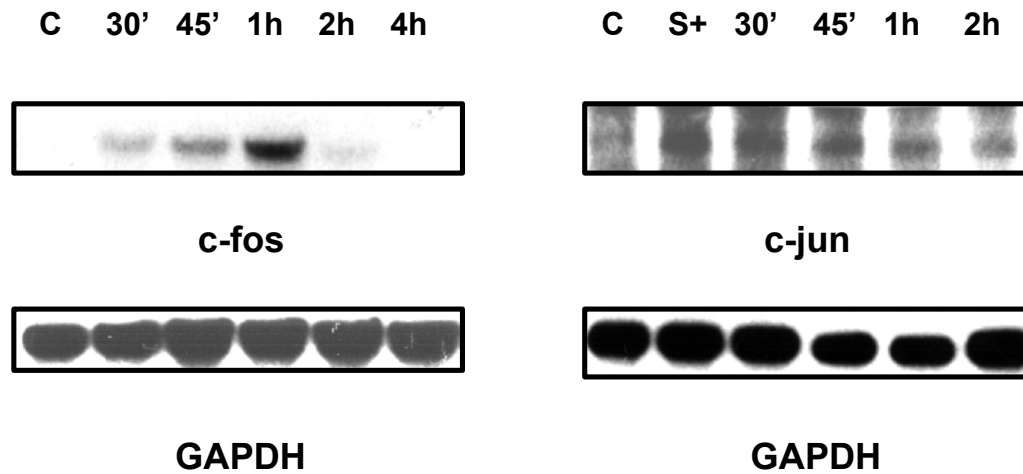
Proliferation of human ASM cells in relation to IL-6.

Growth-arrested human ASM cells were stimulated with IL-6 (0, 1, 5, 10, or 50 ng/ml) for 72 h or 7 days, and cellular proliferation was assessed by thymidine incorporation (*open bars*) or cell counting (*hatched bars*), respectively. Values are represented as dpm/well (*left y-axis*) or mean percentage change in cell number (*right y-axis*) relative to controls  $\pm$  SEM of four measurements from two separate experiments.



#### *Expression of c-fos and c-jun proto-oncogenes in relation to TNF- $\alpha$*

Representative Northern blots showing the expression pattern of the proto-oncogenes c-fos and c-jun in TNF- $\alpha$  stimulated human ASM cells are shown in figure 5.9. Densitometric analysis of the Northern blots revealed that TNF- $\alpha$  induced the expression of mRNAs encoding c-fos and c-jun after 30 min of stimulation. The induction of c-fos mRNA was transient and reached a maximum at 60 min followed by an abrupt decline. However, the expression of c-jun reached a maximum level at 45 min of TNF- $\alpha$  stimulation and levels remained elevated. FBS (10%) also induced the expression of c-jun mRNA.



**Figure 5.9** Northern blot analysis of c-fos and c-jun expression in human ASM cells treated with TNF- $\alpha$ .

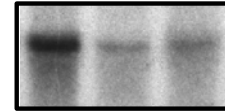
Total RNA samples from control and TNF- $\alpha$  treated human ASM cells at various time points were subjected to Northern hybridisation with radiolabelled cDNA probes. Representative Northern blots for c-fos (*left panel*, 2.2 kb mRNA) and c-jun (*right panel*, 2.3 kb mRNA) are shown. Serum-deprived ASM cells were incubated with TNF- $\alpha$  (50 units/ml) for the times indicated at the top of each panel. Rehybridisation of each filter with a GAPDH cDNA probe (*lower bands*) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from three different individuals. C = control.

Furthermore, in an attempt to examine whether NDGA could block the c-fos expression, we found that at 1 and 10  $\mu$ M concentrations, TNF- $\alpha$ -induced expression levels of c-fos mRNA were decreased by  $60.8 \pm 12.1$  and  $69 \pm 9\%$ , respectively (figure 5.10).

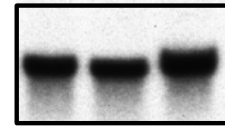
**Figure 5.10** Northern blot analysis of c-fos expression in human ASM cells treated with TNF- $\alpha$  in the presence or absence of NDGA.

Total RNA samples from TNF- $\alpha$ -treated (1 h) human ASM cells in the presence or absence of NDGA were subjected to Northern hybridisation with radiolabelled c-fos probes. A representative Northern blot for c-fos (2.2 kb mRNA, *upper panel*) is shown. Incubation conditions are indicated at the top of the panel. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from different individuals.

<b>TNF-<math>\alpha</math></b>	<b>+</b>	<b>+</b>	<b>+</b>
<b>1 <math>\mu</math>M NDGA</b>	<b>-</b>	<b>+</b>	<b>-</b>
<b>10 <math>\mu</math>M NDGA</b>	<b>-</b>	<b>-</b>	<b>+</b>



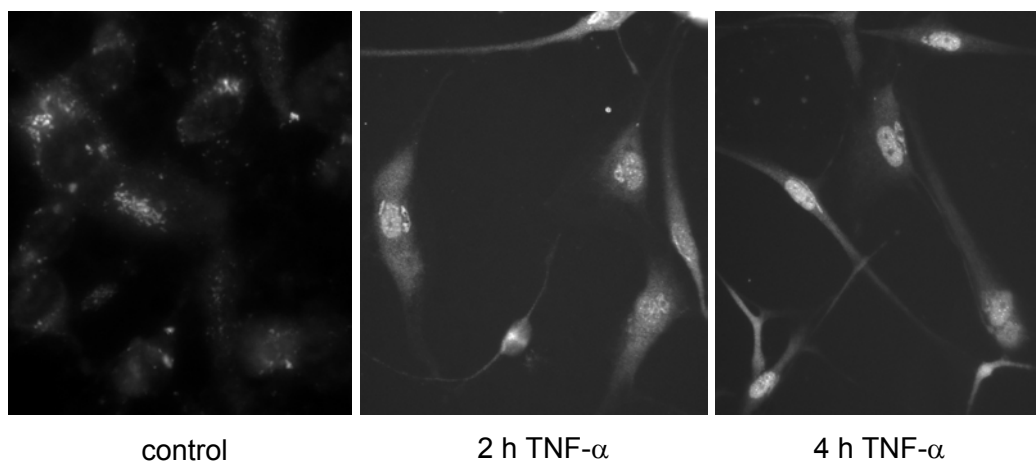
**c-fos**



**GAPDH**

#### *Immunocytochemical localization of C-JUN in human ASM cells*

Figure 5.11 illustrates representative microscopic fields of either serum-deprived or TNF- $\alpha$ -treated human ASM cells stained with antibodies recognising C-JUN protein. At 2 h of TNF- $\alpha$  (50 units/ml) stimulation, C-JUN was localised in the cytoplasm of human ASM cells (figure 5.11, *middle panel*). After 4 h of TNF- $\alpha$  stimulation, the C-JUN was translocated to the nuclei giving rise to prominent staining in the nuclei rather than the cytoplasm (figure 5.11, *right panel*). Untreated cells showed a faint background immunofluorescence in their cytoplasm and nucleus (figure 5.11, *left panel*). Additionally, the negative controls, prepared by omitting the primary antibody C-JUN, showed no immunofluorescence.



**Figure 5.11** Immunocytochemical localisation of C-JUN in TNF- $\alpha$  treated human ASM cells.

Immunoreactive C-JUN was localised in TNF- $\alpha$  treated cells using an affinity purified human C-JUN polyclonal anti-rabbit antibody and FITC-conjugated secondary antibody. Translocation of C-JUN to the nucleus of human ASM cells following 4h of stimulation with TNF- $\alpha$  was observed. Cells were fixed after treatment with serum-deprived medium (*left panel*,  $t=0$ , control) or TNF- $\alpha$  (50 units/ml) for 2 h (*middle panel*) or 4 h (*right panel*), and then subjected to immunofluorescence staining.

## 5.5 Discussion

Our study shows that human ASM cells in culture can be stimulated by TNF- $\alpha$  to express and release a proinflammatory cytokine, IL-6, and that this effect is concentration and time dependent. IL-6 protein and mRNA levels remained very low in unstimulated ASM cells, suggesting that an inducible rather than a constitutive mechanism is involved. The concentrations of TNF- $\alpha$  at which significant IL-6 production by human ASM cells were observed were within the range likely to be present during airway inflammation. [29, 38].

Recently several groups have shown that, in addition to contractile responses, ASM cells are potentially capable of modulating airway inflammation by synthesising and secreting proinflammatory secondary mediators, like IL-8, eotaxin, regulated on activation, normal T cells expressed and secreted (RANTES) and granulocyte-macrophage colony stimulating factor (GM-CSF) [8-12]. Thus ASM cells may also act as “immune-effector” cells in perpetuating airway inflammation. Similarly, our results suggest that human airway smooth muscle may contribute directly to airway inflammation by interacting with TNF- $\alpha$ , a pro-inflammatory cytokine found to be upregulated in symptomatic asthmatic airways, by the production and release of the cytokine IL-6. Stimulation of human ASM cells with IL-1 $\alpha$  transforming growth factor- $\beta_1$

(TGF- $\beta_1$ ), and CD40 ligand also leads to the production of IL-6 [24, 39]. IL-6 is a 21-kD pleiotropic cytokine that is produced by a variety of cells including airway epithelial cells [40], lung fibroblasts [41], macrophages and monocytes [42]. It has a number of pro-inflammatory properties that could be relevant in the development and perpetuation of airway inflammation during asthma, including the terminal differentiation of B cells into antibody-producing cells [43], upregulation of IL-4-dependent immunoglobulin E production [44], and stimulation of cytotoxic T cell differentiation [45],

A potential mechanism for the induction of IL-6 gene expression could involve events such as the activation of the AP-1 transcription factor-binding site on the IL-6 gene. The promoter region of the IL-6 gene has been shown to contain two AP-1 binding sites in addition to a number of other functionally important elements [23]. The transcription factor AP-1 is a heterodimer constituting the oncoproteins resulting from c-fos and c-jun gene translation. AP-1 may be activated by various cytokines including TNF- $\alpha$  via different types of protein tyrosine kinases (PTK) and mitogen-activated protein (MAP) kinases which subsequently activate a cascade of intracellular kinases [46] leading to the upregulation of the IL-6 gene. We have shown in this study that the mRNAs for the c-fos and c-jun proto-oncogenes are upregulated by TNF- $\alpha$  stimulation of human ASM cells *in vitro*. Additionally, we observed that TNF- $\alpha$ -induced c-fos mRNA expression could be inhibited by NDGA. The subsequent induction of IL-6 mRNA and secretion of IL-6 protein were also inhibited. Furthermore, immunoreactive C-JUN was localised in the cytoplasm of cells treated with TNF- $\alpha$  for 2 h, and mainly in the nucleus of cells after 4 h of treatment, suggesting that the induced expression and translocation of AP-1 could play a role in the observed gene expression for IL-6 in human ASM cells.

As corticosteroids are known to have potent anti-inflammatory effects and are clinically effective in suppressing airway inflammation, we also determined whether dexamethasone was capable of inhibiting TNF- $\alpha$  mediated expression and secretion of IL-6 by human ASM cells. We found that IL-6 gene expression and protein secretion by human ASM cells was significantly decreased but not completely abolished by treatment with dexamethasone. The precise mechanism of this downregulation has not been determined in ASM cells, but it has been demonstrated in HeLa cells to be due to the binding of ligand-activated glucocorticoid receptor to the transcriptional regulatory regions of the IL-6 promoter [21]. Zitnik and coworkers [47] and Tobler *et al.* suggested that dexamethasone exerts its action mainly by decreasing the stability of IL-6 mRNA through AUUUA-rich motifs in the 3' untranslated region [48]. Dexamethasone has also been shown to inhibit the production of several other



proinflammatory cytokines, including GM-CSF, IL-8 and RANTES in human ASM cells [8, 10, 12]. This suggests that ASM could be another important therapeutic target for the anti-inflammatory effects of steroids [49]. Surprisingly, the inhibition by dexamethasone is variable for the above mentioned cytokines. GM-CSF production by human ASM cells is completely abolished by 100nM of dexamethasone, whereas the inhibition of RANTES and IL-8 production did not exceed 50% [8, 10, 12]. In our experiments we could decrease the effect of TNF- $\alpha$  on IL-6 production by 83% with 100 nM of dexamethasone.

The molecular basis of dexamethasone-mediated inhibition of GM-CSF gene expression has been reported to involve post-transcriptional destabilisation of GM-CSF mRNA [48]. On the other hand, inhibition of IL-6 and IL-8 gene expression and protein synthesis, on the other hand, have been shown to involve both transcriptional inhibition and post-transcriptional destabilisation mechanisms by dexamethasone [21, 48, 50, 51]. Kwon *et al.* reported that dexamethasone did not affect the TNF- $\alpha$ -induced RANTES mRNA half-life in eosinophils, suggesting that purely transcriptional inhibition mechanisms are responsible [52]. The degree of involvement of transcriptional inhibition and/or post-transcriptional destabilisation mechanisms may account for the variation in the observed dexamethasone-induced inhibition in cytokine expression and secretion by human ASM cells.

CHX (100 nM), a protein synthesis inhibitor, significantly reduced, but did not abolish, IL-6 protein production by TNF- $\alpha$  stimulated human ASM cells. It is very likely that 1  $\mu$ M of CHX was insufficient to completely inhibit *de novo* synthesis of IL-6. Also, we found that the increased release of IL-6 into the conditioned medium was due to the *de novo* synthesised IL-6 and not due to the release of preformed IL-6. This is evident from the ELISA data, which show that ASM cells store a negligible amount of IL-6.

To determine if the remaining protein production was a result of an inhibited mRNA-degradation pathway, ASM cells were incubated with either TNF- $\alpha$ , CHX or a combination of TNF- $\alpha$  and CHX, and IL-6 mRNA expression was assessed by Northern blot analysis. We found that in CHX-treated (1  $\mu$ M) human ASM cells, IL-6 mRNA was expressed to the same extent as in TNF- $\alpha$ -treated cells. The combined treatment with CHX and TNF- $\alpha$  lead to an almost threefold increase in mRNA for IL-6 compared to treatment with either CHX or TNF- $\alpha$  alone. Our results suggest that the inhibition of protein synthesis by CHX may also involve the inhibition of mRNA degradation pathway via a limited synthesis of Rnase activity, as

advocated previously [40]. This can lead to an accumulation of IL-6 mRNA in human ASM cells, which can subsequently lead to higher levels of IL-6 protein in the presence of CHX.

Radiolabelled thymidine incorporation demonstrated that TNF- $\alpha$  (50 units/ml) had no immediate mitogenic effect on human ASM cells in this study, in agreement with Belvisi and coworkers [53] but in contrast to work published by Amrani *et al.* [7]. This inconsistency may be explained by taking into account that Amrani *et al.* included 0.3% or 3% serum in their culture medium, whereas our culture medium was completely free of serum. TNF- $\alpha$  and growth factors (present in serum) activate MAP kinases. MAP kinases are key transducers of extracellular signals in proliferation and differentiation pathways. Signals activated by these varying stimuli may converge to induce proliferation in a synergistic manner, possibly explaining the proliferative response to TNF- $\alpha$ .

Also, Stewart *et al.* found a mitogenic response to TNF- $\alpha$ , at very low concentrations (0.3 to 30 pM) in human ASM cells, whereas at higher concentrations (300 pM), this mitogenic effect was abolished [7]. De *et al.* have reported that IL-6 causes hyperplasia and hypertrophy of cultured guinea pig ASM cells at concentrations comparable to those present in the conditioned medium of TNF- $\alpha$ -treated cells in this study [5]. We found, however, that human recombinant IL-6 at concentrations ranging between 1 and 50 ng/ml was not mitogenic for human ASM cells. Therefore, it could be stated here that the continuing time-dependent accumulation of IL-6 in the cell-conditioned media in this study does not exhibit an autocrine mitogenic effect on the cells. Apparently, the upregulation of c-fos and c-jun, proteins forming the AP-1 transcription factor that is normally associated with cellular proliferation, is not sufficient to cause human ASM cells to proliferate, but is probably involved in other processes such as regulating the inflammatory response through upregulation of proinflammatory cytokines such as IL-6 [46]. A number of cells involved in the inflammatory response in asthma are important sources of proinflammatory cytokines such as TNF- $\alpha$  and come into close proximity to the smooth muscle layer. They may be capable of releasing concentrations of TNF- $\alpha$  that can stimulate AP-1 regulated expression and release of IL-6 from cells. Our data suggests a potentially important role for TNF- $\alpha$  and smooth muscle-derived IL-6 in airway inflammation in asthma. The precise role of IL-6 released from human ASM in the pathogenesis of asthma and/or other pulmonary disorders is unclear, but the capacity of airway smooth muscle to produce IL-6 suggests that the cells could participate in perpetuating or regulating local inflammatory events in the airways.

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**Platelet derived growth factor-AB upregulates the  
expression and secretion of interleukin-6:  
role of NF-IL6**

## 6.1 Summary

Chronic inflammation is a characteristic feature of asthmatic airways contributing to the pathogenesis of airway wall remodelling and bronchial hyper-responsiveness. The infiltration of cells such as eosinophils and lymphocytes into the airway mucosa and their concomitant release of cytokines appear to play a central role in mediating the inflammatory response in the airway. We have recently shown that the pro-inflammatory cytokine TNF- $\alpha$  induces the expression and secretion of interleukin-6 (IL-6) by human airway smooth muscle (ASM) cells. Here, we demonstrate that platelet-derived growth factor (PDGF)-AB enhances NF-IL6 DNA binding activity and upregulates the expression and release of IL-6 by cultured ASM cells. In contrast neither AP-1 nor NF- $\kappa$ B nuclear proteins were activated by PDGF-AB in our ASM cell culture system. Northern blot analysis revealed the induction of messenger RNA (mRNA) encoding IL-6 following PDGF-AB stimulation. IL-6 protein was detected in ASM cell-conditioned medium with levels increasing in a time dependent manner. The secretion of IL-6 was completely inhibited by pretreatment with the glucocorticosteroid dexamethasone. Our data demonstrate that PDGF-AB is a potent inducer of IL-6 in human ASM cells and that the activation of the transcription factor NF-IL6 may be essential for the regulation of IL-6 gene expression.

## 6.2 Introduction

Inflammation of the airway wall is central to the pathogenesis of asthma and contributes to the pathogenesis of airway wall remodelling [1]. Infiltration and activation of inflammatory cells such as eosinophils, mast cells, lymphocytes and neutrophils and the subsequent release of inflammatory mediators by these cells has been proposed to contribute directly or indirectly to changes in airway structure and function. The exact



interactions between inflammatory cell-derived mediators and ASM are not yet well defined, although accumulating evidence suggests that ASM cells participate in ongoing inflammation through the production and secretion of chemokines and cytokines [2]. The structural consequences of persisting airway inflammation include epithelial shedding, basement membrane thickening, goblet cell hyperplasia and hypertrophy as well as increased airway smooth muscle content [3-5]. These structural changes, or airway wall remodelling, have profound consequences for airway function and are at least partly responsible for the presence of airway hyperresponsiveness in asthma [6, 7]. Cytokines and growth factors, released into the airway mucosa by the infiltrating inflammatory cells, have been implicated in the growth and proliferation of airway smooth muscle cells, and may, therefore, be causally linked to the increase in smooth muscle mass observed in the asthmatic airway [8-15].

PDGF is a known airway smooth muscle mitogen [11] and is produced in large quantities by alveolar macrophages and eosinophils, and by epithelial cells and mesenchymal cells of the airways [16-18]. PDGF is the product of two genes, PDGF-A and PDGF-B, encoding two distinct chains, which form three isoforms. The PDGF-AB heterodimer and the PDGF-BB homodimer are the most potent mitogens for human ASM cells, whereas the PDGF-AA homodimer is only weakly mitogenic. Both PDGF receptor- $\alpha$  and - $\beta$  subunits are expressed in human ASM cells in culture [11]. Ligand binding leads to receptor dimerisation followed by tyrosine kinase phosphorylation, and the subsequent signal transduction pathway results in ASM cell proliferation [19, 20].

Interleukin (IL)-6 is a pleiotropic cytokine that has been found to be upregulated in the airways of asthmatics [21]. It has a number of pro-inflammatory properties that could be

relevant to the development and/or perpetuation of airway wall inflammation including mucus hyper-secretion [22], terminal differentiation of B cells into antibody-producing cells [23], upregulation of IL-4-dependent IgE production [24], and stimulation of cytotoxic T-cell differentiation [25], as well as differentiation of immature mast cells [26]. IL-6 induces hyperplasia and hypertrophy of cultured guinea pig ASM cells [9], but does not induce proliferation of human ASM cells [27]. Expression of the IL-6 gene can be induced in human ASM cells following stimulation with TNF- $\alpha$  [27, 28], IL-1 $\beta$  [29], TGF- $\beta$ , IL-1 $\alpha$  and viruses [30]. IL-6 gene expression can be decreased by treatment with corticosteroids [27, 31-33]. The promoter region of the IL-6 gene contains consensus sequences for the transcription factors; activator protein-1 (AP-1), nuclear factor kappa-B (NF- $\kappa$ B), nuclear factor-IL6 (NF-IL6) (also known as CAAT/enhancer binding protein (C/EBP) $\beta$ ) and cyclic AMP-responsive element (CRE). These regulatory sequences appear to play an important role in transcriptional activation of the human IL-6 gene [34-37].

Several investigators have shown that the levels of IL-6 are upregulated in patients with acute severe asthma [21, 38]. Since human airway smooth muscle cells are potent producers of IL-6 we investigated the regulatory effects of PDGF-AB on IL-6 gene expression by these cells by examining the expression and release of IL-6. We also examined the PDGF-AB-induced binding of nuclear proteins to the NF-IL6, AP-1 and NF- $\kappa$ B consensus sequences of the IL-6 promoter as well as the mRNA expression pattern of c-fos. FOS is one of the constituent proteins of the AP-1 transcription factor complex but can also bind to the CRE consensus sequence. Furthermore, we determined whether PDGF-AB-induced secretion of IL-6 by human ASM cells was sensitive to inhibition by the glucocorticosteroid dexamethasone.

### 6.3 Materials and Methods

#### *Materials*

Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), 0.5% trypsin;0.02% EDTA sodium pyruvate, nonessential amino acid mixture, gentamicin, penicillin/streptomycin and amphotericin B were purchased from Life Technologies BV (Breda, The Netherlands). Bovine serum albumin (BSA, fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml), non-enzymatic cell dissociation buffer, insulin, transferrin, ascorbate and platelet derived growth factor-AB (PDGF-AB) were purchased from Sigma BV (Zwijndrecht, The Netherlands). We obtained foetal bovine serum (FBS) from Bio-Whitaker BV (Verviers, Belgium) and [*Methyl*-<sup>3</sup>H]thymidine from Amersham Nederland BV ('s-Hertogenbosch, The Netherlands).

#### *Human airway smooth muscle cell isolation and culture*

Human bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained immediately following surgery of patients with lung carcinoma. ASM cells were isolated and cultured in DMEM containing 10% (v/v) FBS 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) as described earlier [27, 39]. Confluent cells in the 4<sup>th</sup> - 5<sup>th</sup> passage were used for experiments.

#### *Stimulation of airway smooth muscle cells*

Human ASM cells in the fourth or fifth passage were used for all experiments. 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 1μM insulin, 5μg/ml transferrin and 100μM ascorbate for 72 h. Using flow cytometric analysis of human ASM cells stained with propidium iodide, we previously found that 72 h of serum deprivation resulted in approximately 85% of human ASM cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase. Growth-arrested cell monolayers were stimulated with either PDGF-AB or IL-6 in fresh serum-free DMEM in a time dependent manner. In a separate set of experiments, serum deprived cells were stimulated for 24 h with PDGF-AB in the presence of dexamethasone (1μM).

#### *[<sup>3</sup>H]Thymidine incorporation assay*

The effect PDGF-AB on DNA biosynthesis was evaluated by incorporation of [*methyl*-<sup>3</sup>H]thymidine. Cells were transferred into 24-well plates at a seeding density of 3x10<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 serum-free DMEM (control), serum-containing DMEM (+ 10% serum) or DMEM containing PDGF-AB. 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 using a liquid-scintillation counter (Packard 1500 Tri-carb liquid scintillation analyzer, Packard-Becker BV, Delft, The Netherlands).

#### *Cell counting*

Cells were incubated with PDGF-AB (50 ng/ml) for 7 days. Following stimulation, the cell-conditioned medium was removed and the cells were washed twice in PBS and then

detached in 50  $\mu$ l of trypsin-EDTA by incubating at room temperature for 10 min. We then added 50  $\mu$ l PBS to each well and the cells were dispersed by gently pipetting. The resulting cell suspension was added to 10 ml isotonic counting solution (sodium chloride 6.38 g/l, sodium tetraborate 0.2 g/l, boric acid 1.0 g/l, EDTA 0.2 g/l) and the cells were counted in a CASY-1 Coulter counter (Schärfe system, Reutlingen, Germany).

#### *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. 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 [39]. The RNA concentration was estimated by optical density measurements and a DNA/protein ratio of  $\geq 1.8$  was accepted. Samples of total RNA (10  $\mu$ g) were denatured at 65°C in a 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 also described earlier [39]. The filters were air-dried and UV cross-linked in a gene linker (Biorad Laboratories B.V., Veenendaal, The Netherlands). Blots were hybridized with radiolabeled mouse c-fos (2.1 kb fragment), or human IL-6 (300 bp fragment) or a reference house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (American Type Culture Collection, Rockville, USA. [39]. Hybridization signals were quantified by scanning laser densitometry using the Ultrosan XL enhanced laser densitometer (LKB, Bromma, Sweden). Signals were normalized with respect to GAPDH mRNA values and expressed as relative optical density (OD) in stimulated cells versus controls.

*Measurement of IL-6 protein levels by ELISA*

IL-6 protein levels in ASM cell-conditioned medium were determined using a solid-phase sandwich ELISA (Medgenix, Breda, The Netherlands). Samples of cell-conditioned medium were diluted until the level of IL-6 was within the linearity limits of the standard curve of the assay. Subsequently the samples were preincubated with IL-6 capture antibody followed by biotinylated IL-6 detecting antibody. After addition of the streptavidine-peroxidase conjugate (Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands) tetramethylbenzidine (TMB) (ICN Biomedicals Inc., Costa Mesa, CA) was added and the absorbance of the resulting coloured product was measured at 450 nm using an automated spectrophotometer (Biorad Laboratories BV). The concentration of IL-6 was expressed in nanograms per milliliter. The detection limit of the IL-6 ELISA method was 50 pg of IL-6/ml. No correction for cell number variation was made since the cells were serum deprived 16 to 24 h after plating out, allowing insufficient time for proliferation.

*Nuclear protein extraction*

Nuclear proteins were extracted from the cultured human ASM cells and the DNA binding capacity of NF-IL6, AP-1 and NF- $\kappa$ B was determined using the electrophoretic mobility gel shift assay technique (EMSA). Cells were incubated with either serum deprived medium or serum deprived medium containing PDGF-AB for 1.5 h, 4 h and 8 h. The cells were then washed twice in ice cold HBSS, transferred to a centrifuge tube ( $\pm 5 \cdot 10^6$  cells) and pelleted by centrifuging at 2000 rpm for 5 min. The pellet was washed in ice cold HBSS before resuspending in 400  $\mu$ l of solution A (10 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid [Hepes] [pH 7.0], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). After 15

minutes at 4°C, 25 µl of 10% Nonidet P-40 [NP-40] was added, the suspension was vortexed briefly and then centrifuged at 13000 rpm for 30 seconds. The pellet was resuspended in 50 µl of buffer C (20 mM Hepes [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin) by shaking for 15 minutes. After centrifuging at 13000 rpm at 4°C for 5 minutes, the supernatant containing the nuclear proteins were aliquotted in small fractions, frozen in liquid nitrogen and stored at -80°C. Protein quantitation was performed using the Bradford assay [40].

#### *Electrophoretic Mobility Shift Assay*

Double stranded synthetic oligonucleotide probes containing the NF-IL6 consensus sequence (5'-AGTCGCGTTGTGCAATCTG-3'), the NF-κB consensus sequence (5'-AGCTGCGGGGATTTCCCTG-3'), and the AP-1 consensus sequence (5'-AGCTGCGTGACTCAGCTG-3') were used in the gel retardation assay. The consensus sequences for DNA binding of these transcription factors are underlined. The oligonucleotides (100 ng, double stranded) were labeled with [ $\gamma$ <sup>32</sup>P] ATP (3000 Ci/mmol, Amersham), using T4-polynucleotide kinase, and the electrophoretic mobility shift assay was performed using aliquots of nuclear extracts (2 µg) incubated with labelled consensus oligonucleotides under binding conditions (20 mM Hepes (pH 7.9), 60 mM EDTA, 0.6 mM DTT, 2 mM spermidine, 10% glycerol and 2 µg poly (dI-dC) in a total volume of 16 µl). The binding reaction was carried out at 26°C for 25 min, and protein-DNA complexes were analyzed on a 4% polyacrylamide gel. The gels were dried and exposed to radiographic film. Competition experiments were performed, to identify specific bands, by the addition of 100 fold molar excess of competitive unlabelled consensus sequence oligonucleotides prior to addition of the labelled probes.

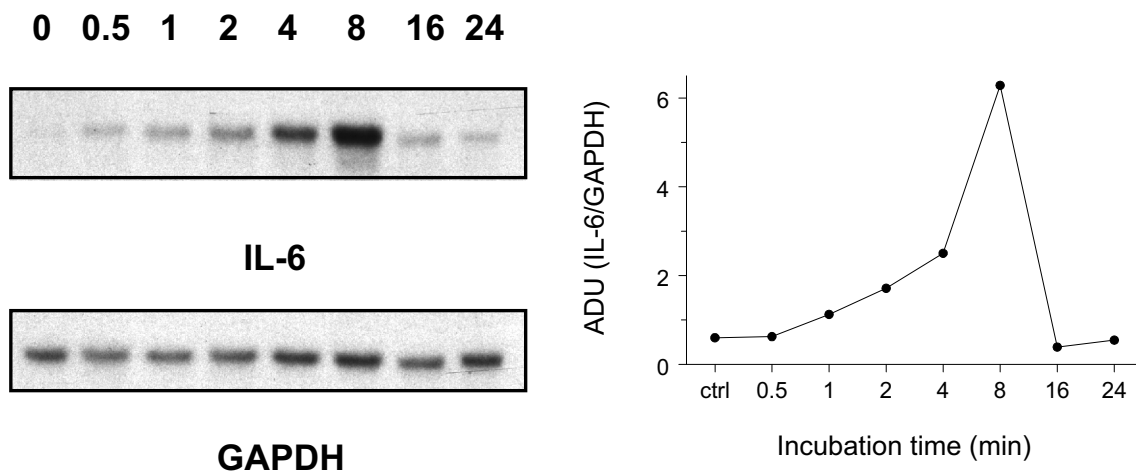
*Statistical Analysis*

Data in the figures are given as mean  $\pm$  SEM of observations from at least 3 experiments. Statistical analysis was performed by using the two-tailed, independent samples *t*-test. Significance was accepted at  $P < 0.05$ .

**6.4 Results**

*IL-6 mRNA expression in relation to PDGF-AB*

A representative Northern blot showing the expression pattern of IL-6 mRNA in PDGF-AB (50 ng/ml) stimulated human ASM cells is illustrated in figure 6.1. Densitometric analysis of the Northern blots revealed that PDGF-AB induced the expression of mRNA encoding IL-6 after 1 h of stimulation leading to a maximal induction at 8 h. IL-6 mRNA levels returned to control levels after 16 h.



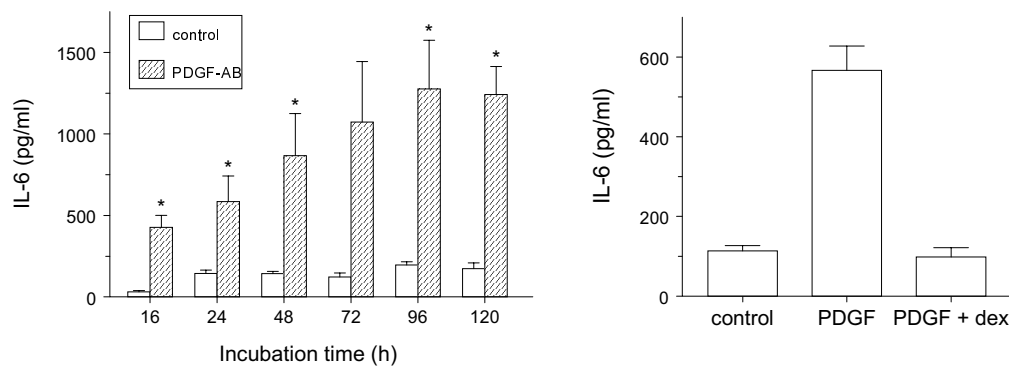
**Figure 6.1** Northern blot analysis of IL-6 mRNA expression.

Human ASM cells were treated with PDGF-AB (50 ng/ml) for the times indicated at the top of each lane (h), and total cellular RNA was extracted and subjected to Northern hybridization using human IL-6 cDNA probes. Rehybridization with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes and shows comparable levels of RNA in each lane. Autoradiographs were scanned and densitometric values were normalised with GAPDH. Values are presented as arbitrary densitometry units (ADU). Results (right) depict the values from the respective blot (left).



### Release of IL-6 protein in response to PDGF-AB

Figure 6.2 shows a time dependent increase in the release of IL-6 protein from human ASM cells in to the cell conditioned media following stimulation with PDGF-AB (50 ng/ml). Conditioned media derived from serum-deprived, untreated ASM cells contained low ( $\pm 100$  pg/ml) levels of IL-6 protein. IL-6 levels were significantly increased after 16 h of stimulation and the levels remained elevated for up to 120 h. IL-6 levels were significantly higher in the conditioned media derived from PDGF-AB stimulated ASM cells compared with untreated cells at all time points studied. The glucocorticoid dexamthasone (1  $\mu$ M) inhibited PDGF-AB-induced IL-6 protein secretion completely ( $98.3 \pm 23.0$  pg/ml) compared to PDGF-AB treated cells ( $566.8 \pm 61.0$  pg/ml). These levels were not significantly different from control levels ( $113.8 \pm 12.9$  pg/ml) at 24 h.

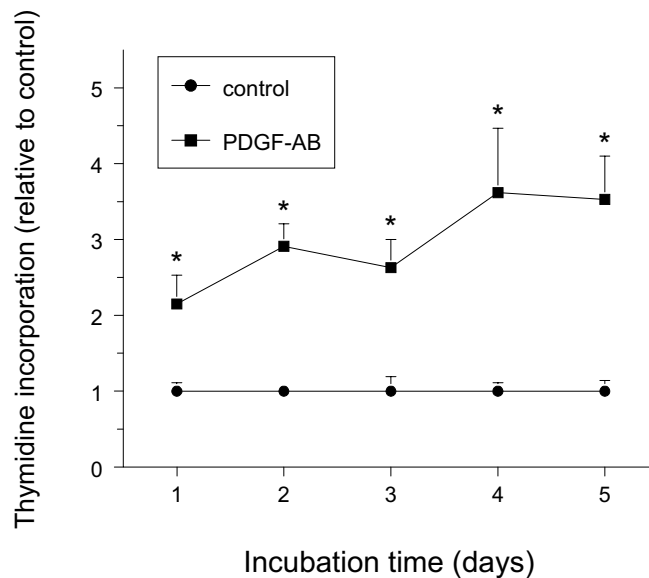


**Figure 6.2** Time dependent production of IL-6 protein by PDGF-AB-stimulated human ASM cells and dexamethasone inhibition of IL-6 protein secretion.

Growth arrested human ASM cells were stimulated with 50 ng/ml PDGF-AB (filled bars) for various times (left). Control cells (open bars) received only serum-deprived medium. Growth arrested human ASM cells were pre-treated with 1  $\mu$ M dexamethasone prior to stimulation with 50 ng/ml PDGF-AB (right). Data represents the mean  $\pm$  SEM of triplicate values from three different experiments using conditioned medium from ASM cells cultured from three different patients. \* $P \leq 0.05$  as compared with respective controls.

*Effects of PDGF-AB on ASM cell proliferation*

Changes in cell number and [<sup>3</sup>H]thymidine incorporation were determined following PDGF-AB stimulation. Treatment of serum-deprived human ASM cells with PDGF-AB (50 ng/ml) induced a significant increase in thymidine incorporation after 24 h and up to 9 days compared to untreated control cells (figure 6.3, left). Furthermore, PDGF-AB stimulation significantly increased ASM cell numbers by 1.5 fold compared to controls after 7 days incubation.



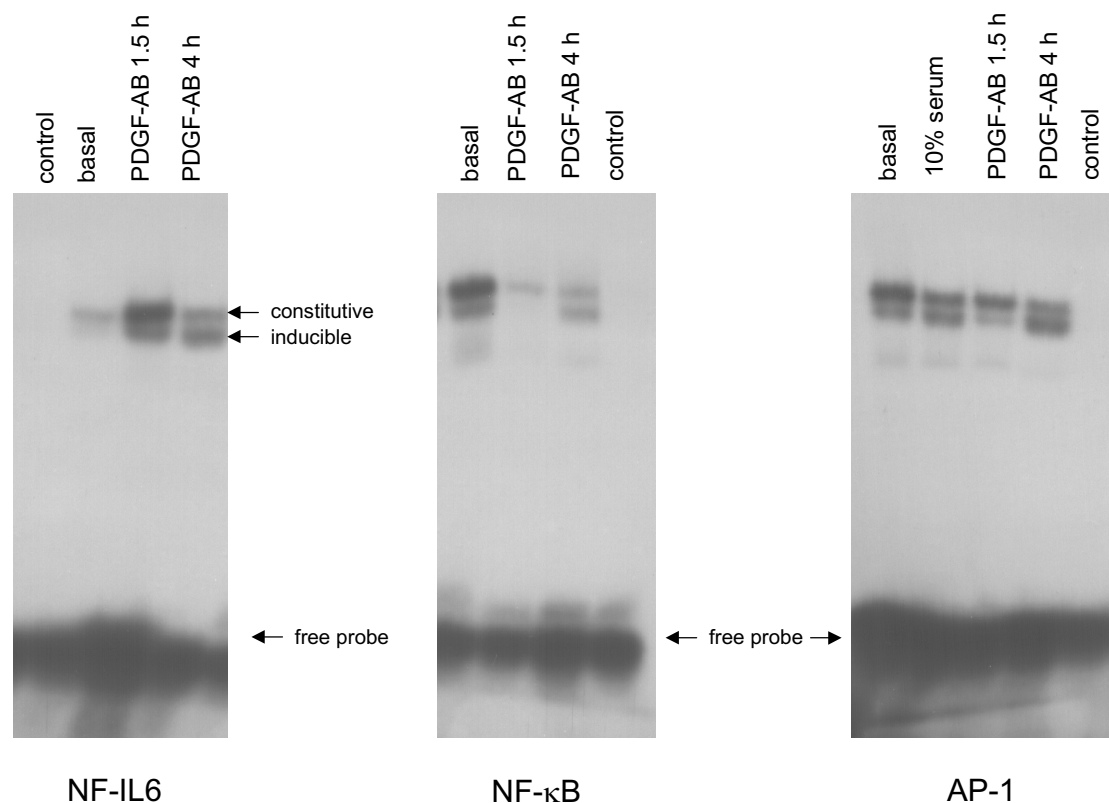
**Figure 6.3** Proliferation of human ASM cells in relation to PDGF-AB.

Growth arrested human ASM cells were stimulated with PDGF-AB (50 ng/ml) for up to 9 days, and cellular proliferation was assessed by thymidine incorporation, values are represented as thymidine incorporation relative to control ± SEM of four measurements from three separate experiments.

*Effect of PDGF-AB on NF-IL6, AP-1, and NF-κB DNA-binding activity*

The transcription factors NF-IL6, AP-1 and NF-κB bind to the human IL-6 promoter and have been shown to fulfil key functions in IL-6 gene regulation. Electrophoretic mobility shift assays were performed for human ASM cells that were either treated with PDGF-

AB for 1.5 h and 4 h or cells that remained in serum-deprived medium (basal). These results demonstrate the formation of an inducible DNA-protein complex for NF-IL6 after PDGF-AB stimulation, which declined after 4h (figure 6.4, left). Furthermore it was shown that PDGF-AB stimulation resulted in a decrease of the NF- $\kappa$ B DNA-protein complex (figure 6.4, middle). The AP-1 DNA-protein complex was not changed following stimulation with 10% serum or PDGF-AB (figure 6.4, right). The protein-DNA complexes for AP-1 and NF- $\kappa$ B were constitutively expressed in unstimulated cells. DNA-protein complex binding was completely inhibited in the presence of excess unlabelled oligonucleotides confirming that we were visualizing the DNA-protein complex of interest.

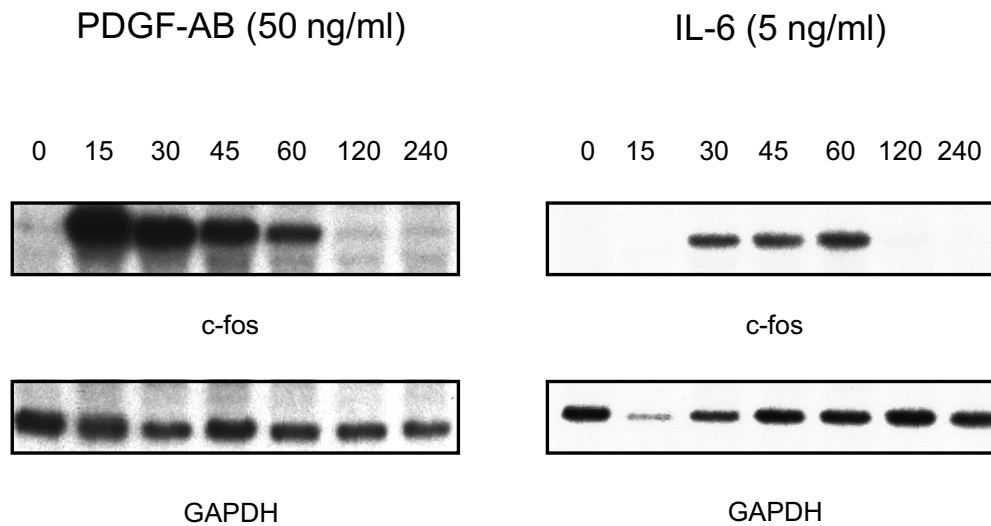


**Figure 6.4** Electromobility gel shift assay.

Using nuclear extracts prepared from human ASM cells treated with PDGF-AB for 1.5 h or 4 h shows DNA-binding activities of NF-IL-6, NF- $\kappa$ B and AP-1. Nuclear extracts from untreated cells in the absence (basal) and presence (control) of 100-fold excess unlabelled oligonucleotides are also shown.

*Expression of c-fos proto-oncogene in relation to PDGF-AB and IL-6*

Representative Northern blots showing the expression pattern of the proto-oncogene *c-fos* in PDGF-AB or IL-6-treated human ASM cells are shown in figure 6.5. Densitometric analysis of the Northern blots revealed a rapid induction in the expression of mRNA; after 15 min PDGF-AB stimulation and after 30 min IL-6 stimulation. The induction of *c-fos* mRNA was transient and reached a maximum at 30 min (PDGF-AB) and 45 min (IL-6) followed by an abrupt decline.



**Figure 6.5** Northern blot analysis of *c-fos* mRNA expression.

Human ASM cells were treated with PDGF-AB (50 ng/ml) or IL-6 (5 ng/ml) for the times indicated at the top of each lane (h), and total cellular RNA was extracted and subjected to Northern hybridisation using a radiolabelled cDNA probe as described in materials and methods. Rehybridisation with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes.

## 6.5 Discussion

In this study we have demonstrated that human ASM cells in culture express and secrete the pleiotropic cytokine, IL-6 in response to PDGF-AB, and that this effect is time dependent. IL-6 mRNA induction was transient and mRNA and protein levels remained very low in unstimulated ASM cells, suggesting that an inducible rather than a constitutive mechanism is involved. Furthermore, our data show that PDGF-AB and IL-6 induced the rapid and transient induction of mRNA encoding the nuclear proto-oncogene c-fos. The FOS protein is one of the constituent proteins of the heterodimeric transcription factor AP-1.

The promoter region of the IL-6 gene contains consensus sequences for the transcription factors NF-IL6, AP-1, NF- $\kappa$ B and CRE. These regulatory sequences appear to play an important role in transcriptional activation of the IL-6 gene [35, 36, 41]. Using gel retardation assays, we show that PDGF-AB increased nuclear proteins interacting with the NF-IL6 sequence, decreased interactions between NF- $\kappa$ B nuclear proteins with their corresponding DNA sequences and had no effect on interactions between AP-1 nuclear proteins and their corresponding DNA sequences. These results suggest that the IL-6 gene is positively regulated via the transcription factor NF-IL6. The induction of c-fos mRNA expression by PDGF-AB is not accompanied with an increase in AP-1 nuclear protein-DNA activity. Nevertheless, FOS also binds to CRE consensus sequences present in the promoter region of the IL-6 gene [37], and it is possible that this particular route may be also important in the regulation of IL-6 gene expression in human ASM cells.

The IL-6 promoter appears to be regulated in a stimulus and cell specific manner. For example, in human lung fibroblasts the transcription factor AP-1 (a junD homodimer) is required for the induction of IL-6 by TGF- $\beta_1$ , whereas CRE and NF- $\kappa$ B are not [42]. TNF- $\alpha$  or IL-1 $\beta$ -induced IL-6 expression in fibroblasts, however, does require NF- $\kappa$ B. Both AP-1 and NF- $\kappa$ B are needed for IL-6 gene regulation in the murine monocyte/macrophage cell line PU5-1.8 [43], and NF- $\kappa$ B, but not AP-1, is required in rheumatoid fibroblast-like synoviocytes [44]. Our findings suggest that NF-IL6 is a major mediator of PDGF-AB-induced IL-6 expression in human ASM cells.

Corticosteroids are known to have potent anti-inflammatory effects and are clinically effective in suppressing airway inflammation. Therefore, we also determined whether dexamethasone was capable of inhibiting PDGF-AB-mediated secretion of IL-6 by human ASM cells. We found that IL-6 protein secretion was completely inhibited by pre-treatment with dexamethasone. We previously found that corticosteroids reduced TNF- $\alpha$ -induced IL-6 expression and secretion by human ASM cells by approximately 80% [27]. The precise mechanism of this downregulation has not been determined in ASM cells, but may involve both transcriptional inhibition and post-transcriptional destabilisation mechanisms [31, 33, 45].

We demonstrated by radiolabelled thymidine incorporation and direct cell counts that PDGF-AB (50 ng/ml) is mitogenic for human ASM cells, in agreement with other publications [10, 11]. Recombinant human IL-6, in concentrations ranging between 1 and 50 ng/ml, however, does not induce ASM cell proliferation in human ASM cells [27]. We can assume that intracellular IL-6 levels increase following PDGF-AB stimulation, and it has been suggested that intracellular IL-6 mediates PDGF-induced

proliferation of fibroblasts, vascular SM cells and mesenchymal cells [46]. We believe, however, that this is not the case for human ASM cells because our previous study shows that, when stimulated with TNF- $\alpha$ , human ASM cells secrete IL-6 protein (intracellular IL-6 levels rise) but cause no proliferation.

Recently we have shown that the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  can induce IL-6 mRNA expression and IL-6 protein synthesis by human ASM cells [27, 29]. Stimulation of these cells with TGF- $\beta$ , IL-1 $\alpha$  and CD40 ligand also results in the production of IL-6 [30, 47]. IL-6 is a 21-kD pleiotropic cytokine with a number of pro-inflammatory properties that could be relevant in the development and perpetuation of airway inflammation during asthma. These include mucus hyper-secretion [22], terminal differentiation of B cells into antibody-producing cells [23], upregulation of IL-4-dependent IgE production [24], stimulation of cytotoxic T-cell differentiation [25] and differentiation of immature mast cells [26]. However, IL-6 has also been shown to possess anti-inflammatory properties including the inhibition of cytokine production by macrophages [48-50] and stimulation of the production of anti-inflammatory molecules such as IL-1 receptor antagonist and soluble TNF receptor p55 [51]. Targeted overexpression of IL-6 in the airways of mice resulted in diminished Th<sub>2</sub> inflammation, eosinophilia and Th<sub>2</sub> cytokine elaboration as well as decreased expression of endothelial vascular cell-adhesion molecule-1 (VCAM-1). Moreover, IL-6 deficiency resulted in Th<sub>2</sub> mediated inflammation, tissue and BAL eosinophilia and Th<sub>2</sub> cytokine and chemokine production [52]. Whether the upregulation of IL-6 in asthmatic airways modulates the inflammatory response in a positive or a negative manner remains to be elucidated.

ASM cells have ample opportunity to encounter PDGF-producing cells in the airway mucosa, eg. activated eosinophils, monocytes, macrophages, endothelial cells, platelets and vascular smooth muscle cells [16-18, 53, 54]. Increased levels of PDGF during chronic inflammation in the airway could contribute to either airway wall remodelling through the stimulation of ASM cell proliferation, or the regulation of local inflammatory events through an increase in ASM-derived IL-6 in the airways. Our results support increasing evidence suggesting that human ASM cells are actively involved in the inflammatory response in the airway. Our data demonstrate that PDGF-AB is a potent inducer of IL-6 in human ASM cells and that activation of the transcription factor NF-IL6 may be essential for the regulation of IL-6 gene expression in this cell type.

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## 6.6 References

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## **Autocrine Regulation of Asthmatic Airway**

### **Inflammation: Role of Airway**

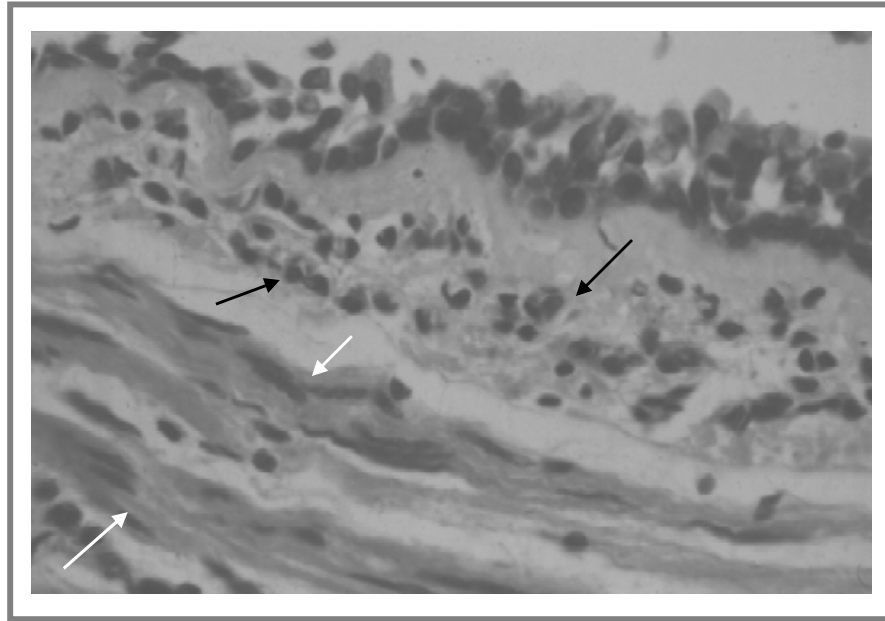
#### **Smooth Muscle**

## **7.1 Summary**

Asthma is a chronic inflammatory disease of the airways. Release of mediators from infiltrating inflammatory cells in the airway mucosa has been proposed to contribute directly or indirectly to changes in airway structure and function. The airway smooth muscle (ASM), which has typically been described as a contractile tissue, has recently been recognised as a rich source of biologically active cytokines, chemokines and growth factors, which may modulate airway inflammation. In this review we discuss the role of ASM cells in the regulation and perpetuation of asthmatic airway inflammation.

## **7.2 Introduction**

Inflammation of the airway wall is a central characteristic of asthma [1-4]. Airways airborne allergens often lead to an accumulation of eosinophils, lymphocytes (predominantly CD4 type), mast cells and macrophages resulting in an inflammatory reaction in the mucosa [1, 5]. Neutrophil numbers can increase during an exacerbation [6]. Release of mediators from these inflammatory cells has been proposed to contribute directly or indirectly to changes in airway structure and function. Important structural changes of inflamed airways include epithelial cell shedding, basement membrane thickening [7], goblet cell hyperplasia (increase in cell number) and hypertrophy (increase in cell size) [8] as well as increase in airway smooth muscle (ASM) content [9, 10]. Consequently, these structural changes form the basis for airway remodelling, a phenomenon believed to have profound consequences for airway function [11]. Figure 7.1 clearly shows infiltrating inflammatory cells in the airway mucosa in close proximity to the ASM layer.



**Figure 7.1** A segmental bronchus section from a 7 year old who died from an acute attack of asthma:

Haematoxylin and eosin stain; original magnification: x400. Infiltration of inflammatory cells (black arrows) into the airway mucosa in close proximity to the ASM in the submucosa (white arrows).

The ASM has been typically described as a contractile tissue, responding to pro-inflammatory mediators and neurotransmitters by contracting, and relaxing in response to bronchodilators. It has recently been recognised, however, that the synthetic function of airway smooth muscle cells may be related to the perpetuation and intensity of airway wall inflammation. A number of recent studies have shown that ASM cells are also a rich source of biologically active cytokines, chemokines and growth factors which may modulate airway inflammation through chemotactic, autocrine or paracrine effects. The expression of adhesion molecules and release of cyclo-oxygenase-derived products by ASM cells may also influence inflammatory

processes in the airways. In this review we discuss the role of ASM cells in the regulation of airway inflammation.

### **7.3 Inflammatory mediators in the airways**

Inflammatory mediators may be generated by resident cells within the airways and lungs, as well as by cells that have migrated into the airway from the circulation. The release of pro-inflammatory mediators can induce airway hyperreactivity and airway wall remodelling [12-19]. This release of mediators may also recruit and activate other inflammatory cells, with generation of further mediators, thus augmenting the inflammatory process. Potential sources of pro-inflammatory mediators in inflamed airways include eosinophils, epithelial cells, lymphocytes, mast cells, macrophages, neutrophils and platelets. Their respective mediators are summarised in table 7.1. The effects of a number of these individual mediators on ASM cell synthetic functions have recently been described. It seems unlikely that one particular mediator is solely responsible for the perpetuation of airway inflammation, a network of mediators probably contributes to the overall inflammatory response.

**Table 7.1** Sources of cytokines, growth factors and inflammatory mediators in inflamed airways.

Source	Inflammatory mediators	Ref
Eosinophil	MBP, EDN, ECP, EPO LTC <sub>4</sub> , PAF, O <sub>2</sub> <sup>-</sup> , MMP-9 IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, TGF- $\beta$ , GM-CSF, TNF- $\alpha$ , MIP-1 $\alpha$ , TIMP-1, PDGF	[20-22]
Epithelium	IL-1 $\beta$ , IL-6, IL-8, GM-CSF, ET-1, FGF, RANTES, MCP, MIP-1 $\alpha$ , TIMP-1, TNF- $\alpha$ , TGF- $\beta$ , PDGF, EGF, PGE <sub>2</sub> , SubsP, LTD <sub>4</sub>	[20, 23]
Macrophage	IL-1, IL-6, IL-10, GM-CSF, TNF- $\alpha$ , prostaglandins, TX's, LT's, PAF, FGF, ET-1, TGF- $\beta$ , PDGF, MCP, IGF, EGF	[20, 23]
Mast cell	IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, GM- CSF, TNF- $\alpha$ , TGF- $\beta$ , histamine, tryptase, chymase, bradykinin, prostaglandins, PAF, LT's, heparin	[20, 22]
Neutrophil	myeloperoxidase, lysozyme, LTA <sub>4</sub> , LTB <sub>4</sub> , IL-1 $\beta$ , IL-6, IL-8, prostaglandins, PAF, TXA <sub>2</sub> , TNF- $\alpha$ , TGF- $\beta$ , elastase, collagenase, MMP-9	[22]
Th <sub>1</sub> -lymphocyte	IL-2, IL-3, GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$	[20]
Th <sub>2</sub> -lymphocyte	IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF	[20]
Plasma/platelet	5-HT, Ang II, EGF, PDGF, IGF-1, TGF- $\beta$ <sub>1</sub> , ET-1, thrombin, LPA	[24, 25]

## 7.4 Cytokine production by human airway smooth muscle

Chronic airway inflammation is orchestrated and regulated by a complex network of cytokines, and these cytokines have numerous and divergent biological effects. ASM cells have been shown to be capable of producing a number of cytokines (Th<sub>1</sub> type: interleukin (IL)-2, granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN)- $\gamma$ , IL-12 and Th<sub>2</sub> type: IL-5, IL-6, GM-CSF) which then have the potential to influence airway inflammation and the development of airway remodelling (table 7.2).



#### **7.4.1 Secretion of Th<sub>1</sub>-type cytokines by ASM cells**

In a study by Hakonarson *et al.* it was demonstrated that sensitised ASM cells can express the Th<sub>1</sub>-type cytokines IL-2, IL-12 and interferon- $\gamma$  (IFN- $\gamma$ ) hours after the initial upregulation of Th<sub>2</sub>-type cytokines [26]. ASM cell-derived IL-2 and IFN- $\gamma$  may play a protective role in the airway considering the results published by Hakonarson and colleagues, whereby exogenous IL-2 or INF- $\gamma$  attenuated atopic serum-induced ASM hyperresponsiveness to acetylcholine. IFN- $\gamma$  may also play a protective role in atopic asthma by functionally antagonising IL-4-driven immunoglobulin isotype switching to IgE synthesis [27]. Inhibition of the proliferation of Th<sub>2</sub> cells, mast cells and eosinophils or promotion of the differentiation of Th<sub>0</sub> cells into those expressing a Th<sub>1</sub> phenotype may also be considered as protective in asthmatic airways [20, 28, 29]. Low levels of IFN- $\gamma$  have been detected in the BAL fluid of patients with stable asthma. Whereas the levels of mRNA for IFN- $\gamma$  were not elevated in BAL from patients with mild asthma [30], supporting the notion that the pro-asthmatic state reflects an imbalance between Th<sub>1</sub>- and Th<sub>2</sub>-type cytokine production. In patients with acute severe asthma, however, serum levels of IFN- $\gamma$  were found to be elevated [20].

#### **7.4.2 Secretion of Th<sub>2</sub>-type cytokines by ASM cells**

The pro-inflammatory cytokines interleukin (IL)-1 $\beta$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) are found in exaggerated quantities in the BAL fluid from symptomatic asthmatics and can cause airway hyperresponsiveness and eosinophilia [12, 31-33]. Cultured human ASM cells stimulated with IL-1 $\beta$  or TNF- $\alpha$  release IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF) [34-39]. IL-6 is a pleiotropic cytokine with a number of pro-inflammatory properties that could be

relevant to the development and perpetuation of airway inflammation during asthma. These include mucus hyper-secretion [40], the terminal differentiation of B cells into antibody producing cells [41], upregulation of IL-4-dependent IgE production [42] and stimulation of cytotoxic T cell differentiation [43], as well as differentiation of immature mast cells [44]. Possible anti-inflammatory properties of IL-6 include the inhibition of macrophage production of inflammatory cytokines [45] and reduced airway responsiveness to methacholine [46]. GM-CSF has been implicated in the activation, proliferation and subsequent survival of infiltrating inflammatory cells such as neutrophils and eosinophils [34, 47]. Elevated levels of GM-CSF have been found in airway biopsies from asthma patients, and its over-expression is associated with pulmonary eosinophilia and fibrosis [23]. Increased levels of IL-6 and GM-CSF have also been detected in the bronchoalveolar lavage (BAL) fluid of asthmatic subjects [12, 32, 48]. IL-6 and GM-CSF expression by human ASM cells can be decreased by treatment with the glucocorticosteroid dexamethasone [34-36], suggesting that these cells may be an important target cell for the anti-inflammatory effects of steroids in asthma therapy [49].

BAL fluid samples isolated from atopic asthmatic patients also reveal significantly increased levels of IL-5 [50]. This cytokine is predominantly produced by infiltrating T cells in asthmatic airways, and possibly mast cells [30] and is involved in the recruitment and subsequent activation of mast cells and eosinophils, that are characteristic of asthmatic airway inflammation. IL-5 promotes mobilisation of eosinophils from the bone marrow. A recent study, however, has shown that human bronchial smooth muscle cells in culture, when passively sensitised with serum from patients with atopic asthma, can also express and secrete IL-5. Treatment of naive

ASM with exogenous IL-5 potentiated its responsiveness to acetylcholine, suggesting that this Th<sub>2</sub>-cytokine may be involved in the pathobiology of asthma [26]. However, it should be borne in mind that the concentrations of IL-5 in these experiments were significantly higher than the concentrations of IL-5 secreted by the sensitised ASM cells into the culture medium. Passive sensitisation of ASM cells in culture also induced the synthesis and release of GM-CSF, IL-1 $\beta$ , IL-6 and IL-8 [26, 38, 51, 52]. The release and subsequent autocrine action of IL-1 $\beta$  is of particular interest considering its pro-inflammatory effects mentioned earlier. The timing and order of secretion of Th<sub>1</sub> and Th<sub>2</sub> cytokines by ASM cells may be an important intrinsic regulatory mechanism. The question remains whether this mechanism is defective in ASM cells in asthmatic airways, thereby leading to an exaggerated Th<sub>2</sub> response.

#### ***7.4.3 Secretion of other cytokines by ASM cells***

IL-11 and leukaemia inhibitory factor (LIF) are classified as IL-6-type cytokines and are produced by fibroblasts and epithelial cells of the airways [62, 63]. IL-11 has a variety of biological properties including the stimulation of tissue inhibitor of metalloproteinase-1 (TIMP-1), inhibition of macrophage/monocyte derived cytokine production [58], and inhibition of nitric oxide (NO) production [57]. A reduction in NO production in the asthmatic airway could have deleterious consequences for airway calibre considering the fact that endogenous NO is partly responsible for maintaining ASM tone. On the other hand, a reduction in NO production (by inducible NO-synthase) in the asthmatic airway could reduce tissue damage and inflammation, depending on the relative amount of NO produced.

**Table 7.2** ASM cell derived inflammatory cytokines, their target cells and effects.

Cytokines	Target	Effect	Ref
IL-1	ASM eo	IL-6, IL-11, LIF, GM-CSF, MCP, eotaxin, PDGF, PGE secretion↑ recruitment	[34, 36, 53-56]
IL-2	T cell eo	proliferation↑ recruitment	[20]
IL-5	eo, mc, T cells ASM	recruitment, activation↑, survival↑ responsiveness↑	[20, 26]
IL-6	mθ goblet cell B cell Cytotoxic T cell fibroblast	proliferation↓ proliferation↑, mucus secretion↑ differentiation to plasma cell differentiation activation	[20]
IL-11	ASM mθ, monocyte	proliferation↑ secretion↓ TIMP-1	[57, 58]
IL-12	T cell	proliferation↑	[20]
IFN-γ	Th <sub>2</sub> cells Th <sub>0</sub> cells B cells  eo, mc ASM  mθ	proliferation↓ → Th <sub>1</sub> IL-4-driven immunoglobulin isotype switching to IgE synthesis↓ proliferation↓ MCP, PGE secretion↑ MHC II expression activation, secretion↑ ICAM-1↑	[20, 59]
LIF	Monocytes Neurons mθ	IL-8 secretion↑ phenotype regulation, tachykinin release↑ differentiation	[60, 61]

LIF is a multifunctional cytokine with the ability to regulate macrophage differentiation. It is also a potent regulator of neuronal phenotype by modulating sympathetic neurons to adopt a cholinergic phenotype. It can also enhance neuronal tachykinin production and differentially regulate the expression of neural muscarinic

receptors. All of these mechanisms can alter airway function and, therefore, may become relevant to the pathogenesis of inflammatory airway diseases [60, 61, 64]. A study by Elias and co-workers [53] showed that transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and/or IL-1 $\alpha$  could stimulate human ASM cells to express and release IL-11, IL-6 and LIF. Also respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV-3) were potent stimulators of IL-11 by human ASM cells. These viruses are known to be important triggers of asthma [65].

## **7.5 Chemokine production by human airway smooth muscle**

Chronic airway inflammation is also regulated by a complex network of chemokines. Chemokines are 8-10 kDa proteins that have been divided into sub-families on the basis of the position of their cysteine residues located near to N-terminus of the protein. These mediators have numerous and divergent biological effects including leukocyte trafficking, degranulation of cells, angiogenesis, hematopoiesis and immune responses [66]. Chemokines produced and secreted by ASM cells may amplify the chemokine signal generated by the infiltrating inflammatory cells in the airway, thereby augmenting the recruitment of eosinophils, neutrophils, monocytes and lymphocytes to the airway (Table 7.3). The accumulation of these inflammatory cells subsequently contributes to the development of airway hyperresponsiveness, local inflammation and tissue injury through the release of granular enzymes and other cytokines. Also, eosinophils are known to produce growth factors such as TGF- $\beta_1$  and platelet derived growth factor (PDGF). These can induce proliferation of fibroblasts and smooth muscle cells *in vitro* [21, 67, 68]), possibly leading to the observed increase in smooth muscle mass in the asthmatic airway.

### 7.5.1 *CC chemokines*

The CC chemokines (or beta subfamily) have two juxtaposed cysteine residues. RANTES (Regulated on activation, normal T cells expressed and secreted) is a potent chemoattractant for monocytes, T lymphocytes and eosinophils [66] and is produced by inflammatory cells and epithelial cells of the airways [69]. Increased levels of RANTES in the BAL fluid and bronchial mucosa of allergic asthmatic patients have been measured [70]. Several studies have demonstrated that human ASM cells are also capable of expressing and secreting biologically active RANTES following stimulation with TNF- $\alpha$  or IL-1 $\beta$  [37, 55, 71]. Stimulation of human ASM cells with IFN- $\gamma$  in combination with TNF- $\alpha$  and/or IL-1 $\beta$  potentiated this effect possibly via upregulation of IFN- $\gamma$  receptor expression [71]. Treatment of the cells with dexamethasone inhibited expression of RANTES mRNA and secretion of RANTES protein whereas the Th<sub>2</sub>-type cytokine IL-10 failed to attenuate RANTES mRNA expression but did inhibit secretion of RANTES induced by a combination of IFN- $\gamma$  and TNF- $\alpha$ . IL-10 could not inhibit TNF- $\alpha$ -induced RANTES secretion from cultured ASM cells [55, 71].

The spectrum of target cells for the monocyte chemotactic proteins (MCP)-1, 2, 3, 4 and 5 includes monocytes, lymphocytes, eosinophils, basophils, dendritic cells and natural killer cells [72]. Cellular sources of MCPs include lymphocytes, monocytes, alveolar macrophages and bronchial epithelial cells. Increased levels of mRNA and protein encoding MCPs have been detected in BAL fluid and bronchial biopsies of patients with asthma [69, 70, 73-75]. Work published by two groups describes the expression and secretion of MCP-1, 2 and 3 by human ASM cells treated with the pro-inflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$  or IL-1 $\alpha$ , although induction

patterns between chemokine mRNA expression after stimulation with the individual cytokines differed [55, 56]. Furthermore, Pype and colleagues show that dexamethasone inhibited MCP mRNA expression and protein secretion, whereas IL-10 had no inhibitory effect. So far no real data have been published showing the secretion of MCP-4, MCP-5 or the weak eosinophil attractant, macrophage inflammatory protein (MIP)-1 $\alpha$ , by ASM cells.

Eotaxin is a potent chemoattractant for eosinophils, basophils and Th<sub>2</sub>-like T lymphocytes. It cooperates with IL-5 *in vivo* to induce eosinophil recruitment; IL-5 promotes mobilisation of eosinophils from the bone marrow, whereas eotaxin recruits eosinophils in the tissue. Moreover, it has the ability to induce mast cell growth. Eotaxin is highly expressed by epithelial cells and inflammatory cells in asthmatic airways and has been measured in increased quantities in BAL fluid from asthmatic subjects [66, 69, 70]. Two recent reports demonstrated that human ASM cells expressed eotaxin mRNA and protein following TNF- $\alpha$  and/or IL-1 $\beta$  stimulation [54, 76]. Neither dexamethasone nor IL-10 inhibited the expression of mRNA encoding eotaxin, although IL-10 did inhibit the release of eotaxin protein into the culture medium, suggesting the inhibition at a translational and/or post-translational level [54].

**Table 7.3** ASM cell derived chemokines, their target cells and effects.

Chemokines	Target	Effect	Ref
IL-8	Neutro eo, T cell	recruitment, activation↑ recruitment	[22]
Eotaxin	eo, T cells, baso mc	recruitment induces growth	[22, 66, 69, 70, 77, 78]
RANTES	eo, T cells, monocytes	recruitment	[22, 66, 69, 70]
GM-CSF	eo, mθ, monocyte, neutro	recruitment, activation↑, proliferation↑ survival↑	[22]
MCP	monocytes, lymphocytes, eo, baso, dendritic cells, NK cells, mθ	recruitment, activation↑ IL-1 secretion↑	[66, 69, 70]
MIP-1α	eo, NK, T cells, monocytes, mc, baso	recruitment, activation↑	[66, 70]

### 7.5.2 CXC chemokines

The CXC chemokines (or alpha subfamily) have two cysteine residues separated by an amino acid residue. IL-8 is an example of a CXC chemokine and is a potent chemoattractant and activator for neutrophils as well as a chemoattractant for eosinophils [18, 22]. It is produced by inflammatory cells and epithelial cells in the airways [79-81] and has been found to be elevated in the BAL fluid from asthma patients [82]. There are several studies demonstrating that human ASM cells, stimulated with TNF-α, IL-1β or IL-1α can express and secrete IL-8 *in vitro* [38, 56, 83]. The study carried out by Herrick and colleagues also showed that atopic/asthmatic serum stimulates ASM cells to express mRNA encoding IL-8. Pang *et al.* showed that bradykinin also stimulates the production of IL-8 in human ASM



cells [84]. Both dexamethasone and IL-10 can inhibit the release of IL-8 protein into the culture medium [83].

## **7.6 Growth factor production by human airway smooth muscle**

Airway smooth muscle cells are also a potential source of growth factors that have been implicated in airway wall thickening and may indirectly influence airway inflammation (Table 7.4). Fibroblast growth factor-2 (FGF-2) is produced by fibroblasts and vascular SM cells *in vitro* and is described as mitogenic for cells of mesenchymal origin [85-87]. Increased concentrations of FGF-2 have been measured in the BAL fluid from asthmatic patients [18]. Rödel and colleagues suggest that this increase in FGF-2 in the airways may be the result of chronic *Chlamydia pneumoniae* infections, supported by their *in vitro* experiments showing that *C. pneumoniae* infection of human ASM cells significantly increased production of FGF-2 and IL-6 [88]. FGF-2 is released after host-cell lysis and may then act as a paracrine growth factor for neighbouring ASM cells as well as upregulating the expression of interstitial collagenase, which mediates extracellular matrix (ECM) turnover. This turnover may support the proliferation of ASM cells *in vivo* leading ultimately to airway remodelling.

TGF- $\beta_1$  can be produced in the lung by a variety of cells, including macrophages, platelets, eosinophils, mast cells, activated T lymphocytes and epithelial cells [85] and it is detected in exaggerated quantities in asthmatic BAL fluid before and after antigen challenge [18]. It is an extremely potent stimulus for the synthesis of ECM components such as collagen and fibronectin leading to tissue fibrosis [89] and it can also inhibit myocyte NO production, potentially resulting in a loss of control of

airway calibre [90]. Modulation of SM cell  $\beta$ -adrenergic receptor number and function by TGF- $\beta_1$  can attenuate the effects of endogenous catecholamines or therapeutically applied  $\beta$ -adrenergic agonists [91]. In some studies TGF- $\beta_1$  expression correlates with basement membrane thickness and fibroblast number and/or disease severity [92-94]. Black *et al.* showed that ASM cells secreted latent TGF- $\beta_1$  into the culture medium [95]. Results from our laboratory demonstrated that also human bronchial ASM cells expressed and secreted significant amounts of TGF- $\beta_1$  in response to the potent vasoconstrictor Angiotensin II [96]. The production of TGF- $\beta_1$  coincided with ASM cellular hypertrophy suggesting an autocrine effect of TGF- $\beta_1$  on ASM cell phenotype. Work by Cohen *et al.* demonstrates that TGF- $\beta_1$  can also modulate EGF-induced DNA biosynthesis in human tracheal ASM cells [97]. However several studies show that exogenous TGF- $\beta_1$  can also stimulate bovine ASM cell mitogenesis [95, 98].

PDGF is produced by activated macrophages, eosinophils, epithelial cells, fibroblasts and smooth muscle cells [21, 85]. De and co-workers reported that ASM cells in culture could also express PDGF following IL-1 $\beta$  stimulation [99]. PDGF is a highly potent mitogen for ASM cells and fibroblasts [23, 67], and has been shown to act as a chemoattractant for fibroblasts as well as a stimulator for collagenase production [85]. However, Vignola *et al.* showed that PDGF-AA, -BB and -AB levels in BAL between controls and asthmatics were not significantly different, inferring that PDGF might not play an important role in the remodelling of asthmatic airways, although it may be involved in fibrotic diseases of the lung.

Insulin-like growth factor (IGF)-2 and IGF-binding protein-2 have also been detected in the conditioned medium of confluent ASM cell cultures [101]. IGF is a mitogen for these cells and IGF-binding protein-2 modulates the bioavailability of IGF by binding to it and thereby decreasing its mitogenic potency. IGF expression is not increased in the airways of asthmatics. More recently in our laboratory we found that bronchial ASM cells are capable of expressing and releasing vascular endothelial growth factor (VEGF), an angiogenic peptide, following stimulation with TNF- $\alpha$ , angiotensin II or endothelin-1 (unpublished observations). These results suggest that ASM cells may be involved in the regulation of vascular remodelling in the airway wall during inflammation [102].

**Table 7.4** ASM cell derived growth factors, their target cells and effects.

Growth factors	Target	Effect	Ref
FGF-2	ASM ECM fibroblast	proliferation $\uparrow$ collagenase $\uparrow$ proliferation $\uparrow$	[85, 86]
PDGF	ASM fibroblast epi	proliferation $\uparrow$ , IL-6 secretion $\uparrow$ recruitment and proliferation $\uparrow$ proliferation $\uparrow$	[23, 67, 85, 100]
IGF-2	fibroblast ASM	collagen $\uparrow$ proliferation $\uparrow$	[85, 101]
TGF- $\beta_1$	ASM  monocyte neutro fibroblast  T cells endo eo	proliferation, hypertrophy $\uparrow$ IL-11, IL-6, LIF secretion $\uparrow$ $\beta$ -adrenergic receptor expression $\downarrow$ recruitment, NO production $\downarrow$ recruitment recruitment, proliferation, collagen $\uparrow$ , fibronectin $\uparrow$ recruitment E-selectin $\downarrow$ survival $\downarrow$ , degranulation $\downarrow$	[23, 53, 85, 96, 97]
VEGF	endo	proliferation $\uparrow$ , migration, tube formation	[102]

These growth factors may be a link between airway inflammation and airway remodelling. Subsequent to hyperplasia or hypertrophy ASM cells remaining in the synthetic/secretory phenotype may increase production of further inflammatory mediators.

## 7.7 Cyclo-oxygenase products in the airway

Cyclo-oxygenase (COX) is the enzyme that converts arachidonic acid to prostaglandins (PG), prostacycline (PGI<sub>2</sub>) and thromboxane (TX) A<sub>2</sub>. COX-1 is the constitutively expressed isoform involved in the production of prostaglandins under physiological conditions [103]. The inducible isoform, COX-2, is expressed in response to pro-inflammatory stimuli suggesting it may play a role in the pathophysiology of asthma [104]. Several studies have shown that ASM cells can express COX-2 and release the prostaglandin PGE<sub>2</sub>, and to a lesser extent PGI<sub>2</sub>, and also the pro-inflammatory TXB<sub>2</sub>, PGF<sub>2α</sub> and PGD<sub>2</sub> in response to pro-inflammatory cytokines [105-110]. The relative contribution of the individual cyclo-oxygenase products on airway inflammation depends ultimately on the presence of their respective receptors on target tissues (Table 7.5). PGE<sub>2</sub> production by ASM cells can be upregulated in the presence of pro-inflammatory mediators such as bradykinin, IL-1β and TNF-α [106, 107, 110, 111], but also by β-adrenoceptor agonists, IFN-γ and agents that elevate cAMP levels [112]. Important anti-inflammatory effects of ASM cell-derived PGE<sub>2</sub> include inhibition of mast cell mediator release, eosinophil chemotaxis and survival, IL-2 and IgE production by lymphocytes [113], inhibition of ASM cell mitogenesis [114, 115] and inhibition of GM-CSF release by ASM cells [110]. These results support the notion that a negative feedback mechanism exists to limit the inflammatory response. However, prostaglandins also have the ability to

induce bronchoconstriction [116], increase mucous secretion from bronchial wall explants [117] and enhance pulmonary airway responsiveness. TXB<sub>2</sub>, a pro-inflammatory and bronchoconstricting mediator, can also be expressed by ASM cells [106], and has been demonstrated to have mitogenic activity on ASM cells and can trigger cysteinyl-leukotriene synthesis [118]. Thromboxane has also been implicated in airway hyperresponsiveness [119].

**Table 7.5** ASM cell derived arachidonic acid metabolites, their target cells and effects.

Cyclooxygenase products	Target	Effect	Ref
PGD	ASM goblet cell	contraction mucous secretion↑	[20, 117, 120]
PGE	ASM goblet cell mc eo T cell	contraction, proliferation↓ GM-CSF secretion↓ mucous secretion↑ secretion↓ recruitment and survival↓ IL-2 secretion↓	[20, 110, 115]
PGF	ASM goblet cell	contraction mucous secretion↑	[20, 117]
Tx	ASM	contraction and proliferation↑	[118, 121]

The cytokine-induced COX-2 activity can be inhibited by the anti-inflammatory steroid dexamethasone and by non-steroidal anti-inflammatory drugs (NSAIDs) [106]. However, the potential therapeutic effects are complicated because the consequences of COX-2 induction and prostaglandin production may be beneficial or deleterious. PGE<sub>2</sub> is an important anti-inflammatory mediator and at low concentrations it acts as

a bronchodilator whereas higher concentrations can lead to bronchoconstriction via the TX receptor [122].

## 7.8 Lipoxygenase products

5-lipoxygenase (5-LO) is the enzyme that converts arachidonic acid to leukotriene (LT) A<sub>4</sub>, which is quickly converted to LTC<sub>4</sub>, LTB<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>. The cysteinyl leukotrienes C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> are known to mediate bronchoconstriction. Expression of mRNA for enzymes of the 5-LO pathway has also been reported following exposure of ASM cells to atopic serum or IL-1 $\beta$ . These include 5-LO, epoxide hydrolase, LTC<sub>4</sub> synthase and  $\gamma$ -glutamyl transpeptidase [123]. 5-LO products can cause tissue oedema and migration of eosinophils and can stimulate airway secretions. Leukotriene D<sub>4</sub> can also stimulate ASM cell proliferation [124]. Whether, under pathological conditions, ASM cells generate relevant amounts of LTs remains to be shown.

## 7.9 T cell interactions with ASM cells

It is well known that adhesion of lymphocytes to endothelial cells, mediated by adhesion molecules and integrins, is necessary for their migration from the blood circulation to areas of tissue injury. The subsequent interactions of the T lymphocytes with ASM cells in the bronchial mucosa have also been investigated [59, 125, 126]. These studies showed that ASM cells constitutively express high levels of CD44, the principal cell surface receptor for hyaluronate [127], and express intercellular adhesion molecule 1 (ICAM-1) and vascular cell-adhesion molecule-1 (VCAM-1) when stimulated with TNF- $\alpha$ . Interactions between ASM cells and activated T-lymphocytes, possibly via specific adhesion molecules, have been shown to stimulate ASM cell DNA synthesis and subsequent ASM cell hyperplasia involved in airway

wall remodelling in inflamed airways. Adherence of anti-CD3-stimulated peripheral blood T cells to ASM cells also markedly upregulated ICAM-1 expression as well as the expression of major histocompatibility complex (MHC) class II antigens. IFN- $\gamma$  stimulation also induced the expression of MHC class II antigens by ASM cells. These studies suggest that ASM cells may also act as antigen presenting cells for pre-activated T lymphocytes in the asthmatic airway. However, the ASM cells were unable to support the proliferation of resting CD4<sup>+</sup> T cells by presenting alloantigen [59, 125].

### **7.10 Therapeutic intervention for airway inflammation**

A large number of cells in the airways, such as eosinophils, mast cells, lymphocytes, neutrophils and ASM cells, contribute to the pathogenesis of inflammatory airway diseases [22, 128-131]. Here we will specifically discuss potential anti-inflammatory interventions that target ASM-driven inflammation. As mentioned earlier, ASM cells are potential targets for glucocorticosteroid therapy. We, and others, have recently demonstrated that the expression and secretion of pro-inflammatory cytokines and chemokines by ASM cells *in vitro* can be inhibited by glucocorticosteroid treatment [34, 35, 49, 54, 55, 71, 83, 87]. Similarly, COX-2 induction and the resulting production of arachidonic acid metabolites are also inhibited by treatment with dexamethasone [34, 36, 106, 107]. On the other hand, suppression of the COX-2 pathway may result in deleterious consequences considering the bronchoprotective properties of PGE<sub>2</sub> in asthmatic airways. Mechanistically, it has been proposed that glucocorticoid receptors interact with transcription factors such as activator protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) that are activated by inflammatory signals. Protein-protein complexes thus formed prevent DNA binding and subsequent

transcription of pro-inflammatory cytokines that amplify inflammation, chemokines involved in recruitment of eosinophils, inflammatory enzymes that synthesise mediators and adhesion molecules involved in the trafficking of inflammatory cells to sites of inflammation. Corticosteroids may also control airway inflammation by increasing the transcription of anti-inflammatory genes such as IL-10, IL-12 or IL-1 receptor antagonist (IL-1ra), the gene products of which appear to be the most potent anti-inflammatory drugs for use in the treatment of airway inflammation [132].

Stewart *et al.* have also demonstrated that pre-treatment with dexamethasone, methylprednisolone and hydrocortisone can inhibit serum, FGF-2 and thrombin-induced human ASM cell proliferation [87, 133]. Beclomethasone and cortisol inhibit bovine ASM cell proliferation in culture [134]. However, in view of the complexity of the mechanisms involved in airway inflammation, a treatment for the inhibition or reversal of airway wall remodelling has yet to be fully validated [16]. In addition, inhibition of metallo-proteinases (MMPs) and growth factors during glucocorticosteroid therapy may eventually lead to the persistence of chronic inflammation by preventing proper wound healing, and thus indirectly enhance airway remodelling.

Novel therapies aimed at reducing the effects of (ASM-derived) chemokines and IL-5 include chemokine receptor antagonists and IL-5 antagonists [66, 135]. Met-RANTES (methionine extension on the amino terminus of RANTES) and Met-Chemokine  $\beta$ 7 (modified form of MIP-4) have been shown to interfere with CCR1 and CCR3 chemokine receptors, thereby inhibiting eosinophil chemotaxis in animal models of airway inflammation and allergy. However, a multi-mechanistic approach would



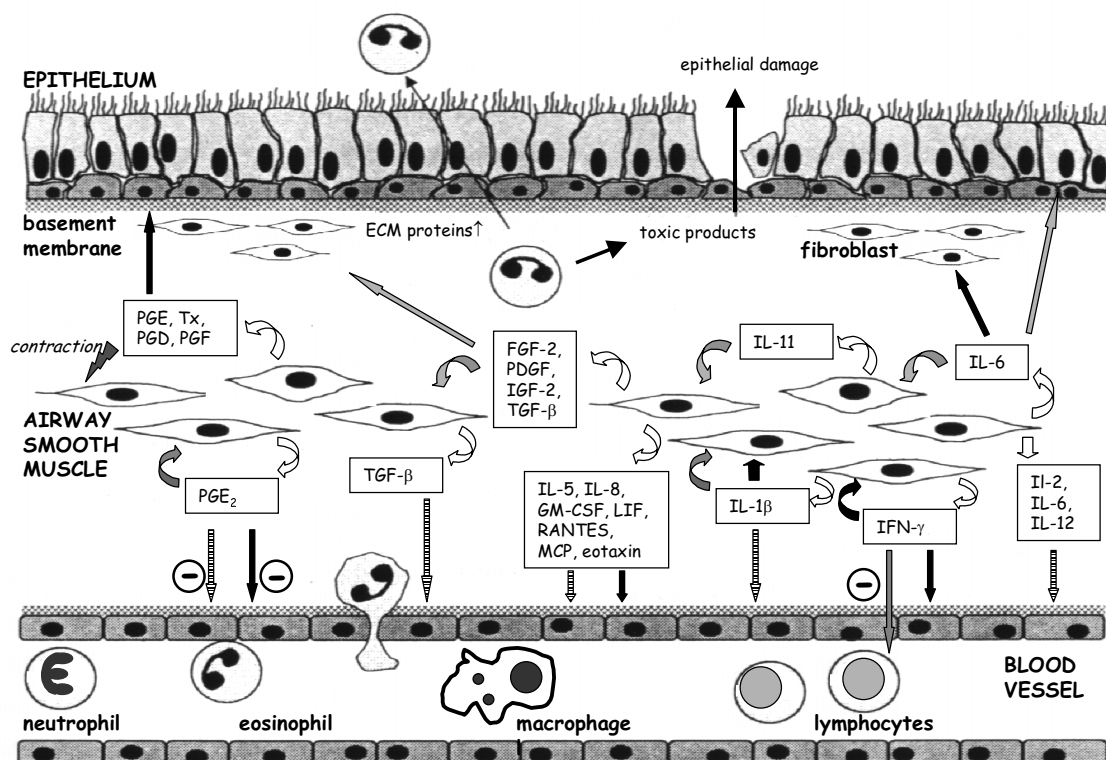
probably be advantageous for the treatment of airway inflammation due to the large number of chemokines and the promiscuous binding pattern for multiple receptors (redundancy), making it unlikely that a single chemokine or chemokine receptor-approach would be beneficial [66]. Similar to the use of chemokine receptor antagonists, the IL-5 antagonist approach should result in decreased eosinophilia in inflamed airways. Anti-IL-5 antibodies were effective in animal models to abrogate eosinophilia suggesting that the IL-5 receptor is a potential drug target [135]. A 19-amino acid peptide that binds to the IL-5 receptor alpha/beta heterodimer complex, with an affinity equal to that of IL-5, has been shown to be a potent and specific antagonist of IL-5 activity in a human eosinophil adhesion assay [136]. However this IL-5 antagonist has not yet been tested *in vivo*.

Our increasing knowledge of the intercellular communication between structural and infiltrating inflammatory cells in the airways, and in view of the role of various cytokines, chemokines and other inflammatory mediators provide an insight into the complex inflammatory processes and may help us to identify novel therapeutic targets.

### **7.11 Consequences for ongoing airway inflammation**

Allergic airway inflammation develops following the uptake and processing of inhaled allergens by antigen-presenting cells such as dendritic cells and macrophages. The subsequent interactions between these cells, T lymphocytes and resident cells leads to a cascade of events contributing to chronic inflammation, bronchospasm, mucus secretion, oedema and airway remodelling. The contemporary viewpoint is that the pro-asthmatic state reflects an imbalance between Th<sub>1</sub>- and Th<sub>2</sub>-type cytokine

production and action with an upregulated Th<sub>2</sub> cytokine response and a downregulated Th<sub>1</sub> cytokine response [137, 138]. It is postulated that the release of preformed cytokines by mast cells is the initial trigger for the early infiltration of inflammatory cells (including T cells) into the airways and that their subsequent activation and release of proinflammatory mediators induces airway hyperreactivity and recruitment of further inflammatory cells. Thus, the recent data showing that ASM cells exposed to an inflammatory environment can express and secrete chemokines, Th<sub>1</sub>- and Th<sub>2</sub>-type cytokines and growth factors provide evidence demonstrating that these structural cells could regulate airway inflammation by influencing the local environment within the airway wall. Prolonged survival of infiltrating inflammatory cells is thought to be a result of delayed apoptosis (programmed cell death), a mechanism that would normally limit tissue injury during inflammation and promote resolution rather than progression of inflammation. Both GM-CSF and IL-5 can reduce eosinophil apoptosis, resulting in persistence of the inflammatory infiltrate and even more tissue damage. The numerous chemokines secreted by ASM cells amplify this effect by recruiting more inflammatory cells to the airway wall (Figure 7.2). TGF- $\beta_1$ , however, can inhibit eosinophil survival and degranulation and may therefore play a role in the resolution of inflammation by stimulating the development of post-inflammatory repair processes in the airway [139]. Also the proinflammatory cytokine-induced release of PGE<sub>2</sub> by ASM cells may limit the inflammatory response through a mechanism whereby the secretion of GM-CSF and, possibly, other cytokines is inhibited [110]. It should be noted that mast cell products may have anti-inflammatory properties too. Heparin and heparan sulphate can modulate cell differentiation, ASM cell proliferation and inflammation [16, 115].



**Figure 2** Schematic impression of the role of ASM cells in airway inflammation.

The release of cytokines, chemokines or growth factors (open arrows) can result in proliferation (grey arrows) of structural and infiltrating inflammatory cells in the airway, or the recruitment (black and white striped arrows) or activation (black arrows) of airway cells.

## 7.12 Consequences for ASM physiology

The recent study by Hakonarson and colleagues [26] showed that cytokine exposure can influence ASM contractile function. They demonstrated that passive sensitisation of ASM strips augments constrictor responses and reduces relaxation responsiveness. These effects are ablated by exposure to the Th<sub>1</sub>-type cytokines IL-2 and IFN- $\gamma$ . Furthermore, exposure of naïve ASM strips to IL-5 and GM-CSF (Th<sub>2</sub>-type cytokines) increased muscarinic responsiveness and impaired ASM relaxation to the  $\beta$ -agonist isoproterenol. This study suggests that ASM contractile function can be

modulated in an autocrine fashion during an inflammatory episode. Work from Dr. Stephens' laboratory show that ragweed pollen-sensitised canine bronchial SM (BSM) displayed altered contractile phenotypes when compared to controls, with increased muscle shortening at maximum velocity. ASM cells obtained from asthmatic patients also showed increased shortening compared to controls [140]. These increases could be attributed to increased actin-activated myosin  $Mg^{2+}$ -ATPase activity. Increased quantity and activity of myosin light chain kinase (MLCK), thereby increasing the actomyosin cycling rate, was also reported [141]. TGF- $\beta$  exposure has been reported to activate MLCK, indicating that stimuli present in inflamed airways may be able to influence actin-myosin cycling [142]. Hautmann and co-workers showed that TGF- $\beta$  exposure also increases SM- $\alpha$  actin, SM-myosin heavy chain and h1-calponin mRNA in ASM cells *in vitro* [143]. A recent review by Solway discusses in more detail the mechanisms whereby a variety of inflammatory stimuli, such as TNF- $\alpha$ , lysophosphatidic acid and smooth muscle mitogens, could enhance the actomyosin cycling rate in ASM cells, thereby influencing ASM physiology in chronically inflamed asthmatic airways [144].

### **7.13 Consequences for airway remodelling**

The continuous process of healing and repair, due to chronic airway inflammation, can lead to airway wall remodelling [13, 15, 17, 130, 145]. The release of cytokines, chemokines and growth factors by ASM cells exposed to an inflammatory environment can result in ASM cell and goblet cell hyperplasia and/or hypertrophy [17, 23, 53, 67, 85, 86, 96, 97, 100]. Proliferation of fibroblasts and the subsequent deposition of ECM components also contribute to airway wall thickening [17].

Components of the ECM can form a reservoir for cytokines and growth factors. Upregulation of TIMP expression can inhibit the degradation of ECM components, resulting in an amplification of this effect. The increase in airway wall thickening can increase bronchial hyperresponsiveness and profoundly affect airway narrowing, with a subsequent increase in resistance to airflow caused by smooth muscle shortening [11].

#### **7.14 Concluding remarks**

The expression and secretion of cytokines, chemokines and growth factors by ASM cells *in vitro* support the notion that ASM cells are actively involved in the inflammatory response in the airway. These biologically active multipotent mediators can act to alter ASM contractility and proliferative responses as well as exaggerating or dampening the inflammatory response by amplifying signals generated by infiltrating inflammatory cells. The interaction between structural ASM cells and cells recruited from the circulation should be further explored in order to understand this complex cellular network and to further define the contributions of the cellular and molecular events involved in the pathogenesis of airway diseases. Whether the endogenous generation of inflammatory mediators by ASM cells enhances ASM hyperresponsiveness, alters ASM function or supports airway remodelling *in vivo* remains to be elucidated. Though the emerging data from various laboratories implicates a regulatory role for the ASM in airway inflammatory responses, there is a clear need to explore the synthetic properties of these cells. Determination of secretory profiles of chemokines, cytokines and growth factors released from ASM cells would give us an insight into the complex mechanisms contributing to chronic

airway inflammation and eventually help us identify potential targets for future drug therapy.

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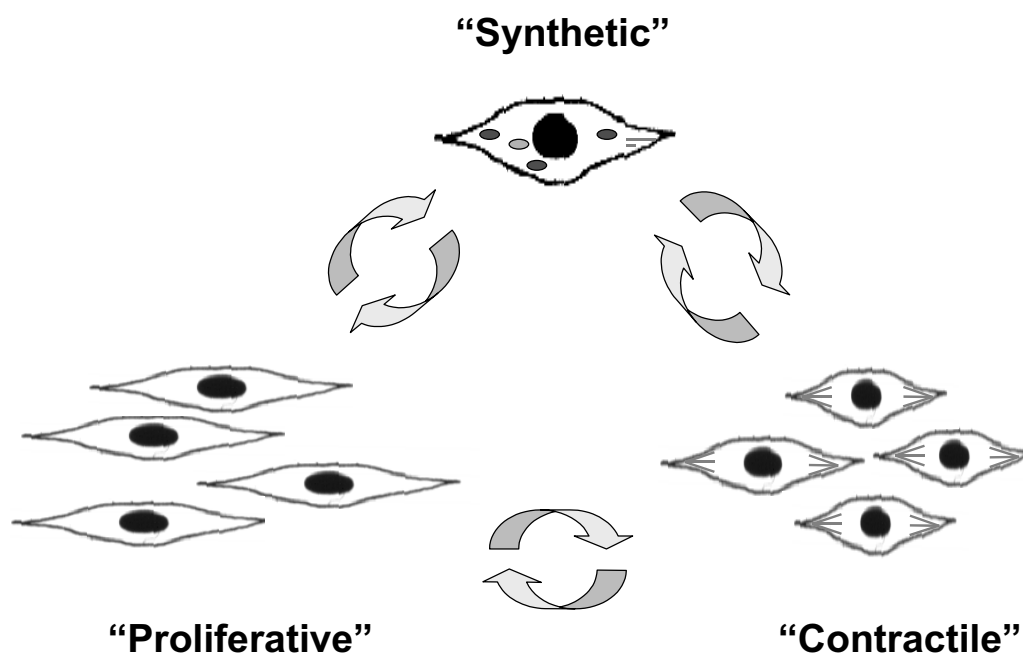
## **General Discussion**

## 8.1 Introduction

Important pathological features of asthmatic airways include inflammatory cell infiltration, epithelial disruption, goblet cell hyperplasia, basement membrane thickening and an increased mass of airway smooth muscle (ASM) [1-6]. A growing body of literature has established that the increase in ASM mass is a result of hyperplasia and hypertrophy of the ASM cells. A large number of cytokines and growth factors released during the inflammatory process in asthmatic airways could be the predominant trigger of ASM proliferation and growth [4, 5]. Furthermore, the thickened ASM layer may exaggerate the effects of ASM shortening on airway narrowing, especially in small airways (diameter < 2mm) [7]. It becomes increasingly clear that ASM cells undergoing hyperplasia/hypertrophy may be phenotypically and functionally altered. These cells may themselves be able to modulate the inflammatory process in the airway through the synthesis and release of cytokines, chemokines or other pro-inflammatory mediators. Furthermore, ASM cells may affect airway wall remodelling through the synthesis and deposition of matrix proteins. As summarised in figure 8.1, the ASM cell could potentially exhibit various phenotypes: a “contractile phenotype” responsible for the regulation of airway calibre, a “proliferative phenotype” allowing an increase in ASM cell numbers and/or size, as well as a “synthetic phenotype” resulting in the synthesis and release of mediators.

To investigate the proliferative and synthetic capacity of human ASM cells we established a cell culture system as a convenient model for studying hyperplasia, hypertrophy and secretory responses to a wide variety of stimuli. The aim of the studies described in this thesis were (i) to identify specific factors involved in stimulating ASM

mitogenesis, (ii) to investigate the expression of immediate early response genes, and (iii) to examine ASM cell secretory capabilities and the underlying mechanisms. We selected a number of pro-inflammatory cytokines, growth factors and contractile agonists reported to be upregulated in asthmatic airways and examined their effects on human ASM cells for varying times.



*Figure 8.1*

Schematic representation of the phenotypic heterogeneity of airway smooth muscle cells.

## 8.2 "Proliferative phenotype"

Smooth muscle in the airway causes variation in airway calibre and airway wall compliance and it has been shown to be necessary for the exaggerated airway narrowing that is observed in asthma. But is ASM cell proliferation important in asthma? A number of studies, but not all, have shown that an increase in airway wall smooth

muscle mass is a pathological feature of severe asthmatic airways [5, 8-10], and this increase, due to hyperplasia and hypertrophy, can influence airway narrowing considerably. This effect is amplified as a result of altered airway wall geometry [7]. Whether the increase in ASM numbers also results in altered smooth muscle function remains uncertain. During proliferation the ASM cells are believed to shift from a contractile phenotype towards a proliferative phenotype, and it is not yet known if the force generating properties of the “extra” ASM cells are altered. Recent investigations show that a high degree of heterogeneity exists in the properties of healthy ASM both at a cellular and tissue level [11-13], but the significance of these findings for asthmatic airways has yet to be evaluated. Smooth muscle from asthmatics has, in some studies, been shown to exhibit reduced responsiveness to smooth muscle relaxants, and a few reports show increased responses to contractile agonists [14-16].

Table 8.1 summarises our data and compares it with some of the published data describing mediators that influence ASM cell mitogenesis *in vitro*. These include the effects of growth factors, cytokines and inflammatory mediators on thymidine incorporation and/or cell numbers. Our experiments were designed to evaluate the individual contributions of various growth factors, cytokines and inflammatory mediators on ASM cell mitogenesis. The studies in this thesis were designed to investigate the relative contributions of a number of pro-inflammatory stimuli on ASM cell proliferation. The results described here show that stimulation of human ASM cells with individual cytokines or inflammatory mediators did not induce mitogenesis. However, it must be stressed that multiple cytokines, growth factors, contractile agonists and inflammatory mediators are probably present simultaneously in asthmatic airways. ASM proliferative responses *in vivo* are most likely the result of the concerted

actions of these stimuli. Although work from other groups has previously shown mitogenic responses to a number of cytokines and contractile agonists, it is important to emphasise that the culture media from these groups often contained serum. Growth factors present in serum activate mitogen-activated protein (MAP) kinases in smooth muscle cells. MAP kinases are key transducers of extracellular signals in pathways leading to cell proliferation and differentiation. Signals activated by cytokines or contractile agonists together with serum may synergistically induce proliferation, possibly explaining the discrepancies between our observations and other reports. Also, in our experiments, we chose 60-72 h of serum free culture prior to stimulation in order to synchronise and growth arrest the cells. Others have reported earlier that 24-48 h of serum deprivation is sufficient for growth arrest. We found, however, that 72 h were required to achieve 85% arrest (cells in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle).

**Table 8.1** Mediators influencing ASM mitogenesis *in vitro*.

Stimulus	Mitogenic effect on human ASM cells in our study	Mitogenic effect on ASM cells in other studies	Species	Ref
FGF-2	+/-	+	Human, bovine	[17, 18]
PDGF	+	+	Human, rabbit	[19, 20]
IL-1 $\beta$	-	+	Guinea-pig	[21, 22]
IL-5	-	nt		[22]
IL-6	-	+	Guinea-pig	[21, 23]
TNF- $\alpha$	-	+	Human	[23-25]
Histamine	-	+	Human, canine	[26, 27]
ET-1	-	+	Human, rabbit, ovine	[28, 29]
Ang II	+/-	nt	Human	[30]

+:mitogenic; -:not mitogenic, +/-: thymidine incorporation increases without increase in cell numbers.

Is the increased ASM mass simply a result of increased ASM cell proliferation? Ebina and colleagues reported both hypertrophy and hyperplasia of ASM cells in asthmatic airways [5]. By applying computer assisted three-dimensional morphometry they analysed digital images of bronchial cross-sections and detected severe hypertrophy of ASM in small peripheral airways. It has been suggested that hypertrophy rather than hyperplasia may result from incomplete growth stimulation, where the cell receives signals to increase both protein and DNA biosynthesis, but fails to complete the cell cycle and actually divide. Alternatively, growth inhibitory factors, such as TGF- $\beta_1$ , may alter the mitogenic response [31, 32]. We demonstrated that Ang II induced hypertrophy of human ASM cells in culture. Ang II induced DNA biosynthesis and protein biosynthesis, with the net effect being accumulation of protein over DNA. The increase in DNA biosynthesis did not coincide with an increase in cell numbers suggesting cellular hypertrophy. We also found that Ang II stimulation of ASM cells resulted in the expression and synthesis of TGF- $\beta_1$ , suggesting an autocrine mechanism regulating ASM cell growth.

Apoptosis, or programmed cell death, of ASM cells could also be a major determinant of ASM cell numbers in the airway. Is it possible that a reduced rate of apoptosis, following injury and repair, results in an “apparent” accumulation of ASM cells in asthmatic airways? Apoptosis is regulated by a complex interplay between cell surface signals, such as those from death receptors (e.g. Fas), survival factors (e.g. PDGF), and the expression of specific intracellular gene products (e.g. Bcl-2). Cell-cell and cell-matrix interactions also regulate the apoptotic response. A recent study by Hamann *et al* showed that human ASM cells express surface Fas, and that cross-linking of Fas resulted in apoptosis [33]. TNF- $\alpha$  stimulation of human ASM cells markedly potentiated

the killing effect of Fas cross-linking but it did not induce substantial apoptosis by itself. Further investigations are necessary for a better understanding of the relationship between apoptosis and the increased ASM mass observed in asthmatic airways.

### 8.3 Immediate early nuclear events

Airway smooth muscle cells constantly respond to a complex environment in which gene expression and consequent cellular phenotype are mediated by growth factors, peptide hormones, neurotransmitters and cytokines that bind to cell-surface receptors. Information from the ligand-receptor interaction is transmitted across the cell membrane and is then relayed to the nucleus where specific gene expression is selectively modulated via transcription factors. Many transcription factors have been identified, but the studies described in this thesis concentrate on the expression of the immediate early response genes encoding *c-fos* and *c-jun* (constituents of the AP-1 transcription factor) and *egr-1* (encodes the EGR-1 transcription factor). These transcription factors are implicated in the regulation of many inflammatory and immune genes that may play an important role in regulating the inflammatory response in asthmatic airways, such as IL-2, IL-5, GM-CSF, IFN- $\gamma$ , FGF-2, 5-LO, TNF- $\alpha$  and fibronectin [34-41]. Transcription factors may be considered as potential targets for the development of drugs aimed at reducing airway wall inflammation or airway wall remodelling.

In all of our experiments the induction of *c-fos*, *c-jun* and *egr-1* was found to be rapid and transient for all stimuli investigated. Table 8.2 summarises our *c-fos* and *egr-1* expression data as well as subsequent cellular responses. The IL-6 promoter region is known to contain a binding site for the AP-1 transcription factor, and an *egr-1* binding site can be found in the fibronectin promoter region. Both AP-1 and *egr-1* transcription

factors are reported in the literature as markers for cell proliferation and/or cell differentiation [27, 35, 42-44].

The studies described in this thesis demonstrate that the expression of *c-fos* and *egr-1* is not always accompanied by ASM cell mitogenesis. Because non-proliferating cells synthesised and secreted IL-6 and expressed mRNA for fibronectin, we hypothesise that the expression of these early response genes may be considered as markers of ASM cell activation.

**Table 8.2** Expression of immediate early response genes and subsequent potential gene regulation in ASM cell cells.

Stimulus	c-fos mRNA	egr-1 mRNA	Proliferation	IL-6 secretion	Fibronectin mRNA
<b><i>Growth factors:</i></b>					
PDGF-AB	+	+	+++	+	+
FGF-2	+	+	+	nt	+
<b><i>Cytokines:</i></b>					
IL-1 $\beta$	+	+	-	+	+
TNF- $\alpha$	+	+	-	+	-
IL-5	+	nt	-	+	nt
IL-6	+	nt	-	nt	nt
<b><i>Contractile agonists:</i></b>					
Ang II	+	+	-	+	nt
ET-1	+	+	-	+	nt
Histamine	+	+	-	+	nt

nt: not tested, +:positive response, -:negative response



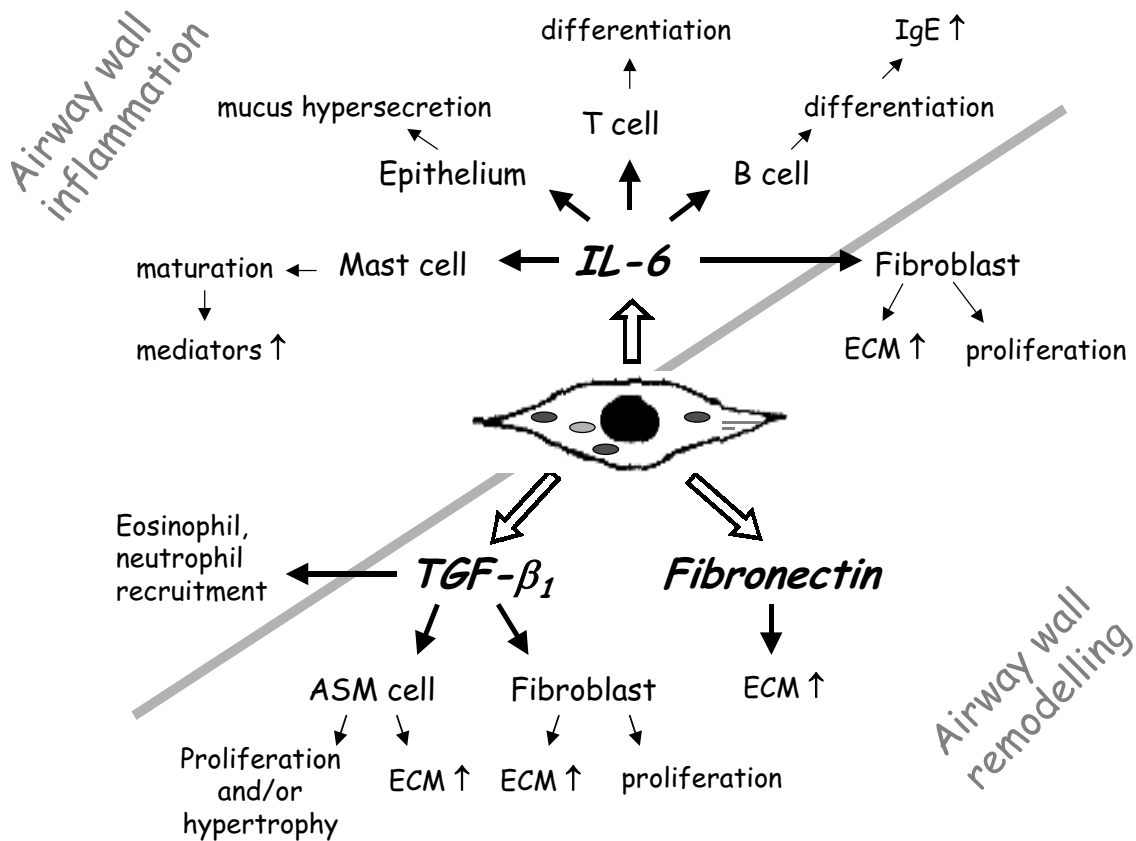
AP-1 and egr-1 transcription factors regulate many of the inflammatory and immune genes that are over-expressed in asthma, it is therefore tempting to speculate that these transcription factors are potential targets for future therapeutic strategies aimed at decreasing/inhibiting airway wall inflammation and airway wall remodelling [43, 44].

#### **8.4 “Synthetic phenotype”**

The role of the “synthetic” ASM cell in airway function remains unclear. Numerous studies provide evidence that the ASM cell could be an important source of pro-inflammatory and bronchoprotective mediators, as well as extracellular matrix components (also *Chapters 2, 3, 4, 5, 6* and reviewed in *Chapter 7*). But are ASM cell secretory capabilities important in asthma? It is an accepted fact that the ASM mass is increased in asthmatic airways, but it has yet to be shown what proportion of the ASM cells are indeed of a “synthetic” phenotype. Furthermore, the relative concentrations of cytokines, chemokines and other mediators released by ASM cells into the airway wall *in vivo* remains to be established, as do the effects of these mediators on airway function and/or the development or perpetuation of airway wall inflammation.

ASM cells may affect airway wall remodelling through the synthesis and deposition of extracellular matrix proteins such as collagen and fibronectin. We showed that ASM cells expressed and released TGF- $\beta_1$  following Ang II stimulation, which further resulted in ASM cell hypertrophy [30]. Kilfeather *et al* demonstrated that TGF- $\beta_1$  stimulates ASM cell mitogenesis [45]. These results suggest that ASM cells can modulate their own growth in an autocrine manner. In addition, Coutts and colleagues recently reported that ASM cell-derived TGF- $\beta_1$  induced the autocrine synthesis of collagen [46]. TGF- $\beta_1$  is also capable of inducing fibroblast proliferation, stimulating

fibroblasts to synthesise collagen, fibronectin, tenascin and proteoglycans, while also inhibiting collagenase and metalloproteinase production, thus decreasing degradation of the existing extracellular matrix [47-51]. It is, therefore, possible that ASM cell-derived TGF- $\beta_1$  plays a role in modifying the structure of asthmatic airways. Also, in our model, ASM cells expressed mRNA encoding fibronectin, an important component of the extracellular matrix, when stimulated with pro-inflammatory mediators. An increase in extracellular matrix proteins surrounding ASM cells could also contribute to the increased thickness of the ASM layer in asthmatic airways.



**Figure 8.2** A schematic representation of the possible effects of ASM cell-derived TGF- $\beta_1$ , IL-6 and fibronectin in the airway.

ASM cells may modulate airway wall inflammation through the synthesis and secretion of IL-6. We have demonstrated that various stimuli induced human ASM cells to express and secrete this pleiotropic cytokine which has a number of pro-inflammatory properties that may be relevant to the perpetuation of inflammation in asthmatic airways. These include mucus hypersecretion, terminal differentiation of B cells into antibody producing cells, upregulation of IL-4-dependent immunoglobulin E production, stimulation of cytotoxic T cell differentiation and maturation of mast cells [52-56].

## **8.5 Interactions with other cells in the airway**

Potential interactions between ASM cells and resident cells of the airway, such as epithelial cells, fibroblasts and goblet cells as well as interactions between ASM cells and infiltrating inflammatory cells in the airway, such as macrophages, T-cells, B-cells, eosinophils and neutrophils are discussed in *Chapter 7*. Studies describing the release of cytokines, chemokines, growth factors and arachidonic acid metabolites by ASM cells *in vitro* suggest that these cells may indeed play a role in the perpetuation of airway inflammation.

## **8.6 Limitations of the model**

The advantage of cell culture is that a defined cell population can be treated with predetermined stimuli in order to investigate parameters of interest whereby experimental conditions have been standardised. In our studies ASM was dissected from fresh macroscopically normal lobar or main human bronchus obtained immediately following surgery of patients with lung carcinoma. Cells were isolated from the tissue using enzyme digestion and cultured for up to the 5<sup>th</sup> or 6<sup>th</sup> passage to

obtain sufficient numbers of cells for the studies. Unfortunately the need to culture the cells to obtain sufficient numbers for experimentation is also the major disadvantage of this approach. Healthy mature ASM cells exist *in vivo* predominantly in a quiescent and fully differentiated “contractile phenotype”. The process of culturing necessitates a transition from a “contractile phenotype” to a “proliferative phenotype”. Although serum deprivation (prior to all experiments) restores the content of contractile proteins to some extent, the cells have undergone at least 5 divisions and differ substantially from the ASM cells in intact airway tissue [57]. Therefore, caution must be exercised when extrapolating from *in vitro* studies to *in vivo* observations. Halayko and colleagues recently reported that canine ASM cells exhibit marked phenotypic plasticity (contractile and synthetic phenotypes present in freshly isolated cell populations) [58], if this also holds true for human ASM cell populations in asthmatic airways, then our cell culture model probably closely mimics the *in vivo* situation.

The patients who donated tissue for our studies were generally elderly and their medical history was unknown to us. Caution is therefore required when comparing our data with data from other investigators who obtained ASM cells from other sources, for example young, road traffic accident victims (e.g. results shown in *Chapter 5* compared to results obtained by Amrani *et al.*[24]). Differences may also exist in experimental results due to differences in the original location of the ASM tissue, e.g. bronchial tissue versus tracheal tissue. Heterogeneity in receptor repertoire has been reported, e.g. receptors for vasoactive intestinal peptide (VIP) are found in high density in the smooth muscle of proximal airways and very few are found in distal airways, whereas tachykinin receptors are more prominent in the distal airways. Also, heterogeneity in innervation

and ASM cell responsiveness between proximal and distal airways could be important for differing ASM cell responses when in culture [11, 59-61].

Furthermore, ASM cells *in vivo* are influenced by multiple mediators in interactions with the surrounding cells and extracellular matrix during the inflammatory process. Our studies were designed, however, to investigate the effects of individual mediators on ASM cells, and interactions with other cell types or with matrix proteins were not explored. Recently, more complex models have been developed to try to take such interactions into account [62, 63].

## 8.7 Conclusions

Evaluating the data presented in this thesis, we can draw the following conclusions:

- The individual pro-inflammatory cytokines (IL-1 $\beta$ , IL-5, IL-6, TNF- $\alpha$ ) and contractile agonists (ET-1, histamine) do not induce ASM cell proliferation *in vitro* at the concentrations investigated.
- Ang II induces DNA and protein biosynthesis with a net accumulation of protein over DNA resulting in human ASM cell hypertrophy.
- Pro-inflammatory cytokines (IL-1 $\beta$ , IL-5, IL-6, TNF- $\alpha$ ), contractile agonists (ET-1, histamine) and growth factors (PDGF-AB, FGF-2) induce the rapid and transient expression of immediate early response genes such as c-fos, egr-1 and c-jun.
- Pro-inflammatory cytokines (IL-1 $\beta$ , IL-5, TNF- $\alpha$ ), contractile agonists (ET-1, histamine) and growth factors (PDGF-AB) can induce the synthesis and secretion of IL-6 and (Ang II) TGF- $\beta_1$  as well as the expression of mRNA (IL-1 $\beta$ , PDGF-AB, FGF-2) encoding fibronectin.

- The glucocorticosteroid dexamethasone inhibits IL-6 mRNA expression and subsequent protein secretion.
- The protein synthesis inhibitor cycloheximide partially inhibits IL-6 protein secretion.
- TNF- $\alpha$ -induced IL-6 gene activation appears to be regulated through the AP-1 transcription factor, whereas the NF-IL6 (C/EBP $\beta$ ) transcription factor seems to play a role in PDGF-AB-induced IL-6 gene expression.

The data described in this thesis suggest a potentially important role for ASM cells in the development and/or perpetuation of airway wall inflammation and remodelling in asthmatic airways, identifying these cells as potential targets for future asthma treatments.

## **8.8 Implications for future research**

The studies described in this thesis contribute to a better understanding of ASM responses to a limited number of cytokines and growth factors implicated in asthma. However, the exact relationship between ASM cell proliferation and secretion in airway wall inflammation and remodelling in asthma remains incompletely understood. This relationship may be studied more adequately in a number of ways:

### *In vitro*

Stimulation: Definition of the composition of the pro-inflammatory environment of ASM cells *in vivo* would enable us to manufacture a “pro-inflammatory cell culture medium”. This, in turn, would enable us to mimic the events during asthmatic episodes in the airway wall more closely.

Co-cultures: It would be of interest to develop more complex *in vitro* models to investigate the interactions between ASM cells and other resident cells of the airways, and interactions with infiltrating cells of the airways. This would enable us to determine the importance of the ASM cell “synthetic phenotype”.

“Asthmatic” ASM cells: ASM cell responses in cells originating from asthma patients (symptomatic versus non-symptomatic) should be compared to responses measured in ASM cell cultures derived from non-asthmatics to ascertain similarities/differences between the models. It may then be possible to induce an “asthmatic” ASM cell phenotype in culture, e.g. through passive sensitisation of non-asthmatic ASM cells in combination with pro-inflammatory stimuli.

#### *Ex vivo*

Does exposure to “asthmatic” milieu alter ASM phenotype in a similar way *ex vivo* as *in vitro*. Development of a ventilated/perfusion airway culture model whereby an “asthmatic” milieu is presented extraluminally to small airways would allow us to measure the intraluminal and extraluminal secretion of cytokines, arachidonic acid metabolites, chemokines, growth factors and mucus production (lavage) separately. We could also follow changes in airway narrowing by monitoring changes in luminal pressure. Candidate end points might be ASM cell, goblet cell and epithelial cell proliferation, ASM cell phenotypic markers, ASM responses to contractile agonists or  $\beta_2$ -adrenoceptor agonists. Such a model would enable us to study the effects of defined stimuli (e.g. inflammatory mediators, cytokines, growth factors) on the development of airway wall remodelling and airway responsiveness. Furthermore, interactions between ASM cells, fibroblasts, epithelial cells and sub-epithelial matrix proteins could be

studied in this model, including possible inhibitory effects of endogenously produced factors such as nitric oxide and PGE<sub>2</sub>.

*In vivo*

Chronic asthma-animal models allow us to study the development of airway wall inflammation and remodelling and also allow us to determine the time of onset of these processes. A number of models already exist, each with their own strengths and weaknesses. In the Brown Norway rat model of allergic bronchoconstriction it has been shown that as few as three allergen challenges can induce measurable increases in the mass of ASM in the airways [64-66]. In the guinea pig model, however, an increase in ASM mass following antigen exposure was not evident [67]. As yet, there is no evidence that a longer duration of antigen challenge coincides with more severe airway wall remodelling. The relative contribution of increased ASM mass or alterations in ASM phenotype in the development of airway hyper-responsiveness should be resolved.



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## Curriculum Vitae

The author of this thesis was born in London, Great Britain, on the 8<sup>th</sup> of March 1967. In 1988, after passing her 'A' levels, she moved to Rotterdam. She spent two years studying the Dutch language – 'in the field', and in 1990 embarked on her first year as a biology student at the University of Leiden. In 1991 she transferred to the Biopharmaceutical Sciences undergraduate course and gained her MSc in 1995 after spending half of her final year working for SmithKline Beecham in Welwyn Garden City (UK). She joined the department of Pharmacology of the Erasmus University Rotterdam in 1996 where she worked on a Dutch Asthma Foundation project entitled "Molecular mechanisms of human airway smooth muscle growth in relation to mediators of asthma", the results of her endeavors are published in this thesis. In 1999 she divided her attention between Rotterdam and the department of Molecular Pharmacology of the University of Groningen where she worked part-time as a junior lecturer/researcher. Since September 2001 she has a post doc position at the department of Pharmacology and Pathophysiology at the University of Utrecht.





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5-HT	5-hydroxy tryptamine
5-LO	5-lipoxygenase
ADU	arbitrary density unit
Ang II	angiotensin II
AP-1	activator protein-1
ASM	airway smooth muscle
AT <sub>1</sub>	angiotensin II type-1 receptor
B <sub>2</sub>	bradykinin receptor
baso	basophil
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	copy DNA
CHX	cycloheximide
CRE	cAMP-responsive element
Cys-LT	cysteinyl leukotriene
dex	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EGF	epidermal growth factor
egr-1	early growth response gene-1
ELISA	enzyme linked immunosorbent assay
endo	endothelial cell
eo	eosinophil
EP <sub>2/4</sub>	prostanoid receptor
epi	epithelial cell
EPO	eosinophil peroxidase
ET	endothelin
FBS	Foetal bovine serum
FcεRII	High affinity receptor for immunoglobulin E
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte/macrophage-colony stimulating factor
H <sub>1</sub>	histamine receptor

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HBSS	Hank's buffered salt solution
ICAM	intercellular adhesion molecule
IFN- $\gamma$	interferon gamma
Ig	immunoglobulin
IGF	insulin like growth factor
IL	interleukin
LIF	leukaemia inhibitory factor
LPA	lysophosphatidic acid
LT	leukotriene
m $\theta$	macrophage
M <sub>1/2</sub>	muscarinic receptor
MAP	mitogen activated protein
MBP	major basic protein
mc	mast cell
MCP	monocyte chemotactic protein
MHC II	major histocompatibility complex II
MIP	macrophage inflammatory protein
MMP-9	metalloprotease-9
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NDGA	nordihydroguaiaretic acid
neutro	neutrophil
NF-IL6	nuclear factor-IL6
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NK	natural killer
NK <sub>2</sub>	neurokinin receptor
NP-40	nonidet-40
OD	optical density
PAF	platelet activating factor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PG	prostaglandin
PMSF	phenylmethylsulfonyl fluoride
PTK	protein tyrosine kinase
RANTES	regulated on activation, normal T cells expressed and secreted

RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SEM	standard error of the mean
SRE	serum responsive element
SubsP	substance P
TGF- $\beta$	transforming growth factor- $\beta$
Th1	T helper cell type 1
Th2	T helper cell type 2
TIMP-1	tissue inhibitor metalloprotease
TNF- $\alpha$	tumour necrosis factor- $\alpha$
TP	prostanoid receptor
TX	thromboxane
VCAM	vascular adhesion molecule
VEGF	vascular endothelial growth factor