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Primary airway fibroblasts in the understanding of asthma and its severity

by

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ABSTRACT

SCHOOL OF MEDICINE

DIVISION OF INFECTION INFLAMMATION AND REPAIR

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PRIMARY AIRWAY FIBROBLASTS IN THE UNDERSTANDING OF ASTHMA AND ITS SEVERITY

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Introduction. Asthma is characterised by both chronic inflammation and a remodelling of the airways. Airway remodelling in asthma includes increase myofibroblast numbers and increased extra cellular matrix (ECM) deposition.

Hypothesis. It is hypothesised that fibroblasts in the severe asthmatic airway are contributing to asthma severity by possessing an increased ability to proliferate, synthesise ECM proteins and propagate the inflammatory process, and that the airway environment in asthma may contributes to this behaviour.

Aims. The aims of this thesis were to use broncho-alveolar lavage (BAL) and primary airway fibroblasts to construct a simple *in vitro* model of the *in vivo* environment of the airways, and by using this model we are able to compare and quantify the mitogenic ability of asthmatic fibroblasts to that of healthy fibroblasts, as well as comparing their ability to induce mRNA synthesis, which would represent an ability to synthesise ECM and pro-inflammatory proteins, in response to the local environment.

Methods. Fibroblasts from 6 healthy, 6 mild asthmatic and 6 severe asthmatic patients were grown from biopsies and challenged with BAL from 6 healthy, 6 mild asthmatic or 6 moderate/severe asthmatic volunteers. The [^{3}H] thymidine incorporation assay and TaqMan real time RT-PCR were used to assess their mitogenic potential and ability to synthesise collagen III mRNA. Interleukin-8 protein levels were also measured in the supernatents of these BAL challenged fibroblasts using ELISA. The phosphorylation status of a variety of MAPKs within healthy, mild asthmatic and severe asthmatic fibroblasts was determined after challenge with moderate/severe asthmatic BAL using the R&D systems MAPK-phosphorylation assay kit.

Results. BAL stimulated [^{3}H] thymidine incorporation in fibroblasts grown from biopsies from healthy and mild asthmatics but not in those from severe asthmatics ($p<0.0001$), indicative of an altered fibroblast mitogenic potential in severe asthma. BAL from those with moderate/severe asthma , however, induced significantly more collagen III mRNA expression by the fibroblasts cultured from severe asthmatics than in fibroblasts cultured from the airways of either healthy or mild asthmatics subjects ($p<0.05$) at 1 hour. IL-8 protein generated by severe asthmatic fibroblasts was significantly decreased compared to that from healthy and mild asthmatic fibroblasts after a 1 and 4 hour challenge with healthy and moderate/severe asthmatic BAL ($p<0.05$). There was also Akt1 and Akt2 phosphorylation in mild and severe fibroblasts after a 30 minute challenge with moderate/severe asthmatic BAL, which was not present in healthy fibroblasts.

Conclusion. Fibroblasts from severe asthma thus have an altered phenotype favouring a synthetic rather than proliferative phenotype. Signalling pathways influencing Akt phosphorylation may be implicated in this process. These findings have relevance to structural airway changes in asthma and processes underlying disease severity.

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Abbreviations

α-SMA	Alpha- Smooth Muscle Actin
ACE	Angiotensin Converting Enzyme
ADAM	A Disintegrin and Metalloprotease
AMV	Avian Myeloblastosis Virus
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
aFGF	Acidic Fibroblast Growth Factor
AIDS	Acquired Immunodeficiency Syndrome
AP	Activator Protein
ASM	Airway Smooth Muscle
AT	Angiotensin Receptor
ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
BDP	Beclomethasone Dipropionate
bFGF	Basic Fibroblast Growth Factor
BHR	Bronchial Hyperresponsiveness
BMP	Bone Morphogenetic Protein
BPD	Bronchopulmonary Dysplasia
BSA	Bovine Serum Albumin
CaM	Calmodulin
CD	Cluster of Differentiation
cdk	Cyclin-dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
CLD	Chronic Lung Disease of the Infant
CNE	Nasopharyngeal Carcinoma Cell
COX	Cyclooxygenase
CPM	Counts Per Minute
CTGF	Connective Tissue Growth Factor
Da	Dalton
DABCO	1,4-diazabicyclo[2.2.2]octane

DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EBUS	Endobronchial Ultrasound
ECE	Endothelin Converting Enzyme
ECM	Extra Cellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil Derived Neurotoxin
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
EMTU	Epithelial Mesenchymal Trophic Unit
ERK	Externally Regulated Kinase
ET	Endothelin
FAST-1	Fork Head Activin Signal Transducer-1
FBM	Fibroblast Medium
FCS	Fetal Calf Serum
FEV_1	Forced Expiratory Volume in One Second
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycans
GDF	Growth and Differentiation Factors
GDP	Guanosine'5 Diphosphate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
G-protein	Guanine Protein
GRB	Growth-factor Receptor Bound Protein
GSK	Glycogen Synthase Kinase
GST	Glutathionine S-transferase
GTP	Guanosine'5 Triphosphate
HGF	Hepatocyte Growth Factor

HIV	Human Immunodeficiency Virus
HRP	Horse-radish Peroxidase
HRCT	High Resolution Computed Tomography
HSP	Heat Shock Protein
HSPG	Heparan Sulphate Proteoglycan
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IgE	Immunoglobulin E
IGF	Insulin-like Growth Factor
IGFBP	IGF Binding Protein
IL	Interleukin
INT	Iodotetrazolium Chloride
IP_3	Inositol Triphosphate
ITS	Insulin Transferrin Selenium
JNK	c-Jun NH_2-terminal Kinase
JBP	JNK-binding Domain
LDH	Lactate Dehydrogenase
LFA	Leukocyte Function-associated Antigen
LO	Lipoxygenase
LPA	Lysophosphatidic Acid
LT	Leukotriene
Mad	Mothers Against Dpp
MAPK	Mitogen Activated Protein Kinase
MB	Membranous Bronchioles
MBP	Major Basic Protein
MCP	Monocyte Chemo-attractant Protein
MEK	MAP-ERK Kinase
MHC	Major Histocompatibility Complex
MLCK	Myosin Light Chain Kinase
MMLV	Moloney Murine Leukemia Virus

MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAD^+	Nicotinamide Adenine Dinucleotide
NF-κβ	Nuclear Factor Kappa-beta
NGF	Nerve Growth Factor
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NTP	Nucleoside Triphosphate
PAI	Plasminogen Activator Inhibitor
PAF	Platelet Activating Factor
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PC20	Dose of Histamine or Methacholine Required to Cause a 20% Decrease in FEV1
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PDGF	Platelet Derived Growth Factor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3-K	Phosphatidylinsitol-3 Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PTK	Protein Tyrosine Kinase Receptor
RANTES	Regulated Upon Activation, Normal T Cell Expressed and Secreted
RDS	Respiratory Distress Syndrome
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
RSK	Ribosomal s6 Kinase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SH2	Src Homology
SNP	Single Nucleotide Polymorphism
ssDNA	Single Strand Deoxyribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
sTNF	Soluble-TNF
TACE	TNF-α Converting Enzyme
TCA	Trichloroacetic Acid
TGF	Transforming Growth Factor
Th	T Helper Cell
TIMP	Tissue Specific Inhibitors of Matrix Metalloproteinase
T-LAK	Human Lymphokine-activated T Killer Cells
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour Necrosis Factor
UBC	Ubiquitin C
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen
WHO	World Health Organisation
WMS	Wilson-Mikity Syndrome

Chapter 1:

Introduction

What is asthma?

Asthma was first described in ancient Greece and originally referred to the state of breathlessness that accompanies an attack, rather than the characterisation of the disease state. The first clinical definition of asthma came from Salter in 1860 where he describes asthma as "paroxysmal dyspnea, generally periodic, with healthy respiration between attacks" (Salter H.H. 1860). This definition was redefined in 1959 by the Ciba foundation as a "widespread narrowing of the bronchial airways which changes in severity over short periods of time, either spontaneously or under treatment" (Ciba foundation guest symposium, 1959). However asthma is now understood to be a complex and multifactorial syndrome comprising multiple variants whose underlying cause is unclear (Haitchi H.M. *et al.* 2003), but whose key pathophysiological characteristics are reversible airway obstruction, bronchial hyperresponsiveness (BHR), airway inflammation and airway remodelling (National Institute of Health and National Heart, 2002). World-wide, the economic costs associated with asthma are estimated to exceed those of TB and HIV/AIDS combined (World Health Organisation 2000).

The strongest risk factors for developing asthma are exposure, especially in infancy, to indoor allergens (such as domestic dust mites, cats and cockroaches) and a family history of asthma or allergy. A study in the South Atlantic Island of Tristan da Cunha, where one in three of the 300 inhabitants have asthma, found children with asthmatic parents were much more likely to develop the condition (World Health Organisation 2000)

The majority of asthma originating in childhood occurs in association with atopy, the predisposition to generate (immunoglobulin E) IgE to common environmental allergens through a (T helper cell-2) Th2 cell dependent mechanism (Haitchi H.M. *et al.* 2003). A variety of stimuli are recognized to initiate asthma attacks, these include environmental allergens, exercise, aspirin and chemical exposure and are known as asthma triggers. When inflamed airways respond to an asthma trigger through bronchoconstriction, the characteristic symptoms of asthma appear namely wheezing, cough, and chest tightness or dyspnea. Chest tightness or dyspnea is the sensation associated with the increased work needed to breathe when the airways are constricted. Wheezing is a reflection of airflow limitation and the asthmatic cough probably results from stimulation of sensory

nerves in the airways by inflammatory mediators, released by a range of inflammatory cells involved in asthma.

Because asthma is a chronic condition, it usually requires continuous medical care. Patients with moderate to severe asthma are prescribed long-term medication daily to control the underlying inflammation and prevent symptoms and attacks. If symptoms occur, short-term medications (inhaled short acting β2-agonists) are used to relieve them (World Health Organisation 2000); however there are no specific treatments to restrict the remodelling of the airway wall. While it seems certain that prolonged exposure to chronic inflammation can initiate the remodelling process, it may also be plausible that the remodelling process is an abnormal response independent of inflammation, and thus should be treated as a separate part of the disease.

The advent of fibre-optic bronchoscopy and it's ability to be performed safely on patients, has provided an opportunity to obtain bronchoalveolar lavage (BAL) fluid and endobronchial biopsies from asthmatic volunteers, these biopsies can be grown and individual cells (e.g. smooth muscle, epithelial cells, fibroblasts) specifically extracted in order to analyse differences between the healthy and disease state (Djukanovic R. *et al.* 1991). Before bronchoscopy the only way to investigate asthmas effect on the lung was via *post mortem* evaluation of fatal severe asthmatics.

The study of BAL has allowed a comprehensive study of inflammatory cells within the lung, and thus given insight into their state of activation and their role in mediator production, enabling a detailed characterisation of the inflammatory response, while assessment of endobronchial biopsies has allowed protein and messenger (ribonucleic acid) RNA production to be determined before and after stimulation with various stimuli, giving an insight into the role of individual cells in the progression of the disease in both the inflammatory and remodelling process within the asthmatic lung.

Incidence and Prevalence

Respiratory disease is the most commonly reported long-term illness in children, accounting for over 40% of all long-term illnesses. Over 80% of long-term respiratory illnesses (and nearly a third of all long-term illnesses) in childhood are due to doctor-diagnosed asthma (Joint Health Surveys Unit. 1999). During childhood the male to

female ratio for asthma is approximately 2:1, however after puberty the disease is more common in the female population, suggesting a role for hormonal factors (Weiss S.T. 1998). In the Western Pacific the incidence of asthma varies from over 50% among children in the Caroline Islands to virtually zero in Papua New Guinea (World Health Organisation 2000).

It is estimated that the incidence of asthma has increased by around 50% over the last 30 years (Bauman A. *et al.* 1992). In recent studies undertaken in children between 13-14 years old it was found that asthma prevalence was highest in the UK, New Zeland Australia and Ireland, with incidence being around 30%. (The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee, 2001). In a follow up study performed by the same group in 2006 it was found that asthma prevalence had slightly decreased in the UK and New Zealand, however the overall global trend showed an increase in the prevalence of asthma (Asher M. I. *et al.* 2006). The lowest rates are found in Indonesia, Albania, Romania and Georgia. Death rates are decreasing in countries where anti-inflammatory medications are available and used effectively, while death rates are increasing in areas lacking proper medical care and access to treatments (Heart Lung Blood Institute 1998). The cause of this increase is unknown however urbanization appears to be correlated with an increase in asthma. The nature of the risk is unclear because studies have not taken into account indoor allergens although these have been identified as significant risk factors (World Health Organisation 2000).

In the UK around 5.1 million people are currently being treated for asthma at a cost of around £854 million per year. Serious or life threatening asthma accounts for around 74,000 hospital emission each year, with 1,500 deaths. Of these deaths around one third will be under the age of 65.

Inflammation and atopy in asthma

Airway inflammation in asthma is due to an immune-mediated process in which inflammatory cells and inflammatory mediators enter airway tissues to cause disease; the bronchi are infiltrated with eosinophils, mast cells, plasma cells, lymphocytes and macrophages, with eosinophilic infiltration being the most striking feature (Sheffer A.L. *et al.* 1995). The bronchial wall may become thickened with tissue oedema, due to

microvascular leakage, which cause short term airway constriction and reduced lung capacity additional to that attributable to smooth muscle constriction. One possible explanation for this migration of inflammatory cells is the release of chemoattractant mediators by signalling cells such as Monocyte Chemoattractant Protein 1 (MCP-1), (interleukin-8) IL-8, eotaxin, Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) and epithelial derived neutrophil attractant-78 (ENA-78). Alternatively, signalling cells may release cytokines which up regulate endothelially expressed adhesion molecules (e.g. intracellular adhesion molecule (ICAM)) that promote the recruitment and migration of cells from the circulation into the focus of tissue inflammation. Mast cells secrete a wide array of pre-formed and newly formed mediators, such as histamine, prostaglandin-D2 (PGD_2), leukotreine-C4 (LTC4), chymase and tryptase (Kumar A. *et al.* 1995), and interleukins such as IL-4, IL-5 (Bradding P. *et al.* 1994) and IL-13 (Burd P.R. *et al.* 1995). IL-4 and IL-13 have the potential to induce the immune switching of B lymphocytes to immunoglobin production, and up regulate the expression of the adhesion molecule vascular cell adhesion molecule-1 (VCAM1) on the endothelial surface and may also inhibit the production of matrix proteins (Adachi Y. *et al.* 1997), while production of IL-5 has been correlated with severity of disease and of airway hyperresponsiveness (Humbert M. *et al.* 1997), although intervention with IL-5 monoclonal antibodies has not led to the clinical benefit expected (Leckie M.J. *et al.* 2003). Airway tissue mast cells have been shown to store preformed cytokines, and there is evidence of upregulated endothelial expression of adhesion molecules in on going asthma and rhinitis (Montefort S. *et al.* 1993). Eosinophils may adhere to these endothelial cells by binding to VCAM1 and ICAM-1 through specific integrin ligands (Very Late Antigen-4 (VLA 4) and Leukocyte Function-Associated Antigen 1 (LFA-1), respectively), which enables their migration to the bronchial mucosa (Bochner B.S *et al.* 1994), an increased expression of ICAM-1 by bronchial epithelial cells has also been noted in allergic asthma (Vignola A.M. *et al.* 1993).

Atopy is a genetic inclination to develop an IgE antibody response to common allergens. About 90% of persons under the age of 30 who have asthma are atopic; therefore atopy may be the strongest risk factor for the development of asthma (Peat J.K. *et al.* 1999).

Concurrently 30% of asthmatics are non-atopic; the majority of these cases are in adult onset asthma and include aspirin sensitive asthma.

When an atopic individual encounters a specific allergen to which they are sensitised the allergen is internalised by antigen presenting cells such as dendritic cells and macrophages. The allergen is processed and short peptide fragments are produced which are combined with class II major histocompatibility complex (MHC) molecules, this is then presented to T helper cells resulting in T cell polarisation and the release of cytokines. T helper cells have been split into two categories Th1 and Th2, in asthma there is a predisposition toward the Th2 class which leads to the release of granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. The up regulation of one set of T helper cells maybe associated with down regulation of the other, although it is now generally recognised that upregulation is a feature of dysregulated control and there may not be reciprocal down-regulation. The selective expansion of the Th2 cells plays a significant role in inducing the IgE synthesis and the eosinophilia associated with allergic asthma. A possible role for Th1 lymphocytes in BHR has been suggested using murine models; however their role in asthma requires further investigation (Cui J. *et al.* 2005).

Without the narrowing of the airways due to inflammation asthma may be a tolerable disease.

Bronchial Hyperresponsiveness (BHR)

The prevalence of BHR in the population varies from 6 to 35% (Burney P.G. *et al.* 1987) and is strongly associated with the presence of respiratory symptoms. Even though BHR is generally accompanied by respiratory symptoms, population studies have shown that it may also occur in subjects without any respiratory symptoms, so-called asymptomatic hyperresponsiveness (Woolcock A.J. *et al.* 1987).

BHR (in the form of exaggerated bronchoconstriction) in reaction to non-specific stimuli is a characteristic feature of the asthmatic patient (Beckett P.A. *et al.* 2003). However BHR cannot be used to diagnose between asthmatic and non-asthmatic individuals as there are individuals with asymptomatic BHR who demonstrate no symptoms of asthma. In symptomatic asthma BHR is thought to be a direct consequence of airway

inflammation, although the sole dependence upon inflammation is questionable. This inflammatory process may directly or indirectly increase smooth muscle contractility, leading to the propensity for rapid changes in airway calibre and hence the symptoms of cough, wheeze, and dyspnoea (Jansen D.F. *et al.* 1997). BHR leads to bronchoconstriction in response to irritant stimuli such as chemicals or fumes, and can cause bronchoconstriction without necessarily causing inflammation, however exposure to an allergen may cause a more prolonged period of bronchoconstriction, accompanied by an immediate inflammatory response which lasts around 1-2 hours followed by a later response at 4-8 hours, characterized by inflammatory cell recruitment to the airways (Cockcroft *et al.* 1977). This late inflammatory response is associated with an increase in BHR. The degree of BHR has been shown to directly correlate with the levels of eosinophils in sputum, numbers of activated Th2 memory cells, mast cells, eosinophils in BAL and activated eosinophils, mast cells, Cluster of Differentiation-4 ($CD4^+$) T-memory cells and $CD8^+$ T cells in bronchial biopsies (Bradley B.L. *et al.* 1991). These cells can produce a range of cytokines, growth factors and inflammatory mediators such as histamine and leukotrienes which activate other cells enhancing the inflammatory process.

BHR can be quantified in the laboratory by the administration of stimulus such as methacholine and histamine (direct stimuli) or allergen, adenosine, cold dry air or exercise (indirect stimuli that cause airway narrowing through the release or mediators from airway cells) (Holgate S.T. 2004). Histamine and methacholine are non-specific stimuli and they are the most commonly used in clinical tests due the ease at which the necessary concentration (PC_{20}) or dose (PD_{20}) can be determined to cause a 20% fall in forced expiratory volume in 1 second (FEV_1) from baseline. When a healthy individual is challenged with methacholine or histamine there is a small amount of bronchoconstriction but the FEV_1 plateaus before the 20% fall is reached, however in the asthmatic airway the concentration dose response curve is steeper and displaced to the left (Holgate S.T. 2004) indicating a lower concentration required to cause a 20% fall. BHR is said to be defined as a PC_{20} for histamine of less than 16mg/ml. The lower the PC_{20} value the greater the level of BHR and the more severe the asthma (Holgate S.T. 2004).

BHR in people without asthma can be fully reversed via deep inspiration, whereas in the asthmatic lung BHR may persist, this may be due to an increase in resting airway smooth muscle (ASM) tone (Wheatley J.R. *et al.* 1989), thus BHR may be more of a problem of limited ASM relaxation than of exaggerated ASM contraction (Skloot G. *et al.* 1995), in addition ASM hypertrophy and hyperplasia may also contribute to BHR. As well as ASM abnormalities the thickening of the airway wall due to remodelling that is observed in asthma may also contribute to BHR. Around 50% of children with asthma do not demonstrate BHR (Pattemore P.K. *et al.* 1990) as the treatment of asthmatic patients with anti-inflammatory medication such as inhaled corticosteroids act to control the underlying basis of BHR, namely the infiltration of inflammatory cells into the airways, thus reducing the inflammatory response upon contact with an allergen.

Genetics of asthma

Asthma and allergy are complex genetic disorders that do not conform to simple Mendelian pattern of inheritance (Sandford A.J. *et al.* 2000), despite this it is still recognised that asthma and allergy run in families (Salter H.H. 1860), however twin and extensive pedigree studies show that BHR is inherited independently of atopy (Los H. *et al.* 2003). Estimates for heritability of asthma suggest that 40-60% of asthma risk is attributed to genetic factors (Manian P. 1997); however development of asthma in a genetically susceptible individual still seems to be linked to environmental factors. Two approaches have been used to investigate the role of genes in asthma prevalence, the first concentrates on genome wide screening followed by positional cloning, while the other concentrates on candidate gene association studies. Both techniques correlate variations in the (deoxyribonucleic acid) DNA sequences of genes with the presence of disease (Carroll W. 2005).

Genetic mapping involves comparing the inheritance pattern of a chromosomal region with a disease or given phenotypic characteristic. This process allows the isolation of a gene solely on the basis of its chromosomal location, without regard to its biochemical function (Carroll W. 2005). In 1983 the term positional cloning was first coined when the bithorax complex was isolated in *Drosophilia* (Spierer P. *et al.* 1983).

Positional cloning and simple Mendelian inheritance allowed the identification of genetic abnormalities in around 40 diseases by the 1990's, however it's use in asthma has been limited by a number of considerations, such as: incomplete penetrance or phenocopy, genetic heterogeneity, polygenic mode of inheritance, high frequency of disease causing alleles and non-allelic transmission mechanisms (Lander E.S. *et al.* 1994). These mechanisms combine to reduce the potential signal from an individual locus and increase the noise from surrounding areas of the genome (Carroll W. 2005). Some areas of the human chromosome have, however, been discovered using genetic mapping that may be important in the genetic susceptibility to asthma. Genes identified to date which may contribute to the development of asthma include:

1. 6p21-24 (MHC complex) – Correlates to the MHC complex and shows a strong linkage to the asthmatic phenotype (Daniels S.E. *et al.* 1996)
2. 11q 13-21 – This region has several potential areas of interest including the Clara cell secretory protein (CC16), the β chain of the high affinity receptor for IgE and the Glutathione S-Transferase (GST)-PI region (Daniels S.E. *et al.* 1996).
3. 20p13 – This area contains a disintegrin and metalloprotease (ADAM 33) gene which will be discussed later (Van Eerdewegh P. *et al.* 2002).
4. 7p14-15 - G-protein coupled receptor for asthma susceptibility (GPRA) (Laitinen T. *et al.* 2001).

ADAM33 is a membrane anchored zinc dependent metalloprotease identified by positional cloning and plays a role in the shedding of cell surface proteins such as cytokines and cytokine receptors (Mullberg J. *et al.* 2000). ADAM33 is expressed in both lung fibroblasts and bronchial smooth muscle, but not in bronchial epithelium or immune cells (Van Eerdewegh P. *et al.* 2002). It is known that other ADAM family members are involved in promoting myogenic fusion and the release of proliferative growth factors (Yoshinaka T. *et al.* 2002), so it is probable that ADAM33 is involved in airway remodelling rather than immune-mediated inflammation (Vercelli D. 2003), it may also play a role in BHR. So far 58 SNPs have been identified in ADAM33; however it is not known which SNPs are responsible for the genetic association with asthma. A number of

SNPs have been linked to the accelerated decline in baseline function over 20 years in chronic asthma (Jongepier H. *et al.* 2003) and to predict increased airway resistance in 3 and 5 year old children born to allergic parents (John S. *et al.* 2003).

Candidate gene studies utilise the effect of single nucleotide polymorphisms (SNPs) on a specific area of the genome thought to be associated with asthma development. There are three main groups of candidate genes, genes encoding for cytokines and chemokines, genes encoding receptors associated with Th2 responses and genes relating to oxidative stress (Carroll W. 2005). SNPs can be categorised into two groups relating to their effect on the asthmatic phenotype, the first group known as functional candidate SNPs can lead to a change in structure, function or expression of a protein product. The second group known as positional candidates are located in a region of the chromosome related to asthma. A functional candidate found in a region of the chromosome related to asthma is known as a 'first order candidate' (Illig T. *et al.* 2002).

Candidate gene studies have so far identified a number of potentially important SNPs that may be involved in asthma, such as:

1. Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that participates in the inflammatory reactions in patients (Winchester E.C. *et al.* 2000). An SNP in the promoter region of TNF-α has been linked with the development of childhood asthma in a variety of populations (Albuquerque R.V. *et al.* 1998 and Winchester E.C. *et al.* 2000)
2. IL-13 is a Th2 cytokine that triggers isotype switching from IgG to IgM or IgE in B cells (Punnonen J *et al.* 1997). Two SNPs associated with IL-13 have been found which relate to asthma and BHR.
3. Oxidative stress is thought to play an important role in the inflammatory process which underlies asthma (Barnes P.J 1990); the GST family of genes deals with the products of oxidative stress (Hayes J.D. *et al.* 2000). GST-PI is expressed in the lung; to date two SNPs have been found (Hu X. *et al.* 1997 and Watson M.A *et al.* 1998) which may contribute to asthma. GST-PI is also an interesting candidate as it is located on chromosome 11q13, which is a region which has strong linkage with the asthmatic phenotype (Daniels S.E. *et al.* 1996).

Other potential areas of interest include genes regulating the arginase pathway, with over expression of genes encoding molecules involved in the metabolism of arginine being found in the asthmatic lung (Kocyigit *et al.* 2004). The enzyme arginase I is involved in the production of polyamines (e.g. putrescine, spermidine) and proline which control cell proliferation and collagen production. Low intake of manganese (Mn) is associated with more than a fivefold increased risk of bronchial reactivity. It is also known that nitric oxide (NO) production in asthmatics is significantly higher than in the healthy population. There is a reciprocal pathway between arginase and nitric oxide synthase (NOS) for NO production, arginase activity also correlates with concentrations of Mn, so lower Mn concentrations could cause lower arginase activity (as Mn is required for arginase activity and stability) favouring the up regulation of NO in asthma (Kocyigit *et al.* 2004).

Also of note are genes located on chromosome 1 (A1 adosine receptor - Banerjee S.K *et al.* 2002), 2 (IL-R1N - Gohlke H. *et al.* 2004 and DPP10 - Allen M. *et al.* 2003), 12 (neuronal NOS - Gao P.S. *et al.* 2000) and 14 (PTGDR - Kabashima K. *et al.* 2003)

In total there have been around 80 genes spanning 9 chromosomes currently identified which may play a role in asthma, demonstrating the difficulties that are faced when attempting to isolate an area that can be targeted for therapeutic strategies.

Wound Repair

The general principles of wound healing can be split into 3 phases, phase one involves homeostasis and inflammation, phase two is repair and proliferation and the third phase is concerned with maturation or wound resolution. After injury there is initial bleeding and endothelial damage leading to the activation of the coagulation cascade and platelets. The coagulation cascade causes the formation of the protease thrombin which converts fibrinogen to fibrin, fibrin along with platelets act to form a temporary matrix or 'plug' which seals the wound. Platelets also adhere to damaged endothelium and release adenosine diphosphate (ADP), promoting platelet clumping and the release of several factors including transforming growth factor-beta (TGF-β), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) which activate and attract inflammatory cells and cause the proliferation and activation of fibroblasts. Vasoactive

amines such as histamine and serotonin are also released from dense bodies found in platelets. Accompanying this is vasoconstriction and an influx of neutrophils into the wound which clear any bacteria present, but also release pro-inflammatory cytokines such as IL-6 and IL-8. These cytokines in addition to others cause the migration of leukocytes from the vascular basement membrane such as eosinophils, masts cells and monocytes from the blood vessels, adhesive interactions between leukocytes and the vascular endothelium are mediated by LFA-1 and ICAM, respectively. These interactions help determine the rate of inflammation and granulation tissue formation. Once a monocyte migrates from a blood vessel it is known as a macrophage. As the macrophages migrate into the wound the neutrophils begin to depart and the macrophage becomes the predominant cell in the wound. The macrophage begins to phagocytose necrotic debris from bacteria and also releases chemo attractants which cause the migration of mesenchymal cells to the area of injury. The damaged matrix is removed by collagenases and elastases secreted by mast cells, neutrophils and eosinophils. If inflammation becomes chronic migrating monocytes can converge into multinucleated giant cells.

Once the wound has been cleared of debris the repair process can initiate. The process of repair involves differentiation, migration and proliferation of structural cells such as fibroblasts, epithelial cells and also the return of blood supply by capillaries (angiogenesis). Macrophages, mast cells and eosinophils release cytokines such as PDGF, IL-1, TNF-α and TGF-β which lead to the recruitment and activation of the structural cells. For healing to occur in a wound, fibrin from the original clot must be removed from the inflamed tissue, metalloproteinases release from e.g. inflammatory cells enhances fibrin clot dissolution. After around 3 days mesenchymal cells may transform into fibroblasts, and along with fibroblasts already present can begin to synthesise extra cellular matrix (ECM) proteins such as fibrin strands, tenascin and fibronectin on which cells can migrate. Fibrin also provides the structural support for cellular constituents of inflammation. The fibroblasts release collagen which acts to strengthen the wound, the collagen fibres are aligned according to the stress being applied to the wound. Adequate oxygen delivery is a prerequisite to the hydroxylation of lysine and proline during collagen synthesis, so it is essential that the blood supply is restored to the area. This is

achieved by angiogenesis; the capillaries follow an oxygen gradient to the centre of the wound which is an area of low oxygen tension.

Fibroblasts proliferation and capillary migration cause the production of granulation tissue which is highly resistant to infection due to the presence of leukocytes. Fibroblasts within the granulation tissue differentiate into myofibroblasts expressing α-smooth muscle actin (α-SMA) which allows contraction of the wound. Epithelial cells begin to migrate to the wound hours after the initial injury following stimulation with growth factors such as bFGF, acidic fibroblast growth factor (aFGF) and vascular endothelial growth factor (VEGF), once in place the epithelia flatten and migrate across the open wound in order to close it; epithelialization also requires oxygen and is stopped by contact inhibition. The epithelial cells release transforming growth factor-alpha (TGF-α) and TGF-β which can enhance wound closure by promoting the synthesis of ECM such as collagen by myofibroblasts. The constant cell migration requires continual synthesis and degradation of ECM, degradation occurs via the serine protease plasmin and the matrix metalloproteases, these enzymes can be synthesised by inflammatory cells such as neutrophils and macrophages, or by structural cells such as myofibroblasts and epithelial cells. After three to five days the degradation of ECM is slowed and the synthesis of new ECM is up-regulated, with specific increase in collagen I and III. The final stage of healing is the maturation phase in which the wound undergoes constant alterations known as remodelling. Collagen fibres are degraded by matrix metalloproteinases (MMPs) and resynthesised, but the amount of collagen present in the wound does not change. The myofibroblasts and newly synthesised collagen align along lines of tension within the wound and scar tissue is formed, which has around 80% the original strength of the skin it has replaced. After wound resolution myofibroblasts undergo apoptosis (Desmouliere A. 1995)

Remodelling in the asthmatic airway

Current understanding of asthma defines it as an inflammatory disorder of the airways involving mast cells, T cells and eosinophils (Bousquet J. *et al.* 2000). As previously described inflammation is a beneficial non-specific response to tissue injury which leads to wound resolution and the restoration of normal tissue function. Asthma represents a

chronic Th2 mediated inflammatory process of the airways, followed by a continual repair mechanism, whose end result is a characteristic altered structure known as remodelling of the airways, leading to a reduced baseline airway calibre and exaggerated airway narrowing (Chiappara G. *et al.* 2001). Reversible airway narrowing results in irreversible airflow obstruction in a subgroup of individuals (Brown P.J. *et al.* 1984), the subgroup of asthmatic subjects with irreversible airflow obstruction is of importance because they experience considerable morbidity and account for a high percentage of the health costs of asthma (Little S.A. *et al.* 2002). *In vitro* studies have shown that inflammatory cells have the potential to induce features of remodelling through the release of proteases, cytokines and inflammatory mediators. Although remodelling is thought to be a consequence of inflammation, there is an indication that it may be an independent process, and even the primary event in asthma development, itself contributing to the development and persistence of airway inflammation (Warner J.O. *et al.* 2000)

Airway remodelling in asthma was first observed in fatal cases (Huber H.L. *et al.* 1922), and has since been confirmed in *post mortem* examinations by Carroll N. *et al.* 1993 and Kuwano K. *et al.* 1993. The remodelling of the airways in asthma is thought to be irreversible in the short term and leads to a progressive decline in lung function culminating in a degree of fixed airflow obstruction. Mathematical models indicate that modest airway wall thickening results in disproportionally severe airway narrowing due to ASM shortening (James A.L. *et al.* 1989), especially if the wall thickening is localized in peripheral airways. Airway wall thickening and a loss of lung recoil can partially explain the persistence of BHR observed in steroid treated asthmatic patients. Remodelling is generally believed to be a disadvantageous process; however it may prove to have a protective function.

The term remodelling encompasses a number of structural changes involving all areas of the airway, including the epithelium, fibroblasts, mucous glands, blood vessels, smooth muscle, airway nerves and changes in sub-basement membrane thickness and ECM deposition and degradation.

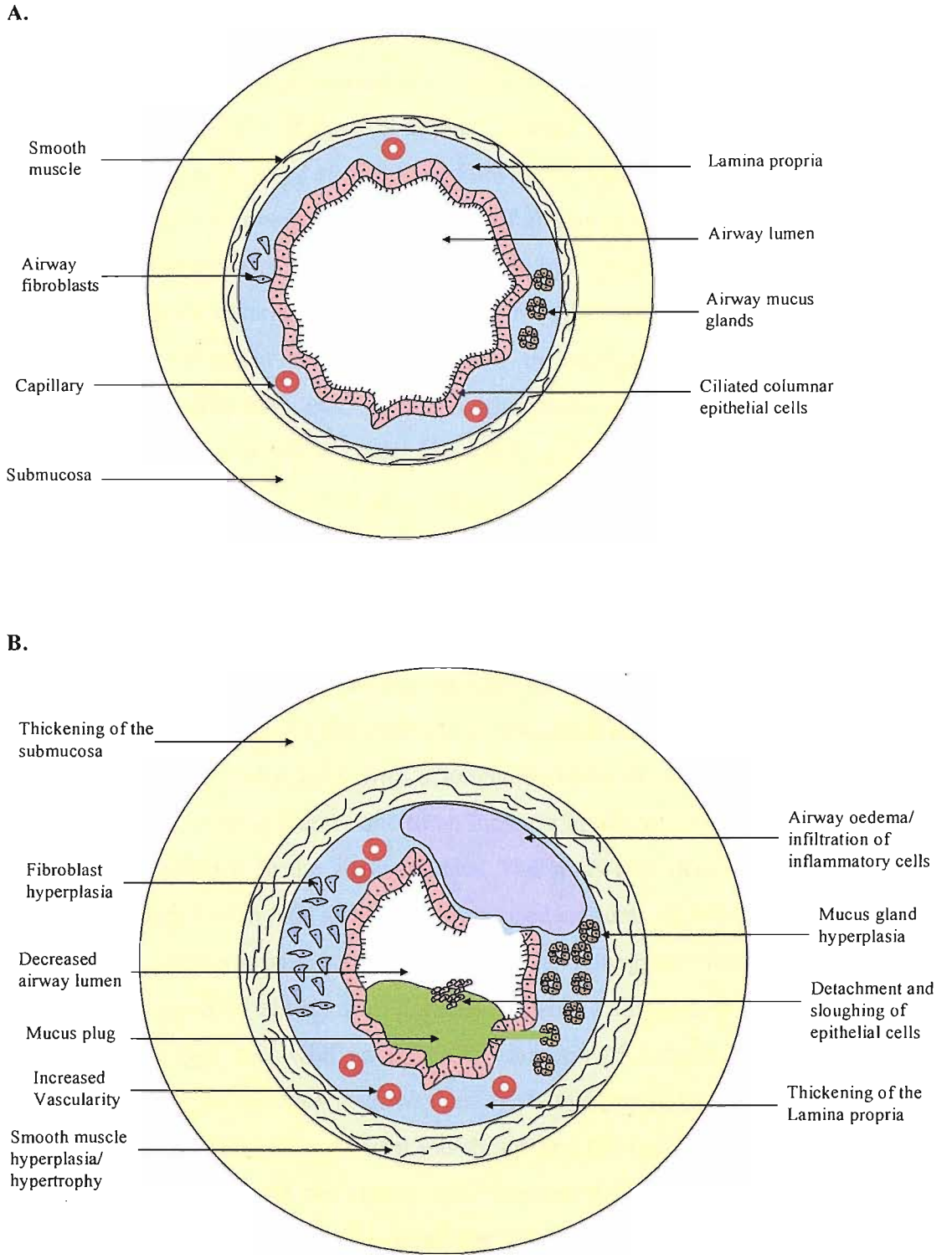


Fig 1.1 Comparison between healthy (A.) and asthmatic (B.) airways. The asthmatic airway undergoes extensive remodelling including, a thickening of the sub-mucosa and lamina propria, smooth muscle, mucus gland and fibroblast hyperplasia, infiltration of inflammatory cells, airway oedema, increased vascularity, detachment and sloughing of airway epithelia and increased mucus secretion. All of these events contribute to a decreased airway lumen and exacerbation of asthma symptoms.

Consequences of airway wall thickening

On the basis of histological analyses in individuals with fatal asthma, airway wall area has been reported to be increased from 50% to 300% compared with non-asthmatic control subjects, in individuals with nonfatal asthma increases from 10% to 100% have been shown (James A. L. *et al.* 1989 and Kuwano K. *et al.* 1993), suggesting a potential link between disease severity and the degree of airway narrowing and the severity of the accompanying symptoms such as BHR. A study by Carroll N. *et al.* 1993 compared the airway dimensions in large and small airways in both fatal and nonfatal cases of asthma. The study showed that in cartilaginous airways, the cases of fatal asthma had greater ($p < 0.05$) total wall, inner wall, outer wall, smooth muscle, mucous gland and cartilage areas than did control and nonfatal cases. The inner wall area was greater in the fatal and nonfatal cases than in the control cases ($p < 0.05$) in the small cartilaginous airways and membranous bronchioles (MB). In small MB (perimeter < 2 mm), the total and outer wall areas were greater ($p < 0.05$) in cases of fatal and nonfatal asthma than in control cases (Carroll N. *et al.* 1993).

A study by Bai T.R. *et al.* 2000 on *post mortem* young individuals who died of asthma ($n = 14$, range 17–23 yr), and older individuals with fatal asthma ($n = 13$, range 40–49 yr) indicated that older individuals with a longer duration of disease have an increase in airway wall area, predominantly due to an increase in adventitial area, as well as greater *post mortem* reduction in the airway lumen. The study also demonstrated that both old and young subjects with fatal asthma had increased amounts of ASM compared to control subjects, with greater amounts in older individuals. Young individuals with fatal asthma did not have increased airway wall dimensions when compared to age-matched normal subjects, suggesting that with an increased duration of asthma there is an on going remodelling with an increase in airway tissue, both internal to the epithelial basement membrane and between the basement membrane and the outer border of the ASM layer (Bai T.R. *et al.* 2000). If the airway wall internal to the layer of smooth muscle is thickened by remodelling then contraction of the ASM would cause an increased narrowing of the airway, which would be represented as BHR. However this increase in thickness wouldn't cause a drop in the baseline FEV_1 when the ASM is relaxed (James A. L. *et al.* 1989)

When the lungs of a healthy individual are challenged with increasing doses of a non-specific contractile stimulus, a plateau is reached at which the airways will no longer narrow. The plateau is interpreted to mean that the airway smooth muscle is activated maximally and has shortened as much as it can against the elastic load of the airway. Once this plateau has been reached any further addition of stimulus will cause no additional muscle shortening. In an asthmatic patient the airways may respond to levels of stimulant at which the healthy individual would be unresponsive, this phenomenon is known as hypersensitivity. In the asthmatic airway the plateau effect may be abolished altogether, this is known as hyperreactivity. It is the ability of the airways to narrow excessively, with an elevated or abolished plateau which accounts for the morbidity and mortality associated with asthma (Sterk P.J. *et al.* 1989). It is now widely recognised that ASM is the major source of acute airway narrowing in asthma (Lambert R.K. *et al.* 1997), and there is general consensus that shortening of the ASM is the primary cause of excessive airway narrowing during an asthmatic attack (Dulin N.O. *et al.* 2003), with airway swelling, caused by oedema and inflammatory cell infiltration, and the plugging of the airways with mucous amplifying the severity of the episode (Lambert R.K. *et al.* 1997). The proposed increase in ASM shortening observed in asthma has a variety of possible explanations such as a decrease of the static load against which the muscle shortens, through an uncoupling of elastic forces of the lung parenchyma from the ASM (Kuwano K. *et al.* 1993), there may be an increased ASM shortening velocity, this may be important as a large proportion of smooth muscle contraction occurs within the first few seconds, therefore the faster the muscle can shorten, the more it will shorten (Ma X. *et al.* 2002).

The Perturbed equilibria of myosin binding is another theory which suggests that the response to both periodic stretch from tidal breathing and deep inhalations may be impaired in asthma due to the remodelling process, and this impairment may be the proximal cause of the loss of plateau and the resulting morbidity of the disease (Moore B.J. *et al.* 1998). In the healthy individual both tidal breathing and deep inspirations cause bronchdilation of the airways. In the healthy individual lung inflations strain ASM with each breath; this causes the detachment of the myosin head from the actin filament which has formed during muscle contraction. This detachment occurs more rapidly than it

would do in an unstressed muscle and serves to depress the active force of the muscle, making breathing easier during tidal breaths (Fredberg J.J. 2004). The thickening of the airways in asthma reduces tidal muscle strains and permits myosin binding to reach unperturbed levels causing a stiffening of the ASM, manifesting as a rigidity of the airways in asthmatic subjects (Colebatch H.J. *et al.* 1973).

The development of high resolution computed tomography (HRCT) has provided a potential non-invasive technique for the measurement of both airway wall thickness and airway narrowing in asthmatic individuals (Little S.A. *et al.* 2002). Within the airway features of 100–200 μm size can be identified, allowing assessment of small airways in the region of 1.5–2 mm diameter (King G.G. *et al.* 1999). Consequently, HRCT scanning is increasingly being used as a research tool for studying obstructive lung disease (Lynch D.A. *et al.* 1993). Measurements of canine lung with HRCT (McNamara A.E. *et al.* 1992), and asthmatic subjects have shown a varying degree of success. Awadh N. *et al.* 1998 investigated near fatal asthma and control groups and showed good intraobserver (the differences in interpretation by an individual making observations of the same phenomenon at different times) and interobserver (the differences in interpretation by two or more individuals making observations of the same phenomenon) agreement, however when measuring airway wall thickness in chronic asthma Little S.A. *et al.* 2002 showed a discrepancy in interobserver measurements. Subsequently HRCT may prove to be too inaccurate and time consuming to provide a useful clinical role. Another potential draw back is the high dose of radiation resulting from a full HRCT scan, which is thought to be around 8mSv, the equivalent of 400 chest radiographs (European Commission, Referral Guidelines for Imaging, Report 118), which may provide an ethical problem (Shaw T.J. *et al.* 2004).

Endobronchial ultrasound (EBUS) also allows identification of airway wall structures within the asthmatic airway and delivers a much lower dose of radiation 0.6 mSv per patient (ImPACT1; Bence Jones Offices, St Georges Hospital, London, UK). Shaw T.J. *et al.* 2004 postulated that endobronchial ultrasound is a valid technique for the assessment of cartilaginous airway wall remodelling in respiratory diseases, such as asthma.

Current evidence from both *post mortem* studies and *in* vivo studies suggest that there is a definitive thickening of the airway in asthmatic individuals when compared to control groups. This thickening affects the way in which the asthmatic lung reacts not only to bronchoconstrictor stimuli but also to the environment of the outside world on a daily basis, and involves changes in the components of the whole airway wall.

Epithelial changes

The epithelial surface of the bronchi acts as a physical and functional barrier to external agents, regulates a variety of biologic reactions including protection against chemical and microbiological injuries and modulates the repair processes through the secretion of extracellular matrix proteins and the interaction with other cells involved in the same processes, namely the interstitial fibroblasts (Thompson A.B. *et al.* 1995). The epithelium adopts a stressed phenotype when exposed to potentially damaging environmental agents, characterised by the upregulated expression of proinflammatory transcription factors including, nuclear factor kappa beta (NF-κB) (Hart L.A. *et al.* 1998) , activator protein-1 (AP-1) (Demoly P. *et al.* 1992) and STAT-1 (signal transducer and activator of transcription) (Sampath D. *et al.* 1999). STAT-1 is a downstream regulator of the epidermal growth factor (EGF) receptor (EGFR), which is upregulated throughout the asthmatic airway epithelium, the degree of upregulation tends to correlates with the severity of asthma (Puddicombe S.M. *et al.* 2000). It is suggested that the epithelium in the asthmatic airway is predisposed to adopt the stress phenotype upon contact with environmental or inflammatory agents (Holgate S.T. *et al.* 2000). This hypothesis seems to be supported by the increased production of inflammatory mediators such as GM-CSF, RANTES and IL-8 from asthmatic epithelial cells in response to diesel exhaust particle exposure, than in control subjects (Devilia J.L. *et al.* 1999). The bronchial epithelium in the asthmatic airway also shows a predisposition to injury and prolonged tissue repair (Holgate S.T. *et al* 2003) which is mediated by the increased production of PDGF, enothelin-1 (ET-1) and bFGF, this altered phenotype supports the drawn out inflammatory response witnessed in asthma.

In the asthmatic airway there is an increased shedding of the columnar epithelial cells depending on disease severity, suggesting cellular fragility or weak intercellular

attachments between adjacent epithelial cells and/or to basal epithelial cells and the basement membrane (Knight D. 2001). In *post mortem* studies a stripping of pseudostratified ciliated epithelium down to the basal layer occurs over a large percentage of the asthmatic lung, the loss of bronchial epithelium and its ciliated layer prevents the clearance of mucus and allows the access of allergens and environmental factors to the bronchial smooth muscle and sensory nerve receptors (Salvi S.S. *et al.* 2001). High shear stress due to airway obstruction resulting from smooth muscle constriction and subepithelial ECM deposition at or near the epithelial layer may explain the pronounced epithelial sloughing that occurs in asthma (Wiggs B.R. *et al.* 1997). Clumps of shed epithelial cells, known as Creola bodies, are a characteristic finding in the sputum of untreated asthmatic patients. The eosinophil has been considered to play an important role in this shedding of epithelial cells in asthma via the release of MMP9, eosinophil cationic protein (ECP), major basic protein (MBP) or eosinophil peroxidase. MBP is found in large quantities in the asthmatic lung and is particularly cytotoxic to epithelial cells (Salvi S.S. *et al.* 2001).

Airway epithelial cells express a variety of growth factors with the potential to stimulate proliferation and collagen synthesis by fibroblasts, continued damage to the epithelium affects cell to cell interactions between the epithelium and the underlying fibroblast sheath, this leads to the activation of the Epithelial Mesenchymal Trophic Unit (EMTU), amplifying the structural changes in the airways creating a microenvironment for sustaining chronic inflammation.

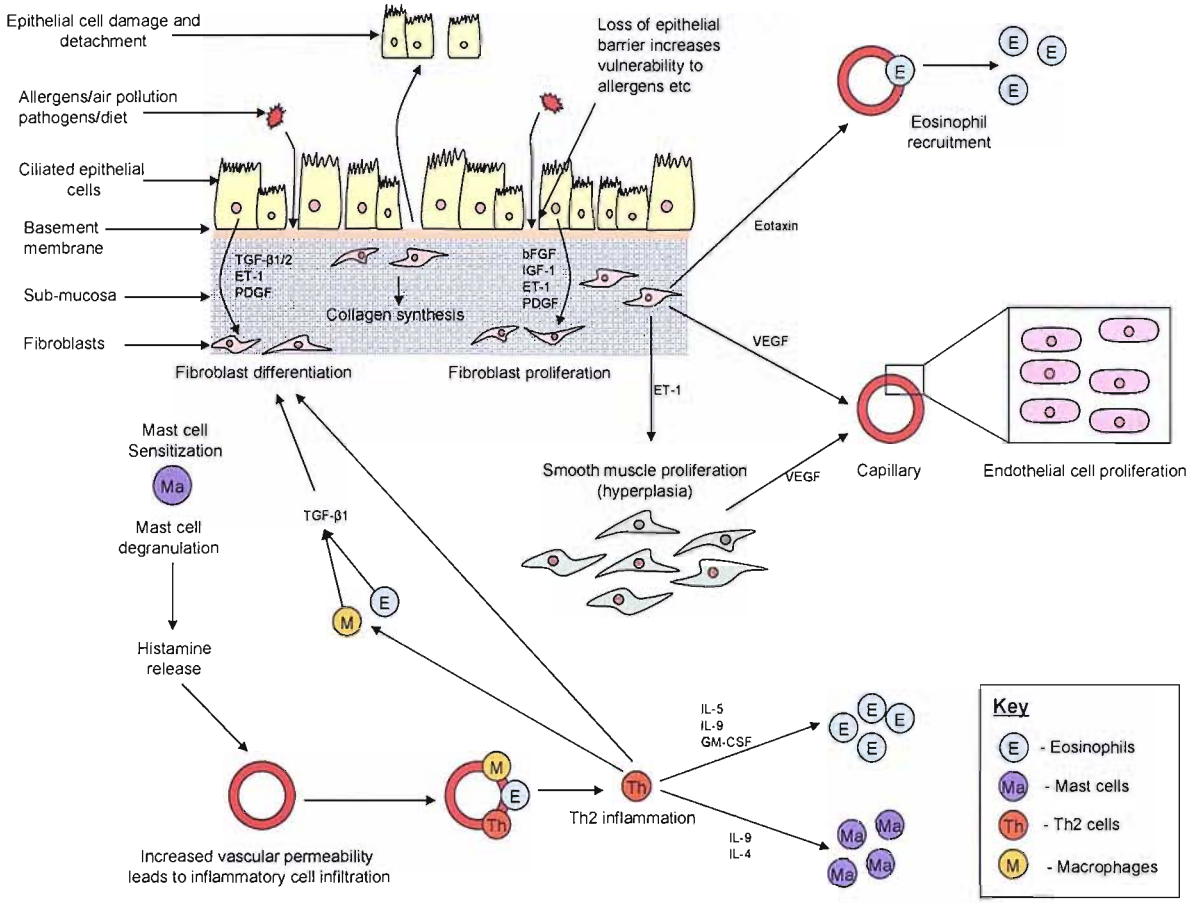


Fig 1.2 Representation of the epithelial mesenchymal trophic unit. In asthmatic airways hyper-sensitive epithelial cells may be damaged by environmental agents, damage to the epithelial layer leads to the release of factors such as TGF-β, ET-1 and PDGF which cause fibroblast differentiation and ET-1, bFGF, IGF-I and PDGF which can lead to fibroblast proliferation. Activated fibroblasts release mediators that stimulate smooth muscle proliferation, angiogenesis and eosinophil recruitment, as well as synthesising increased levels of collagen. Exposure to environmental agents may lead to the activation of mast cells through dendritic cell antigen presentation or direct cross-linking of IgE receptors on the mast cell surface. Activation of mast cells causes the release of histamine which causes vascular permeability and infiltration of inflammatory cells. Influx of Th2 cells stimulates macrophages and eosinophils to release TGF-β, which perpetuates fibroblast differentiation. In these ways epithelial damage and subsequent repair helps propagate the remodelling process in asthmatic airways.

Smooth muscle hypertrophy/hyperplasia

Within the asthmatic airways it has been reported that there is a significant ASM hypertrophy (increase in cell size) and hyperplasia (increase in cell number), which can be categorised into two patterns. In type one there is an increase in ASM hyperplasia in the large central airways, while in type two there is mild hyperplasia in the large airways but hypertrophy throughout the entire bronchial tree, especially in peripheral airways (Ebina M. *et al.* 1990). As already mentioned there are functional abnormalities present within the ASM of the asthmatic airway, which contribute to the BHR and the severity of asthma. However it is unknown whether airway hyperresponsiveness is due to fundamental changes in smooth muscle itself, or whether it is linked to the surrounding milleau of inflammatory mediators, chemokines and cytokines (Leckie M.J. *et al.* 2000). Studies using detailed computer models of human airways indicate that increased airway smooth muscle mass is by far the most important abnormality responsible for excessive airway lumen narrowing and increased compliance of the airway wall in severe asthma (Lambert R.K. *et al.* 1993 and Bramely A.M. *et al.* 1994).

The hyperplasia within the airway has been noted in *post mortem* analysis of fatal cases of severe asthma (Bai T.R. *et al.* 2000), it has been suggested that this increase may be due to an autocrine loop in which the ASM can regulate its own proliferation (Chiappara *et al.* 2001), a decrease in ASM apoptosis, through the migration of mesenchymal cells to ASM bundles or stimulation via paracrine cell signalling. It has been noted that factors within the BAL from asthmatic patients who underwent allergen challenge are proliferative for ASM; these factors have been shown to be below 10kDa in size (Naureckas E.T. *et al.* 1999), suggesting potential candidates such as TGF-β1, PDGF and EGF. EGF has already been shown to be upregulated in the asthmatic airway (Amishima M. *et al.* 1998), Panettieri R.A. *et al.* 1996 showed that EGF can stimulate ErbB-2 phosphorylation and stimulates phosphatidylinositol 3-Kinase (PI3-K); both pathways have been shown to cause the proliferation of ASM (Krymskaya V.P. *et al.* 1999a and Krymskaya *et al.* 1999b). Key signalling mechanisms have been identified as the p38 mitogen activated protein kinase (MAPK) and the p21-activated kinase 1 pathways (Hocking D.C. 2004); MMP-2 has also been shown to play a role in ASM proliferation (Johnson S. *et al.* 1999). MMP-2 is released from cells within the airway including

fibroblasts and macrophages and as well as contributing to ASM proliferation also plays a role in airway wall remodelling, via the breakdown of the ECM. ASM may also release MMP-2 and connective tissue growth factor (CTGF) (Burgess J.K. *et al.* 2003) upon stimulation with TGF-β, suggesting that ASM may play a role in airway wall remodelling, MMP-2 could be one factor responsible for autocrine regulation of ASM proliferation (Johnson S. *et al.* 1999). Johnson P.R. *et al.* 2000 showed that ASM can produce a variety of ECM proteins such as collagen I, III, IV, laminin β1 and fibronectin upon exposure to asthmatic serum, and that the profile of these proteins can be altered by exposure to atopic asthmatic serum (Johnson P.R. *et al.* 2000). Johnson P.R. *et al.* 2000 also showed that treatment with beclamethasone reduced ASM cell number but did not affect the production of ECM proteins from untreated cells.

Hypertrophy of ASM in the asthmatic airway may be due to continued bouts of bronchospasm, a reduced inhibitory control or accumulation of plasma in the environment surrounding the ASM (Beckett *et al.* 2003). TGF-β, IL-1β and cardiotrophin (Zhou D. *et al.* 2003) have been suggested as potential hypertrophic influences. Woodruff P.G. *et al.* 2004 has suggested however that hypertrophy may not be present within the asthmatic airways, and this is an area which still requires research.

Mucus secreting elements

The surface of the columnar epithelial cells within the airway is covered with a protective mucus layer composed mainly of mucin glycoproteins which are produced by goblet cells (Yoshisue H. *et al.* 2004). In a healthy airway the mucus is propelled out of the lung by ciliated epithelial cells to remove debris. In asthma there is goblet cell hyperplasia, with a greater proportion of the airway wall being occupied by goblet cells in the asthmatic airway than in the healthy (Carroll N. *et al.* 1993). Goblet cell hyperplasia is particularly marked in the small airways of patients with fatal asthma (Aikawa T. *et al.* 1992). Goblet cell hyperplasia has been reported in allergic asthma but not in non-allergic asthma (Ordonez C.L. *et al.* 2001 and Shahana S. *et al.* 2005). The increase in goblet cell number leads to excessive production of mucus that, together with the inflammatory exudate forms sticky tenacious plugs that block the airways. (Wanner A. *et al.* 1988). The epithelial damage and impaired cilial function in asthma leads to defective mucus

clearance. Factors believed to be instrumental in goblet cell hyperplasia include IL-13 (Grunig G. *et al.* 1998 and Wills-Karp M. *et al.* 1998) and the EGFR (Takeyama K. *et al* 1999). More recently it has been suggested that MMP/ADAM may play a pivotal role in goblet cell hyperplasia in lung epithelial cells via the cleavage of TGF-α (Yoshisue H. *et al.* 2004).

Within the asthmatic airway there is also an abnormal distribution of both submucosal glands and goblet cells, with mucus secreting glands being found in the peripheral bronchioles, where they are normally absent.

There is submucosal gland enlargement in fatal asthma (Dunnill M.S. *et al.* 1969), and also dilatation of gland ducts which is referred to as bronchial gland ectasia (Cluroe A. *et al.* 1989). Continued exposure to allergens leads to goblet cell metaplasia and an increase in the production of mucus in animal models (Salmon M. *et al.* 1999), and has been proposed to initiate the mucus gland remodelling seen in asthma.

There has been no definite correlation made between hyperplasia of mucous glands and the severity of asthma (Cho S.H. *et al.* 1996).

Increased Vascularity in asthma

Increased vascularity and increased vascular dilation have been witnessed in patients who have died from severe asthma (Roche W.R. *et al.* 1989, Djukanovic R. *et al.* 1990 and Wilson J.W. *et al.* 1993). When Carroll N.G. *et al.* 1997 compared blood vessels in large cartilaginous airways in patients with fatal asthma they found that both the number and area of large blood vessels was increased, however the number and area of the small blood vessels was decreased, when compared with non-fatal asthma and control patients.

The increase in large blood vessels observed in fatal asthma may be due to vascular congestion which is associated with severe asthma. Carroll N.G. *et al.* 1997 also assessed the area occupied by blood vessels in the airway submucosa of subjects with fatal asthma and discovered that this was similar to patients with nonfatal asthma and healthy control patients. Blood vessels were found to have been distended by 80% of their maximal estimated area, both findings have also been observed by other groups (Chu H.W. *et al.* 2001). However Li X. *et al.* 1997 suggested that angiogenesis is present in the submucosa of asthma and may be a component of the chronic inflammatory response in mild to

moderate asthma, this theory is supported by the observation that TNF-α secreted by mast cells (Bradding P. *et al.* 1994) can cause angiogenesis. Angiogenesis within the submucosa may also be driven by factors released from bronchial structural cells such as VEGF from ASM (Knox A.J. *et al.* 2001) and PDGF (Ishikawa F. *et al.* 1989) or bFGF from endothelial cells (Tsuboi R. *et al.* 1990).

Angiogenesis is thought to contribute to both persistent hyperresponsiveness (James A.L. *et al.* 1989) and also airflow obstruction observed in the asthmatic airway (Van Schayck C.P. *et al.* 1991). Increased vascularity accompanied with consequent thickening of the airway wall, may lead to the narrowing of the bronchial lumen, increased airway resistance, and decreased forced expiratory flow rates (McFadden E.R. 1990).

Airway wall cartilage degeneration

In a study of three cases of fatal asthma and four cases of non-fatal asthma, it was found that there was a greater extent of cartilage degeneration in the asthmatic subjects, accompanied by increased levels of perichondrial fibrosis (Haraguchi M. *et al.* 1999), however this has not been reported in other studies (Dunhill M.S. *et al.* 1969 and Takizawa T. *et al.* 1971). It is unknown which proteinase is responsible for cartilage degradation in the airways, if any, however likely candidates could be neutrophil elastases or cathepsin B/L. Cartilage degradation could contribute to airway narrowing due to a loss of structural rigidity, which would be amplified by increased smooth muscle contraction in the asthmatic airway.

Extra cellular matrix deposition and fibrosis

The estimated physiological turnover of total ECM in the human lung is 10–15% per day (Dunsmore S.E. *et al.* 1996). Rather than the ECM being a passive support for structural cells, its role in cell-cell signalling, wound repair, cell adhesion and tissue function is a dynamic one. In the lungs the ECM is composed primarily of proteoglycans, laminin, fibronectin, elastin and most importantly collagen, which is the most abundant protein in animal tissues. Collagen exists in several forms including collagen I, II, III IV and V, collagen I and III form long fibrillar arrays with collagen I being utilised in bone and

skin. Collagen II is found in cartilage, while collagen IV is predominant in basement membranes where it forms a two dimensional cross linked structure.

Proteoglycans are long protein chains with glycosaminoglycans (GAGs) side chains, which are polysaccharides formed from repeating disaccharide units. Proteoglycans can be up to 95% polysaccharide by weight and contain one to one hundred GAG chains. The GAGs are highly negatively charged which allows them to bind positively charged ions, growth factors, cytokines and trap water molecules to form hydrated gels. The hydrophilic nature of the GAGs provides a high osmotic pressure, allowing resistance to compressive loads, and providing mechanical support to the extracellular matrix.

Proteoglycans are a diverse group of macromolecules ranging from 10 to >500kDa, and the proteoglycan composition of the ECM can affect its biological function. In addition to being components of the extracellular matrix, some proteoglycans are cell surface proteins that function in cell adhesion. Proteoglycans interact with both collagen and other matrix proteins to form gel-like networks in which the fibrous structural proteins of the extracellular matrix are embedded, for example the proteoglycan perlecan binds to both type IV collagen and laminin.

The ECM also contains elastic fibres, these elastic fibres are composed primarily of a protein called elastin, which is cross linked into a network by covalent bonds formed between the side chains of lysine residues (similar to those found in collagen). The function of elastin within the lungs is to provide recoil after expiration. Fibronectin is the principal adhesion protein of connective tissues, and is a dimeric glycoprotein consisting of two polypeptide chains. Adhesion molecules within the ECM bind the components of the matrix to one another and to the surface of cells. Fibronectin has binding sites for both collagen and GAGs, so it crosslinks these matrix components. However, fibronectin is primarily responsible for the attachment of cells to the ECM. Fibronectin may also bind to cell surface receptors to modulate their function. Laminin is another adhesion protein, however its functions are more closely related to collagen IV. Like collagen IV, laminins can form two dimensional cross linked polymers and provide structural support for the components of the ECM. Laminins also have binding sites for cell surface receptors and type IV collagen. In the ECM laminin, collagen IV and perclan form cross linked networks within the basal lamina. As well as a structural role laminins also play a part in

lung morphogenesis and leukocyte recruitment. The major cell surface receptors responsible for the attachment of cells to the ECM are the integrins. The integrins bind components of the extracellular ECM, including collagen, fibronectin and laminin. Integrins also serve as anchors for the cytoskeleton; the linkage of the cytoskeleton to the ECM is responsible for the stability of cell matrix junctions. Distinct interactions between integrins and the cytoskeleton are found at two types of cell to matrix junctions, focal adhesions and hemidesmosomes. Focal adhesions attach a variety of cells, including fibroblasts, to the ECM; hemidesmosomes are specialized sites of epithelial cell attachment to the basal lamina.

One of the prominent structural changes associated with asthma involves enhanced matrix deposition and remodelling within the subepithelial region of the airway wall (Roberts C.R. 1995). In patients with severe persistent asthma the airway undergoes irreversible structural changes with a thickening of the subepithelial basement membrane contributed to by an increased deposition of fibronectin (Roberts C.R. 1995) and collagen I, III, V, tenascin, hyaluronan, versican and laminin (Roberts C.R. *et al.* 1998 and Laitinen A. *et al.* 1997), but decreased deposition of collagen IV and elastin (Bousquet J. *et al.* 1992). In the healthy airway the subepithelial layer measures between 5-8μm, while in asthma the thickness ranges from 10-15μm (Roberts C.R. 1995). The imbalance between degradation and deposition of ECM components may be due to increased synthesis of ECM by structural cells within the airway, decreased levels of ECM degrading enzymes such as MMPs, or up regulation of the tissue-specific inhibitors of MMP (TIMP).

It has been proposed that increased deposition of fibronectin in the airway walls of asthmatic patients may alter the distribution, deposition or arrangement of collagen, affecting the mechanical properties of the airway wall account for the tensile stiffness reported in asthma. Ohke M. *et al.* 2001b showed that levels of fibronectin in the plasma of patients with asthma were significantly higher than in healthy controls.

Huang J. *et al.* 1999 showed that proteoglycan deposition was increased in mild atopic asthmatic airways, and that the increase in proteoglycans was related to the degree of airway hyperresponsiveness. Westergren-Thorsson G. *et al.* 2002 showed that altered proteoglycan metabolism by bronchial fibroblasts in the asthmatic airway may contribute

to the increased proteoglycan deposition in the bronchial mucosa, and linked airway hyperresponsiveness observed in asthma to the production of the proteoglycans; perlecan, small heparan sulphate proteoglycan (HSPGs), biglycan, hyaluronan and versican. Ludwig M.S. *et al.* 2004 suggested that the increase in mechanical stress within the asthmatic airway may be responsible for the increase in proteoglycan production, especially decorin and versican. Although decorin has not been related to airway hyperresponsiveness, it has been shown to be able to modulate the function of TGF-β, an important regulator of airway remodelling (Minshall E.M. *et al.* 1997). EGF and LTD4 have been shown to regulate the production of proteoglycans, especially versican, by bronchial smooth muscle within the asthmatic airway (Potter-Perigo S. *et al.* 2004). Hyaluronan and versican deposited between the smooth muscle and epithelium could account for some of the pre-load of the asthmatic airways and would oppose smooth muscle shortening, increasing airway resistance.

The distribution of laminin in the airway wall of asthmatics and healthy control patients was assessed, laminin was identified in the superficial margin of the basement membrane blood vessels, and smooth muscle, and the thickness was significantly greater in patients with chronic and occupational asthma than in the healthy controls (Altraja A. *et al.* 1996). In severe chronic asthma there was a discontinuous laminin staining along the epithelial margin of the basement membrane (Altraja A. *et al.* 1996). In animal models dexamethasone has been shown to decrease the expression of laminin in the asthmatic lung (Christie P.E. *et al.* 2004).

In both asthma and chronic bronchitis it has been shown that there is an imbalance between elastases and its inhibitor (α_1-protease inhibitor) in sputum, and that the level of elastases correlated with a decline-e in FEV_1 (Vignola A.M. *et al.* 1998). Elastases have the ability to degrade elastin; evidence of elastin degradation in the asthmatic airways has been observed (Bousquet J. *et al.* 1992) suggesting a role for elastase in the reduced lung function witnessed in asthma. Also of note is the abnormal distribution of elastic fibres within the asthmatic airway, with fibres often being patchy, tangled and thickened (Mauad T. *et al.* 1999). Bundles of elastic fibres in severe asthmatics appear to be hypertrophied as a result of increased collagen and myofibroblasts matrix deposition occurring during exaggerated elastic fibre deposition (Carroll N.G. *et al.* 2000). Loss of

elastic recoil has been shown in chronic persistent asthma and the increased electrolysis within the airways may play a role in observed exaggerated airway narrowing. Wiggs B.R. *et al.* 1997 has suggested that an abnormal folding of the airway mucosa arising from subepithelial fibrosis and altered smooth muscle contraction may contribute to airway obstruction.

The thickened subepithelial basement membrane in asthma was found not to be due to a thickened basal lamina, but to be composed of a thickened layer of matrix, containing fibrillar collagen under an epithelial basal layer (Roche W.R. *et al.* 1989). The thickening of the subepithelial matrix due to the deposition of collagen correlated strongly with myofibroblasts number (Brewster C.E.P. *et al.* 1990). The deposited collagen in the asthmatic airways is more densely packed than normal, denser packing of collagen fibrils coupled with the more general thickening of the basement membrane could stiffen the subepithelial matrix contributing to the increase in airway stiffness in the asthmatic airway, opposing the effect of smooth muscle shortening on airway narrowing (Roberts C.R. 1995).

The role of the fibroblast in airway remodelling

Airway fibroblasts are situated beneath the epithelial cells within the ECM, however they are not necessarily stationary and can migrate in and out of tissue, for example in a study by Larsen K. *et al.* 2004 activated and mobile fibroblast-like cells were found in BAL fluid of subjects with mild asthma. In the healthy lung fibroblasts and the epithelial layer communicate via the release of cytokines and growth factors to maintain lung ECM homeostasis. However in the asthmatic airway there appears to be altered cell to cell communication, and also a change in the fibroblast phenotype to become a more synthetic cell (myofibroblasts), which present features similar to smooth muscle cells, with increased contractile properties and expression of α-SMA. The fibroblast may play a role in inflammation via the release of inflammatory mediators including RANTES and IL-6, which aid in the recruitment of Th2 cells, eosinophils, macrophages and neutrophils.

Accompanying altered fibroblast phenotype is myofibroblast hyperplasia with myofibroblast numbers correlating to the thickness of the basement membrane. The

myofibroblast can contribute to ECM thickening by releasing fibronectin, collagen I, III, V and tenascin (Jeffery P.K. *et al.* 2000), and a range of proteoglycans (Ludwig M.S. *et al.* 2004). Myofibroblast hyperplasia has been shown to be controlled by a variety of factors released by both inflammatory cells and structural cells, suggesting that both chronic inflammation and the remodelling of the airways perpetuate the altered myofibroblast phenotype. For example IL-13 (Ingram J.L. *et al.* 2003) and IL-4 (Bergeron C. *et al.* 2003a) released from Th2 cells has been shown to stimulate myofibroblast proliferation. IL-4 has also been shown to cause the increased expression of collagen III from myofibroblasts when challenged with BAL (Bergeron C. *et al.* 2003b and Batra V. *et al.* 2004), IL-4 may also down regulate the production of MMP2 leading to a decrease in collagen III degradation but an increase in collagen III production (Bergeron C. *et al.* 2003b). Other factors linked to an increase in collagen III synthesis include bFGF, TGF-β1 (Batra V. *et al.* 2004), insulin-like growth factor-1 (IGF-1) (Hoshino M. *et al.* 1998a) and IL-11 (Tang W. *et al.* 1996). A decrease in the production of MMP2 may also decrease the rate of degradation of fibronectin, laminin and collagen IV and V (Xu J. *et al.* 2002). It has been shown that fibronectin acts as a growth factor for fibroblasts (Bitterman P.D. *et al.* 1983), increased levels of both fibronectin (Meerschaert J. *et al.* 1999) and TGF-β1 (Redington A.E. *et al.* 1997) have been found in the BAL from asthmatic patients, increased levels of fibronectin in BAL could help up regulate the proliferation of airway fibroblasts. As well as the increased synthetic capabilities of myofibroblasts they also exhibit an increased contractile ability which may contribute to increased airway narrowing.

Mediators of airway remodelling

Transforming growth factor-β (TGF-β)

The TGF-β family is one of the largest families of secreted multifunctional peptides. The family includes three isoforms of TGF-β, activins and inhibins, growth and differentiation factors (GDF), and bone morphogenetic protein (BMP) (Seppa R. 2002). It was first identified as a protein secreted from sarcoma cells that promoted normal rat kidney cells to grow in soft agar (Moses H.L. *et al.* 1981 and Roberts A.B. *et al.* 1981) and was named transforming growth factor because of this ability to induce a transformed

or tumour cell characteristic in normal cells (Seppa R. 2002). The TGF-βs are initially synthesized as large precursor proteins with a signal sequence and a large pro-domain, the larger precursor protein is proteolytically cleaved to release the 25kDa mature peptide which contains seven cysteine residues which participate in intermolecular and intramolecular disulfide bonds (Flaumenhaft R. *et al.* 1993 and Seppa R. 2002).

The mammalian TGF-β family comprises three isoforms denoted as TGF-β1, TGF-β2 and TGF-β3 which signal through serine/threonine TGF-β receptors. There are six known TGF-β receptors (RI-VI), however most research has concentrated on TGF-βRI, RII and RIII. TGF-βRIV and RV are known to bind TGF-β; however no downstream signalling has been observed (Segarini P.R. *et al.* 1992). The main route, by which the TGF-β family signals is through heteromeric protein complexes, composed of type I and type II serine/threonine kinase receptors. These receptors are composed of a cysteine-rich extracellular domain, a single transmembrane region and an intracellular kinase domain. The type I receptor consists of a juxtamembrane domain rich in glycines and serines (the GS domain). A complex of both type I and type II receptors are required to generate a response to TGF-β (Seppa R. 2002). When the TGF-β ligand binds to the TGF-β type II receptor on the cells surface (Wrana J.L. *et al.* 1994), each bound type II receptor sequesters a type I receptor, these receptor pairs are then able to form a heterotetrameric complex comprising two type I and two type II receptors. The type II receptor can then phosphorylate the GS domain of the type I receptor, thus activating the type I serine/threonine kinase, and subsequently the downstream targets of the type I receptor, transducing the signal to the nucleus (Seppa R. 2002). TGF-βRIII is comprised of either betaglycan or endoglin, depending on which cell type the receptor is being expressed. This receptor is responsible for primarily binding TGF-β2 and presenting the ligand to the type II receptor, as TGF-β2 has a weak affinity for the type II receptor alone and can only signal via interaction with betaglycan (Blobe G.C. *et al.* 2001). In contrast, TGF-β1 can signal directly through TGF-βRII (Boxall C. *et al.* 2006).

TGF-β mediates intracellular signalling via Smad proteins. The name Smad was coined by combining the names of two findings, the first of which was by Sekelsky J.J. *et al.* 1995 during a genetic screen in *Drosophila*. The screen was designed to identify mutations that could modify the dpp mutant phenotype; the gene isolated from this screen

was called Mothers against Dpp (*mad*). The second finding was made by Savage C. *et al.* 1996, whilst working with the *C. elegans* worm. The researcher was able to identify three proteins sma2, sma3 and sma4 (known as sma due to their small size) which shared homology with the *Drosophila* Mad protein, and were also identified in a genetic screen as components of a TGF-β like signalling pathway in worms. These families of conserved TGF-β signalling molecules were named Smads, a combination of the *Drosophila* and *C. elegans* nomenclature. Smads which interact with receptors (Smad2 and Smad3) reside in the cytoplasm in an inactive state. When the TGF-βRI is activated it serine phosphorylates Smad2 and Smad3, these Smads then bind Smad4, and the complex translocates to the cell nucleus. Once the phosphorylated Smad complex reaches the nucleus it can bind directly to the Smad-binding element of various genes, such as junB, c-jun and IgA (Jonk L.J. *et al.* 1998 and Wong C. *et al.* 1999), or mediate gene transcription via functional co-operation with other transcription factors, such as Fork Head Activin Signal Transducer-1 (FAST-1) (Zhou S. *et al.* 1998).

Once the ligand binding process is complete, and the signal propagated, the TGF-β receptors are typically endocytosed. In the case of fibroblasts, heteromeric TGF-β receptors are internalised whereas homomeric TGF-β receptors are recycled back to the membrane (Anders R.A. *et al.* 1996 and Anders R.A. *et al.* 1997).

TGF-β in asthmatic airway remodelling

Transforming growth factor-β is known to increase the production of ECM proteins such as collagen from fibroblasts (Varga J. *et al.* 1986, Fine A. *et al.* 1993 and Eickelberg O. *et al.* 1999), whilst also suppressing the activation of ECM proteases such as MMP and collagenase (Overall C.M. *et al.* 1989 and Bullard K.M. *et al.* 1997). It is also accepted that TGF-β is a major contributor to fibroblast myofibroblast differentiation. In the asthmatic airways TGF-β levels in epithelial cells have been shown to correlate to fibroblast number and airway thickness (Vignola A.M. *et al.* 1997, Hoshino M. *et al.* 1998a and Sagara H. *et al.* 2002). In studies by Khalil N. *et al.* 1991 and 1996 it was shown that TGF-β 2 and 3 are expressed in macrophages, epithelial and endothelial cells in the healthy lung, but that TGF-β1 was only expressed in macrophages and epithelial cells in fibrotic lungs. In patients with severe asthma TGF-β1 mRNA was also shown to

be localised in eosinophils (Ohno I. *et al.* 1992 and Minshall E.M. *et al.* 1997) and macrophages (Vignola A.M. *et al.* 1997), a finding supported when TGF-β levels were assessed in bronchial biopsies, although these studies suggested TGF-β2 was the prominent TGF-β isoforms in eosinophilic asthma (Balzar S. *et al.* 2005). The presence of TGF-β isoforms in epithelial cells and inflammatory cells supports the possibility of the EMTU being involved in airway remodelling in asthma.

The use of animal models has shown that TGF-β is required for airway remodelling, with increased collagen mRNA and protein deposition seen in BALB/C mice treated with TGF-β, in this study collagen deposition was accompanied by airway hyper reactivity (Kenyon N. *et al.* 2003), although treatment of mice with TGF-β1 after sensitisation with sheep red blood cells (SRBC) lead to decreased mast cell mediated hyper reactivity in mice skin (Meade R. *et al.* 1992). Transforming growth factor-β has also been shown to be important in airway inflammation, with TGF-β1 gene disruption in mice leading to extensive multi-organ inflammation, particularly in the lung, resulting in tissue necrosis and death (Shull M.M. *et al.* 1992 and Geiser A.G. *et al.* 1993). There is also evidence from murine studies that TGF-β mediates mast cell differentiation (Wright S.H. *et al.* 2002), and that increased TGF-β levels correlate to eosinophil and neutrophils numbers which infiltrate the airways post allergen challenge in sheep (Collie D.D. *et al.* 2001). These animal studies support a role for TGF-β in early inflammatory responses.

Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor exists as dimers of polypeptide chains, A, B, C and D, which may conjugated as either PDGF-AA, BB, AB, CC or DD. The PDGF receptors are tyrosine kinase receptors, PDGF-α and PDGF-β, which dimerise after ligand binding. dimerisation of the receptor leads to autophosphorylation of tyrosine residues and subsequent activation of the receptors enzymatic activity, whilst also creating docking sites for signal transduction proteins, Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, which transduce signals into the cell. The PDGF receptors are able to form homodimers (α-α and β-β) or heterodimers (α-β), depending on which PDGF dimer binds, PDGF-α is able to bind all PDGF dimers, while PDGF-β will only bind PDGF-BB and PDGF-AB. The PDGF family is able to regulate a variety of cellular processes

including mitogenesis, angiogenesis, hyperplasia, chemotaxis, differentiation and protein synthesis.

PDGF in asthmatic airway remodelling

The role of PDGF in asthma has been investigated by numerous researchers, resulting in multiple roles being assigned, however there is believed to be no direct correlation with PDGF expression and airway structural changes in asthma (Aubert J.D. *et al.* 1994). Platelet derived growth factor may be released by epithelial cells (Shimizu S. *et al.* 2000), eosinophils (Ohno I. *et al.* 1995), macrophages (Taylor I.K. *et al.* 1994 and Pan P.H. *et al.* 2001), fibroblasts (Ingram J.I. *et al.* 2004) and smooth muscle (Crowley S.T. *et al.* 1995), and may therefore play a role in airway remodelling in asthma. In work by Taylor I.K. *et al.* 1994 and Pan P.H. *et al.* 2001 it was shown that PDGF levels were not increased in macrophages from asthmatic patients compared to healthy controls; however PDGF was shown to be increased in eosinophils from asthmatic compared to controls (Ohno I. *et al.* 1995). Platelet derived growth factor has been shown to cause differentiation of fibroblasts into an intermediate type of myofibroblasts, which possessed a more stretched cell shape and lamellipodia protrusions (Malmström J. *et al.* 2003), and also increase asthmatic but not healthy fibroblast proliferation (Dubé J. *et al.* 1998). However its role in promoting collagen synthesis from fibroblasts has been debated with work by Dubé J. *et al.* 2000 showing that PDGF alone is not able to cause an increase in collagen synthesis from fibroblasts, while Lewis C.C. *et al.* 2005 showed that PDGF-BB significantly increased collagen I production in severe asthmatic fibroblasts compared to healthy. There may also be a role for PDGF in smooth muscle hyperplasia seen in the asthmatic airway as up-regulation of the PDGFR-α by bFGF was shown to increase smooth muscle proliferation (Bonner J.C. *et al.* 1996). Work in animal models has shown that PDGF is important in the remodelling events caused by exposure of mice to diesel exhaust particles; when mice were treated with anti-PDGF-β neutralising antibodies before exposure to diesel exhaust particles there were reduced levels of airway resistance, elicited by acetylcholine, and airway wall thickening (Yamashita N. *et al.* 2001). There is also evidence that low expression of PDGFR-α may a contributing factor

to non-allergic childhood asthma (Wu L.S. *et al.* 2006). These observations suggest that PDGF is an important regulator of asthmatic airway remodelling.

Endothelin-1 (ET-1)

Endothelin was first discovered by Hickey K.A. *et al.* 1985 and identified as a peptidic endothelium-derived constricting factor, its structure was later classified Yanagisawa M. *et al.* 1988. The identification of ET-2 and ET-3, the other known members of the ET family, was made by Inoue A. *et al.* 1989. The ET receptors were subsequently identified, ETA was discovered by Arai H. *et al.* 1990, and ETB by Sakurai T. *et al.* 1990. The enzymes responsible for post-secretional processing of ET were identified as endothelin-converting enzyme-1 (ECE-1) (Takahashi M. *et al.* 1993 and Xu D. *et al.* 1994) and ECE-2 (Emoto N. *et al.* 1995).

The ET receptors are members of the G-protein coupled receptors family; the ETA receptor has a higher affinity for ET-1 and ET-2 than ET-3, while the ETB receptor has an equal affinity for all three ET isoforms. Endothelin-1 can be secreted by endothelial cells (Hickey K.A. *et al.* 1985), macrophages (Ehrenreich H. *et al.* 1990), fibroblasts (Kawaguchi Y. *et al.* 1994, Hasdai D. *et al.* 1997, Richter A. *et al.* 2001 and An S.J. *et al.* 2006), bronchial smooth muscle (Goldie R.G. *et al.* 1995 and Fukuroda T. *et al.* 1996) and epithelial cells (Sun G. *et al.* 1997). Enothelin-1 is able to mediate multiple cellular events including vasoconstriction, proliferation and differentiation.

Endothelin-1 in asthmatic airway remodelling

The presence of both ETA and ETB has been shown in the airways of mammalians (Goldie R.G. *et al.* 1996); however no significant variation in ETA or ETB density was seen between healthy and asthmatic lungs (Knott P.G. *et al.* 1995). There is evidence suggesting that ET-1 levels in BAL from asthmatics are increased compared to controls (Mattoli S. *et al.* 1991, Sofia M. *et al.* 1993 and Redington A.E. *et al.* 1997), and also that ET expression in the epithelial and endothelial cells in bronchial biopsies is greater in asthma than in control biopsies (Springall D.R. *et al.* 1991). However, as with PDGF, no relationship was seen between raised ET levels and airway remodelling or inflammation. When ET-1 was first discovered it was shown to cause smooth muscle contraction

(Turner N.C. *et al.* 1989), and bronchoconstriction (Uchida Y. *et al.* 1988), and it is now believed that ET-1 is able to increase smooth muscle proliferation, either alone (Janakidevi K. *et al.* 1992) or in conjunction with EGF (Panettieri R.A. *et al.* 1996). The release of ET-1 from epithelial cells and fibroblasts may be route through which smooth muscle hyperplasia is perpetuated in the asthmatic airway, but ET-1 may also be able to increase smooth muscle constriction, contributing to airway narrowing. Supporting this proposal is work by Chalmers G.W. *et al.* 1997, where aerosolised ET-1 was shown to elicit bronchoconstriction in asthmatics but not in non-asthmatics, suggesting that asthmatic airways are hyper-sensitised to ET-1.

It was shown that ET-1 released from epithelial cells post-allergen challenge was able to induce fibroblast to myofibroblast differentiation (Sun G. *et al.* 1997), and also that ET-1 was able to cause increased collagen I and III production as well as decrease MMP-1 production in fibroblasts from sclerosis (Shi-Wen X. *et al.* 2001). However this finding was not supported by Dubé J. *et al.* 2000, who found that ET-1 alone did not cause increased collagen production from fibroblasts, in this study collagen production was only increased when combinations of TGF-β, PDGF and ET-1 were used, suggesting that the presence of multiple factors is required to activate proliferation and collagen synthesis from healthy and asthmatic fibroblasts. Therefore ET-1, PDGF and TGF-β may be important contributing factors to airway remodelling in asthma.

Insulin-like Growth Factor-I (IGF-1)

The IGFs were first discovered in 1957 (Salmon W.D. *et al.* 1957) when they were named sulphation factors, it was not until Rinderknecht E. *et al.* 1976 identified two factors in serum which resembled the structure of pro-insulin that the terms IGF-1 and IGF-1 were coined. Insulin-like growth factor-I, also known as somatomedin C, is a 70 amino acid long protein with a molecular weight of around 7.6kDa. The IGF receptors are tyrosine kinase receptors located on the cell membrane of almost all cells; there are two IGF receptors IGF-1R and IGF-1R, IGF-1R has a higher affinity for IGF-1 than IGF-1. The IGF-1R comprises two extra-cellular α-subunits, containing hormone binding sites, and two membrane-spanning β-subunits, encoding an intracellular tyrosine kinase. Ligand binding activates the receptor kinase, leading to receptor autophosphorylation and

tyrosine phosphorylation of multiple substrates. Insulin-like growth factor-IIR has no intrinsic tyrosine kinase activity but a 500 fold greater affinity for IGF-1 than IGF-1, its primary function is to sequester IGF-1 to prevent its binding to IGF-1R. Around 95% of the IGFs are bound to high affinity IGF-binding protein (IGFBP) when in circulation, and there are 6 members of this family IGFBP-1-6, the most common complex formed is IGF-1 or IGF-1 with IGFBP-3. The purpose of the IGFBP appear to be 3 fold, they act as IGF transports, prevent degradation and regulate interaction of IGFs with their receptors. Insulin-like growth factors have a range of cellular affects including cell proliferation, differentiation, apoptosis and transformation (Jones J.I. *et al.* 1995). Insulin-like growth factor-I is able to regulate proliferation by stimulating cyclin-D1 expression, which causes the cells to proceed into S phase from G1 phase more rapidly (Furlanetto R.W. *et al.* 1994). The release of IGF-1 has been shown from macrophages (Rom W.N. *et al.* 1988 and Homma S. *et al.* 1995), epithelial cells (Cambrey A.D. *et al.* 1995 and Zhang S. *et al.* 1999), fibroblasts (Homma S. *et al.* 1995, Horio T. *et al.* 2005 and Giacco F. *et al.* 2006), smooth muscle (Homma S. *et al.* 1995, Zwaka T.P. *et al.* 2003), endothelial cells (Homma S. *et al.* 1995), mononuclear phagocytes (Homma S. *et al.* 1995) and alveolar type II cells (Homma S. *et al.* 1995).

IGF-1 in asthmatic airway remodelling

When bronchial biopsies from healthy and asthmatic volunteers were compared there was an increase in IGF-1 staining seen in the bronchial mucosa in asthmatics compared to healthy subjects (Hoshino M. *et al.* 1998b). Insulin like growth factor-I released from epithelial cells has been shown to cause fibroblast proliferation (Cambrey A.D. *et al.* 1995 and Zhang S. *et al.* 1999), and collagen synthesis (Chetty A. *et al.* 2006). There is also evidence suggesting that IGF-1 can cause rabbit smooth muscle proliferation, and this was exaggerated when added in combination with LTD4 (Cohen P. *et al.* 1995), but other work has suggested that IGF-1 only increases smooth muscle proliferation when added to cells in addition to PDGF (Gosens R. *et al.* 2003). There may also be a role for IGF-1 in promoting epithelial cell proliferation (Oyamada H. *et al.* 2000). In animal studies when ovalbumin sensitised mice were challenged with creatine it was found that hyperresponsiveness, eosinophilic inflammation, airway collagen, elastin content, smooth

muscle thickness and IGF-1 positive cells were increased in the airway (Vieira R.P. *et al.* 2007), suggesting the presence of cells able to secrete IGF-1. In support of this observation mice sensitised with ovalbumin exhibited increased expression of IGF-1 in the airway; when these mice were exposed to neutralising antibodies to IGF-1 there was a marked reduction in airway resistance, airway inflammation and airway wall thickening (Yamashita N. *et al.* 2005). When bronchial biopsies from asthmatic volunteers were taken before and after 6 months of treatment with beclomethasone dipropionate (BDP), it was seen that in biopsies taken after treatment there was an improvement in asthma symptoms such as peak flow and airway responsiveness compared to the placebo group. There was also a significant decrease in the thickness of the lamina reticularis, number of activated eosinophils, T-lymphocytes and fibroblasts accompanied by a reduction in the expression of IGF-1 in the BDP treated patients. In addition to these observations a significant correlation was seen between IGF-1 expression and collagen deposition. These observations suggest that corticosteroid treatment may reduce the thickness of the lamina reticularis by reducing IGF-1 expression and inflammatory cell infiltration, contributing to preventing airway remodelling (Hoshino M. *et al.* 1998a). These findings suggest that IGF-1 may be important in regulating airway remodelling events within the airway.

Hypothesis

Asthma is characterised by both chronic inflammation and a remodelling of the airways. Airway remodelling in asthma includes increase myofibroblast numbers and increased ECM deposition. It is hypothesised that fibroblasts in the severe asthmatic airway are contributing to asthma severity by possessing an increased ability to proliferate, synthesise ECM proteins and propagate the inflammatory process, and that the airway environment in asthma may contributes to this behaviour. The aims of this thesis were to use broncho-alveolar lavage and primary airway fibroblasts to construct a simple *in vitro* model of the *in vivo* environment of the airways, and by using this model we are able to compare and quantify the mitogenic ability of asthmatic fibroblasts to that of healthy fibroblasts, as well as comparing their ability to induce mRNA synthesis, which would represent an ability to synthesise ECM and pro-inflammatory proteins, in response to the local environment.

Aims

1. To investigate the responses of fibroblasts from healthy, mild asthmatic and severe asthmatic fibroblasts to challenge.
2. To determine whether BAL from subjects with asthma has different effects on fibroblasts to BAL fluid from healthy controls, and whether disease severity affects these observations.
3. Assess factors, pathways and intra-cellular MAPK which may be responsible for these responses.

Chapter 2:

Methods

Culture of Fibroblasts from Bronchial Biopsies

Biopsies were taken from the bronchial wall of healthy, mild asthmatic or severe asthmatic volunteers during bronchoscopies. The biopsies were immediately transferred to a 7ml bijoux tube (Fisher Scientific, Leicestershire, UK) containing 3ml of culture medium (FBM) containing DMEM (Sigma Aldrich, Dorset, UK) supplemented with 1% sodium pyruvate, 1% non-essential amino acids (both Sigma Aldrich, Dorset, UK), 1% penicillin, streptomycin, L-glutamine (Invitrogen, Paisley, UK), 10% foetal calf serum (FCS) (Invitrogen, Paisley, UK). To the FBM 1:1000 50mg/ml gentamicin (Sigma Aldrich, Dorset, UK) and 1:500 fungizone (Invitrogen, Paisley, UK) were added as the biopsies were obtained from a non-sterile environment. The biopsies were then scored down into a 6 well plate (Fisher Scientific, Leicestershire, UK) using a scalpel. To score the biopsy down the biopsy was cut into 3-4 pieces and then these pieces cut gently into the plate. Once scored down 3ml of the supplemented FBM was slowly pipetted onto the side of the well to minimise the amount of biopsy lifting from the plate. The 6 well plate containing the biopsy was then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the supplemented FBM was changed once a day for the first 2 weeks. Cells would generally begin to grow out of the biopsy after 3-5 days; however some biopsies could take up to 10-14 days to begin to grow out cells. After 2 weeks the FBM was no longer supplemented with gentamicin and fungizone, and was changed once a week until the cells were confluent in the well.

Once the cells reached confluence they were lifted using 2ml of a 1% trypsin/EDTA (Invitrogen, Paisley, UK) solution, after washing the cells twice with 3ml HBSS (Invitrogen, Paisley, UK) without calcium or magnesium. The cell suspension was transferred to a 50ml Falcon tube (Greiner Bio-One Ltd, Gloucestershire, UK); along with 5ml of FBM to inactivate the trypsin/EDTA, the well was then washed with 3ml of FBM to collect any remaining cells. The cell suspension was centrifuged at 500xg, 20°C for 5 minutes to pellet the cells. The supernatant was poured off and the pellet resuspended in 5ml FBM, which was then used to seed a 75cm^2 culture flask (Triple Red Limited, Buckinghamshire, UK), along with 10ml of FBM per flask.

The cells were then grown to confluence in the flask, after which they were passaged by lifting the cells with 2ml of a 1% trypsin/EDTA (Invitrogen, Paisley, UK) solution, after

washing the cells twice with 5ml HBSS. As before the cell suspension was transferred to 50ml Falcon tube along with 5ml of FBM, and the flask was washed with 5ml FBM. The cell suspension was then centrifuged at 500xg, 20°C for 5 minutes to pellet the cells. After centrifugation the supernatant was poured off and the pellet resuspended in 5ml FBM. From this cell suspension a 75cm^2 culture flask was seeded using 1ml of cell suspension and 12ml FBM per flask, and three 1.8ml cyovials (Alpha Labs, Hampshire, UK) were used to freeze cells down in liquid nitrogen. To each cryovial 900µl of cell suspension was added to 100µl of DMSO (Sigma Aldrich, Dorset, UK), the vials were stored at -80°C in a gel pack overnight then transferred to liquid nitrogen the next day. The fibroblasts were passaged in this way up until passage 7.

Fibroblast Cell Culture

The fibroblasts were grown from bronchial biopsies obtained from healthy, mild asthmatic or severe asthmatic volunteers during bronchoscopies. Cells frozen in liquid nitrogen were thawed rapidly in a water bath at 37°C and transferred immediately into 10ml of FBM, the cell suspension was centrifuged at 500xg, 20°C for 5 minutes to pellet the cells. The supernatant was poured off and the cells resuspended in 5ml FBM, this was then used to seed a 75cm^2 culture flask along with 10ml FBM. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air; the culture medium was changed twice weekly.

Once the cells reached confluence they were lifted using 2ml of a 1% trypsin/EDTA solution, after washing the cells twice with 5ml HBSS without calcium or magnesium. The cell suspension was transferred to a 50ml Falcon tube along with 5ml of FBM to inactivate the trypsin/EDTA, the flask was then washed with 10ml of FBM to collect any remaining cells. The cell suspension was centrifuged at 500xg, 20°C for 5 minutes to pellet the cells. The supernatant was poured off and the pellet resuspended in FBM, a 1 in 3 split was used to reseed 75cm^2 culture flasks, along with 10ml FBM per flask.

To freeze cells down in liquid nitrogen for storage the cells would passaged as before and split 1 in 5 with FBM along with 10% DMSO and stored in a cryovial. The cells were frozen at -80°C in a gel pack overnight then transferred to liquid nitrogen the next day.

Code	Male/ Female	Age	Disease	Atopic	PC20 Mg/ml	Disease Duration (years)	FEV1 (%Pred)	Drug	Miscellaneous Info
FB4	Female	45	None	No	>9	0	103	None	-
FB15	Female	28	None	No	>9	0	108	None	-
FB19	Male	48	None	No	>9	0	109	None	-
FB21	Female	23	None	No	>9	0	99	None	-
FB23	Female	47	None	No	>9	0	100	None	-
MJ3	Male	26	None	No	>9	0	98	None	-
FB2	Female	42	Mild Asthma	No	3.28	25	94	400µg BDP + SAβ2-agonist prn	Diabetic
FB3	Male	35	Mild Asthma	No	1.16	31	89	SAβ2-agonist prn	-
FB17	Female	48	Mild Asthma	No	0.2	44	84	400µg BDP + SAβ2-agonist prn	-
FB18	Female	40	Mild Asthma	No	1.1	33	110	400µg BDP + SAβ2-agonist prn	-
FB22	Male	22	Mild Asthma	No	0.83	10	101	SAβ2-agonist prn	-
MJ1	Male	22	Mild Asthma	No	-	-	85	SAβ2-agonist prn	-
MJ5	Female	60	Severe Asthma	-	-	-	41.7	5000µg BDP + LAβ2-agonist	-
MJ6	Male	70	Severe Asthma	Yes	-	-	69.3	7200µg BDP + LAβ2-agonist	-
MJ8	Female	62	Severe Asthma	Yes	-	-	44.4	2000µg BDP + LAβ2-agonist	-
MJ10	Female	53	Severe Asthma	No	-	-	68.2	320mg Ciclesonide + LAβ2-agonist	-
MD11	Female	54	Severe Asthma	Yes	-	-	56	1250µg BDP + LAβ2-agonist	-
MD12	Female	56	Severe Asthma	Yes	-	-	56	2000µg BDP + 20mg oral steroid + LAβ2-agonist	-

Table 2.1 Fibroblast patient data.

SA = Short Acting
LA = Long Acting
- = Unknown Detail
BDP = Beclomethasone dipropionate equivalent.
PRN = Pro re nata (as required).

Processing of Bronchoalveolar lavage

Bronchoalveolar lavage was obtained from bronchoscopies performed on healthy, mild asthmatic, moderate asthmatic and severe asthmatic volunteers. During the bronchoscopy the bronchioles were washed with 0.9% saline, and then this washing was recovered. The recovered lavage was passed through a 100μm strainer (BD Biosciences, Bedford, USA) into 50ml Falcon tubes to remove the mucus. After the mucus was removed the lavage was spun at 500xg for 10 minutes at 4°C to pellet any cells or debris. The supernatant was poured off into 50ml Falcon tubes and the pellet(s) resuspended in 1% phosphate buffered saline (PBS) (Invitrogen, Paisley, UK) for differential cell counting to asses the number of lymphocytes, macrophages, neutrophils and eosinophils present in the BAL. The supernatant was then aliquoted into 1.5ml eppendorphs (Alpha Labs, Hampshire, UK) and 15ml Falcon tubes (Greiner Bio-One Ltd, Gloucestershire, UK) and stored at -80°C.

Code	Male/ Female	Age	Disease	Atopic	PC20 Mg/ml	FEV1 (%Pred)	Drug	IL-8 (pg/ml)
MA7B	Male	26	None	No	>9	102	None	103.3
MA10	Female	20	None	No	>9	110	None	53.12
WM10	Male	35	None	No	>9	112	None	158.6
WM18	Male	18	None	No	>9	108	None	65.49
WM25	Male	22	None	Yes	>9	112	None	72.95
WM26	Female	20	None	No	>9	92	None	96.51
716	Female	50	Mild Asthma	Yes	0.62	91	SAβ2-agonist prn	24.65
WM9	Female	37	Mild Asthma	Yes	0.21	79	SAβ2-agonist prn	40.61
WM15	Female	19	Mild Asthma	Yes	0.53	76	SAβ2-agonist prn	95.18
WM16	Male	28	Mild Asthma	Yes	0.2	84	SAβ2-agonist prn	333
WM20	Male	21	Mild Asthma	Yes	>9	104	SAβ2-agonist prn	172.5
WM24	Male	19	Mild Asthma	Yes	6.2	107	SAβ2-agonist prn	34.52
WM27	Male	21	Mild Asthma	Yes	3.8	97.2	SAβ2-agonist prn	157.1
702	Female	28	Moderate Asthma	Yes	2	115	400µg BDP + LAβ2-agonist	60.9
705	Male	36	Severe Asthma	Yes	-	72	1500µg BDP + LAβ2-agonist	36.07
711	Female	20	Moderate Asthma	Yes	0.25	93	1000µg BDP + LAβ2-agonist	81.46
719	Female	40	Moderate Asthma	Yes	0.41	102	50µg BDP + LAβ2-agonist	28.6
722	Male	43	Moderate Asthma	Yes	3.8	91	200µg BDP + LAβ2-agonist	36.76
725	Female	44	Moderate Asthma	Yes	0.46	96	400µg BDP + LAβ2-agonist	36.76
MJ6	Male	70	Severe Asthma	Yes	-	69.3	7200µg BDP + LAβ2-agonist	41.5
MD10	Female	47	Severe Asthma	Yes	-	81.9	1000µg BDP + LAβ2-agonist	101.6
DS016	Female	19	Severe Asthma	Yes	1.15	125.3	800µg BDP + LAβ2-agonist	6.814

Table 2.2 Bal patient data.

SA = Short Acting
LA = Long Acting
- = Unknown Detail
BDP = Beclomethasone dipropionate equivalent.
PRN = Pro re nata (as required).

CellTiter 96® Aqueous Non-radioactive MTS Assay

The CellTiter 96® Aqueous non-radioactive MTS assay (Promega, Hampshire, UK) was used to assess mitogenesis. The wells were collagen coated with 1:1000 vitrogen collagen solution (3.1mg/ml) (Nutacon, The Netherlands) for one hour before the cells were seeded. The cell pellet obtained after the lifting of the cells was resuspended in FBM and a cell count was performed using a haemocytometer, the cells were centrifuged again at 500xg at 22°C for 5 minutes. The pellet was resuspended in FBM to give 25,000 per ml, and then 100µl of this cell suspension was transferred to the relevant wells on the 96 well plates (Marathon laboratory supplies, London, UK) for 24 hours. The cells were serum deprived with 100µl of 1% ITS medium consisting of DMEM F-12/HAM no phenol red (Sigma Aldrich, Dorset, UK), 1% non-essential amino acids, 1% sodium pyruvate, 1% bovine serum albumin (BSA) (Sigma Aldrich, Dorset, UK), 1% penicillin, streptomycin, L-glutamine, 0.2% fungizone and 1% ITS - insulin (5mg/L), transferrin (5mg/L) and selenium (5µg/L) (Sigma Aldrich, Dorset, UK), which halted cellular proliferation, causing the cells to enter the G_0 phase of the cell cycle. Following serum deprivation for 24 hours the stimuli were added for 72 hours. After the challenge with stimuli the water blank in row 1 was removed and replaced with 100µl of 1% ITS media, then to these media blanks and the remaining test wells 20µl of the assay substrate was added. The plates were incubated for 60 minutes, and then read at 490nm on the Dynatech MR700 microplate reader (Molecular devices, Wokingham, UK), the substrate was left on the plates and the incubation continued for a further 60 minutes, after which the plates were read again.

[^{3}H] Thymidine Incorporation Assay

For the cell mitogenesis assay using the incorporation of [^{3}H] thymidine as an indicator of DNA synthesis, cells were seeded in flat bottomed 96 well plates, which were either coated with a 1:1000 vitrogen collagen solution or left uncoated. The plates were seeded with either 2,500 or 5,000 cells per well and were left to seed for 24 hours, at which point the culture medium was removed and cells were washed twice with 200µl of DMEM, then were serum deprived for 24 hours with 1% ITS medium. After serum deprivation cells were challenged with growth factors or BAL. After challenge 0.5µCi of [^{3}H]

thymidine (Amersham Biosciences, Buckinghamshire, UK) was added to each well for 24 hours. Following the incubation with [^{3}H] thymidine the plates were processed; the serum free medium plus stimuli was removed and the cells were fixed with 100µl of 5% trichloroacetic acid (TCA) (BDH Laboratory supplies, Poole, UK) at 4°C for 10 minutes. After fixing the cells were washed twice with 200µl of 5% TCA, followed by 2 washes with 200µl of 100% methanol (Sigma Aldrich, Dorset, UK), after the second wash of methanol the plates were left for 5 minutes in order for the methanol to evaporate. Once dry the acid insoluble material was dissolved in 50µl per well of 0.2M sodium hydroxide (NaOH) (Sigma Aldrich, Dorset, UK). 40µl per well was transferred to a fresh non-sterile white 96 well plate (Perkin Elmer, Beaconsfield, UK), and to this 160µl of Microscint 40 (Perkin Elmer, Beaconsfield, UK) was added. The plate was sealed with a plate seal (Alpha Laboratories, Hampshire, UK), and read on the Packard Bioscience microplate scintillation and luminescence counter. The results were presented as counts per minute (CPM), and compared to a baseline provided by the ITS control.

The stimuli used so far have been:

basic Fibroblast Growth Factor (bFGF) (Preprotech, London, UK)
Epidermal Growth Factor (EGF) (Preprotech, London, UK)
Transforming Growth Factor β_1 (TGF- β_1) (Preprotech, London, UK)
Transforming Growth Factor β_2 (TGF- β_2) (Preprotech, London, UK)
Tumour Necrosis Factor α (TNF-α) (Preprotech, London, UK)
BAL from healthy, mild asthmatic and moderate/severe asthmatic volunteers

As well as the stimuli various inhibitors have been used to investigate the mitogens effects on the fibroblast and try to link this to activity present in the BAL of the various disease categories:

Pertussis Toxin (Sigma Aldrich, Dorset, UK) – Prevents the G-protein α-subunit $G_{i/o}$ from interacting with receptors, thus blocking its coupling and activation.

Genistein (Sigma Aldrich, Dorset, UK) – Inhibitor of protein tyrosine kinases (PTK).

Wortmannin (Sigma Aldrich, Dorset, UK) – Potent and specific PI3-K inhibitor.

*SU5402 (*Merck Biosciences, Nottingham, UK) – Inhibits fibroblast growth factor receptor 1 (FGFR1).

AG1478 (Merck Biosciences, Nottingham, UK) – Inhibitor of epidermal growth factor receptor kinase.

PD 123-319 di(trifluoroacetate) (Sigma Aldrich, Dorset, UK) - Potent and selective non-peptide angiotensin II receptor-2 (AT2) antagonist.

Proteinase K (Sigma Aldrich, Dorset, UK) - A stable and highly reactive serine protease belonging to the subtilisin family which is able to digest a variety of proteins and nucleases.

Etanercept (Wyeth Laboratories, Berks, UK) - binds specifically to both TNF-α and TNF-β preventing binding to cell surface TNF receptors.

Pan-specific TGF-β neutralising antibody (R&D systems, Oxfordshire, UK) - selectively neutralises the biological activity of human TGF-β1 but shows less than 2% reactivity to human TGF-β2 and human TGF-β3 (Wang H. *et al.* 2003).

U0126 (Merck Biosciences, Nottingham, UK) - Inhibitor of map-externally regulated kinase (ERK) kinase1 (MEK1) and MEK2.

SB203580 (Merck Biosciences, Nottingham, UK) - Cell-permeable inhibitor of p38 MAP kinase.

JNKI1 (Merck Biosciences, Nottingham, UK) - Blocks c-Jun NH_2-terminal kinase (JNK) signalling by preventing the activation of the transcription factor *c-jun*.

The inhibitors were added at varying concentrations depending on their IC_{50} obtained from the manufacturer, and doses which had been used in previous published studies. The inhibitors were used in concentrations at varying doses to assess whether there was a dose dependent inhibitory relationship and also to ensure the inhibitor was used at the optimum concentration. Before addition of the inhibitors the serum free media was removed and replaced with 90µl of fresh 1% ITS medium, to this 10µl of the inhibitor was added for varying incubation times before addition of the challenge. Proteinase K

and Etanercept were added directly to the BAL fluid for 1 hour before challenge, and this kept at room temperature.

The BAL was also fractionated using the Millipore filter devices (Millipore Bioscience, Hertfordshire, UK). The BAL was fractionated into six sizes using three different filter types, the YM3 filter fractionated the BAL into sizes of <3kDaltons (kDa) and >3kDa, the YM30 filter separated the BAL into fractions of <30kDa and >30kDa, and the YM100 filter separated the BAL into fractions of <100kDa and >100kDa. The BAL was pooled into disease groups of healthy BAL and asthmatic BAL, to the YM3 filter 0.5ml of BAL was added at a time as this was all the filter could hold, the filter plus BAL was then centrifuged for two hours at 9,400xg at 4°C, the YM100 filter could also only hold 0.5ml of BAL, but this was spun for 30 minutes at 9,400xg at 4°C, the YM30 filter was larger and could hold 1ml of BAL, this was spun for 30 minutes at 1,500xg for 30 minutes. After spinning the BAL was separated into two fractions the fractionated part below the filter and the retentate which was still above the filter. The filtrate consisted of the factors in the BAL below the filter size while the retentate contained the factors above the filter size. To collect the retentate the filter was inverted and pulse spun into a new tube and resuspended with phosphate buffered saline (PBS). This process was repeated until the required amount of BAL was fractionated, and then the BAL was added to the plate as previously described.

Direct Cell Counting Using Trypan Blue

Assessing the effect of growth factors and BAL fluid has on primary lung fibroblasts proliferation can be assessed by direct cell counting. Trypan blue is a diazo dye which passes through the damaged cell membranes of dying/dead cells, staining them blue. The dye will not pass through the membrane of a viable or living cells, therefore trypan blue may be used to assess cell viability during a cell count. Fibroblasts from primary cultures were seeded in 6 well plates at 100,000 cells/well and left for 48 hours in FBM. After seeding the plates were washed twice with 2ml of DMEM and then serum deprived with 1ml of 1% ITS medium for 24 hours. After serum deprivation cells were challenged with either growth factors or BAL fluid or control medium for 96 hours. Once challenge was complete cells were lifted with 0.5ml trypsin/EDTA per well and transferred to a 1.5ml

microtubes containing 0.5ml FBM. The cells were spun at 500xg for 5 minutes at 22°C to pellet the cells. The cells were resuspended in 100µl of DMEM, the cell suspension was added to trypan blue at a 1:5 dilution (20µl trypan blue 80µl cell suspension) just prior to the count, using a haemocytometer. Before counting 3 squares of the haemocytometer were chosen at random and all samples counted using those 3 squares only, in an attempt to reduce bias. Cell counts used in the results sections consist of viable cells only, numbers of dead cells were negilible.

Lactate Dehydrogenase (LDH) Assay

The lactate dehydrogenase (LDH) in cell supernatants was measured to assess cell viability using the cytotoxicity detection kit (LDH) (Roche applied Biosciences, East Sussex, UK). LDH is a stable cytosolic enzyme that is released upon cell lysis. To assess the cytotoxicity of the inhibitors used cells were seeded in 96 well, flat bottomed culture plates at 5,000 cells per well. The cells were left to seed for 24 hours before being washed with 200µl of DMEM, and serum deprived with 100µl of 1% ITS medium for 24 hours. After serum deprivation the media were removed and replaced with 90µl of 1% ITS, to this 10µl of inhibitor was added. There were 3 concentrations of each inhibitor on the plate, which consisted of the concentration of the inhibitor which was used for the inhibition assays, and a dose 10 fold below and 10 fold above that dose.

As a negative control 100µl of 1% ITS medium was used, for the positive control 5µl of the lysis solution provided in the kit was added to wells containing cells plus 100µl of ITS medium only for 30 minutes at room temperature on a shaker. The LDH reaction mix was prepared, according to the manufacturers instruction, which contained the dye solution (iodotetrazolium chloride (INT) and sodium lactate) and the catalyst (diaphorase/NAD^+). To all the wells 100µl of the reaction mix was added, and the plates then incubated for 30 minutes, in the dark, at room temperature. The reaction was stopped with 50µl of stop solution per well and the plate was read at 490nm on the Dynatech MR700 microplate reader (Molecular devices, Wokingham, UK). The cytotoxicity of each inhibitor was determined using this equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental value} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100\%$$

IL-8 ELISA

The Biosource human IL-8 ELISA kit was used to quantitate the production of IL-8 protein from supernatants collected during analysis of cellular mRNA expression. The initial step of the assay involved the coating of 96 well plates (nunc, Roskilde, Denmark) with anti-human IL-8 antibody. The antibody was diluted 1:1000 in coating buffer, 0.05M carbonate/bicarbonate (Sigma Aldrich, Dorset, UK) pH 7.4. Plates were incubated with 100µl of the coating solution overnight at 4°C. After coating the plates were washed with 400µl wash buffer, PBS/0.1% Tween20 Sigma Aldrich, Dorset, UK), and blocked with 300µl 0.5% BSA in PBS for 2 hours at room temperature. During the incubation the supernatants were thawed and the IL-8 standards prepared by serial dilutions in assay buffer PBS/0.5% BSA/0.1% Tween20. After blocking the plates were aspirated and washed twice in 400µl wash buffer, 100µl of the IL-8 standard or supernatant was pipetted into designated wells and to this 50µl of biotinylated anti-human IL-8 detection antibody, diluted 1:2500 in assay buffer, was added. The plates were incubated for 2 hours on a shaking platform at room temperature, after which they were washed 5 times in 400µl wash buffer. To the plates 100µl of streptavidin-HRP solution, diluted 1:6000 in assay buffer, was added and incubated for 30 minutes on a shaking platform at room temperature. The plates were again aspirated and washed 5 times with 400µl wash buffer, after which 100µl of the TMB (3, 3, 5, 5 tetramethylbenzidine) substrate solution, (containing 0.11M sodium acetate buffer plus 200µl 6mg/ml TMB and 1.2µl 30% H_2O_2 per 12ml), was added to the plates, this was again incubated for 10 minutes on a shaking platform at room temperature. To halt the reaction 100µl of 2M H_2SO_4 was added to the plates, the plates were read at 450nm on the molecular devices Thermomax micro-plate reader. The amount of IL-8 in the supernatant was determined by comparison to a typical standard curve constructed using serial dilutions of the IL-8 standard. The amount of IL-8 protein in BAL was determined in the same manner, as BAL was added at a 1:3 dilution a third of the value measured in the BAL was subtracted from the amount of IL-8 protein measured in the supernatant.

Fibroblast Characterisation

Fibroblasts were characterised by staining for mesenchymal and myofibroblast markers, staining for the mesenchymal protein vimentin was used to show cells were not of epithelial origin, whereas negative staining for α-SMA indicated that cells were not of smooth muscle origin, or had not differentiated into myofibroblasts. Confluent fibroblasts at an early passage (2-4) were trypsinised and seeded in 8 well chamber slides (nunc, Roskilde, Denmark) at 100,000 cells per well. The cells were allowed to seed and grow to ~90% confluence for 1-2 days before the media was removed and 1ml of 100% ice cold methanol added to each well, the slides were left in methanol for 10 minutes at -20°C to fix the cells, care was taken during fixing as the methanol strips adhesion molecules from the cell surface allowing the cell monolayer to detach and fold if slides were tilted too greatly. After 10 minutes the methanol was removed from the slides and they were left to dry, once dry slides were stored at -20°C or stained straight away.

If slides had been stored at -20°C they were allowed to thaw, if not then the chamber was removed using the slide separator and key. Slides were washed in 1x PBS for 2 minutes then blocked with FBM supplemented with 1% BSA for 30 minutes at room temperature. After blocking the slides were washed with 1x PBS for 2 minutes, the PBS was removed and the area in between the chambers was dried to prevent antibodies running into adjacent wells. The primary antibodies were diluted in 1x PBS/3% BSA and incubated for 2 hours, the dilutions were:

- α-SMA (2ml of mouse ascites fluid) (Sigma Aldrich, Dorset, UK) - 1:100
- Vimentin (1mg/ml) (Abcam, Cambridgeshire, UK) - 1:200
- IgG1 (1μg/ml) (Dako, Cambridgeshire, UK) - 1:100
- IgG2a (1mg/ml) (Sigma Aldrich, Dorset, UK) - 1:200

Also on each slide there was a 1x PBS only control in addition to the IgG1 and IgG2a isotype controls. The isotype controls are antibodies directed against non-human antigens, and are a measure of non-specific primary antibody binding that is unrelated to the target antigens. After incubation with the primary antibodies the slides were washed four times with 1x PBS for 5 minutes, the secondary antibody, goat-anti mouse FITC

(Jackson ImmunoResearch Laboratories, Pennsylvania, USA) was prepared using a 1:100 dilution with PBS/3% BSA and incubated on all wells for 1 hour at room temperature in a light protected container. The slides were then washed four times in 1x PBS for 2 minutes and the nuclei counter stained with a 1:5000 dilution of sytox-orange (Invitrogen, Paisley, UK) for 5 minutes at room temperature in a light protected chamber. Slides were then mounted with Mowiol/DABCO (Sigma Aldrich, Dorset, UK) and coversliped.

Human Phospho-MAPK Array Kit

The human phospho MAPK array kit (R&D systems, Oxfordshire, UK) detects the phosphorylated state of nine MAPKs and nine serine/threonine kinases using capture antibodies spotted on a nitrocellulose membrane.

Primary bronchial fibroblasts from six healthy, six mild asthmatic and six severe asthmatic patients were trypsinised and counted as in previous experiments, each fibroblast cell line was resuspended to give 100,000 cells per ml. Three 75cm^2 flasks were seeded with 1ml of either 6 healthy fibroblasts, 6 mild asthmatic fibroblasts or 6 severe asthmatic fibroblasts. Seven ml of FBM was added to each flask to give a final volume of 13ml then the flasks were left to grow to confluence. Once confluent the cells were washed twice with 5ml DMEM then serum deprived with 10ml of 1% ITS media for 24 hours. The cells were challenged for 1 hour with BAL from severe asthmatic volunteers, diluted 1:3 in 1% ITS media to give a final volume of 5ml per flask. After challenge the cells were lysed using 2ml of the lysis buffer provided in the kit and incubated on the Stuart Scientific Gyro-rocker rocking platform at 40 r.p.m, 2-8°C for 30 minutes. After lysis the supernatant was removed to two 1.5ml microtubes and centrifuged at 15,800xg for 5 minutes at 4°C, after centrifugation the supernatant was removed into two fresh 1.5ml tubes and stored at -80°C.

The tubes were thawed and a Bradford protein assay (Bio-Rad, Hertfordshire, UK) was performed to assess the total protein content of the supernatant. The Bio-Rad protein assay is based on the Bradford protein assay and is a dye binding assay in which a colour change of coomassie brilliant blue dye occurs in response to various concentrations of protein. The coomassie blue dye binds to basic and aromatic amino acid residues. To construct standard curve serial dilutions of 10mg/ml BSA were prepared in sample buffer

provided in the protein assay kit. After which 90µl of sample buffer was added to wells on a 96 well plate. To the wells 10µl of either the BSA standard or supernatant and 60µl of the protein assay dye reagent was added. The plates were incubated for 15-30 minutes then read at 570nm. The total protein content of the samples was determined by plotting a standard curve from the absorbance of the BSA standard, and from this the protein content of the supernatants could be determined. The protein content of the supernatants was determined to be between 800-900µg/ml.

The nitrocellulose membranes provided in the human phospho-MAPK array kit were removed from their protective sheets with flat tipped tweezers and placed into array buffer on a rocking platform for 1 hour. The optimum protein concentration for the phospho-MAPK array is 200-300µg/ml, the supernatant was thawed and 250µl added to 1.25ml of array buffer, to give between 130-150µg/ml of protein per sample. This was the maximum that could be used in the case of my samples as the maximum volume of lysates allowed to be used was 250µl per 1.5ml. After 1 hour the nitrocellulose membranes were removed from the array buffer and incubated overnight in the presence of the diluted supernatant on a rocking platform at 2-8°C. The membranes were washed three times in 20ml of wash buffer for 10 minutes then incubated with detection antibody on a rocking platform at room temperature for 2 hours. The membranes were again washed three times in 20ml of wash buffer for 10 minutes and then incubated with streptavidin-HRP for 30 minutes at room temperature on a rocking platform. After incubation the membranes were washed and exposed to chemiluminescent reagents (Amersham Biosciences, Buckinghamshire, UK) for 5 minutes before exposure to x-ray film. The spots on the membranes were also visualised on the BioRad Versa Doc imaging system, model 400.

RNA Extraction

RNA extraction was performed using the TRI® Reagent (Sigma Aldrich, Dorset, UK) RNA Isolation Reagent according to the manufacturers protocol, this system is used to extract RNA from tissues, cells cultured in monolayer, or cell pellets. TRI Reagent® is an improved version of the single-step total RNA isolation reagent developed by Chomczynski (Chomczynski P. *et al.* 1995). The RNA isolation method based on this

reagent is widely used and proven for RNA applications (Chomczynski P. *et al.* 1987). The cells were plated into a sterile 12 well plate (Marathon laboratory supplies, London, UK) at 50,000 cells per well (1ml of 50,000 cells per ml) in FBM. The cells were allowed to seed for 24 hours, then serum deprived with 500µl 1% ITS medium after 2 washes with 1ml DMEM, for a further 24 hours. After 24 hours 250µl of the relevant stimuli was added, which was either BAL, 1ng/ml TGF-β1 or 10ng/ml TNF-α. The stimuli were added at various time-points over a 24 hour period (24 hours, 12 hours, 6 hours, 4 hours, 2 hours and 1 hour) to asses mRNA expression over this period, after which two time-points, 1 and 4 hours, were chosen for subsequent work. To asses the role of receptors, individual factors and intra-cellular signalling molecules, cells were pre-incubated with the same inhibitors used to assess cell mitogenesis before challenge with BAL, TGF-β1, TGF-β2 and TNF-α. After challenge the stimuli and medium were removed into a RNase free 1.5ml microtube (Alpha Laboratories, Hampshire, UK) and spun at 850xg for 5 minutes at 4°C, after spinning the supernatant was aliquoted into 96 well cell culture plates and stored at -20°C for future analysis. The cells were lysed with 0.5ml of Trizol per well for 5 minutes, the cell lysate was then transferred into a 1.5ml RNase free eppendorph and either stored at -80°C for future processing or transferred to ice for immediate extraction.

To the cell lysate 100µl of 100% molecular grade chloroform (Sigma Aldrich, Dorset, UK) was added and the mixture vortexed for 10-15 seconds and left on ice for 10-15 minutes to allow the separation of the clear aqueouss phase, which contained the RNA and forms the upper layer, from the organic phase, which consists of the DNA and protein and forms the lower pink layer. The samples were then centrifuged at 15,800xg for 15 minutes at 4°C, after which the upper aqueous layer was transferred to a fresh RNase free 1.5ml microtube, care was taken not to disturb or collect any of the lower organic layer. To this 250µl of 100% molecular grade isopropanol (Sigma Aldrich, Dorset, UK) was added to the aqueous phase, along with 20mg/ml glycogen (Sigma Aldrich, Dorset, UK) and the RNA precipitated overnight at -20°C.

After 24 hours the samples were briefly vortexed to resuspend any mRNA which may have precipitated and bound to the side of the tube, then centrifuged at 15,800xg for 15 minutes at 4°C to pellet the RNA, after which the supernatant was removed into a

separate container in order to dispose of the isopropanol safely. The pellet was washed with 1ml of 80% molecular grade ice cold ethanol (Sigma Aldrich, Dorset, UK), which was freshly prepared with DEPEC water, the pellet was resuspended via a brief vortex and centrifuged at 15,800xg for 15 minutes at 4°C. The ethanol was removed and the pellet left to dry in a culture hood for 1 hour or until the ethanol has completely evaporated, as the ethanol will interfere with the reverse transcription process. Once the ethanol has evaporated 10µl of RNase free water was used to resuspend the pellet, and the sample was heated to 65°C for 5 minutes on a heating block (Jencons, Leighton Buzzard, UK) to destroy the secondary RNA structure. RNA transcripts can exhibit significant secondary structure that affects the ability of the RNA dependent DNA polymerase (reverse transcriptase, RT) to generate transcripts (Buell G.N. *et al.* 1978). This can affect RT-PCR quantification and should be minimised when comparing or quantifying diverse mRNA populations (Shimomaye E. *et al.* 1989).

The quality of RNA was evaluated by reading the absorbance of 1µl of RNA in 99µl of RNase free water at 260nm and 280nm. A value of around 1.8 suggests good quality RNA, the quantity of RNA was also determined spectrophotometrically by reading the absorbance at 260nm, using the Beckman DU530 UV/visible light spectrophotometer.

Reverse Transcription of RNA

The RNA was DNase treated using Promega's DNase system (Promega, Hampshire, UK), according to manufacturers instruction, RNase-free DNase is a preparation of deoxyribonuclease I that degrades single-stranded or double-stranded DNA to produce 3′-hydroxyl oligonucleotides. For every RNA sample 1µl of DNase buffer was mixed with 1µl DNase enzyme (1U/µl), and 2µl of this was added to each tube. The tubes were briefly vortexed and pulse centrifuged then incubated at 37°C for 30 minutes. After the incubation 1µl stop solution was added to each tube, the tubes were vortexed and pulse centrifuged before being incubated at 65°C for 10 minutes. The reverse transcription was performed using the Primer Design oligo-dT reverse transcription kit (PrimerDesign, Hampshire, UK) using the manufacturers protocol. The Reverse Transcription System reverse transcribes RNA into cDNA, the MMLV Reverse Transcriptase synthesizes single-stranded cDNA from total or poly(A)+ RNA. For each RNA sample the following

reagents were mixed: 5.2µl RNase free water, 4µl 5x buffer, 2µl oligo dT primer and dNTP mix and 0.8µl MMLV enzyme (Reverse Transcriptase). 12µl of the reverse transcription mastermix was added to each RNA sample, the tubes were then vortexed and pulse centrifuged before being put onto the Perkin Elmer DNA thermal cycler 480 at 42°C for 60 minutes to facilitate reverse transcription, the sample was then incubated at 2°C to inactivate the AMV-RT for 5 minutes. The samples were diluted 1:6 (100µl RNase free water) to give a final volume of 122µl, and stored at -80°C.

Quantitative Real Time Polymerase Chain Reaction (PCR)

For real time quantitative PCR the TaqMan® QPCR core kit (Eurogentech, Romsey, UK) was used according to the manufacturers instructions. The iCycler iQ real-time PCR detection system (Bio-Rad, Hertfordshire, UK) was used, along with Thermo-Fast® 96 PCR Plate Non-Skirted (ABgene, Epsom, UK).

The three genes which were investigated were; collagen III (PrimerDesign, Hampshire, UK), CTGF (Eurogentech, Romsey, UK) and IL-8 (Eurogentech, Romsey, UK). Their primer and probe sequences are shown in table 2.1.

Gene	Probe	Forward Primer	Reverse Primer
Collagen III α1 chain (perfect probe)	5'-CTC-GCA-GTC-CAG-GAG-CAC-CAT-TAG-CAC-CAT-AAT-GCG-AG	5'-GTC-CCG-CTG-GCA-TTC-CTG	5'-CTC-TCC-TTT-GGC-ACC-ATT-CTT-AC
CTGF (TaqMan probe)	5'-CAC-GTT-TGG-CCC-AGA-CCG-AAC-TAT-GA	5'-GCG-GCT-TAC-CGA-CTG-GAA	5'-GGA-CCA-GGC-AGT-TGG-CTC-T
IL-8 (TaqMan probe)	5'-CTG-CAA-AGA-GAG-CCA-CGG-CCA-G	5'-AAG-GAA-CCA-TCT-CAC-TGT-GTG-TAA-A	5'-TTA-GCA-CTC-GTT-GGC-AAA-ACT-G

Table 2.3 Primer and probe sequences for TaqMan analysis

The quantity of the target gene expression is measured relative to a perfect probe standard house keeping gene, which in this case was Ubiquitin C (UBC) and phospholipase A2 (A2) (PrimerDesign, Hampshire, UK). This standard uses two probes for 2 different house keeping genes, UBC uses the CY-5 probe which is read at 695nm, while A2 uses

the FAM probe and is read at 490nm, the average of the two readings is used, the target genes are read using the FAM probe also at 490nm.

The TaqMan mastermix contains: 1X reaction buffer (KCl, Tris-HCl), 3mM $MgCl_2$, 200μM dNTP Mix, PCR enzyme (0.025U/μl Taq Polymerase). The PCR reaction for the standard house keeping genes was setup so each sample contained 12.5μl TaqMan mastermix, 6.5μl RNase free water, 1μl probe (4pmol forward and reverse primer and 3 pmol probe), each PCR reaction for the target genes had 7.5μl of the TaqMan mastermix, 1.5μl RNase free water, 1μl of probe (4pmol forward and reverse and 3pmol probe), and 5μl of the reverse transcribed cDNA. The final volume in each standard well was 25μl, while for the test genes it was 15μl. The plates were sealed then read on the iCycler iQ RT- PCR Detection System, before reading the plates were briefly vortexed and centrifuged at 300xg for 1 minute at 22°C, the plates were ran using the following cycles: For the UBC/A2 standard - 95°C for 10 minutes to activate the Taq polymerase (Hot GoldStar), 42 cycles of 95°C for 15 seconds (denaturing), 50°C for 30 seconds (data collection) and 15 seconds at 72°C (annealing/extending).

For the target genes using the perfect probe- 95°C for 10 minutes to activate the Taq polymerase (Hot GoldStar), 42 cycles of 95°C for 15 seconds (denaturing), 50°C for 15 seconds (data collection) and 15 seconds at 72°C (annealing/extending).

For the target genes using TaqMan probes - 95°C for 4 minutes to activate the Taq polymerase (Hot GoldStar), 42 cycles of 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing/extending).

Principles of the Polymerase Chain Reaction (PCR)

The basic purpose of a PCR is to make a large number of copies of a specific DNA fragment (gene). The discovery of this technique is accredited to Kary Mullis in 1983, and the first publication of the work was a 1985 article in *Science* on the detection of the mutation causing sickle cell anaemia in whole genomic DNA (Saki R.K. *et al* 1985). Mullis was later to receive a Nobel Prize in 1993 for the discovery. Although Mullis had laid the foundations of PCR the process still relied on a polymerase isolated from *E.Coli.*

This *E.Coli* polymerase became inactive after heating and had to be replaced after every cycle. It was David Gelfand and his associates who in 1988 discovered purified (Saki R.K. *et al* 1988) and subsequently cloned (Lawyer F. *et al* 1993) a heat stable polymerase in bacterium *T. aquaticus* allowing a complete PCR amplification to be performed without having to open the reaction tube, the polymerase was known as Taq polymerase. The new polymerase also allowed the DNA synthesis step to be performed at a higher temperature which reduced the non-specific products which had been present while using the *E.Coli* polymerase.

The PCR reaction has 3 steps:

1. Denaturation at 95°C - In this step the double stranded DNA is melted to give two single strands of DNA (ssDNA), in this step all enzymatic reactions are also stopped i.e. the extension from the previous cycle.

2. Annealing of primers at 60°C – The forward and reverse primers (short oligonucleotides with the capacity to bind to complementary strands of DNA or RNA) attach specifically to the ssDNA via ionic bonds, the more specific the primer is for the target ssDNA the stronger the ionic bond.

3. Extension at 72°C – The Taq polymerase attaches to the 3' end of the primer and reads the DNA in a 3' to 5' direction adding complementary deoxynucleotide bases as it progresses producing a complementary strand of DNA (cDNA)

As both strands of DNA are copied, and each cycle the number of strands doubles then there is an exponential increase in the number of copies of the DNA; 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 etc.

PCR has many applications such as genetic fingerprinting, paternity testing, genotyping of specific mutations and the detection of hereditary disease. It has also proven to be an invaluable tool in allowing scientists to explore the intra-cellular workings of mammalian and bacterial cells to determine their role in various diseases, while also allowing an insight into viral diseases.

Reverse Transcription-PCR (RT-PCR)

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of low-abundance messenger RNA (mRNA), often obtained from limited tissue samples (Bustin S.A. 2000). The principles are similar to PCR, however instead of using double stranded DNA as your template RNA is used, in particular mRNA. The technique exploits the 5'-3' nuclease activity of the AmpliTaq Gold DNA polymerase to cleave a specific probe sequence that is annealed to the cDNA between two primers. The probe contains a reporter dye covalently bound to the 5' end and a quencher bound to the 3' end. Cleavage of this probe during the PCR reaction leads to fluorescence of the reporter dye, and this can be detected and quantified. mRNA is a copy of the information carried by a gene on the DNA, the role of mRNA is to move the information contained in DNA to the translation machinery. mRNA has a 5 ' cap composed of a 5' to 5' triphosphate linkage between two modified nucleotides: a 7-methylguanosine and a 2 ' O-methyl purine. This cap serves to identify this RNA molecule as an mRNA to the translational machinery; in addition most mRNA molecules contain a poly-adenosine (poly-A) tail at the 3' end.

The reverse transcription of mRNA uses a reverse transcription enzyme; the enzyme requires the presence of primers and can use either specific primers, hexamer primers or oligo-dT primers. The primers anneal to the RNA and the reverse transcription enzyme attaches deoxynucleotides to the 3' end of the primer.

The use of mRNA-specific primers decreases background priming, random hexamer primers will anneal to segments of any RNA type (messenger, transfer and ribosomal) whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analysed from a small sample of RNA (Bustin S.A. 2000), as it will specifically anneal to the mRNA's ploy-A tail.

Quantitative RT-PCR Techniques

There are currently four techniques available that detect amplified product with about the same sensitivity (Wittwer C.T. *et al.* 1997). They use fluorescent dyes and combine the processes of amplification and detection of an RNA target to permit the monitoring of PCR reactions in real-time during the PCR. Their high sensitivity eliminates the need for

a second-round amplification, and decreases opportunities for generating false-positive results (Morris T. *et al.* 1996). The simplest method uses fluorescent dyes that bind specifically to double-stranded-DNA. The other three rely on the hybridisation of fluorescence-labelled probes to the correct amplicon (segment of DNA/RNA in between the forward and reverse primers), these RT-PCR procedures remove the need for post-PCR Southern analysis or sequencing to confirm the identity of the amplicon (Bustin S.A. 2000).

The four techniques are:

1. **Molecular Beacons** – Uses a hairpin structure to keep the fluorescent marker and quencher in proximity. The quencher is a non-fluorescent chromophore that dissipates the energy that it receives from the fluorophore as heat (Bustin S.A. 2000). When molecular beacons encounter a complementary target at the annealing temperature, they undergo a conformational transition, this separates the fluorophore and the quencher, leading to fluorescence which can be detected (Bustin S.A. 2000).

2. **DNA binding dyes** – This assay uses SYBR green dyes ability to bind to any dsDNA (double stranded DNA). In solution the unbound dye has little fluorescence, but when it binds to dsDNA during the polymerisation phase of PCR the fluorescence can be measured. The fluorescence is measured at the end of the elongation step of every PCR cycle as the signal is lost during the denaturing phase. The assay relies on the specificity of the primers as the SYBR green dye will bind to any dsDNA.

3. **Hybridisation probe** - This method uses two hybridisation probes to maximise specificity (Wittwer C.T. *et al.* 1997). The probe at the 3' end is a flourescin donor and emits green fluorescent light when excited by the detectors light source, at the 5' end is an acceptor fluorophore. In solution the two probes are apart but during the annealing phase the two probes attach to

their target sequence and align in a head to tail arrangement, becoming in close proximity to each other. When the acceptor fluorophore and the flourescin donor come together they emit a light at longer wavelength which can be detected. The level of fluorescence is proportional to the amount DNA synthesized.

4. **Hydrolysis probes** - The TaqMan assay utilises the 5' nuclease activity of the DNA polymerase to hydrolyse a hybridisation probe bound to its target amplicon (Bustin S.A. 2000). The assay requires the annealing of 3 oligonucleotides to the DNA, a forward primer, reverse primer and a probe. The probe has a fluorescent reporter dye on the 5' end and a quencher at the 3' end. As the 5' exonuclease activity of Taq and polymerase is double strand specific (Heid C.A. *et al.* 1996), unbound probe remains intact and no reporter fluorescence is detected. When the Taq polymerase reaches the bound probe it cleaves the probe at the 5' end separating the reporter dye and 3' quencher, accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The 3' end of the probe is blocked to prevent extension of the probe.

The increase in fluorescent emission can be read by the Bio-Rad iCycler iQ real-time PCR detection system during the course of the reaction, with the fluorescence being as a direct consequence of gene amplification, it is able to multiplex four different fluorophores per sample tube. The iCycler measures the background fluorescence during calibration, before any probe has been cleaved and uses this to calculate the fluorescence at the end of every cycle.

$$\text{Fluorescence} = \text{Fluorescence at end of cycle} - \text{Baseline fluorescence}$$

Or

$$\Delta Rn = \Delta Rn^{+} - \Delta Rn^{-}$$

This ΔRn is plotted against cycle number, during the early cycles this value does not exceed the baseline. A threshold value is chosen which is said to be 10 times the standard deviation from the baseline between cycle 3 and 15. When the fluorescence exceeds this threshold it is known as the threshold cycle (C_t), the C_t value decreases linearly as the target gene number increases, and this can be used as a quantitative measure of gene expression. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (Gibson U.E. *et al.* 1996).

Quantification of RT-PCR data

There are two strategies used to quantify the results obtained by real-time RT-PCR, these are the standard curve method and the comparative threshold method.

Standard Curve Method

In this method a standard curve is constructed using known concentrations of RNA, this curve may then be used to extrapolate concentrations of unknown mRNA targets. Construction of a standard curve will allow the determination of an absolute copy number in the sample, however the instability of RNA can introduce variability into the standard curve. In addition to the use of RNA standards involves the construction of cDNA plasmids, which then have to be *in vitro* transcribed into the RNA standards and accurately quantitated, it is for these reasons that cDNA plasmids are the preferred standards for standard curve. However, the use cDNA plasmids introduces error in the form of variations in the efficiency of the reverse transcription step, therefore the use of this standard method will only present information on relative changes in mRNA expression. This variation can be corrected by normalization to a housekeeping gene.

Comparative Ct Method

The more favoured quantitation approach is the comparative Ct method, which involves comparing the Ct values of the samples of interest with a control or non-treated sample. The Ct values of both the control and the sample of interest are normalized to an appropriate endogenous housekeeping gene.

The comparative Ct method is also known as the [delta][delta]Ct method, where [delta][delta] Ct = [delta]Ct, sample - [delta]Ct, reference

In this equation [delta] Ct sample is the Ct value for any sample normalized to the housekeeping gene and [delta] Ct reference is the Ct value for the calibrator also normalized to the housekeeping gene.

In any PCR reaction after each cycle the amount of DNA doubles, for example after two cycles we have 2 X 2 times as much, after 3 cycles 2 X 2 X 2 times as much or 2^3 times as much, after 4 cycles 2 X 2 X 2 X 2 times as much or 16 times (2^4) as much. Thus, after N cycles there will be 2^N times as much. The [delta][delta] Ct values are therefore converted into fold change values to represent the amount of target gene mRNA present in the original sample:

$$\text{Fold change in target gene} = 2^{[\text{delta}][\text{delta}]\text{Ct}}$$

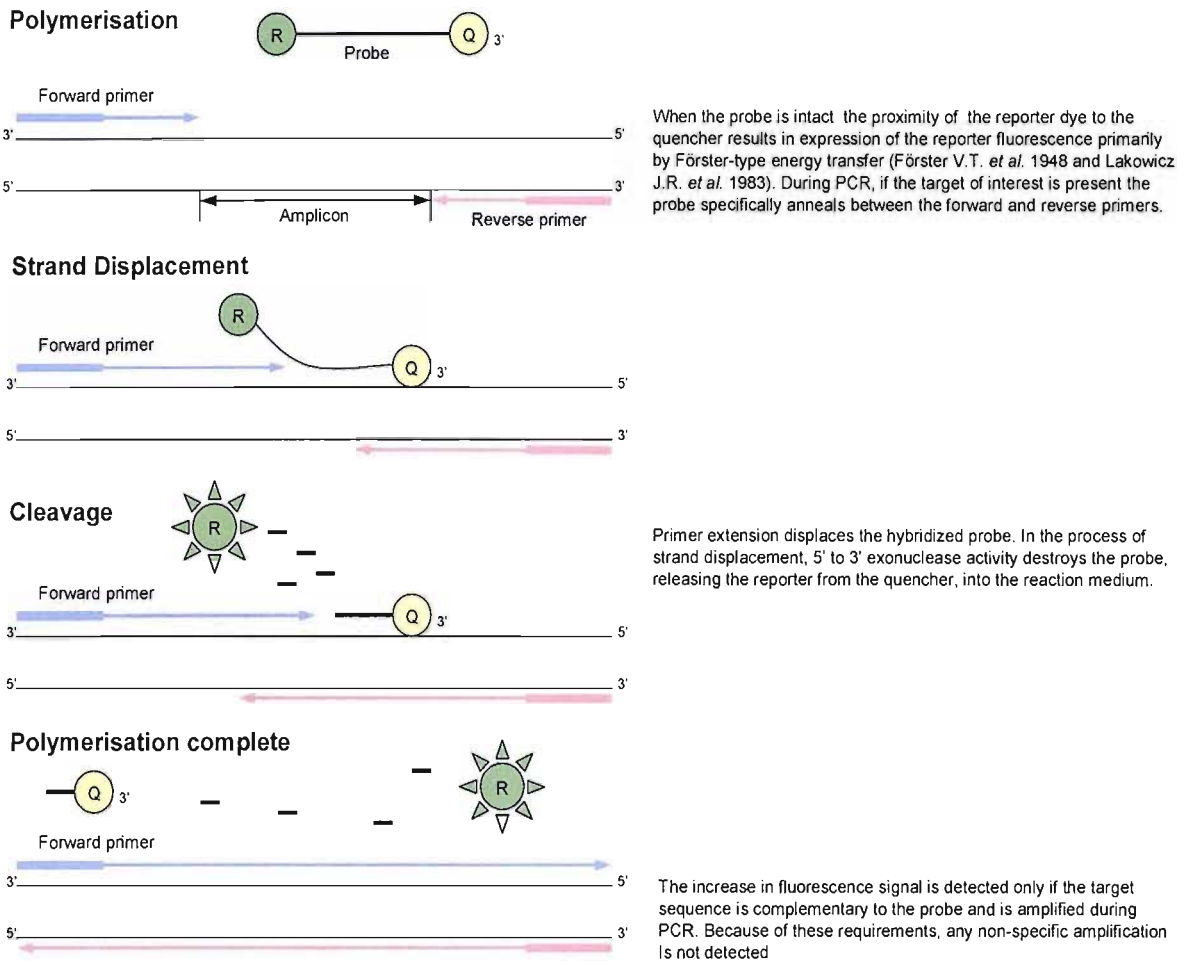


Fig 2.1 Overview of the principle behind TaqMan RT-PCR.

Primers

Forward and reverse primers used in RT-PCR are around 15-20 bases in size and bind to separate exons, minimising false positives arising from amplification of contaminant cDNA. Other considerations when designing primers are the melting temperature (T_m), and the amplicon length. The T_m of the forward and reverse prime shouldn't vary by more than 1-2°C, and should be around 58-60°C, preferably this value will be 10°C lower than the T_m of the probe, these temperatures are the most crucial for TaqMan primers. The primer concentration should be within the 50-200nM range, if the primer concentration is too high there may be mis-priming and an accumulation of non-specific product, whereas too low a concentration would only be a problem if it were rate limiting as the target copy number should have been calculated well before primer supply was exhausted. Non-

specific priming can be minimised by selecting primers with only one or two guanidine or cytosines in the last 5 nucleotides at the 3' end, this instability will make the primers less likely to hybridise transiently and available for non-specific extension by the DNA polymerase (Bustin S.A. 2000). It is also important that the 3' ends of the forward and reverse primers are not complementary as this would lead to primer dimmer formation. The creation and amplification of primer dimmers reduce the efficiency of the PCR reaction by reducing the availability of primers to the template molecule, leading to a decreased sensitivity or failure of the PCR

Probes

Most probes are around 30 base pairs long and have a T_m around 5-10°C higher than the primers, placing their T_m between 68-70°C, allowing annealing of the probe during the extension phase of the PCR cycle. The guanidine/cytosine content of the probe should be 50/50, and for TaqMan analysis there should be no guanidine at the 5' end as this will quench the signal even after cleavage. The concentration of probe used should be around 100nM.

Chapter 3:

Preliminary work

Introduction

Fibroblast hyperplasia is a characteristic observation within the asthmatic airways (Gabbrielli S. *et al.* 1994), with myofibroblast numbers correlating to the thickness of the basement membrane (Brewster C.E. *et al.* 1990). The thickening of the basement membrane contributes to asthma severity by restricting airflow during normal respiration and also by exacerbating asthma attacks. To investigate whether fibroblasts from the asthmatic airway possess an increased ability to proliferate than fibroblasts from healthy volunteers fibroblasts cultured from healthy, mild asthmatic and severe asthmatic bronchial biopsies were challenged with growth factors and BAL from healthy, mild asthmatic and moderate/severe asthmatic volunteers. The use of BAL from healthy and asthmatic volunteers was intended to investigate whether the asthmatic airway environment could affect fibroblast mitogenesis.

The proliferative response can be measured by directly counting an increase in cell number, however this approach is labour intensive and assays such as the MTS assay and the [^{3}H] thymidine incorporation assay have been developed as high throughput indicators of cellular proliferation, without counting an actual increase in cell number. Before the responses of healthy and asthmatic fibroblasts could be compared it was important to investigate which of these assays would provide us with the most reproducible data. The MTS and [^{3}H] thymidine incorporation assays were performed under a range of conditions, using known fibroblast mitogens such as bFGF (Chambard J.C. *et al.* 1987, Inoue Y. *et al.* 2002 and Chen Y. *et al.* 2003), EGF (Chambard J.C. *et al.* 1987 and Huang N.N. *et al.* 1993) and low concentrations of TGF-β1 (McAnulty R.J. *et al.* 1997), as well as FCS, as FCS contains many mitogens and it was assumed that it would initiate fibroblast mitogenesis.

As bronchial biopsies will often contain fibroblasts along with populations of epithelial and smooth muscle cells, it was important to characterise the cells present in the cultures grown out of these biopsies, as described in the methods section. The fibroblasts cultures were stained for α-SMA and a mesenchymal marker protein vimentin, cells staining positive for vimentin were likely to be of fibroblast or smooth muscle and not of epithelial origin, while cells staining positively for α-SMA were likely to be of myofibroblast or smooth muscle origin. Therefore cells staining positively for vimentin,

but negatively for α-SMA were likely to be of fibroblast origin. It is, however, difficult to differentiate between myofibroblasts and smooth muscle cells when cells have stained positively for α-SMA and vimentin. The characterisation of fibroblasts from epithelial cells, smooth muscle and myofibroblasts using the identification of vimentin and α-SMA proteins is a well documented technique (Kuhn C. *et al.* 1991, Ludwicka A. *et al.* 1992, Touhami A. *et al.* 2005 and Kaarteenaho-Wiik R. *et al.* 2007).

Methods

CellTiter 96® aqueous non-radioactive MTS assay

The CellTiter 96® aqueous non-radioactive MTS assay described in the methods section was used to assess fibroblast mitogenesis after stimulation with growth factors. The stimuli were incubated on the plates for 72 hours before addition of the MTS substrate. Plates were either collagen coated with 1:1000 vitrogen solution for one hour or left uncoated. Cells were serum deprived with 1% ITS medium. The fibroblasts used were primary cell lines grown from biopsy from healthy individuals, and were seeded at 2,500 cells per well. Plates were read at 490nm at two time-points 60 or 120 minutes. For all plates 1% ITS media only wells were used as controls. Columns represent the mean data with standard error bars.

[^{3}H] thymidine incorporation assay

The effect of growth factors on healthy and mild asthmatic primary human fibroblasts grown from biopsy was analysed using the [^{3}H] thymidine incorporation assay, as described in the methods section. Plates were collagen coated with a 1:1000 vitrogen collagen solution for one hour or left uncoated, and cells seeded at either 2,500 or 5,000 cells per well. For all plates ITS only wells were used as controls. Columns represent the mean data with standard error bars.

Direct Cell Counting

The effect of growth factors on fibroblast proliferation was assessed using direct cell counting, as described in the methods section. Cells were seeded at 100,000 cells/ml in 6 well 1:1000 vitrogen collagen coated plates, and challenged with bFGF, EGF, TGF-β1

and FCS. Cells treated with 1% ITS media only were used as negative controls. Cells were counted using trypan blue at a 1:5 dilution, each well was counted 3 times. Counts consisted of viable cells only. Bars represent column means and standard error bars.

Characterisation of primary fibroblasts

Primary airway fibroblasts were characterised by staining for vimentin and α-SMA as described in the methods section, cells were seeded at 100,000 cells per well. For negative controls PBS only, IgG1 and IgG2a were used to check for non-specific secondary antibody binding and to assess the level of primary antibody binding unrelated to the target antigens.

Statistics

The Wilcoxon signed rank test was used to analyse the effect of growth factors and inhibitors on fibroblast mitogenesis with both the MTS assay and the [^{3}H] thymidine incorporation assay. For all assays each treatment was repeated at least in triplicate. To assess dose response to challenge the Kruskal-Wallis and Mann Whitney tests were performed, $p < 0.05$ indicates significance.

Aims

1. To compare two established *in vitro* mitogenesis assays to determine which would provide the most reproducible results, and hone the assay to discover optimal conditions for assessing fibroblast mitogenesis, using known growth factors.
2. To characterise the purity of the primary cultured fibroblast populations by visualising vimentin and α-SMA proteins using monoclonal antibodies.

Results

The effect of growth factors on healthy fibroblast [^{3}H] thymidine incorporation and MTS metabolism when cells were seeded at 2,500 cells/well.

The data in fig 3.1 and 3.2 show the response of primary airway fibroblasts obtained from healthy donors, seeded in non-collagen coated wells at 2,500 cells per wells, and challenged with growth factors. The responses were assessed using the MTS metabolism assay and the [^{3}H] thymidine incorporation assay. For the MTS assay one healthy fibroblast cell line was used and the data represent the data from one experiment performed in duplicate, for the [^{3}H] thymidine assay two healthy cell lines were used and the data represent the findings from two assays performed individually.

Statistical analysis of these data could not be performed as there were insufficient fibroblast numbers used, however it can be observed that both of these assays show a poor response to increasing doses of challenge. Of the two assays the [^{3}H] thymidine incorporation assay provided the greatest cellular response to challenge when compared to the control. In fig 3.2 there is around a 7 fold increase in [^{3}H] thymidine incorporation after challenge with FCS compared to the control, and this is likely to be indicative of cells preparing to divide, suggesting that the [^{3}H] thymidine assay is able to show cells responding to a mitogenic challenge. The MTS metabolism seen in fig 3.1 however, is unlikely to correlate to an increase in cell number, as the absorbance at 490nm seen in challenged cells often does not show an increase in absorbance vastly greater than that seen in unchallenged cells. As the absorbance at 490nm directly correlates to an increase in the metabolism of MTS, which should represent the number of cells present, around a doubling in the absorbance at 490nm, compared to the control, would be required to indicate an increase in cell number. This is not observed even in the largest response seen in fig 3.1, where the cells are challenged with 8% FCS, suggesting that there is little increase in cell number at the time measured. The initial data suggests that the [^{3}H] thymidine incorporation assay is a more sensitive assay than the MTS metabolism assay.

The data showed little dose response and in an attempt to improve the quality of the data it was decided to coat the plates with 1:1000 vitrogen collagen solution. When cells bed down onto plastic wells it is likely that they lay down matrix proteins to help anchor them to the surface, this process requires the cells to produce proteins, and thus the cells may

take longer to begin mitogenesis after exposure to challenge. The aim of collagen coating the wells was to encourage the cells to enter mitogenesis more readily.

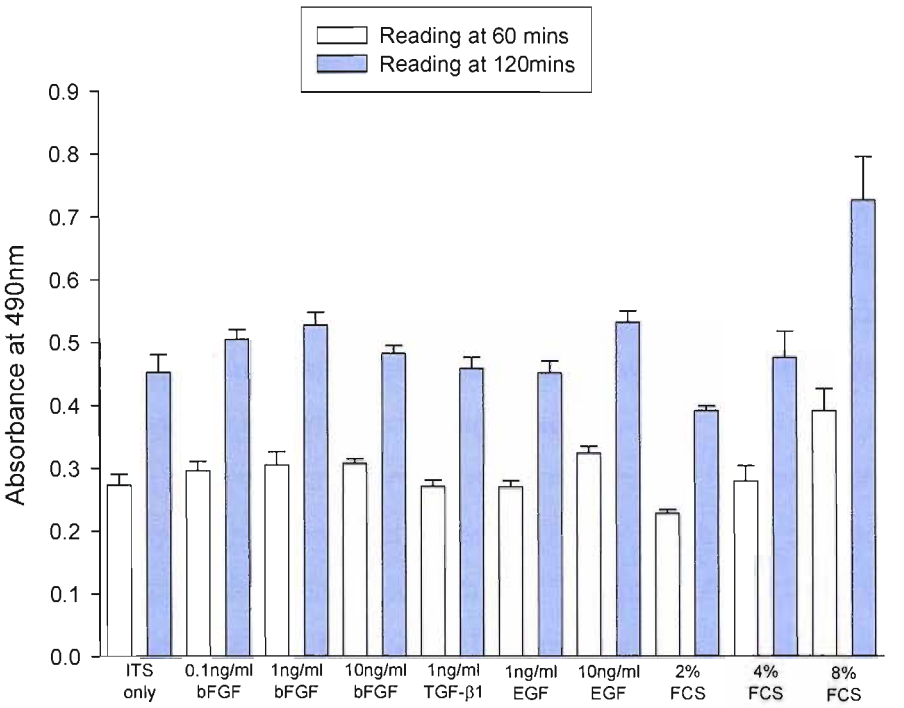


Fig 3.1 MTS metabolism assay used to assess the effect of growth factors on healthy lung fibroblasts (n = 1). Wells were seeded at 2,500 cell/well in non-collagen coated wells.Stimuli was left to incubate on the plate for 72 hours before addition of MTS substrate. The plate was read at 60 and 120 minutes at a wavelength 490nm. The data represents the mean from 1 experiment, repeated in duplicate.

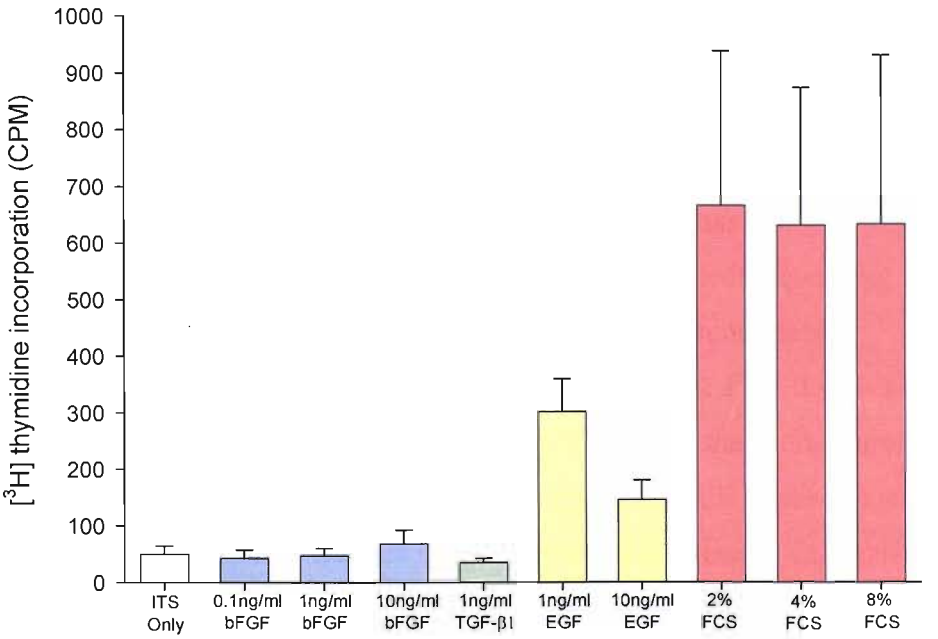


Fig 3.2 The effect of growth factors on healthy primary fibroblast (n = 2) mitogenesis assesed using the [^{3}H] thymidine incorporation assay. Cells seeded at 2,500 cells per well in non-collagen coated wells. Stimuli was incubated for 24 hours before addition of [^{3}H] thymidine. The data represents the mean from 2 experiments.

The effect of growth factors on healthy fibroblast [^{3}H] thymidine incorporation and MTS metabolism when cells were seeded at 2,500 cells/well in collagen coated plates.

Fig 3.3 and 3.4 show the effect of various growth factors assessed by the MTS and [^{3}H] thymidine incorporation assays on healthy fibroblasts, when cells were seeded at 2,500 cells per well on collagen coated plates. For the MTS assay two healthy cell lines were used and the data represents the findings from two assays performed individually, for the [^{3}H] thymidine assay one healthy fibroblast cell line was used and the data represents the data from one experiment performed in duplicate.

Collagen coating the wells gave a more stable response to increasing doses of challenge from the [^{3}H] thymidine incorporation assay (fig 3.4), although statistical analysis was not able to be performed on these data (fig 3.3 and 3.4) as there were insufficient fibroblast numbers. The data from the [^{3}H] thymidine incorporation assay show around a 4 to 8 fold increase in [^{3}H] thymidine incorporation after challenge when compared to the controls, and this is likely to be suggestive of potential cellular division. However collagen coating the wells did not improve the sensitivity of the MTS assay, with poor dose response observed after challenge. As with fig 3.1, there was little increase in absorbance at 490nm observed in challenged cells compared to the control wells, suggesting that the MTS assay is unsuitable for assessing fibroblast proliferation under the conditions being used in these assays. It may be that increasing the incubation time with challenge may increase the sensitivity of the MTS assay, as unlike the [^{3}H] thymidine assay the MTS assay is more reliant on an actual increase in cell number. In this respect the MTS assay is a more suitable assay for assessing cellular proliferation, however the [^{3}H] thymidine assay is a well established technique and, from the above work, appears to be a more sensitive and reliable method in my hands.

On the evidence of the above data it was decided that the [^{3}H] thymidine incorporation assay performed in collagen coated wells was providing the more convincing data, and that this assay would be used in future work. The assay still required optimisation as the dose response to challenge was not ubiquitous to all challenges. In an attempt to provide more consistent data it was decided to seed the cells at a higher density on collagen coated wells. Seeding the cells at a higher density would provide a mitogenic response

from a greater number of cells, and was hoped to provide more consistent data than that witnessed after seeding cells at 2,500 cells/well.

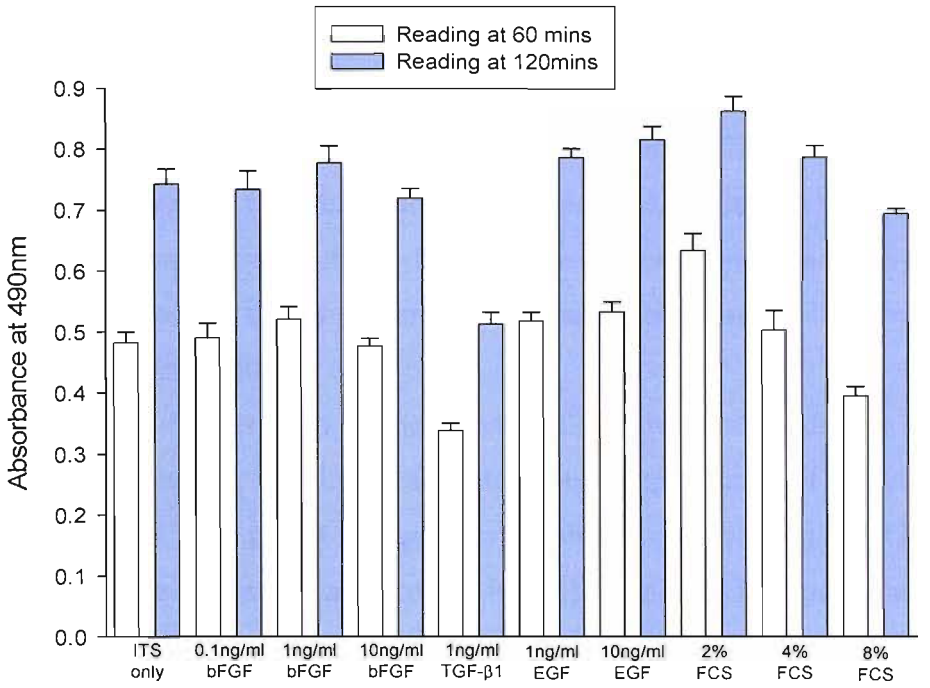


Fig 3.3 MTS metabolism assay used to assess the effect of growth factors on healthy lung fibroblasts (n = 2). Wells were collagen coated with 1:1000 vitrogen solution for 1 hour and cell seeded at 2,500 cell/well. Stimuli was left to incubate on the plate for 72 hours before addition of MTS substrate. The plate was read at 60 and 120 minutes at a wavelength 490nm. The data represents the mean from 2 experiments.

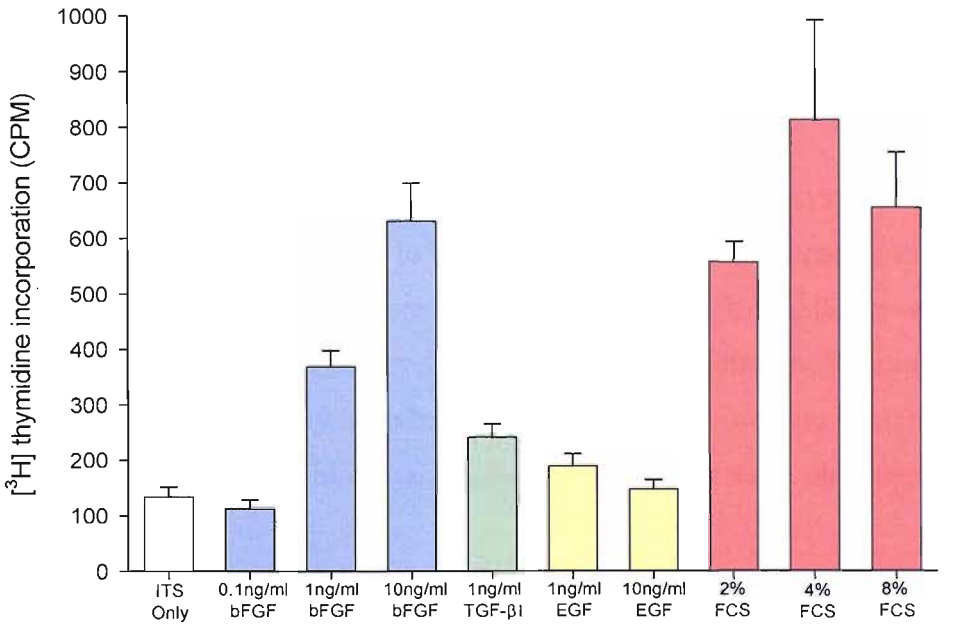


Fig 3.4 The effect of growth factors on healthy primary fibroblast (n = 1) mitogenesis assesed using the [^{3}H] thymidine incorporation assay. Cells seeded at 2,500 cells per well 1:1000 vitorgen collagen coated wells. Stimuli was incubated for 24 hours before addition of [^{3}H] thymidine. The data represents the mean from 1 experiment, repeated in duplicate.

The effect of growth factors on healthy and mild asthmatic fibroblast [^{3}H] thymidine incorporation when cells were seeded at 5,000 cells/well in collagen coated plates.

In figs 3.5 and 3.6, healthy fibroblasts and mild asthmatic fibroblasts were seeded at 5,000 cells per well in collagen coated wells, and then challenged with various growth factors. Both figures represent the data from two experiments, one using two healthy fibroblast cell lines and the other two mild asthmatic fibroblast cell lines, with the assays being performed individually.

The data in these figures show on the whole that cells were responding to challenge in a dose dependent manner, and that these findings were reproducible. The figures show that both bFGF and FCS are potent mitogens for fibroblasts, and that 1ng/ml TGF-β1 does not appear to be able to lead to an increase in [^{3}H] thymidine incorporation at this dose. These observations are supported by figure 3.4, however it was not until figs 3.5 and 3.6 that there was a more consistent response to dose. The data also suggest that healthy fibroblasts possess a greater mitogenic potential, around double, than do mild asthmatic fibroblasts, however this observation would require further scientific scrutiny.

The assays also show that EGF is not eliciting a dose response in mild asthmatic fibroblasts, and this may be due to either experimental variation or an altered response from mild asthmatic fibroblasts. If this work were repeated then there may be a dose response to EGF observed from mild asthmatic fibroblasts, however this may also represent altered behaviour from mild asthmatic fibroblasts.

Nevertheless, it was decided that the conditions used in these assays were providing data of a high enough quality for them to be used for future work, comparing the responses of healthy, mild asthmatic and severe asthmatic fibroblasts to challenge with BAL. To investigate whether the data observed from the [^{3}H] thymidine incorporation assays (fig 3.5 and 3.6) could reasonably be extrapolated into an actual increase in cell number direct cell counting using trypan blue was performed after cells were challenge with growth factors.

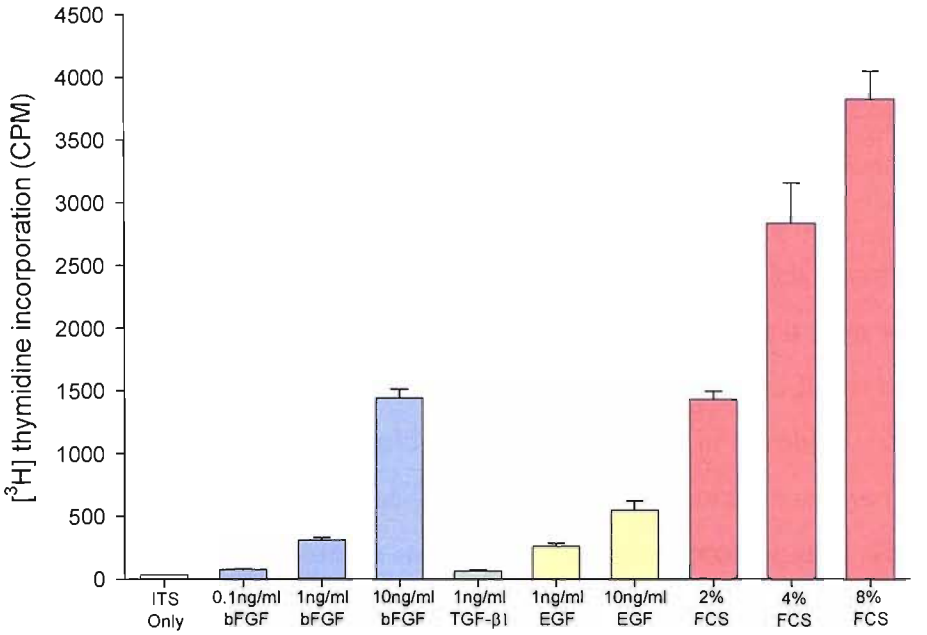


Fig 3.5 The effect of growth factors on healthy primary fibroblast (n = 2) mitogenesis assesed using the [^{3}H] thymidine incorporation assay. Wells were collagen coated with 1:1000 vitrogen solution for 1 hour, and cells seeded at 5,000 cell per well. Stimuli was incubated for 24 hours before addition of [^{3}H] thymidine. The data represents the mean from 2 experiments.

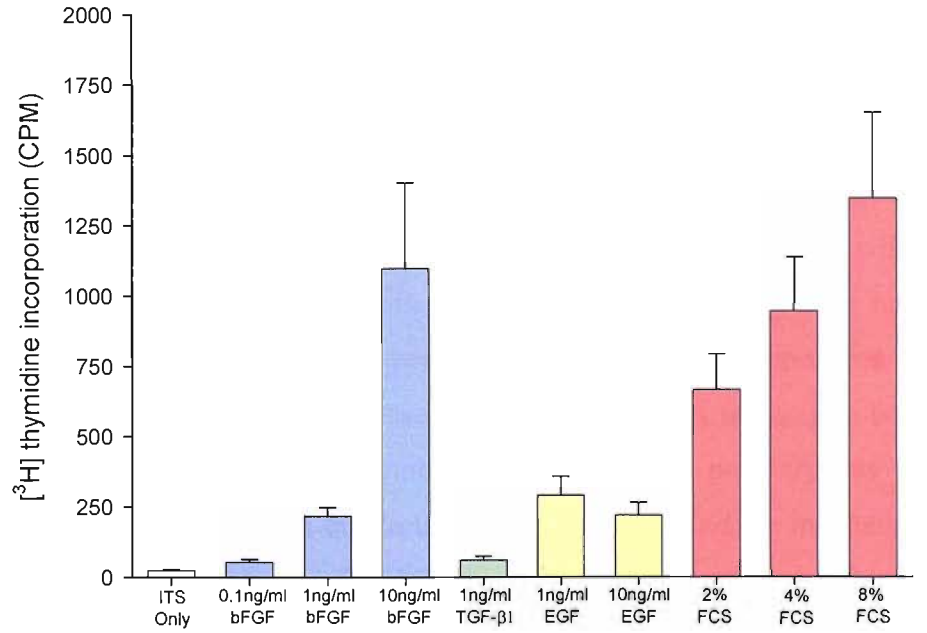


Fig 3.6 The effect of growth factors on mild asthmatic primary fibroblast (n = 2) mitogenesis assesed using the [^{3}H] thymidine incorporation assay. Wells were collagen coated with 1:1000 vitrogen solution for 1 hour, and cells seeded at 5,000 cell per well. Stimuli was incubated for 24 hours before addition of [^{3}H] thymidine. The data represents the mean from 2 experiments.

The effect of growth factors on healthy fibroblast proliferation, assessed via direct cell counting, when cells were seeded at 100,000 cells/well.

Fig 3.7 shows the effect of growth factors on primary lung fibroblast (1 healthy, 1 mild asthmatic and 1 severe asthmatic) proliferation, assessed by direct cell counting utilising trypan blue, the assays were performed individually and the observations pooled, counts include viable cells only, no statistical analysis could be performed.

It was important to determine whether the challenges which were mitogenic for fibroblasts in figs 3.5 and 3.6 would also be fibroproliferative, as it is an accepted that not all cells that incorporate [^{3}H] thymidine will go on to proliferate. It can be seen from the data that bFGF, EGF and FCS could all elicit an increase in fibroblast number compared to the control, however, as with the [^{3}H] thymidine incorporation assays (fig 3.5 and 3.6) 1ng/ml TGF-β1 did not cause an increase in fibroblast proliferation. There was also a good dose response to EGF and bFGF, with the response to FCS being slightly more variable. The data therefore show that the mitogenic response seen in figs 3.5 and 3.6 could be translated into an increase in cell number, however the data also show that the [^{3}H] thymidine assays were more sensitive than direct cell counting. Data from the [^{3}H] thymidine assays show that there was up to a 40 fold increase in [^{3}H] thymidine incorporation after challenge, whereas with the same challenges there was only a 1.5 to 2.5 fold increase in cell number (fig 3.7). This is likely due to a number of considerations; firstly the [^{3}H] thymidine assay is only a marker of newly synthesised DNA, with only a proportion of cells incorporating [^{3}H] thymidine then going on to complete mitosis. It is probable that some of the cells which incorporate [^{3}H] thymidine will then undergo apoptosis or necrosis, due to events such as random DNA copy error or cellular stress. Therefore the large magnitude of increased [^{3}H] thymidine incorporation seen in figs 3.5 and 3.6, where a 30-40 fold increase can be observed was unlikely to be shown in data looking at an increase in cell number. However this is not only due to not all cells completing mitosis, but also due to the size of the wells and the incubation time used in the direct cell counting assays (fig 3.7). As the cells were seeded at 100,000 cells per well it was physically impossible for them to increase their cell number more that 3-4 times, as contact inhibition once they reached confluence would prevent further division, in addition to this the 96 hour incubation is likely to have limited the cells to only 2-3

divisions. Taking this into consideration the direct cell counting data was more a conformation that the effects of each challenge in the [^{3}H] thymidine assays were translating into actual increases in cell number, rather than a verification of the relative magnitudes of stimulation.

It may also of been pertinent to have performed the MTS assay under the same conditions that the direct cell counting were performed, this is because the direct cell counting had shown that the cells could increase their number at 96 hours. If the MTS assay had been performed in conjunction with the direct cell counting and still shown no significant increase in absorbance at 490nm then it would further confirm that the assay was not suitable for this particular study.

Although it is still difficult to interpret findings from the [^{3}H] thymidine assays in terms of extrapolation out to an increase in cell number, the data from figs 3.5, 3.6 and 3.7 suggest that in my assays an increase in [^{3}H] thymidine incorporation after challenge is likely to relate to an impending increase in cell number, albeit an increase of a lower magnitude than the [^{3}H] thymidine assay suggests.

Although the [^{3}H] thymidine assay had been chosen as the most suitable assay by which to assess the mitogenic responses of healthy, mild asthmatic and severe asthmatic fibroblasts after BAL challenge, it was still necessary to ensure that the cells to be used in future work contained a high fibroblast percentage. It is an unfortunate consequence of obtaining fibroblasts from bronchial biopsy that cultures of fibroblasts will contain varying levels of myofibroblasts, smooth muscle and possibly epithelial cells. Staining the cells for vimentin and α-SMA allows the rough quantitation of the relative percentages of fibroblasts to myofibroblasts/smooth muscle, however, as already mentioned, differentiation between myofibroblasts and smooth muscle is difficult. To ensure cells used for future work would be of primarily fibroblast origin cells were stained for α-SMA and vimentin.

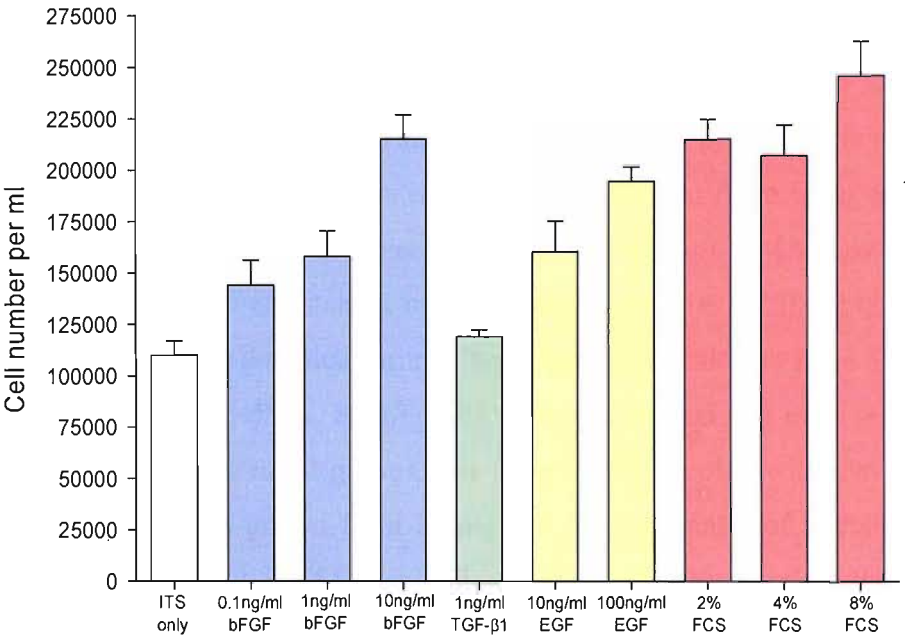


Fig 3.7 Graph showing the effect of growth factors and FCS on human primary lung fibroblast (n = 3) proliferation assessed via direct cell counting using trypan blue, after a 96 hour incubation period. The data represents the mean from 3 experiments.

Characterisation of cells in culture by staining for α-SMA and vimentin.

The characterisation of fibroblasts using α-SMA and vimentin staining is shown in fig 3.8. It can be seen from the negatively stained PBS control that there is no non-specific secondary antibody binding occurring during the staining process. The negative staining shown in the IgG1 and IgG2a controls also indicates that there is no primary antibody binding that is unrelated to the target antigens. Staining for α-SMA showed that there are generally fewer positively stained cells in healthy cultures of fibroblasts than in mild asthmatic or severe asthmatic cultures; however in all cultures there is predominantly negative staining for α-SMA. Staining for vimentin showed that there are predominantly positively stained cells in all groups, this observation coupled with the α-SMA staining suggests that the cells grown from biopsy are predominantly of fibroblast origin, with little or no epithelial cells and few myofibroblasts or smooth muscle cells. It is difficult to accurately quantify the percentage of cells stained positively for α-SMA, however there was around 90-95% positive staining for vimentin in all groups in the fibroblasts characterised in this study. The levels of α-SMA staining varied between the groups with around 0-10% positive α-SMA staining in the healthy fibroblasts, 10-20% positive staining in the mild asthmatic fibroblast group, and 20-30% positive staining in the severe asthmatic fibroblast group. The higher levels of α-SMA stained cells in the asthmatic groups does present a problem when it comes to analysing any data produced from subsequent assays, as you aren't comparing groups comprised of exactly the same cell populations. Nevertheless, fibroblasts are still the principle cell present in our cultures, and to this end the responses witnessed in future work will likely be due, in the majority, to fibroblast activity. However, consideration to the contribution of myofibroblasts and smooth muscle will have to assessed when analysing any data.

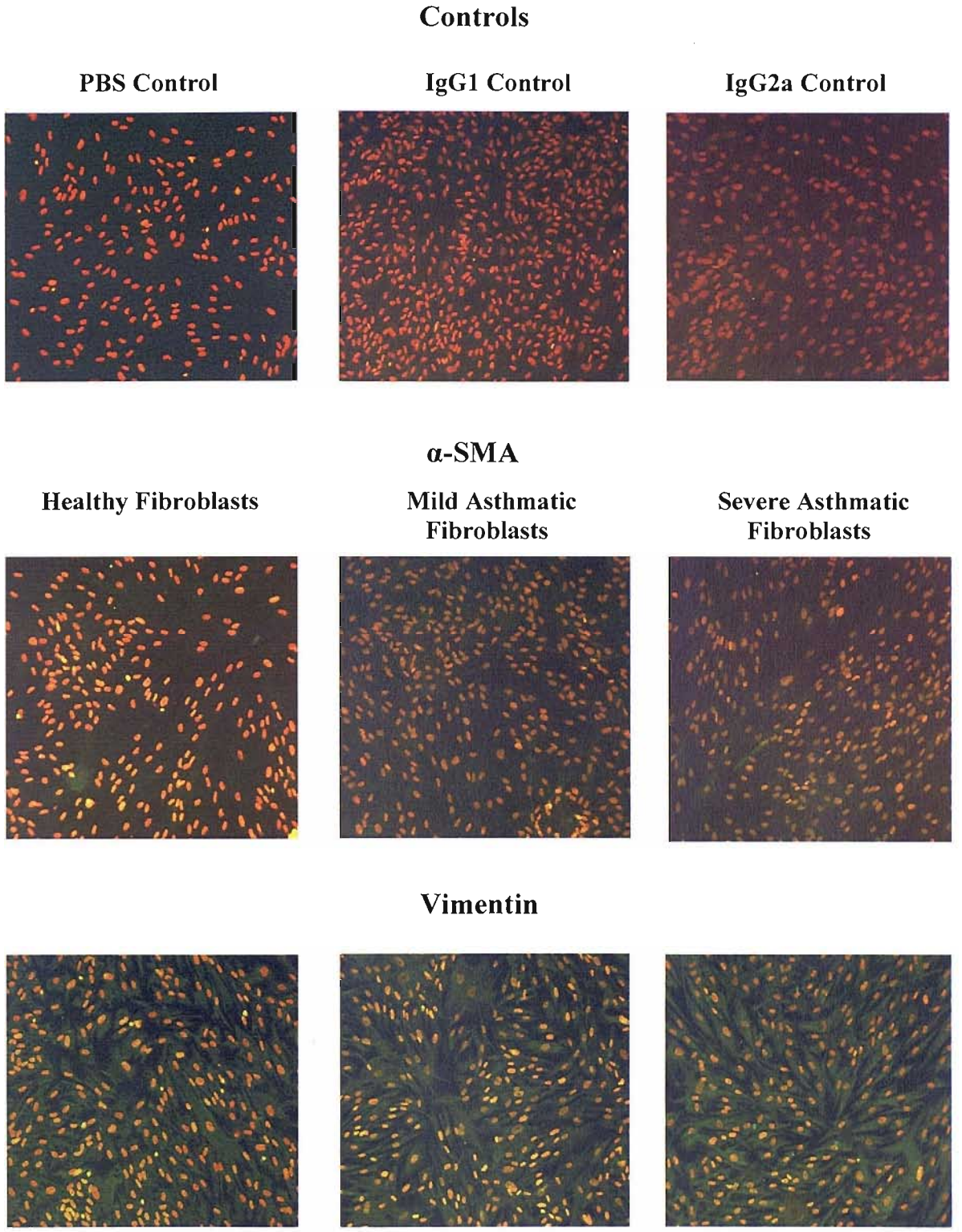


Fig 3.8 Fibroblast characterisation. Red staining represents nuclei, while green staining either represents staining of α-SMA or vimentin positive cells.

Discussion

These experiments were designed to establish the most reproducible assay by which to assess the mitogenic/proliferative response of fibroblasts after challenge with growth factors, and also to characterise the cells being used as predominantly of fibroblast origin. The MTS assay measures the quantity of MTS converted to soluble formazan (Barltrop J.A. *et al.* 1991) by the mitochondrion (Martinez E.J. *et al.* 1999); this is measured colourmetrically at 490nm absorbance and is directly proportional to the number of living cells in culture (Cory A.H. *et al.* 1991 and Riss T.L. *et al.* 1992) and the time of incubation of the cells with MTS (Promega technical bulletin). The [^{3}H] thymidine assay can be used to assess the amount of DNA synthesis after challenge, and is thus an indirect indicator of cellular proliferation. The assay takes into account the fact that RNA does not contain thymidine, cells will therefore utilise little [^{3}H] thymidine when they are in the G1 phase of the cell cycle (normal cell growth), but rather the incorporation of [^{3}H] thymidine will be primarily due to DNA synthesis during the S phase of the cell cycle. The incorporation of the radio-labelled thymidine can be measured via the addition of a scintillation fluid, and correlates to the amount of newly synthesised DNA. Both these assays are well established techniques for measuring fibroblast proliferation (Cory A.H. *et al.* 1991, Dube J. *et al.* 1998, Silvestri M. *et al.* 2001, Bunger C.M. *et al.* 2002, Scaffidi A.K. *et al.* 2002, Xu J. *et al.* 2002, Ouyang P. *et al.* 2004a and Ouyang P. *et al.* 2004b)
Although the two assays used to assess mitogenesis in the chapter are techniques widely used by other researchers, they each have limitations in that neither assay is a direct measure of an increase in cell number/proliferation. In the case of the MTS assay, primary fibroblast cell lines with more active mitochondria may be able to reduce more MTS into formazan than other fibroblasts, meaning the amount of formazan produced wouldn't be a direct measure of cell number, but rather of cellular activity. With regard to the [^{3}H] thymidine assay, the incorporation of thymidine is due to cells priming to undergo mitosis in the S phase. It is not a measure of an increase in cell number because not all cells synthesising DNA will undergo mitosis, some may apoptose/necrose. Also requiring consideration is that the incubation time of 24 hours in these experiments was not be long enough for fibroblasts to complete the cell cycle, therefore the incorporated [^{3}H] thymidine may not have been destined for cellular division. For these reasons it was

necessary to perform direct cell counting in conjunction with the MTS and [^{3}H] thymidine assay to assess whether an increase in thymidine incorporation or MTS reduction would directly correlate with an increase in cell number.

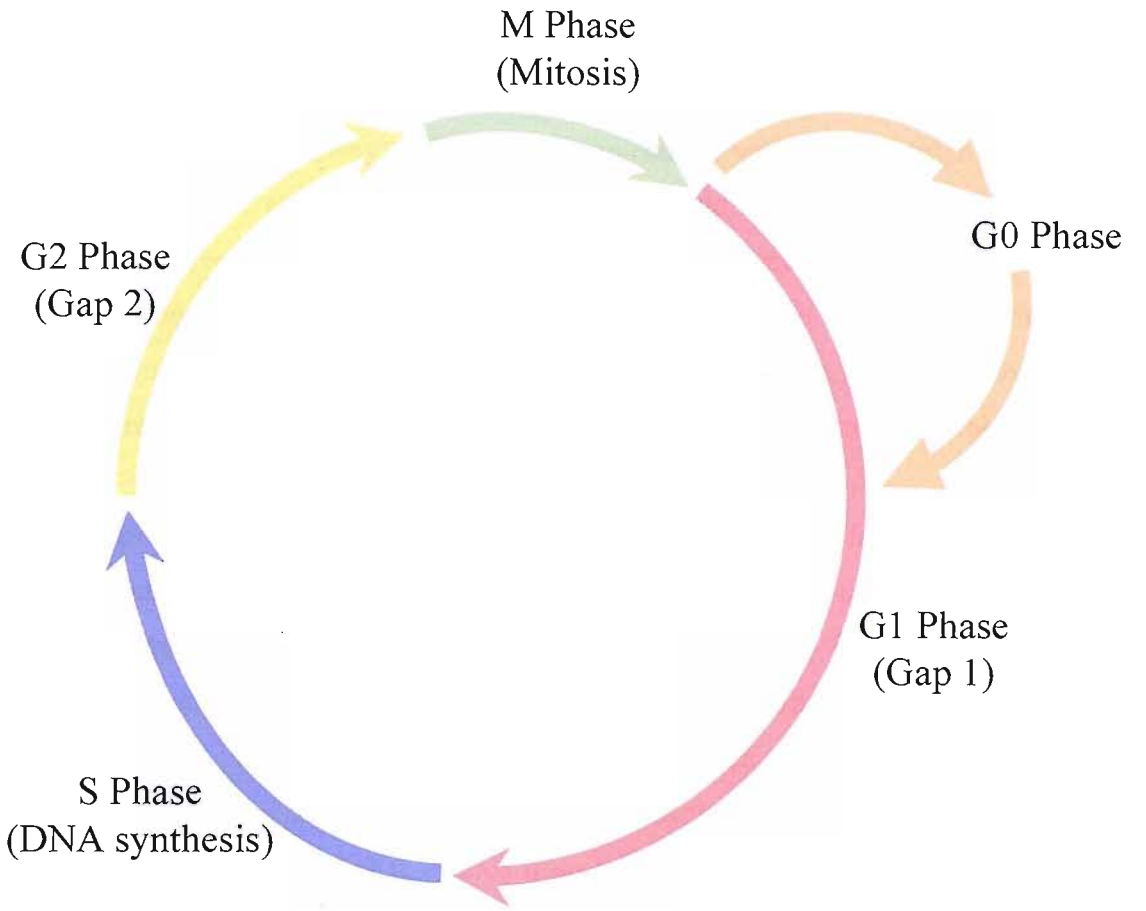


Fig 3.9 The cell cycle. The M or mitosis phase is where nuclear division occurs to produce two identical daughter cells. Mitosis consists of prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Following mitosis, the daughter cells may re-enter the G1 phase, or go into G0 phase. G1 phase is the major period of cell growth due to protein synthesis, where as cells in G0 are said to be quiescent, meaning cell growth and replication stops. Cells in G0 may eventually re-enter G1 or they may die. The S phase is where DNA synthesis occurs. G2 phase follows successful completion of DNA synthesis, at the end of this phase there is a control checkpoint which determines whether the cell can proceed onto M phase and divide. The G2 checkpoint prevents cells from entering mitosis with DNA damage obtained since the last division or during the production of newly synthesised DNA. This provides an opportunity for DNA repair and prevents the proliferation of damaged cells.

Taking into account the data shown in figs 3.5 and 3.6 it was decided that the [^{3}H] thymidine assay provided the most reproducible data, along with good dose responses compared with the MTS assay, which was not as consistent in my hands (fig 3.1 and 3.3).

The data from the [^{3}H] thymidine assay, along with the data from direct cell counting (fig 3.7) after challenge with growth factors, shows good correlation suggesting that the [^{3}H] thymidine assay may be used as a marker of cellular proliferation. MTS and [^{3}H] thymidine assays are used instead of direct cell counting as they are more rapid, allowing higher throughput, and are less susceptible to human error with regard to quantifying data. This is because they utilise automated counting systems, in the form of a measure of absorbance with the MTS assay, and a scintillation counter with the [^{3}H] thymidine assay. It was thought that collagen coating the wells would more accurately reflect the environment *in vivo*, and also provide a partial matrix for the fibroblasts to use in order to 'bed down' more rapidly, this may allow the cells to move more swiftly into the mitogenic cycle. The data suggested that collagen coating the wells had led to a more stable dose response to stimuli, and that these data were also more reproducible (figs 3.4 to 3.6).

During the preliminary work the effect of cell number per well and incubation time were also investigated to optimise these conditions. It was found that the most reliable and consistent conditions were to seed the cells at 5,000 cells per well, and incubate the stimuli for 24 hours (data not shown). The use of a 24 hour incubation period with stimuli utilising the [^{3}H] thymidine incorporation assay has been used by other researchers including Kraft M. *et al.* 2001, Liu Y. *et al.* 2002 and Kobayashi E. *et al.* 2003.

Other observations of potential relevance from these preliminary experiments were that there appeared to be a decrease in proliferative potential, for both healthy and mild asthmatic fibroblasts, as the passage (or cell age) increased, this observation may be explained by the findings of Lorenzini A. et al. 2002, who suggest that the Raf/MEK/ERK and the PI3-K/Akt pathways, which are essential for cellular proliferation are down regulated in senescent cells. Also of note was that mild asthmatic and severe asthmatic fibroblasts appeared to generally have a decreased mitogenic potential when growing to confluence in the culture flasks, and were more difficult to lift from the flasks with trypsin/EDTA than healthy fibroblasts. The presence of myofibroblasts in the asthmatic cultures may partially explain their reduced ability to reach confluence, and also their reluctance to detach from the culture flasks, as myofibroblasts possess a decreased ability to proliferate (Cazes E. et al 2001 and Ramos C et al. 2001) and are also

more synthetic than fibroblasts. It may therefore be the case that the myofibroblasts are laying down increased levels of ECM protein in the flask, and that this ECM protein deposited by the asthmatic fibroblasts could bind the cells to the flask with a greater affinity than that experienced by healthy fibroblasts. However this observation has not undergone scientific scrutiny.

Although all fibroblasts used in this study were stained to assess their phenotype, only examples representative of the disease groups are produced in fig 3.8. The levels of α-SMA staining suggest that the populations are predominantly of fibroblast origin, and are not myofibroblasts or smooth muscle. The positive staining for vimentin suggests that the cells are not of epithelial origin, these characteristics are important as future work would not be representative of fibroblast behaviour if the cell lines used were heavily contaminated with smooth muscle, epithelial cells or myofibroblasts. It is, however, a consequence of using primary cultures grown from biopsy that there will be some contamination of fibroblasts populations with myofibroblasts. Although the cells stained positively for vimentin and showed little α-SMA staining suggesting that they were indeed fibroblasts and not smooth muscle, this could be further qualified by staining for desmin or caldesmon, both of which are smooth muscle markers (Touhami A. *et al.* 2005). The positively α-SMA stained cells in the cultures of fibroblasts may be of smooth muscle origin, and this must be taken into account when analysing future data. Nevertheless it may be the case that as fibroblasts are grown through passages that because myofibroblasts possess a decreased proliferative potential the percentage of myofibroblasts decreases as they are outgrown by the fibroblasts present in the cultures, this could be checked by periodically staining the cultures for α-SMA positive cells.

The work in this chapter has allowed the honing of the [^{3}H] thymidine assay and the characterisation of the fibroblasts which will be used, giving a good foundation for future work.

Chapter 4:

Mitogenic effects of BAL on fibroblasts *in vitro* using the [^{3}H]-thymidine incorporation assay

Introduction

From the work performed in chapter three we were able to proceed and investigate the mitogenic responses of fibroblasts cultured from healthy, mild asthmatic and severe asthmatic volunteers. To compare the mitogenic response of these fibroblasts, cells were challenged with BAL, and two previously used mitogens, 10ng/ml bFGF and 8% FCS. BAL fluid obtained from the lungs via bronchoscopy can give a useful insight into the biological processes occurring within the airways, and has been used to study a range of pulmonary diseases including asthma. It is appreciated that the site of sampling is imprecise, as the recovered fluid will represent fluid returning from the alveoli, small airways and intermediate sized bronchi beyond the 5^{th} to 7^{th} airway divisions, since the bronchoscope is wedged within the airways at this level and fluid inserted more distally prior to its recovery. Furthermore the recovered fluid will not only contain cellular products, secreted into the airways as part of the inflammatory process, but will also contain products of exudation that have moved into the airway space as part of the inflammatory process. An additional consideration is that the collection of BAL includes considerable dilution. For these studies 120ml of physiological saline was inserted into the airways and then suctioned, after a 10 second dwell time, to sample the endobronchial lining fluid. It is thus an extrapolated consideration that the effect the factors in the BAL have *in vitro* mirrors the effect the have *in vivo*. Nevertheless BAL remains the best way of sampling the endobronchial lining fluid, and provided the procedure is the same in all groups helps provide comparative data between health and disease. Many studies have demonstrated the value of BAL measures in differentiating asthma from non-asthma, indicating that this sampling method identifies disease specific airway changes reflective of on-going inflammation.

Thus the recovery and use of BAL fluid provides the best available method for exposing the fibroblasts to the environmental milieu that they might have been exposed to *in vivo*. This allows the preliminary exploration of possible abnormal epithelial-mesenchymal signalling which may be occurring within the airway, which is believed to contribute to the remodelling process within the asthmatic lung (Holgate S.T. *et al.* 2004). If the epithelial-mesenchymal signalling in the asthmatic airway is abnormal then one way in which we can investigate this is through the comparison of the effect of healthy and asthmatic BAL on fibroblast mitogenesis. It has been shown

in previous studies that fibroblast mitogens such as bFGF (Burgess W.H. *et al.* 1989) are raised in the BAL of asthmatics (Redington A.E. *et al.* 2001). Therefore if fibroblast hyperplasia in the asthmatic airway is driven by the increased levels of mitogens such as bFGF, then challenging fibroblasts from all groups with BAL from asthmatics should lead to increased fibroblast mitogenesis. Previous studies using asthmatic BAL have shown it to be mitogenic for airway smooth muscle (Naureckas E.T. *et al* 1999). However if it is the case that fibroblasts in the asthmatic airway have an increased ability to proliferate, then asthmatic fibroblasts should possess an increased mitogenic response independent of stimuli. The use of 10ng/ml bFGF and 8% FCS, which were shown in the previous chapter to be potent fibroblast mitogens (figs 3.5 and 3.6), will allow a simple, direct comparison of the mitogenic potential of the fibroblasts, as BAL is a complex mixture of factors, some of which will stimulate, while some may inhibit fibroblast mitogenesis.

Methods

The effect of BAL and individual factors on fibroblast mitogenesis

The [^{3}H] thymidine incorporation assay described in the methods section was used to assess fibroblast mitogenesis after stimulation with BAL and two previously used mitogens, 10ng/ml bFGF and 8% FCS. For each experiment using BAL both an unstimulated (1% ITS only) and stimulated (8% FCS) control were used. The BAL was obtained from healthy, mild asthmatics or moderate/severe asthmatic volunteers and added at a 1:3 dilution on the plate, this was incubated for 24 hours before addition of [^{3}H] thymidine. The fibroblasts of similar passages were primary fibroblasts grown from biopsy from healthy, mild asthmatic or severe asthmatic volunteers. Box-and-whisker plots represent 36 individual data points, and show boxes that extend from the 25th percentile to the 75th percentile, with a horizontal line representing the median, the whiskers extend down to the 10th percentile and up to the 90th percentile, with each outlier shown as an individual point outside the plots, bars in the bar charts represent column means and standard error bars.

Direct Cell Counting

The effect of BAL on fibroblast proliferation was also assessed using direct cell counting, as described in the methods section. Cells were seeded at 100,000 cells/ml

in 6 well plates, and challenged with healthy BAL, mild asthmatic BAL and moderate/severe asthmatic BAL, and also 10ng/ml bFGF and 8% FCS. Cells treated with 1% ITS media only were used as a negative control. Cells were counted using trypan blue at a 1:5 dilution, each well was counted 3 times. Counts consisted of viable cells only. Box-and-whisker plots represent 18 individual data points and show boxes that extend from the 25th percentile to the 75th percentile, with a horizontal line representing the median, the whiskers extend down to the 10th percentile and up to the 90th percentile, with each outlier shown as an individual point outside the plots, bars in the bar charts represent column means and standard error bars.

The effect of BAL fractions on fibroblast mitogenesis

BAL was fractioned using the Millipore filters into molecular weights of <3kDa, >3kDa, <30kDa, >30kDa, <100kDa and >100kDa, the fractions of BAL were then added to the fibroblasts along with unfractionated BAL to asses the mitogenic effect of the individual fractions. Bars in the bar charts represent column means and standard error bars.

Statistics

The Wilcoxon signed ranks test was used to assess the effect BAL, bFGF and FCS on fibroblast mitogenesis compared to the ITS control. The Mann-Whitney test along with Bonferroni adjustments were used to analyse the effect of different BAL on fibroblast DNA synthesis. All the treatments were performed at least in triplicate, $p < 0.05$ indicates significance.

Aims

1. To asses the effect of recovered BAL and individual mitogens on human lung fibroblasts grown from biopsy.
2. To investigate differences between BAL recovered from healthy airways and asthmatic airways of different asthma severity on fibroblast mitogenic responses.
3. To evaluate the mitogenic behaviour of primary fibroblasts cultured from healthy and asthmatic airways to explore disease and disease severity related differences in response to stimulation.
4. Analyse the effect of BAL fractions on fibroblast mitogenesis.

Results

The effect of BAL on healthy fibroblast mitogenesis and proliferation.

The data from figs 4.1 and 4.2 show the effect of healthy, mild asthmatic and moderate/severe asthmatic BAL fluid on healthy fibroblast mitogenesis, assessed by the [^{3}H] thymidine incorporation assay and healthy fibroblast proliferation, assessed by direct cell counting of viable (non-stained) cells using trypan blue. Both figures show that after challenge with BAL there is a significant increase in DNA synthesis, and cell number ($p < 0.0003$), compared to the ITS control. There was however, no significant change in fibroblast DNA synthesis or cell number when comparing the effects of different BAL fluids. This suggests that either there is a similar balance of stimulatory and inhibitory factors present in the BAL fluid from the three groups; or that the concentration of factor(s) in BAL leads to a maximal response from the fibroblasts. To test this, serial dilutions of the three different BAL fluids could be used, this would investigate whether in fact it is that the BAL from each group has a similar balance of stimulatory and inhibitory factors, or whether we are using BAL at a dilution at which no differential effect can be witnessed. Given this data it is not possible to determine whether the same factors in each BAL are responsible for these observations, however to investigate whether fibroblasts from mild asthmatic and severe asthmatic donors respond in a similar manner these cells were challenged with the same BAL.

For direct cell counting only 1 healthy, 1 mild asthmatic and 1 severe asthmatic fibroblast line was chosen, and this was due to time constraints Choosing which cell line to use for direct cell counting was difficult as two considerations had to be taken into account, firstly it was inappropriate to choose a cell line which was responding poorly to stimuli, as these cells were unlikely to give data of sufficient quality to undergo any kind of critical assessment. Secondly, choosing a cell lines whose response to challenge were at the top of each group would also skew the data as there would be little variation in response to challenge. It was therefore decided to choose cell lines which whose responses were more representative of the average response of the groups. It would have been more interesting to perform direct cell counting on six or more cell lines from each group, and this would be provide a more complete assessment of the direct proliferative response of each group. Nevertheless, this was the best way in which I could proceed with this work at the time.

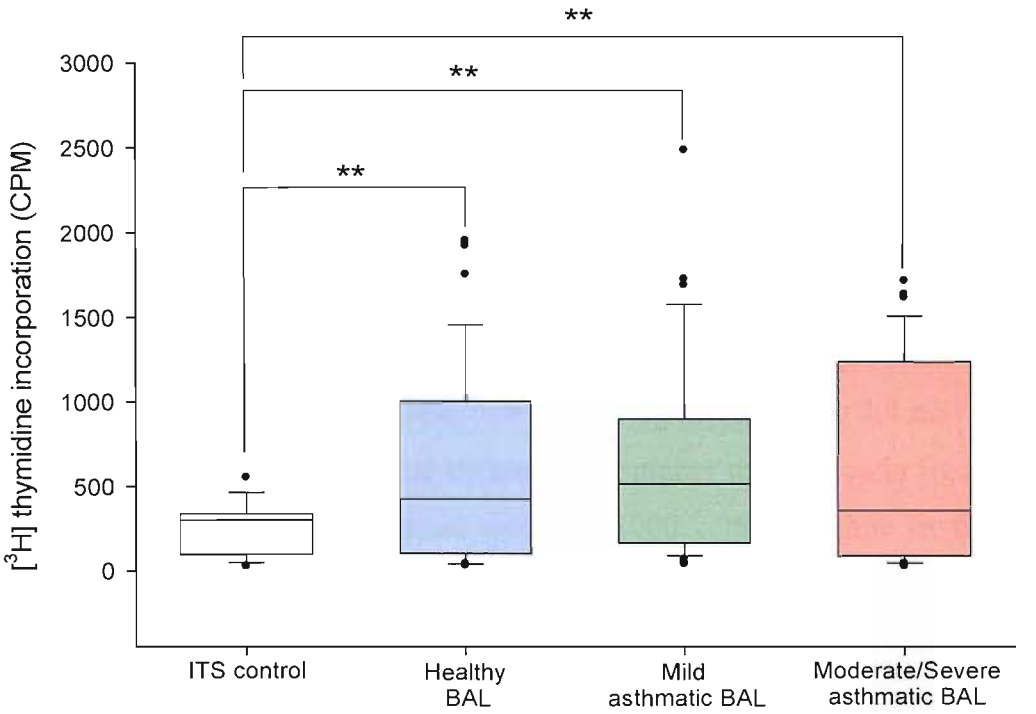


Fig 4.1 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on healthy primary human lung fibroblast (n = 6) mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. ** = $p < 0.0003$

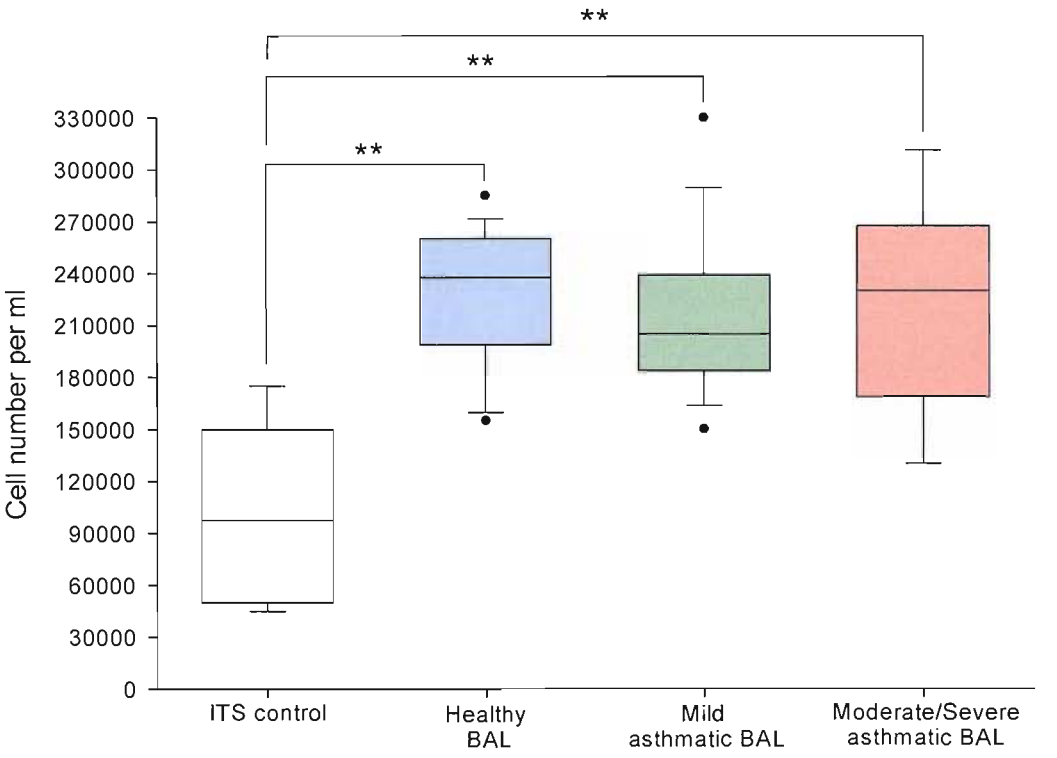


Fig 4.2 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on healthy primary human lung fibroblasts (n = 1) proliferation, assessed via direct cell counting using trypan blue after a 96 hour incubation period. ** = $p < 0.0003$.

The effect of BAL on mild asthmatic fibroblast mitogenesis and proliferation.

In figs 4.3 and 4.4 when mild asthmatic fibroblasts are challenged with the same BAL as the healthy cells in figs 4.1 and 4.2. It can be observed that there is a significant increase in fibroblasts DNA synthesis ($p < 0.03$ or 0.0003) but not cell number compared to the ITS control. As with the data in figs 4.1 and 4.2 there was little variation in response from mild asthmatic fibroblasts to the different BAL fluids, and it is likely that if this study were to be extended then a significant increase in cell number would be observed in fig 4.4. The lack of significant in fig 4.4 may be due to the cell number in the unstimulated control being higher than it was in fig 4.2. In fig 4.2 the cells unstimulated numbered around 90,000 cells/ml, while in fig 4.4 the unstimulated cells were counted at around 180,000 cell/ml. This is liable to be due to the cells being seeded at a higher density, due to either counting or pipetting error, and if this work were repeated then the unstimulated cell number would likely be lower, at around the seeding density of 100,000 cells/ml. Although the unstimulated fibroblast cell numbers/ml were higher in fig 4.2 than in fig 4.4, the BAL challenged fibroblasts increased their cell number to a magnitude relative to those in fig 4.2, at around 210,000 to 240,000 cells/ml, suggesting that mild asthmatic BAL had a similar ability to increase cell number as does healthy BAL. Following on from this work the affect of BAL was determined on severe asthmatic fibroblasts.

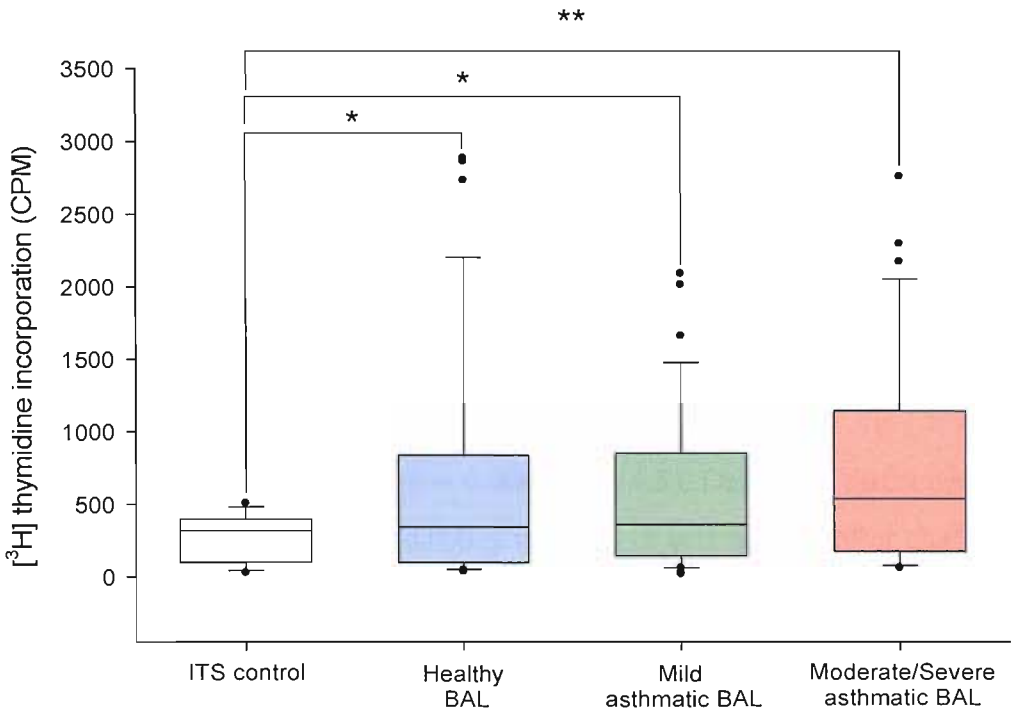


Fig 4.3 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on mild asthmatic primary human lung fibroblast (n = 6) mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. * = $p < 0.03$ ** = $p < 0.0003$

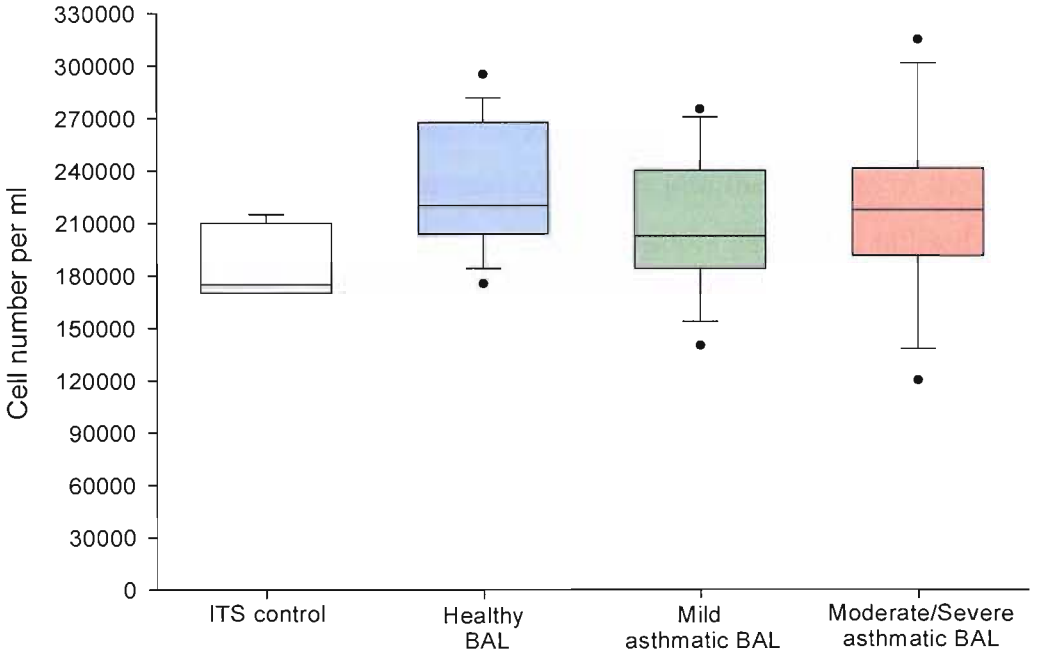


Fig 4.4 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on mild asthmatic primary human lung fibroblast (n = 1) proliferation, assessed via direct cell counting using trypan blue after a 96 hour incubation period.

The effect of BAL on severe asthmatic fibroblast mitogenesis and proliferation.

In figs 4.5 and 4.6 where the effect of different BAL fluids on severe asthmatic fibroblast DNA synthesis and proliferation was compared, it was seen that there was a significant increase in [^{3}H] thymidine incorporation after challenge with mild asthmatic BAL ($p < 0.0003$), when compared to the ITS control, however significance was not observed after challenge with healthy or moderate/severe asthmatic BAL. There was also a significant increase in DNA synthesis after challenge with mild asthmatic BAL when compared to challenge with healthy ($p < 0.03$) and moderate/severe asthmatic BAL ($p < 0.0003$) (fig 4.5). Data from direct cell counting (fig 4.6) show a significant ($p < 0.0003$) increase in cell number after challenge with BAL compared to the ITS control, however no significant variation in cell number after challenge with different BAL was observed.

These data propose that there may be some mis-regulation of severe asthmatic fibroblast mitogenesis, with mild asthmatic BAL leading to a significant increase in fibroblast DNA synthesis when compared to challenge with healthy and severe asthmatic BAL in fig 4.5, however this significant increase does not translate into a significant variation in cell number with the same challenges in fig 4.6. These data propose that it is possible the incubation time used is missing the mitogenic potential of healthy and moderate/severe asthmatic BAL, as in fig 4.6 the three BAL fluids are able to elicit a similar proliferative response from the severe asthmatic fibroblasts after a 96 hour challenge. One possible explanation for this may be that there are factors present in healthy and moderate/severe asthmatic BAL which are initially inhibiting the entry of severe asthmatic fibroblasts into the S phase of the cell cycle, where DNA is synthesised. However after these factors have been utilised the severe asthmatic fibroblasts are able to enter the cell cycle and this is represented as an increase in proliferation observed in fig 4.6.

That fibroblasts from severe asthmatics appear to have some sort of variable response to challenge, suggests that they may be responding to factors present in BAL in an altered manner than do fibroblasts from healthy or mild asthmatic volunteers. To assess the responses of fibroblasts from severe asthmatics with respect to those from healthy and mild asthmatic donors, the cellular mitogenic and proliferative responses were compared after BAL challenge.

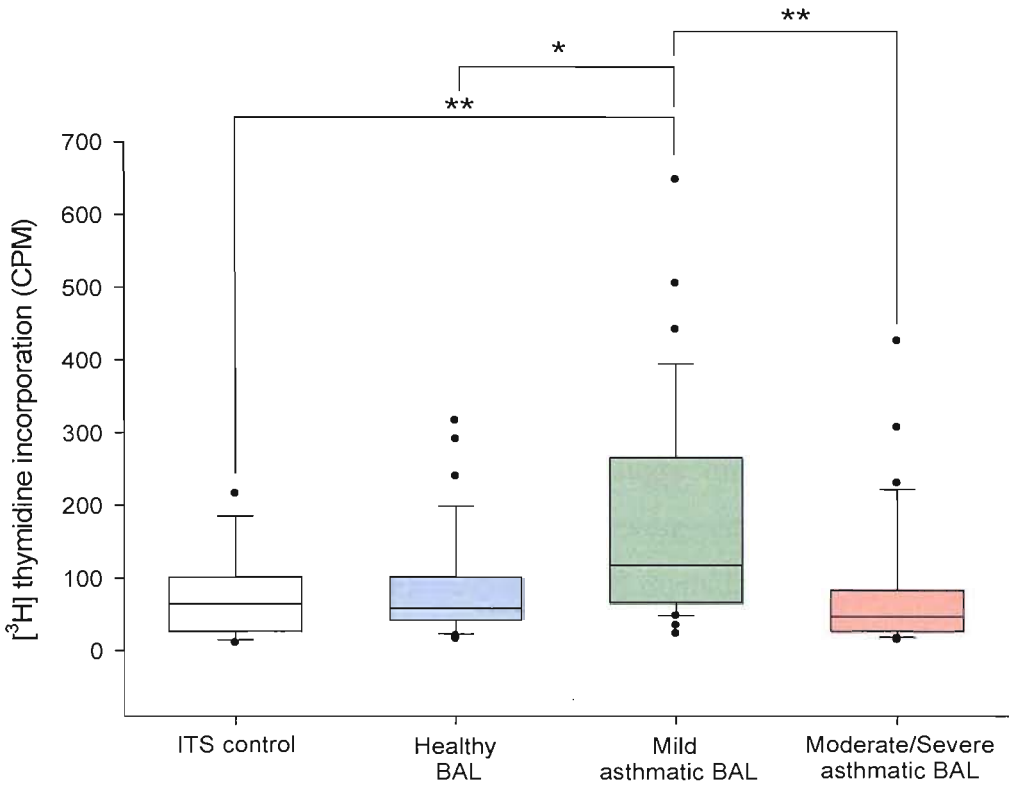


Fig 4.5 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on severe asthmatic primary human lung fibroblast (n = 6) mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. * = $p < 0.03$ ** = $p < 0.0003$

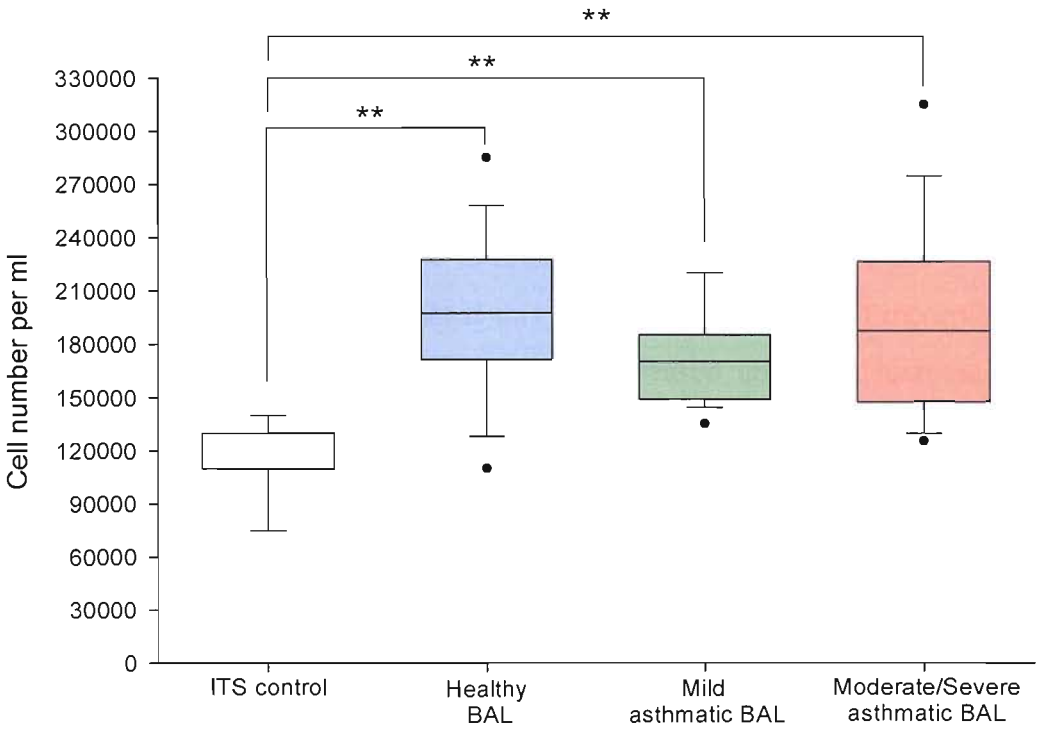


Fig 4.6 Graph showing the effect of BAL from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) subjects on severe asthmatic primary human lung fibroblast (n = 1) proliferation, assessed via direct cell counting using trypan blue after a 96 hour incubation period. ** = $p < 0.0003$.

Comparison of healthy, mild asthmatic and severe asthmatic fibroblast mitogenesis and proliferation after challenge with healthy BAL.

The comparison between the mitogenic and proliferative responses of fibroblasts from healthy, mild asthmatic and severe asthmatic donors to challenge with healthy BAL is shown in figs 4.7 and 4.8. The data indicates that both healthy and mild asthmatic fibroblasts are able to significantly increase their incorporation of [^{3}H] thymidine and cell number compared to the control ($p < 0.03$ and $p < 0.0003$ respectively), however fibroblasts from severe asthmatics are only able to significantly increase their cell number after challenge with BAL ($p < 0.003$) suggesting that they can respond to mitogenic stimuli. The data also show that severe asthmatic fibroblasts have a significantly decreased ability to incorporate [^{3}H] thymidine than do healthy or mild asthmatic cells ($p < 0.0003$), but that there is no significant variation in their ability to increase their cell number (fig 4.8). There was little variation between the responses of healthy and mild asthmatic fibroblasts, and as eluded to earlier, this may be due to the factor(s) in the BAL being at a concentration which leads to a maximal response from the healthy and mild asthmatic fibroblasts. By diluting down the BAL we may be able to investigate whether the healthy and mild asthmatic fibroblasts possess a similar mitogenic potential. Data from earlier figures (fig 3.5 and 3.6) where fibroblasts were challenged with growth factors and FCS suggested that healthy fibroblasts may possess a slightly greater mitogenic potential, however this would require further investigation, using larger numbers of fibroblasts.

The decreased mitogenic potential from severe asthmatic fibroblasts is not, however, mirrored by a significant decrease in their ability to increase their cell number after challenge, although it is likely that if this study were increased to incorporate larger numbers of fibroblasts, then a significantly decreased ability to increase their cell number compared to healthy and mild asthmatic fibroblasts would be observed. This is because although severe asthmatic fibroblasts are able to increase their cell number compared to the control, if the data in fig 4.8 is analysed then it can be seen that the median level of cells counted is below that of the healthy and mild asthmatic fibroblasts.

To determine whether this response was due to challenge with healthy BAL, or whether it was cellular based, the same cells were challenged with BAL from mild and moderate/severe asthmatics.

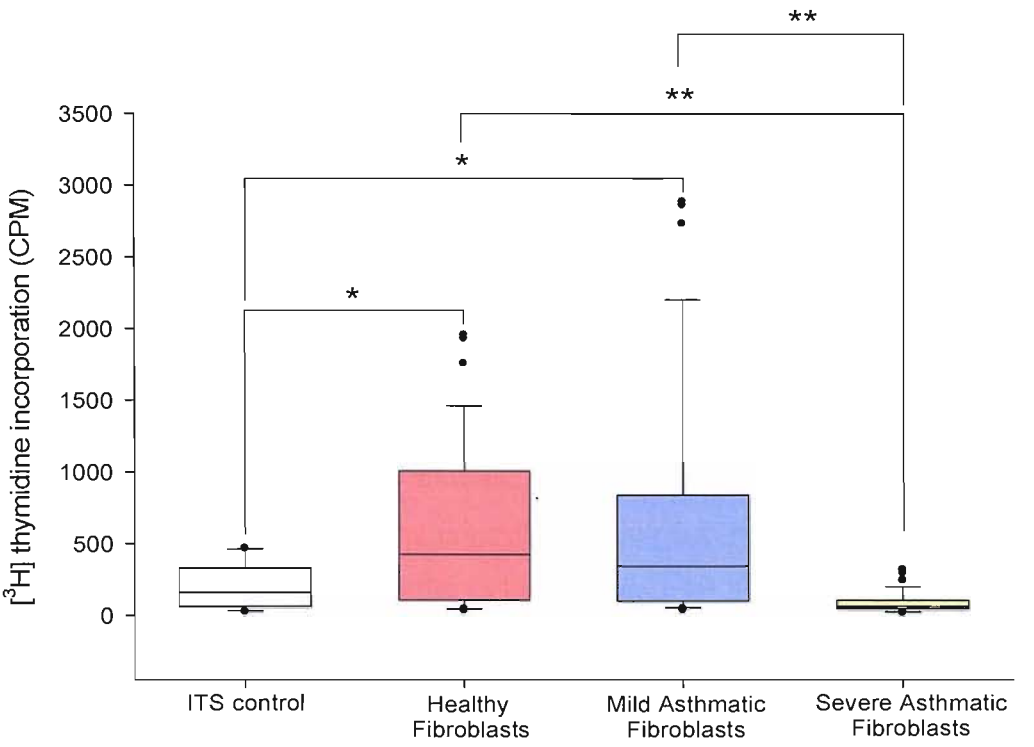


Fig 4.7 Graph showing the effect of BAL from healthy (n = 6), subjects on healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) human primary lung fibroblast mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. * = $p < 0.03$ ** = $p < 0.0003$

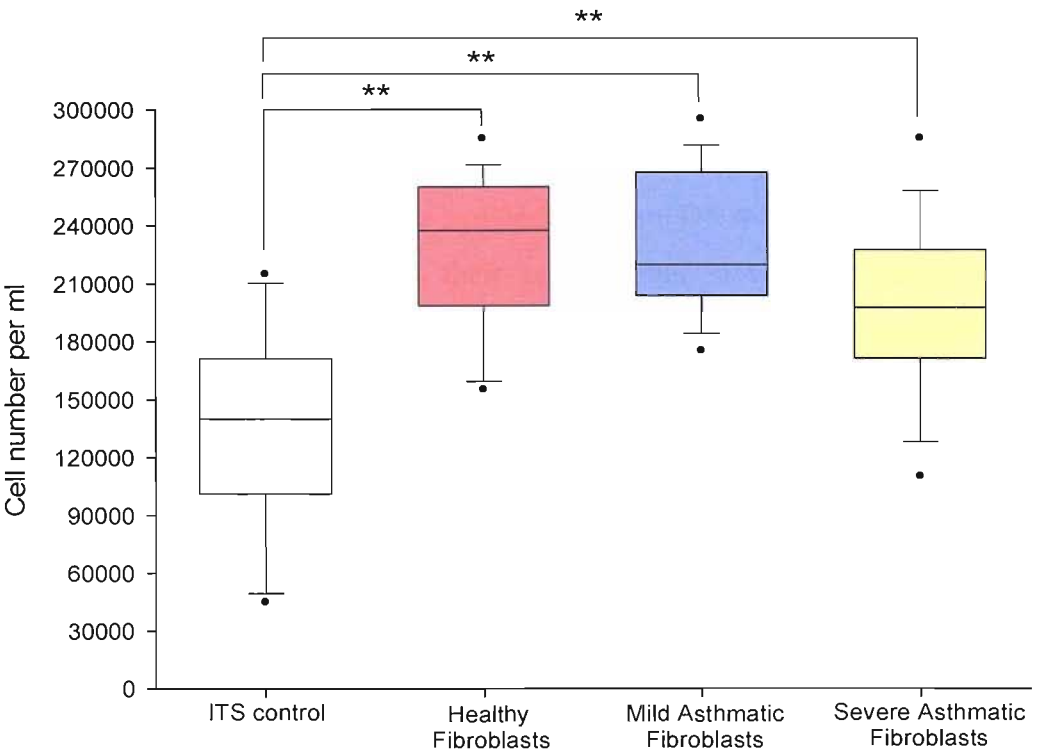


Fig 4.8 Graph showing the effect of BAL from healthy (n = 6), subjects on healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) human primary lung fibroblast proliferation assessed via direct cell counting using trypan blue, after a 96 hour incubation period. ** = $p < 0.0003$

Comparison of healthy, mild asthmatic and severe asthmatic fibroblast mitogenesis and proliferation after challenge with mild asthmatic BAL.

The effect of mild asthmatic BAL on healthy, mild asthmatic and severe asthmatic fibroblast [^{3}H] thymidine incorporation and proliferation is shown in figs 4.9 and 4.10. The data show that after challenge with mild asthmatic BAL both healthy and mild asthmatic fibroblasts are able to significantly increase their incorporation of [^{3}H] thymidine and cell number compared to the control ($p < 0.03$ and $p < 0.0003$ respectively). The data also show that severe asthmatic fibroblasts are not able to significantly incorporate [^{3}H] thymidine or increase their cell number after a challenge with BAL, when compared to the control, and that severe asthmatic fibroblasts also have a significantly decreased ability to incorporate [^{3}H] thymidine than do healthy or mild asthmatic fibroblasts ($p < 0.0003$).

Although severe asthmatic fibroblasts were not shown to significantly increase their cell number compared to the control cells the median counts for the challenged cells was around 40,000 cells/ml higher than the unchallenged cells (140,000 compared to 180,000 cells/ml). Therefore if this work were to be extended then severe asthmatic fibroblasts would likely be shown to be able to significantly increase their cell number after a 96 hour challenge with mild asthmatic BAL compared to unchallenged cells.

That there is relatively little [^{3}H] thymidine incorporation from severe asthmatic fibroblasts after a 24 hour challenge with BAL, followed by a 24 hour incorporation with 0.5μCi [^{3}H] thymidine, but that the cells are likely to be able to proliferate suggests that possibly the cells are synthesising new DNA before incubation with [^{3}H] thymidine, and then increasing their cell number slowly, or that the cells are synthesising new DNA at a time-point towards the end or after we incubate them with [^{3}H] thymidine. However it may also be that as mentioned earlier, factors in the BAL are preventing the cells entering the S phase of the cell cycle by elongating the G1 phase. There is previous work suggesting a role for TGF-β in the lengthening of the G1 phase (Nakamura T. *et al.* 1985), and thus delaying the entry of rat hepatocytes into the S phase of the cell cycle, and this observation has been supported by subsequent work (Lin P. *et al.* 1987 and Smeland E.B. *et al.* 1987).

To investigate whether TGF-β is arresting the entry of severe asthmatic fibroblasts into the S phase of the cell cycle, BAL could be treated with a TGF-β neutralising antibody, and then the progression of healthy, mild asthmatic and severe asthmatic fibroblasts through the cell cycle could be monitored at various time-points. This

coupled with using the [^{3}H] thymidine incorporation assay at varying time-points before and after 24 hours, should help identify whether fibroblasts from severe asthmatics do incorporate [^{3}H] thymidine at a different times than healthy or mild asthmatic cells, and also if TGF-β is regulatory in this process.

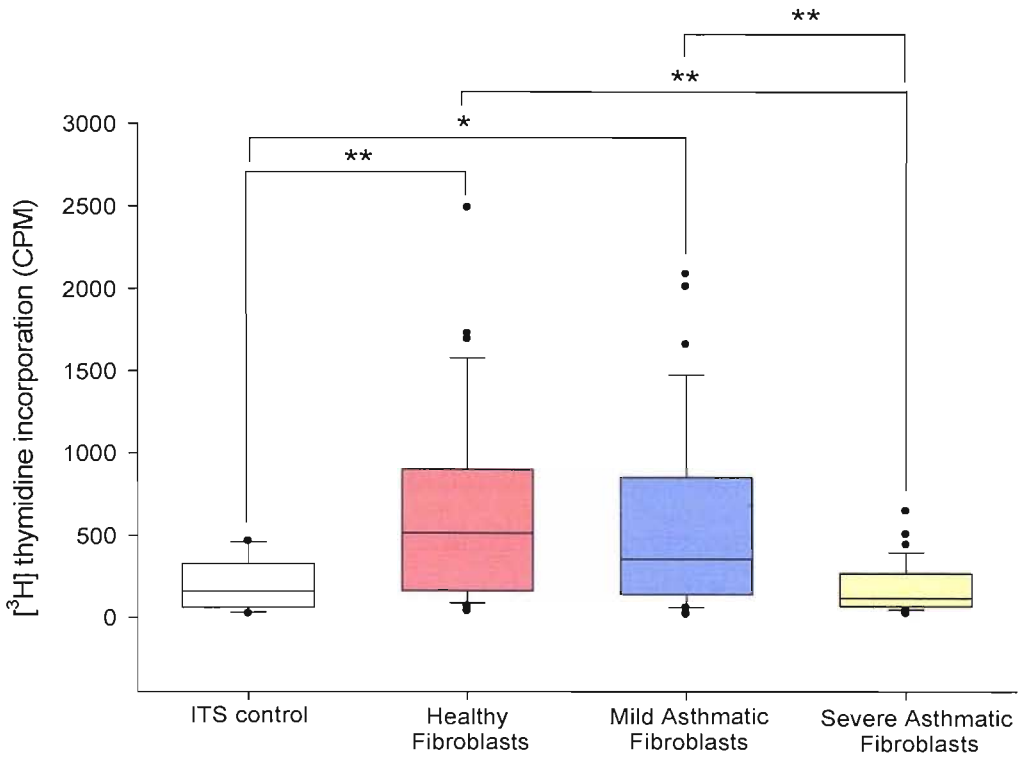


Fig 4.9 Graph showing the effect of BAL from mild asthmatic (n = 6) subjects, on healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) human primary lung fibroblast mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. * $p < 0.03$ ** $p < 0.0003$

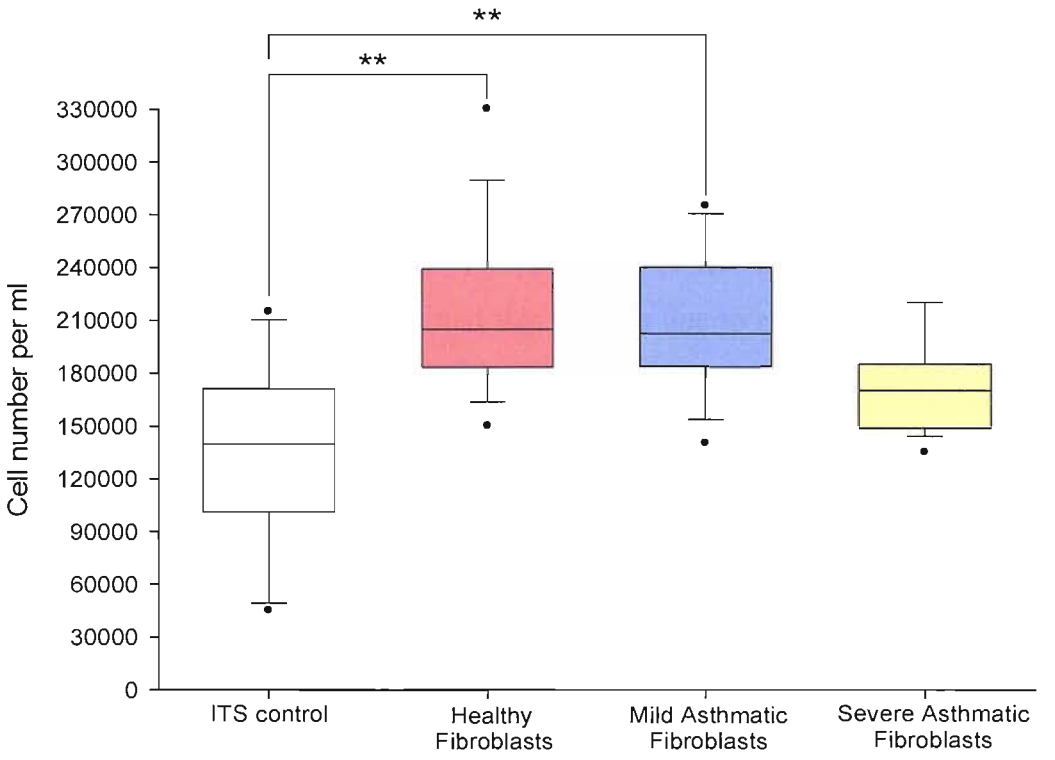


Fig 4.10 Graph showing the effect of BAL from mild asthmatic (n = 6), subjects on healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) human primary lung fibroblast proliferation assessed via direct cell counting using trypan blue, after a 96 hour incubation period. ** $p < 0.0003$

Comparison of healthy, mild asthmatic and severe asthmatic fibroblast mitogenesis and proliferation after challenge with moderate/severe asthmatic BAL.

When healthy, mild asthmatic and severe asthmatic fibroblasts were challenged with moderate/severe asthmatic BAL in fig 4.11 there was a significant increase in [^{3}H] thymidine incorporation from both healthy and mild asthmatic fibroblasts ($p < 0.03$) compared to the control. There was no disparity in response to challenge between healthy and mild asthmatic fibroblasts; however there was a significant increase in [^{3}H] thymidine incorporation from healthy and mild asthmatic fibroblasts compared to severe asthmatic cells ($p < 0.0003$). That there was no significant increase in proliferation from healthy, mild asthmatic or severe asthmatic cells in fig 4.12 is likely due to the low numbers of fibroblasts used, and if this study was extended it is probable that significance would be observed. The box plots in fig 4.12 show that the fibroblasts have a similar proliferative potential after challenge with moderate/severe asthmatic BAL as they did after challenge with healthy and mild asthmatic BAL (fig 4.8 and 4.10), both of which challenges show fibroblasts do possess the ability to significantly increase their cell number compared to the control. It is likely that due to there being less data points than in the [^{3}H] thymidine incorporation assays, that a few outliers have rendered the data statistically non-significant in this case.

The data comparing the mitogenic and proliferative responses of these fibroblasts after BAL challenge has shown that severe asthmatic fibroblasts have a significantly decreased ability to incorporate [^{3}H] thymidine than do healthy or mild asthmatic fibroblasts, and that this is likely to translate into a decreased ability to proliferate if the study were to be extended. The data has been unable to allude to any differences between the BAL fluid however, and this may be due to reasons already mentioned. To investigate whether severe asthmatic fibroblasts decreased ability to incorporate [^{3}H] thymidine related to the cells themselves rather than the BAL challenge, cells were subjected to a simpler challenge. Cells from each group challenged with 10ng/ml bFGF and 8% FCS, and it was hoped that the use of this simpler challenge would allow the investigation of whether the decreased ability to proliferate is a product of a decreased proliferative potential of the cells, or whether it is merely a response to the complex mixture of factors present in BAL.

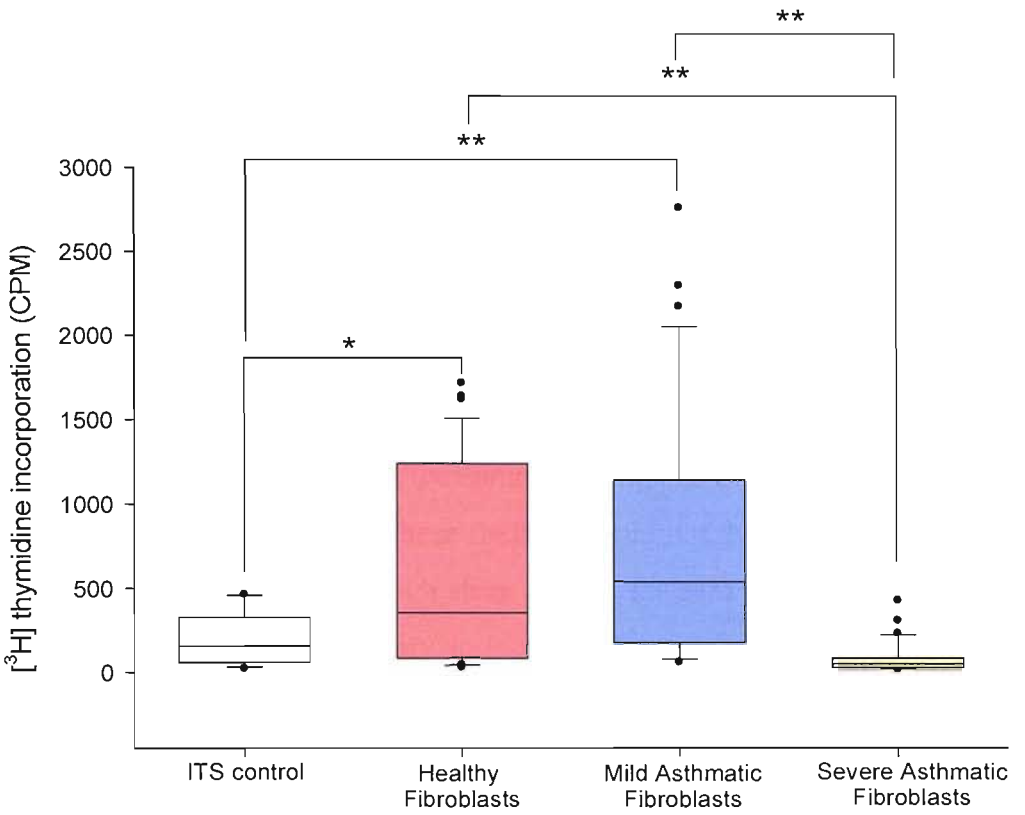


Fig 4.11 Graph showing the effect of BAL from moderate/severe asthmatic (n = 6) subjects, on healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) human primary lung fibroblast mitogenesis, assessed via the [^{3}H] thymidine incorporation assay after a 24 hour incubation period. * = $p < 0.03$ ** = $p < 0.0003$

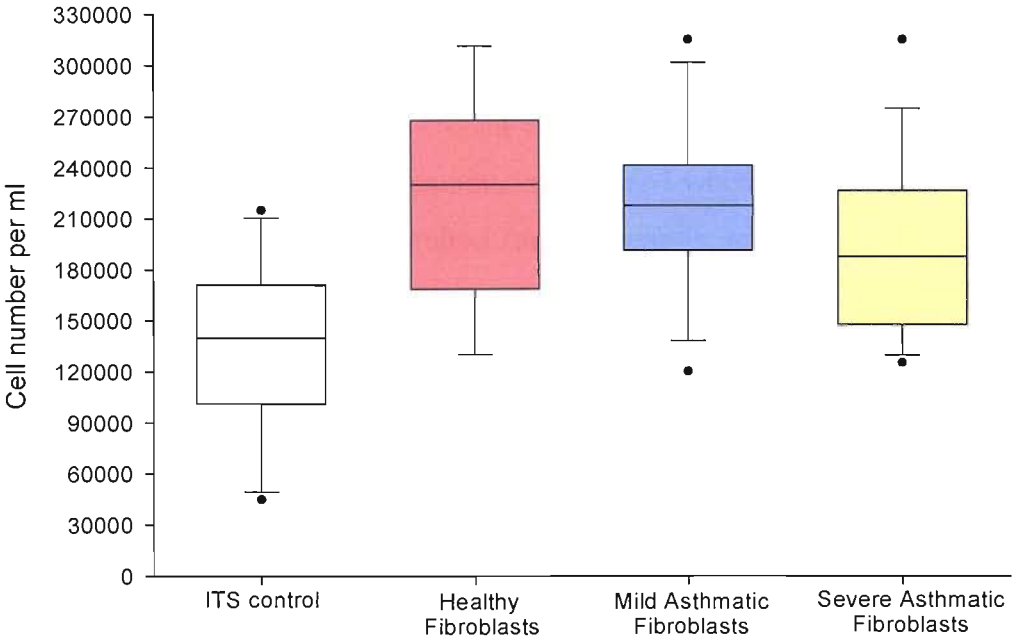


Fig 4.12 Graph showing the effect of BAL from moderate/severe asthmatic (n = 6), subjects on healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) human primary lung fibroblast proliferation, assessed via direct cell counting using trypan blue, after a 96 hour incubation period.

The effect of bFGF and FCS on healthy, mild asthmatic and severe asthmatic fibroblast mitogenesis and proliferation.

Figs 4.13 and 4.14 show comparisons between the response of fibroblasts from healthy, mild asthmatic and severe asthmatic volunteers after challenge with 10ng/ml bFGF and 8%FCS. Data in fig 4.13 show that severe asthmatic fibroblasts have a decrease ability to incorporate [^{3}H] that do healthy or mild asthmatic fibroblasts after a 24 hour challenge with 10ng/ml bFGF and 8% FCS. This observation was supported by data in fig 4.14, where fibroblasts from a severe asthmatic have a decreased ability to proliferate, when subjected to the same challenge as in fig 4.13, than did healthy and mild asthmatic fibroblasts. These findings could not, however, undergo statistical scrutiny as there were not enough data points. To gain more confidence in these observations it would be necessary to increase the number of fibroblasts used from each group.

The decreased mitogenic and proliferative potential observed from the severe asthmatic fibroblasts after challenge with BAL and growth factors may be due to either the cells transforming into myofibroblasts more readily than the healthy or mild asthmatic fibroblasts, or that these cells simply respond to mitogenic stimuli in a down regulated manner. It is difficult to determine which of these responses is more likely however, as bFGF has been shown to be able to facilitate fibroblast-myofibroblast differentiation (Rapraeger A.C. *et al.* 1991), and it is likely that there are also factors present in FCS which are able to transform fibroblasts into myofibroblasts. Even so, both of these responses suggest that fibroblasts from severe asthmatics are phenotypically different in their fundamental mitogenic response to stimuli than are healthy or mild asthmatic cells, and whether this manifests itself as a down regulation of receptors required for mitogenesis, an up regulation of receptors involved in other cellular processes or fibroblast-myofibroblast differentiation is an area which requires further study.

Another possible explanation for the decreased mitogenic and proliferative responses seen in figs 4.13 and 4.14 is that the fibroblasts may respond to the same concentration of challenge in an altered manner. The usual cellular proliferative response to stimuli is known to follow a typical curve, culminating in a saturated plateau at which point any increase in dose will lead to no further increase in proliferation. It may be that severe asthmatic fibroblasts reach this plateau at a lower dose of challenge than healthy or mild asthmatic fibroblasts, and also that the

maximum proliferative response of severe asthmatic fibroblasts is lower than that of healthy or mild asthmatic cells. Whether this is down to down regulated mitogenic receptors or other factors is an area which has not been explored in this work, and requires further exploration. This could initially be investigated by using a wider range of challenge concentrations could be used to determine where the saturation point of each cell type is, and then comparing the maximal proliferative response to stimuli at the optimum dose.

In an attempt to isolate factors present in BAL which lead to mitogenesis, BAL from healthy and asthmatic donors was fractionated at a range of molecular weights.

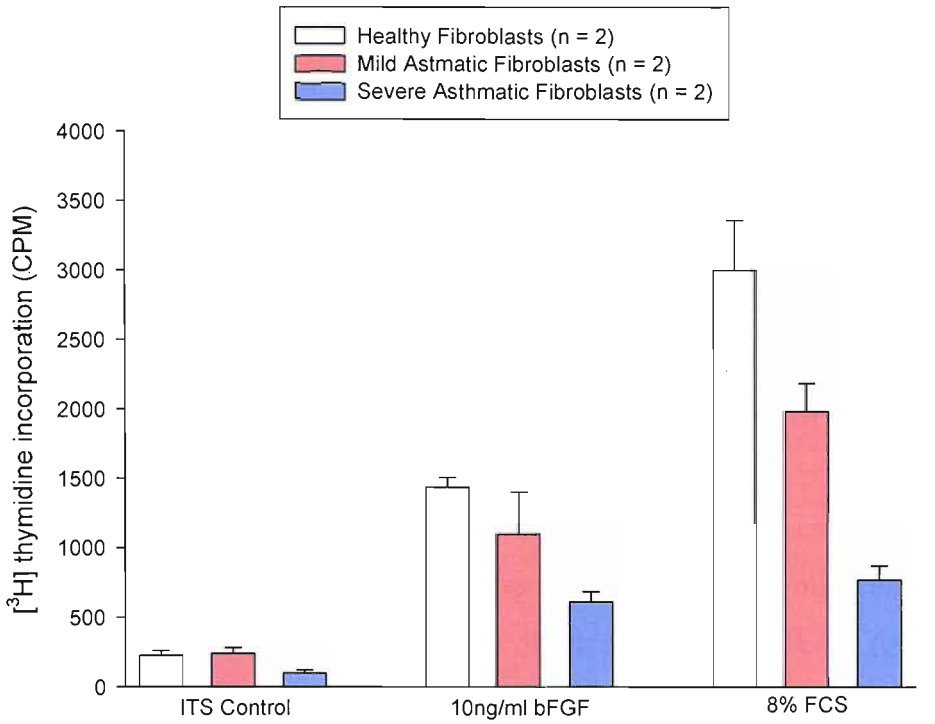


Fig 4.13 Fibroblasts from 2 healthy, 2 mild asthmatic and 2 severe asthmatic volunteers were seeded at 5,000 cells/well on 1:1000 vitrogen collagen coated wells. Fibroblasts were challenged with 10ng/ml bFGF or 8% FCS and their response assessed with the [^{3}H] thymidine incorporation assay.

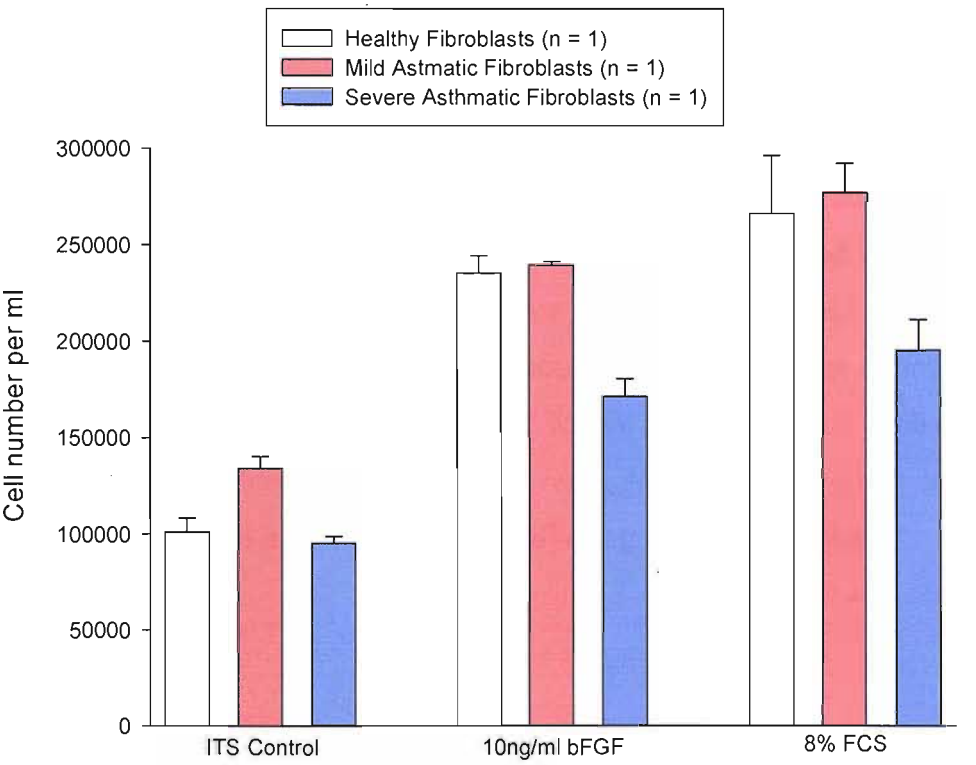


Fig 4.14 Fibroblasts from 1 healthy, 1 mild asthmatic and 1 severe asthmatic volunteer were seeded at 100,000 cells/well on 1:1000 vitrogen collagen coated wells. Fibroblasts were challenged with 10ng/ml bFGF or 8% FCS and their response assessed via direct cell counting utilising trypan blue

The effect of BAL fractions on fibroblast mitogenesis.

Fractionation of the BAL in figs 4.15 and 4.16 showed that in both healthy and asthmatic BAL the >3kDa and >30kDa had the greatest ability to induce [^{3}H] thymidine. In healthy BAL the >3kDa fraction effected a 100% increase in DNA synthesis, while challenge with the >30kDa fraction led to a 150% increase in DNA synthesis, compared to the unfractionated sample (fig 4.15). In the asthmatic BAL fractions challenge with the >3kDa caused a 65% increase in DNA synthesis, while challenge with the >30kDa fraction elicited a 90% increase in DNA synthesis, compared to the unfractionated asthmatic BAL sample (fig 4.16).

The data suggest that the majority of the pro-mitogenic factors present in BAL are likely to lie between 30-100kDa. This implicates a range of known mitogens which are present in BAL including TGF-B (~44kDa), tryptase (~31kDa), IL-17 (~31kDa), HGF (~100kDa), TNF-α (~51kDa), plasmin (~83kDa), thrombin (~35kDa) and factor Xa (~55kDa). It may also be that IGF-1 (~7kDa) is involved in the mitogenesis elicited by the fractionated BAL, as *in vivo* IGF-1 is bound to an IGF-binding protein (IGF-BP) normally IGF-BP3 (~30kDa), to render it biologically inactive. Therefore in the BAL IGF-1 may be bound to IGF-BP3, giving it a molecular weight of around 37kDa. Complete fractionation of a complex mixture of proteins such as BAL is difficult, as smaller proteins will often bind to larger proteins; therefore care must be taken when analysing the effect of these fractions. It is tempting to focus in only on proteins known to lie within the molecular weight range identified, however to exclude the importance of other factors in BAL, known to be raised in asthmatics, such as such as bFGF would short sighted, since as already mentioned, these factors may be bound to larger proteins. A more balanced approach would be to analyse the effects of proteins known to lie in the molecular weight range identified, such as TNF-α and TGF-β, and in addition to this also investigate the role of other factors of interest, which are present in BAL, such as bFGF using selective inhibitors.

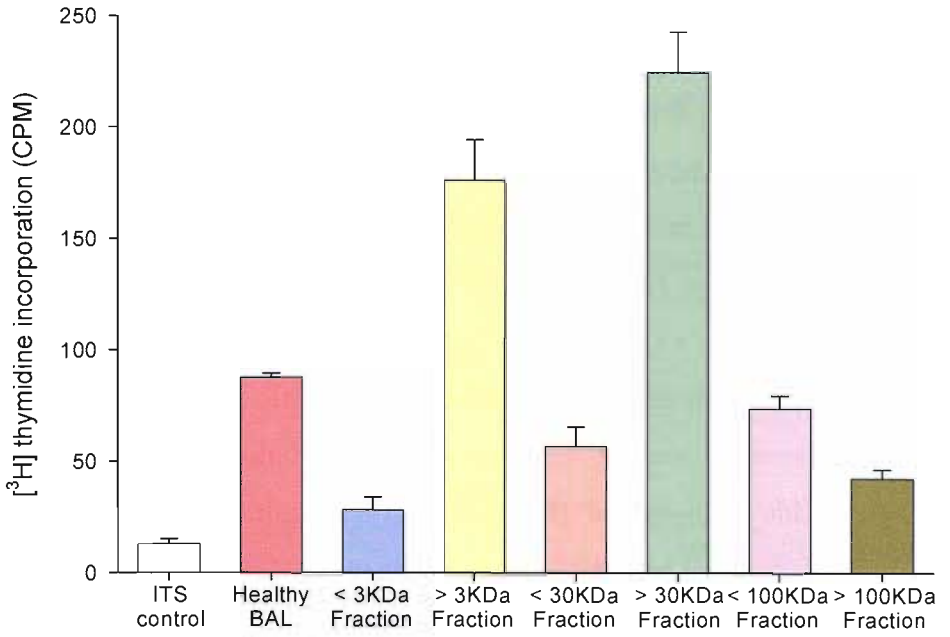


Fig 4.15 Effect of fractionated BAL on healthy fibroblast (n = 1) mitogenesis after 24 hour challenge with healthy BAL (n = 3). Mitogenesis was assessed with the [^{3}H] thymidine incorporation assay, each treatment was repeated in triplicate.

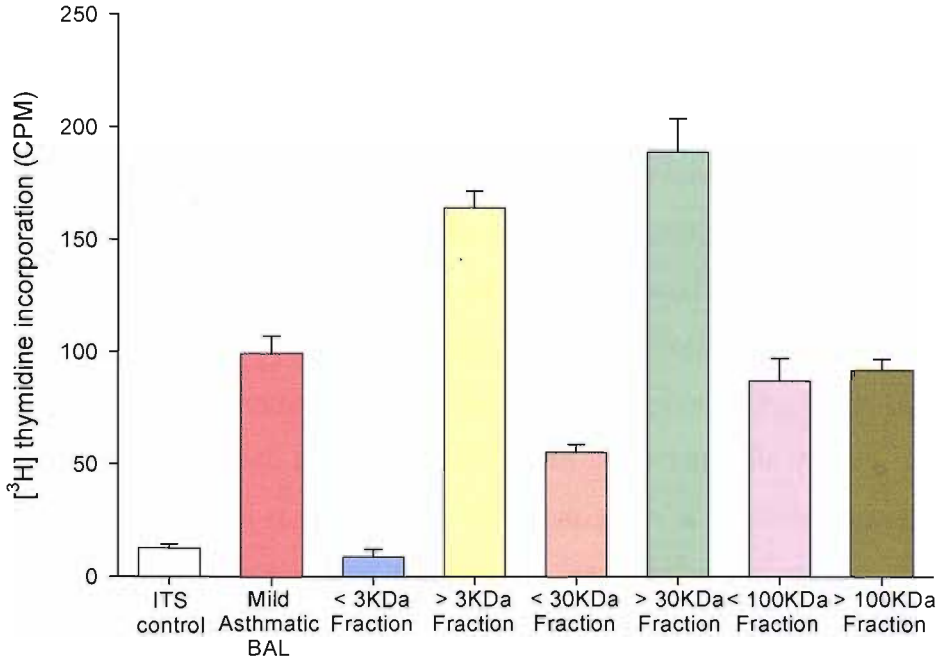


Fig 4.16 Effect of fractionated BAL on healthy fibroblast (n = 1) mitogenesis after 24 hour challenge with mild asthmatic BAL (n = 3). Mitogenesis was assessed with the [^{3}H] thymidine incorporation assay, each treatment was repeated in triplicate.

Discussion

The study of BAL fluid to understand cellular mechanisms and underlying pathology in pulmonary disease is a well established technique (Filippow V.P. *et al.* 1984, Lemaire I. *et al.* 1986, Hein J. *et al.* 1988 and Walters E.H. *et al.* 1991), and has been used to investigate diseases such as bronchitis (Linden M. *et al.* 1990 and Ross D.J. *et al.* 2004), sarcoidosis (Roth C. *et al.* 1981, Chretien J. *et al.* 1985, Grunewald J. *et al.* 1999, Ebe Y. *et al.* 2000 and Terashita K. *et al.* 2006), cystic fibrosis (Esther C.R. Jr *et al.* 2005) and alveolitis (Begin R. *et al.* 1985). Studies undertaken *in vitro* assessing the effect of biological airway fluid on cellular proliferation have been conducted on pulmonary diseases including acute respiratory distress syndrome (Marshall R.P. *et al.* 2000), asbestosis (Mutsaers S.E. *et al.* 1998), bronchiolitis obliterans (Jonosono M. *et al.* 1999) and asthma (Naureckas E.T. *et al.* 1999 and Leung S.Y. *et al.* 2004)

The ability of fibroblasts from healthy, mild asthmatic and severe asthmatic fibroblasts to respond to BAL was assessed using the [^{3}H] thymidine incorporation assay and direct cell counting. The model works on the principal hypothesis that BAL fluid is representative of the airway environment, containing factors primarily secreted by both inflammatory and epithelial cells. These stimuli are able to stimulate the fibroblasts residing in the lamina propria, and therefore a simplified *in vitro* model of the *in vivo* environment can be constructed.

Interactions between the epithelium and mesenchyme play a crucial role in organogenesis, growth, morphogenesis and cytodifferentiation (Saxen L. 1977), and these interactions have been extensively studied in the lung (Wessels N.K. 1977, Goto Y. *et al.* 1999, Swartz M.A. *et al.* 2000, Knight D. 2001 and Myerburg M.M. *et al.* 2007). Work by Myerburg M.M. *et al.* 2007 showed that the presence of sub-epithelial fibroblasts were required for the differentiation of human bronchial epithelial cells into mature ciliated cells. A view supported by Goto Y. *et al.* 1999 who suggests that growth and differentiation of epithelial cells *in vivo* is likely to be mediated by epithelial-mesenchymal interaction, in a soluble factor(s)-mediated manner. It is also worth noting the role of the surrounding milleau in which the fibroblasts and epithelial cells reside, as interactions between the cells and cytokines, ECM and integrins may also regulate the function of both epithelial cells and fibroblasts (Knight D. 2001).

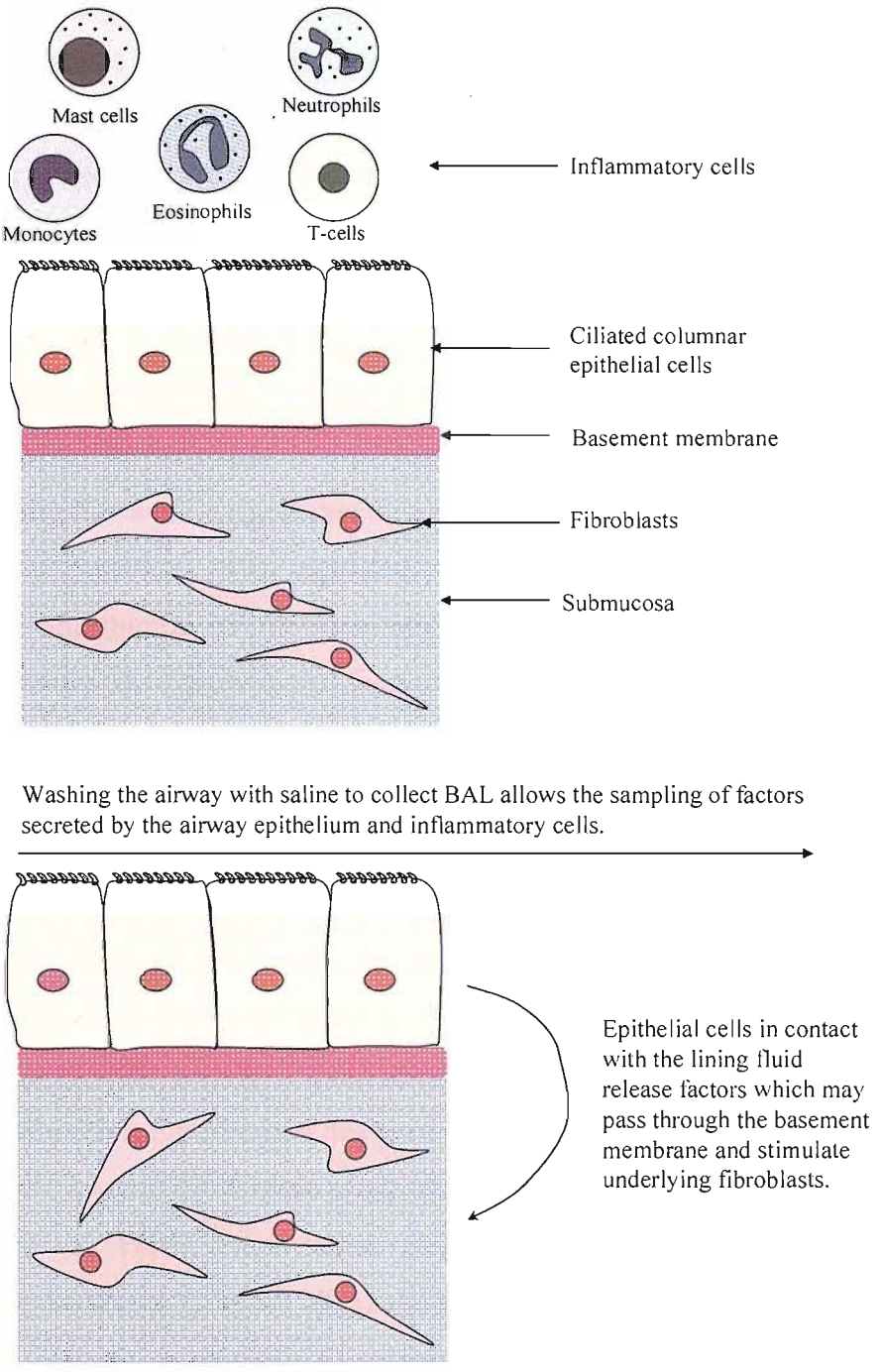


Fig 4.17 Illustration of proposed epithelial-fibroblast signalling within the lung.

These studies suggest that there is likely to be epithelial-fibroblast interactions *in vivo* during normal lung homeostasis, and also as a result of trauma or environmental changes. Therefore collection of BAL, which is likely to contain factors secreted directly from the epithelial cells, as well as resident inflammatory cells, should be a valid method by which to obtain a snap-shot of the local environment of the lung at a given time. From the previously mentioned studies it is reasonable to infer that there are indeed interactions between epithelial cells and fibroblasts occurring within the

bronchus, and that the *in vitro* model proposed can represent a basic imitation of this communication. It is recognised, as with most *in vitro* models, that this model does have inherent limitations. For example fibroblasts in the lung will possess a 3D morphology, and will be exposed to mechanical stress, which has been shown to stimulate collagen III production, amongst other factors and cytokines (Swartz M.A. *et al.* 2000).

The previously described model was used to investigate whether fibroblast/myofibroblast hyperplasia observed in the asthmatic airway is a result of an altered bronchial environment, or a consequence of altered fibroblast behaviour.

The data from this work suggest that the proliferative response of the severe asthmatic fibroblasts is somehow down regulated, and that this is independent of the stimuli used. The contribution of the bronchial environment was explored, however as already mentioned, observations from this work required further investigation. Nevertheless there was little significant variation in the proliferative potential of the different BAL fluids, this does not mean that the BAL fluids are composed of identical factors, but rather it suggests that at the dilutions we were using in this work, the pro and anti-proliferative balance of factors is similar. To identify whether the BAL from healthy, mild asthmatic and moderate/severe asthmatic volunteers was of similar composition inhibitors could be used to assess which pathways, factors and intra-cellular MAPKs each BAL fluid utilises.

Abnormal fibroblast proliferation has been observed in a range of pulmonary diseases, such as acute respiratory distress syndrome (Marshall R.P. *et al.* 2000), scleroderma (Ohba T. *et al.* 1994), emphysema (Holz O. *et al.* 2004), idiopathic interstitial pneumonia (Jakubzick C. *et al.* 2004) and asthma (Harrison N.K. *et al.* 1995, Dube J. *et al.* 1998, Zhang S. *et al.* 1999, Akers I.A. *et al.* 2000, Kraft M. *et al.* 2001 and Xu J. *et al.* 2002). However the studies undertaken assessing fibroblast proliferation in asthma have focused on specific factors, such as MMP2 in Xu's study and IL-4, IL-13 in Krafts study, with little work focusing on the net effect of BAL on fibroblast proliferation. The proliferative potential of asthmatic BAL has been investigated on ASM by Naureckas E.T. *et al.* 1999 and this researcher showed that asthmatic BAL possessed an increased mitogenic activity for human ASM when compared to BAL from healthy volunteers, and that this activity was increased after volunteers were allergen challenged. That this work suggest that ASM has an increased mitogenic potential when challenged with asthmatic BAL has implications in our work, as there

are α-SMA positively stained cells in our severe asthmatic fibroblast cultures, that we cannot rule out being ASM. There was evidence of an increase mitogenic response from severe asthmatic fibroblasts after challenge with mild asthmatic BAL (fig 4.5) however this was not observed after challenge with healthy or moderate/severe asthmatic BAL. That the BAL was obtained from mild asthmatic subjects in the study by Naureckas E.T. *et al.* 1999 suggests that the increased mitogenesis observed from mild asthmatic BAL challenged severe asthmatic fibroblasts may be due to the presence of ASM, and not due to the fibroblasts themselves. Although it may be possible that ASM is contributing to the [^{3}H] thymidine incorporation seen in fig 4.5, it is still worth noting that the severe asthmatic fibroblasts challenged with mild asthmatic BAL still had a significantly lower ability to incorporate [^{3}H] thymidine, than did healthy or mild asthmatic cells (figs 4.9). Therefore even taking into account the possible contribution from a small population of ASM, it is still probable that the majority of the mitogenic and proliferative responses seen in the above figures are down to the contribution of fibroblasts.

From my data it seems unlikely that fibroblast/myofibroblast hyperplasia witnessed in asthma is due to fibroblasts possessing an increased ability to proliferate, however it may be due to an altered lower airway environment, although the data do not wholly support or dismiss this. It therefore may be that fibroblasts in the asthmatic airway are maintained after they respond to airway injury, and this leads to a gradual increase in their numbers over time, or that the fibroblasts seen in the airway have migrated there from other areas of the body. Another explanation for our findings may be that due to ongoing damage occurring within the severe asthmatic airway that the response mechanisms for proliferation are already stimulated in severe asthmatic fibroblasts, and therefore these cells are not able to respond to additional stimuli to the same degree as fibroblasts from healthier volunteers. It would be difficult to assess this as the growth medium used for normal cell culture in this study was supplemented with 10% FCS, a potent fibroblast mitogen, however if fibroblasts could be grown in low stimuli conditions, then the time taken for fibroblasts from healthy, mild asthmatic and severe asthmatic fibroblasts to proliferate could be measured, either by measuring how long they take to reach confluence in a culture flask, or by using direct cell counting to quantify increases in cell number. This would allow a baseline comparison to be performed on the proliferative potential of all 3 groups.

Another consideration which must be taken into account is the donor age of the fibroblasts used in this study. On average donors characterised as severe asthmatics are around 25 years older than donors from healthy or mild asthmatic volunteers. This is another unfortunate consequence of using primary cultures of fibroblasts, as in general, patients presenting problematic severe asthma tend to be older than healthy or mild asthmatic donors. That the fibroblasts are from older donors may account for their decreased mitogenic potential, because as mentioned in the previous chapter senescent cells tend to have a decreased proliferative potential (Lorenzini A. *et al.* 2002). It therefore is a reasonable assumption that fibroblasts from older donors may have a decreased baseline proliferative potential than fibroblasts from younger donors. However, even taking this into account, it is unlikely that all of the decreased mitogenic potential shown from severe asthmatic fibroblasts is due to cell age. In future to correct this, fibroblasts from similar age groups could be used so donor age is no longer a consideration.

Fractionation of the BAL in figs 4.15 and 4.16 show that in both healthy and mild asthmatic BAL factors which lead to an increase in mitogenesis lie between 30kDa and 100kDa. As already eluded to this leaves a large number of potential candidates, however it has allowed a narrowing of the search. This work does not show any variation between healthy and mild asthmatic BAL in its composition using these filters. The findings from our work do fit in with other studies investigating wound blister exudates ability to cause fibroblast proliferation. This study suggested that the mitogenic factors lay between 30kDa to 300kDa, and from these observations is was postulated that the mitogenic factors were bound to high molecular weight substances such as serum proteins in these exudates (Inoue M. *et al.* 1996). This is likely to be what is occurring in our fractionated samples, and therefore fractionation of the BAL may not be the most suited way in which to isolate factors from the BAL responsible for BALs proliferative potential. It was therefore decided to pursue an alternate route and use both broad spectrum receptor inhibitors, and also inhibitors against individual receptors, factors and intra-cellular pathways. From the fractionation work it was decided to investigate the roles of TGF-β and TNF-α, and from previous literature investigating fibroblast proliferation it was decided to investigate the roles of G-protein coupled and tyrosine kinase receptors, receptors for bFGF, EGF and angiotensin II, and also PI3-K, MEK1/2, p38 and JNK intra-cellular MAPKs.

In conclusion the above data suggest that the fibroblasts ability to respond to mitogenic stimuli in BAL is down regulated in severe asthma; however there appears to be little variation in the mitogenic potential of the local environment of healthy, mild and moderate/severe asthmatic lungs. These observations indicate that there may be altered severe asthmatic fibroblast response to stimuli.

Chapter 5:

Investigation of mitogenic pathways involved in BAL mediated fibroblast mitogenesis

Introduction

The work from the [^{3}H] thymidine incorporation assays in the previous chapter had suggested that fibroblasts from severe asthma possesed a significanlty decreased proliferative potential than did healthy or mild asthmatic fibroblasts. There was little variation in BALs mitogenic potential between the three groups, and as already discussed this could have been due to a variety of reasons. It is likely that the composition of healthy BAL is similar to that of asthmatic BAL for the most part, however, due to events such as inflammatory cell infiltration and epithelial shedding, it is also probable that there are factors present in asthmatic BAL which are not present in healthy BAL. It was therefore decided to investigate through which mitogenic pathways and factors each BAL fluid signalled, to determine whether there was any difference in factors and pathways utilised by asthmatic BAL, compared to healthy BAL.

To investigte the mitogenic pathways, factors and MAPKs utilised by BAL a range of inhibitors were used. Initially broad spectrum inhibitors against receptors known to be involved in fibroblast proliferation were blocked. The receptors chosen were G-protein coupled and tyrosine kinase receptors, along with an intra-cellular kinase, PI3-K, which both pathways signal through. Then to study the contribution of individual factors inhibitors against the receptors for bFGF, EGF and angiotensin II were used, along with blocking antibodies against TNF-α and TGF-β. These factors were chosen from previous work where BAL was fractionated (figs 4.15 and 4.16) and also from previous literature, which suggested these factors able to elicit fibroblast proliferation (Carpenter G. *et al.* 1976, Chambard J.C. *et al.* 1987, Elias J.A. 1988a, Neuss M. *et al.* 1994, Thannickal V.J. *et al.* 1998, Chen Y. *et al.* 2003, Hafizi S. *et al.* 2004, Ouyang P. *et al.* 2004b, Motoki T. *et al.* 2005, Pelaia G *et al.* 2007), and that they were also present in BAL (Redington A.E. *et al.* 1997, Currie A.E. *et al.* 2001, Redington A.E. *et al.* 2001 and Howarth P.H. *et al.* 2005). The blocking of various MAPKs was used to investigate whether there was any evidence of a differential mitogenic signalling response from fibroblasts challenge with asthmatic BAL, the MAPKs chosen were MEK1/2, an upstream target of ERK1/2, p38 and JNK, all of which have been implicated in fibroblast proliferation (Laine P. *et al.* 2000, Chou F.P. *et al.* 2002, Stockand J.D. *et al.* 2003, Mandal S.K. *et al.* 2005 and Parkinson D.B. *et al.* 2004).

Before this work could be undertaken, however, it was important to ensure the inhibitors to be used were selective for their targets, and that they were not cytotoxic to the cells at the concentration to be used. The determination of cytotoxicity was performed using the LDH assay. The LDH assay is a rapid and simple method able to quantitate cytotoxicity by measuring the activity of LDH released from damaged cells, as cell death is classically evaluated by the quantification of plasma membrane damage. Upon cell membrane damage LDH is rapidly released into the cell culture supernatant and LDH activity can be determined by a coupled enzymatic reaction. The LDH assay is a well established technique for determination of lung fibroblast death (Hayden L.J. *et al.* 1990 and Chen F. *et al.* 2006).

Methods

The effect of broad spectrum and selective inhibitors on fibroblast mitogenesis

The [^{3}H] thymidine incorporation assay was used to assess the ability of genistein, wortmannin and inhibitors of individual factors to inhibit fibroblast mitogenesis after challenge with specific growth factors as described in the methods section. Genistein, pertussis toxin and wortmannin were pre-incubated with the cells for 4 hours before challenge, while SU5402, AG1478 and PD123-319 were pre-incubated for 1 hour before challenge The pan-specific TGF-β antibody and the soluble TNF-α receptor, Etanercept, were incubated with the challenge for 1 hour before addition to the cells. Fibroblasts were challenged for 24 hours before addition of [^{3}H] thymidine. Columns represent the mean data with standard error bars.

LDH Assay

To determine the cytotoxicity of inhibitors the LDH assay was used as described in the methods section, fibroblasts were seeded at 5,000 cells per well in collagen coated wells and with inhibitors at 3 concentrations. The inhibitors were added at the concentration at which they would be used for future work and also a dose 10 fold above and 10 fold below for 24 hours before the addition of the LDH detection reagent. The plates were read at 490nm. Data represents the mean of 3 values per plot, and bar represent the mean data with standard error bars.

The effect of broad spectrum and selective inhibitors on BAL induce fibroblast mitogenesis

Broad spectrum inhibitors were used to highlight a pathway(s) involved in fibroblast mitogenesis, two inhibitors were chosen; pertussis toxin and genistein. The inhibitors were incubated with the cells from 2 healthy, 2 mild asthmatic and 2 severe asthmatic volunteers for 4 hours before addition of BAL, at concentrations determined by previous experimentation and published work. The role of individual factors within BAL on fibroblast mitogenesis were investigated via the use of SU5402, AG1478, Etanercept, pan-specific TGF-β antibody and PD123-319. Inhibitors of intra-cellular messengers were also chosen to investigate fibroblast mitogenesis further, these included wortmannin, U0126, SB203580 and JNKI1. Proteinase K was used to determine the overall role of proteins in BAL mitogenesis. These inhibitors were incubated for 1 hour either with the cells or with the BAL, before addition of BAL, at concentrations depending on previous experimental data and publications. The BAL was either added individually or pooled before addition; groups were defined as healthy, mild asthmatic or moderate/severe asthmatic and the BAL was added at a 1:3 dilution. Box-and-whisker plots show boxes that extend from the 25th percentile to the 75th percentile, with a horizontal line representing the median; the whiskers extend down to the 10th percentile and up to the 90th percentile, with each outlier shown as an individual point outside the plots.

Statistics

The Mann-Whitney test was used to analyse the effect of BAL on fibroblast DNA synthesis compared to the ITS control. The Wilcoxon signed ranks test was used to assess the effect of inhibitors on BAL induced mitogenesis. All the treatments were performed at least in triplicate, $p < 0.05$ indicates significance.

Aims

1. To ensure the concentration of inhibitors to be used was not cytotoxic.
2. To determine whether the inhibitors to be used were effective against their targets.
3. To investigate which pathways, factors and MAPKs are involved in BAL mediated mitogenesis.
4. To determine whether there are differences in the mitogenic signalling pathways and factors utilised by the different BAL groups.

Results

Investigating the selectivity of pertussis toxin, genistein, wortmannin, SU5402, AG1478 and PD123-319 using the [^{3}H] thymidine incorporation assay.

Figs 5.1 and 5.2 show data investigating the selectivity of pertussis toxin, genistein, wortmannin, SU5402, PD123-319 and AG1478 with regard to inhibiting the mitogenic effects of angiotensin II, bFGF and EGF. Angiotensin II utilises a G-protein coupled receptor while both bFGF and EGF signal through tyrosine kinase receptors, and are known to signal through PI3-K, therefore blocking these pathways with a broad spectrum G-protein coupled receptor inhibitor (pertussis toxin), a tyrosine kinase receptor inhibitor (genistein) and an inhibitor of PI3-K (wortmannin) should inhibit angiotensin II, bFGF and EGFs mitogenic potential. By blocking the receptors for angiotensin II, bFGF and EGF using PD123-319 (angiotensin II), SU5402 (bFGF) and AG1478 (EGF) we could also investigate how selective these receptors inhibitors are, by challenging the cells with angiotensin II, bFGF and EGF.

The data in the figures show that genistein, wortmannin, SU5402 and AG1478 could inhibit the mitogenic potential of bFGF and EGF, albeit to varying degrees. Statistical analysis could not be performed on these data as there were insufficient experiments, however the figures do show that both SU5402 and AG1478 could cause a large level of inhibition after challenge with bFGF and EGF respectively. Pre-incubation of the cells with genistein and wortmannin lead to a degree of inhibition that was less than observed with SU5402 and AG1478, however the two compounds were still able to inhibit the actions of bFGF and EGF. The data also show that angiotensin II is unable to elicit a significant increase in fibroblast DNA synthesis when added alone, and therefore it is uncertain whether pertussis toxin and PD123-319 were able to inhibit its actions. However both pertussis toxin and PD123-319 have been extensively used to assess the role of G-protein coupled and angiotensin II receptors in previous studies. Although challenging the cells with angiotensin II alone was unable to lead to an increase in [^{3}H] thymidine incorporation in the above figures, it was still decided to investigate its role in BAL mediated mitogenesis as angiotensin II is believed to play a role in chronic asthma (Ramsay S.G. *et al.* 1997), and little work had focused on its role in BAL.

Although the inhibitors used above are well established compounds their selectivity is a consideration. Although the inhibitors used on the whole are able to inhibit their targets

they may also affect other pathways. For example SU5402 inhibits both basic and acidic FGF (aFGF), as well as weakly inhibiting the tyrosine phosphorylation of the PDGF receptor (Mohammadi M. *et al.* 1997), and AG1478 effects the phosphorylation of ERK by angiotensin II (Liu W. *et al.* 1999 and Eguchi S. *et al.* 1998). Therefore observations made whilst using all the inhibitors employed in this study must take into account the possibility that the effects witnessed may also be contributed to by the inhibition of alternate factors or pathways. It would also have been pertinent to have investigated the selectivity of the various inhibitors by using challenges which were not related to the inhibitors actions. For example, to check the selectivity of SU5402 (bFGF inhibitor) we could have challenge fibroblasts with EGF in the presence and absence of SU5402. This is an important consideration because, as already mentioned, some of the inhibitors used in the study are able to affect the receptors or intra-cellular MAPKs utilised by other factors. This will have to be taken into account when analysing the data from the inhibition of BAL mediated mitogenesis.

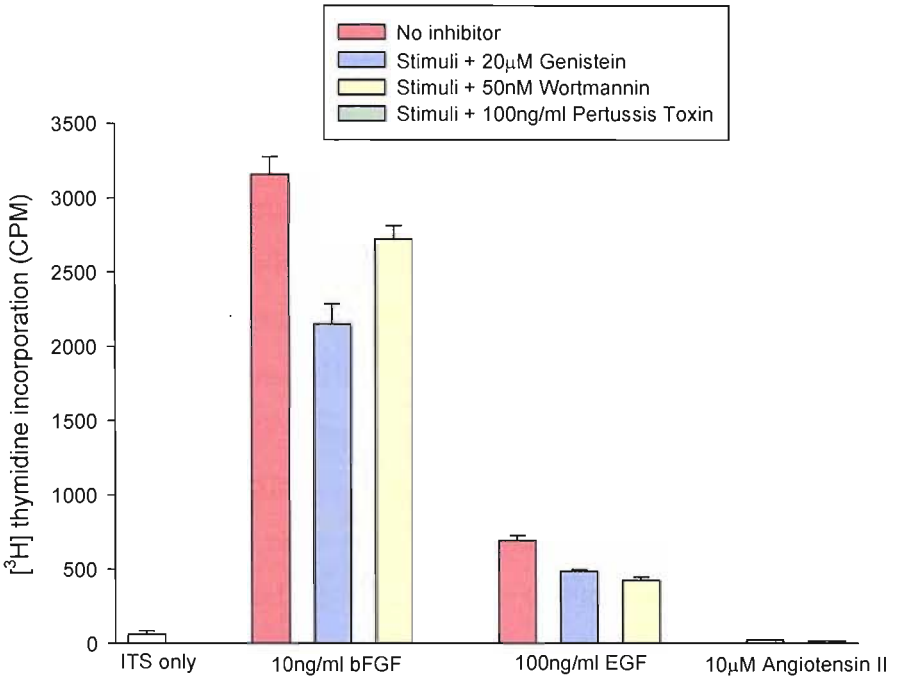


Fig 5.1 [^{3}H] thymidine incorporation assay analysing the effect of pertussis toxin, genistein and wortmannin angiotensin II, bFGF and EGF mediated fibroblast (n = 1) mitogenesis. Primary lung fibroblasts were seeded at 5,000 cells per well in collagen coated wells, cells were pre-incubated with inhibitors for 4 hours before challenge.

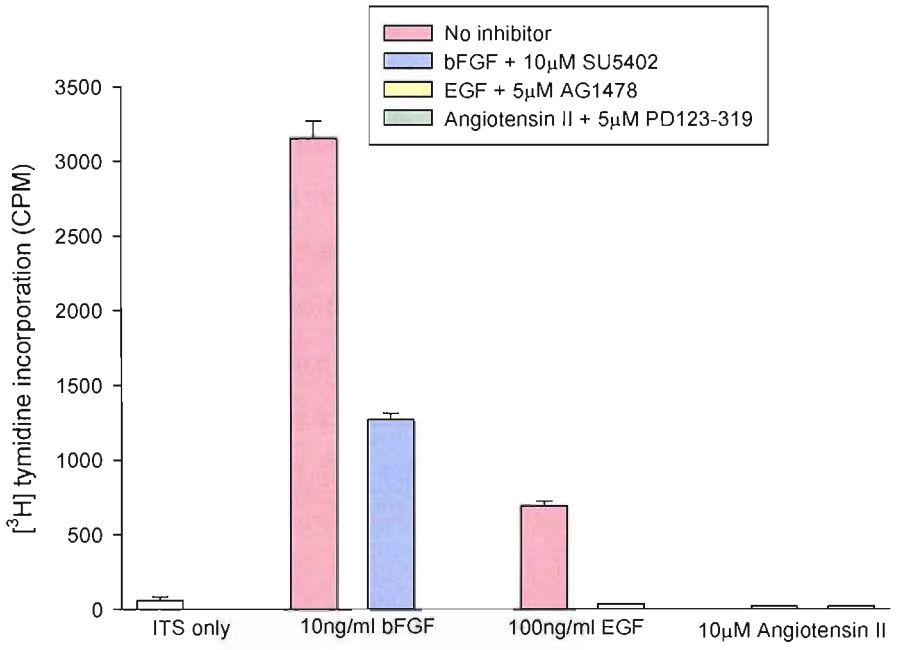


Fig 5.2 [^{3}H] thymidine incorporation assay analysing the effect of specific angiotensin II,bFGF and EGF receptor inhibitors on angiotensin II, bFGF and EGF mediated fibroblast mitogenesis. Primary lung fibroblasts (n = 1) were seeded at 5,000 cells per well in collagen coated wells, cells were pre-incubated with inhibitors for 1 hour before challenge.

Investigating the selectivity of the pan-specific TGF-β antibody and Etanercept.

The ability of a neutralising antibody directed against human TGF-β1, TGF-β2 and TGF-β3 (pan-specific TGF-β antibody), and a soluble TNF receptor able to inactivate TNF-α (Etanercept) to inhibit thymidine incorporation after challenge was investigated in fig 5.3. The graph shows that challenging the cells with 1ng/ml TGF-β1, 1ng/ml TGF-β2 and 10ng/ml TNF-α did not lead to an increase in [^{3}H] thymidine incorporation compared to the ITS control. The decreased [^{3}H] thymidine incorporation observed in the above figure is unlikely to represent TGF-β and TNF-α inhibiting [^{3}H] thymidine incorporation, and is more likely to be an experimental variation. If this work were to be repeated then it may be that the unstimulated cells would incorporate less [^{3}H] thymidine than the cells challenged with TGF-β and TNF-α, and this would also be difficult to interpret as an increase in fibroblast mitogenesis. This was not unexpected as previous work (figs 3.5 and 3.6) had indicated that TGF-β1 added alone did not lead to a significant increase in [^{3}H] thymidine incorporation from fibroblasts. These two factors were chosen for investigation from previous work where BAL was fractionated, and although these factors may not increase fibroblast mitogenesis alone it was believed, based on previous publications, that their co-operation with other factors in BAL could account for some of the mitogenic potential of BAL. The data show that the TGF-β antibody and Etanercept do not affect the level of fibroblast [^{3}H] thymidine incorporation. However as the individual challenges had not lead to an increase in [^{3}H] thymidine incorporation alone, the selectivity of the inhibitors to these factors is difficult to ascertain. Nevertheless these inhibitors have been shown to be selective by previous researchers, and that they don't appear to cause a large increase in [^{3}H] thymidine incorporation, or a decrease on top of the one already exerted by the challenge with TGF-β1, TGF-β2 and TNF-α, suggest that they are likely to not affect the subsequent data to a level which would affect its interpretation.

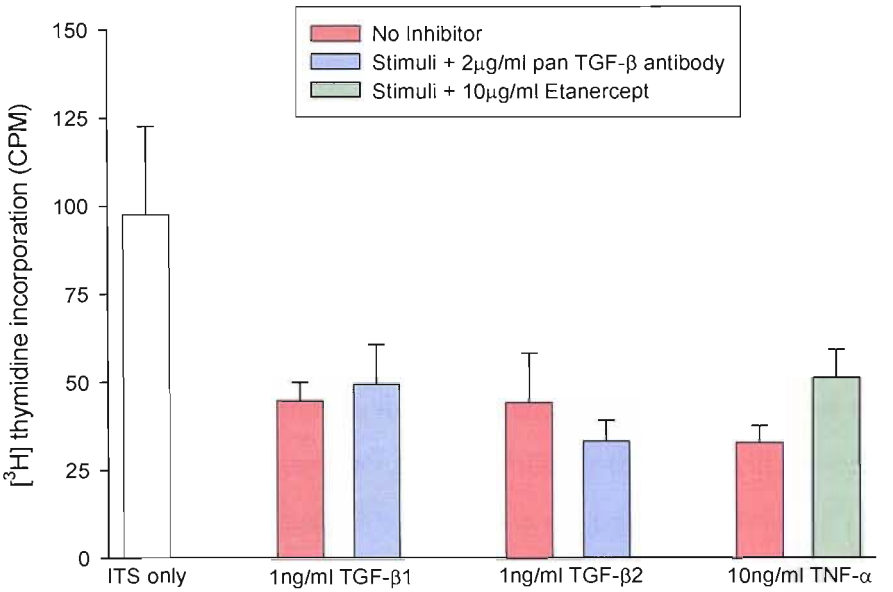


Fig 5.3 The effect of a pan TGF-β antibody on fibroblast mitogenesis assessed by the [^{3}H] thymidine incorporation assay. Primary lung fibroblasts from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) volunteers were seeded at 5,000 cells per well. Cells were challenged with 1ng/ml TGF-β1, 1ng/ml TGF-β2 or 10ng/ml TNF-α. The TGF-β antibody and Etanercept were incubated with TGF-β1, TGF-β2 or TNF-α for 1 hour before a 24 hour challenge.

Investigation of the affect of the inhibitors on fibroblast [^{3}H] thymidine incorporation.

Fig 5.4 shows data determining the effect of individual inhibitors on fibroblast mitogenesis in the absence of a challenge. The graph illustrates that only wortmannin, SU5402 and AG1478 caused a decrease in [^{3}H] thymidine incorporation, all other inhibitors caused an increase. This was most marked when cells were incubated with 10μM JNKI, with the amount of thymidine incorporation increasing around 360%. No significance can be demonstrated with these data as there are not enough values to perform statistical analysis. Although these data show that some inhibitors may lead to an increase in [^{3}H] thymidine incorporation, it is unlikely that this will adversely affect the observations after BAL challenge. This is because the relative increase in [^{3}H] thymidine incorporation compared to challenge with BAL or growth factor challenge is small. The above graph appears to have a large relative increase in [^{3}H] thymidine incorporation because it is compared to unchallenged cells; however the maximal increase is still only around 90 cpm. If this is compared to BAL challenge, around 500 cpm, or challenge with growth factors, around 500-4000cpm, it is unlikely that the use of these inhibitors will contribute significantly to an increase or decrease in [^{3}H] thymidine incorporation which would affect any data to be obtained.

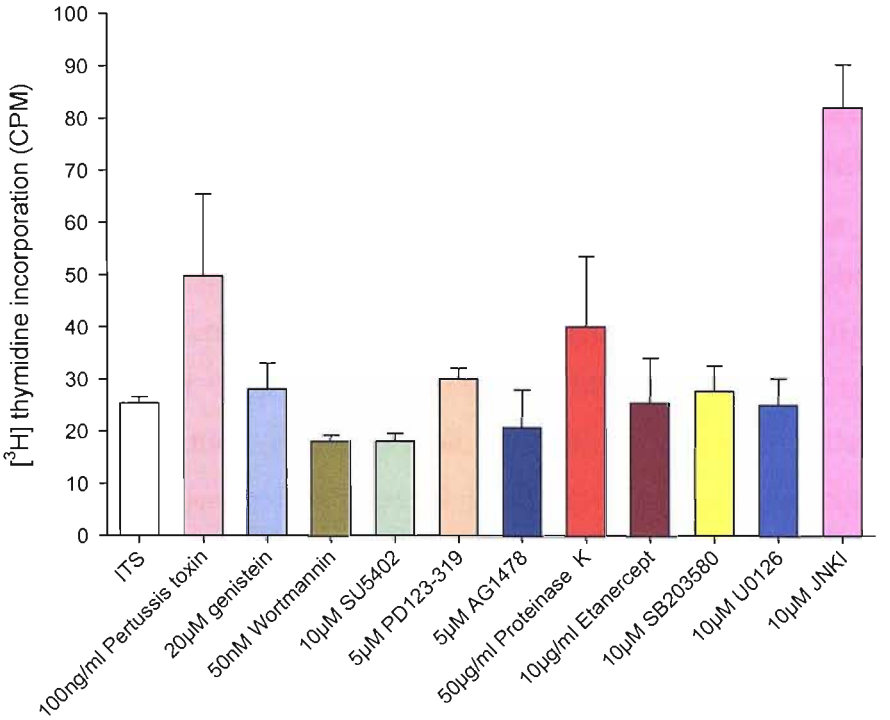


Fig 5.4 Graph showing the effect of individual inhibitors on primary lung fibroblast (n = 1) mitogenesis without challenge, assessed by the [^{3}H] thymidine assay. Cells were seeded at 5,000 cells per well in collagen coated plates. Inhibitors were incubated for 24 hours with the cells before addition of [^{3}H] thymidine.

Determination of the cytotoxicity of the inhibitors.

To ensure the concentrations of inhibitors to be used to investigate the role of broad spectrum receptor inhibitors, individual factors and intra-cellular messenger proteins were not cytotoxic to primary cultures of fibroblasts LDH assays was performed (fig 5.5). The inhibitors were added to the cells at concentrations corresponding to the dose of inhibitor that would be used for future investigation, and a dose 10 fold above and below. For each dose the percentage of cytotoxicity was calculated. Data from fig 5.5 show that SU5402 and JNKI at 100μM caused 21% and 14% of fibroblasts treated to have substantial damage to their cell membrane, most likely resulting in cellular death. This damage was at the upper dose for these inhibitors however, and at the experimental dose it can be seen that all inhibitors cause less than 3% cytotoxicity.

It was important to ensure that the inhibitors we were going to be using would not cause large levels of cellular death at the concentrations to be used in future work. This is because if incubating the fibroblasts with the inhibitors was leading to cell death then there would be a decrease in [^{3}H] thymidine incorporation compared to challenged cells, due to there being fewer cells able to synthesise DNA, but this may be interpreted as an inhibition of the action of a pathway, factor or MAPK. By showing that the inhibitors are not leading to large levels of cell death we can say that if there is a decrease in [^{3}H] thymidine incorporation that it will be due to the actions of the inhibitor, and not due to cellular death.

The above work had indicated that the concentration of inhibitors to be used was not cytotoxic. The dose of inhibitors to be used was decided from previous publications and also the construction of dose responses, which are not shown in this thesis. To gain more confidence in the effect the inhibitors could be having on fibroblast mitogenesis vehicle controls could also have been performed, although the data in fig 5.4 show that the inhibitors are not affecting the fibroblasts ability to incorporate [^{3}H] thymidine to a level which would affect the data to an unacceptable level.

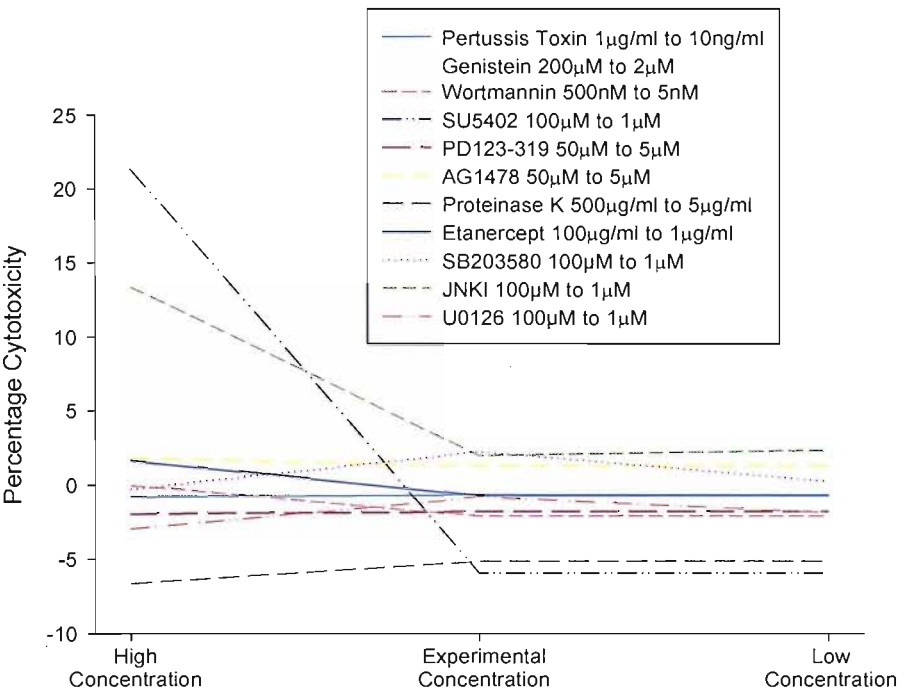


Fig 5.5 Graph showing the percentage cytotoxicity of individual inhibitors when incubated with primary lung fibroblasts (n = 1) over a 24 hour period. Cytotoxicity was determined using the LDH assay, cells were seeded at 5,000 cells per well in collagen coated plates. The plates were read at 490nm. The data represents the mean of 2 experiments.

The role of G-protein coupled receptors, tyrosine kinase receptors and PI3-K in fibroblast mitogenesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL.

It was initially decided to investigate the roles of G-protein coupled receptors, tyrosine kinase receptors and the intra-cellular messenger protein PI3-Ks role in BAL mediated mitogenesis. Fibroblasts from 2 healthy, 2 mild asthmatic and 2 severe asthmatic donors were challenged with BAL pooled from healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) volunteers in the presence or absence of the inhibitors, which were pre-incubated with the fibroblasts for 4 hours before challenge. Box plots contain 18 values each as each inhibitor treatment was performed in triplicate for challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL on 6 fibroblast cell lines.

The data in figs 5.6, 5.7 and 5.8 show that healthy, mild asthmatic and moderate/severe asthmatic BAL is able to significantly increase [^{3}H] thymidine incorporation compared to the ITS control ($p < 0.03$), and that the inhibition of G-protein coupled receptors with pertussis toxin leads to a consistently significant ($p < 0.05$) inhibition of this fibroblast mitogenesis, almost to baseline values, and that this is also independent of challenge with the different BAL. This suggests that G-protein coupled receptors are playing an important role in fibroblast proliferation, and that also their stimulation is ubiquitous in healthy or asthmatic BAL mediated fibroblast mitogenesis. This is not surprising as mitogenic factors such as LPA, PAF, leukotrienes and histamine are known to be present in BAL and signal through G-protein coupled receptors, however it was still interesting to investigate whether the BAL from asthmatics was utilising these receptors in an altered manner. However pertussis toxin only inhibits G-protein coupled receptors linked to the sub-unit, therefore to get a complete assessment of G-protein coupled receptors involvement in BAL mediated mitogenesis other G-protein coupled receptor inhibitors would need to be used, these include either broad spectrum inhibitors or inhibitors of other α sub-units. Examples of suitable candidates include [D-Arg1, D-Trp5, 7, 9, Leu11] SP, a substance P analogue and broad spectrum G-protein coupled receptor antagonist (although its exact specificity is as yet unknown) or BIM-46174, which inhibits the dissociation of the $G\alpha/G_{\beta/\gamma}$ heterotrimeric complex (Prevost G.P. *Et al.* 2006).

When cells were pre-incubated with wortmannin and genistein there was variable levels of inhibition. When cells were challenged with asthmatic BAL genistein was able to significantly ($p < 0.05$) inhibit fibroblast mitogenesis (figs 5.7 and 5.8), whereas wortmannin was inhibitory to mitogenesis after challenge with healthy and mild asthmatic BAL ($p < 0.05$) (figs 5.6 and 5.7). The data in the figures show that the overall inhibition of mitogenesis exerted by wortmannin and genistein is not as great as that seen when using pertussis toxin and this suggests that tyrosine kinase receptors and PI3-K may play a lesser role in fibroblasts mitogenesis than do G-protein coupled receptors. It is likely that if the fibroblast numbers were increased then more significance would be shown as the general trend is that treating the cells with genistein and wortmannin decreases the level of fibroblast [^{3}H] thymidine incorporation, without achieving significance. It would be interesting to use more potent tyrosine kinase and PI3-K inhibitors, such as SU6668 or LY294002 to further investigate their roles in fibroblast mitogenesis. Nevertheless, although PI3-K is a kinase utilised by many mitogenic pathways, that its inhibition does not lead to a more consistently significant inhibition of fibroblast [^{3}H] thymidine incorporation is not surprising as the mitogenic signal is likely to be propagated by many other intra-cellular kinases.

That the inhibition of fibroblast mitogenesis is so complete after incubation with pertussis toxin suggests either that G-protein coupled receptors are the predominant mitogenic signalling pathway stimulated by BAL challenge, or that their inhibition is affecting signalling through other pathways. There is evidence of G-protein coupled receptors interacting with tyrosine kinase receptors, where the activation of the IGF-1 receptor was required for thrombin induced smooth muscle mitogenesis (Delafontaine P. *et al.* 1996). The large level of inhibition seen when pre-incubating cells with pertussis toxin may therefore be due to both the inhibition of G-protein coupled receptors and also the removal of the co-stimulatory effects of G-protein coupled receptors on other receptor pathways. That this is not observed when using genistein may suggest that when there is mitogenic synergy between tyrosine kinase pathways and G-protein coupled receptors that tyrosine kinase pathways merely enhance the signal through G-protein coupled receptors and are not essential for mitogenic signalling through G-protein coupled receptors to occur.

The above work lead us to investigate the roles of individual factors of interest, these included bFGF, EGF, TNF-α, TGF-β and angiotensin II. It has been shown that bFGF is raised in the BAL of asthmatics (Redington A.E. *et al.* 2001) with TNF-α and TGF-β identified as potential targets from the fractionation of BAL (figs 4.15 and 4.16). Angiotensin II was chosen as it signals through G-protein coupled receptors and has been implicated in asthma (Ramsay S.G. *et al.* 1997), as has EGF (Puddicombe S. M. *et al.* 2000).

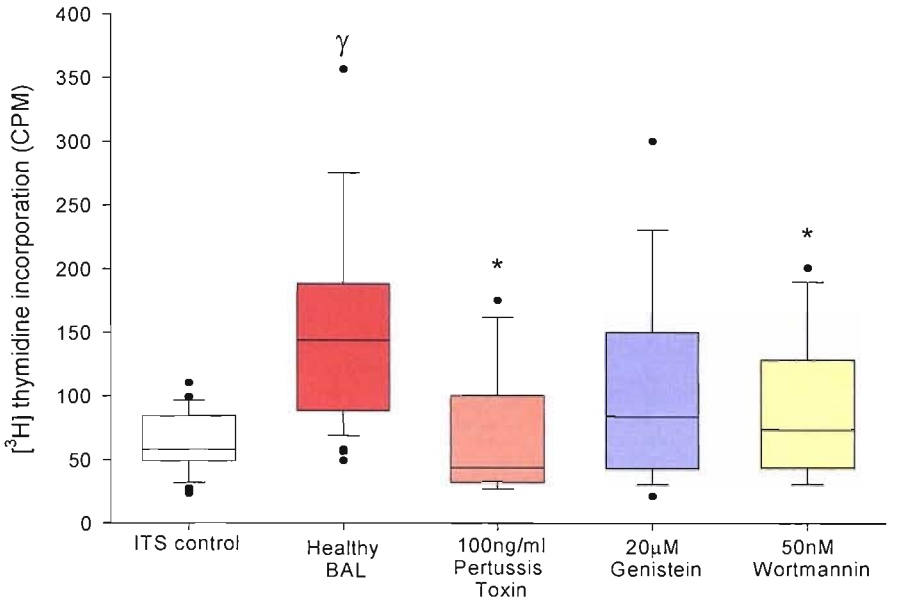


Fig 5.6 Graph showing the effects of inhibitors on healthy BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 4 hours before addition of BAL. γ = $p < 0.03$ vs ITS control * = $p < 0.05$ vs. stimulation with BAL.

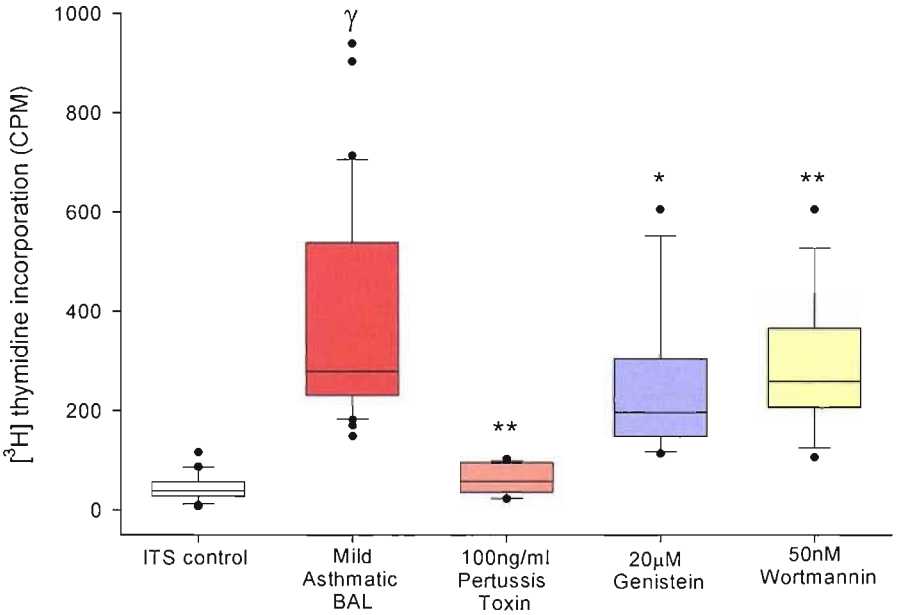


Fig 5.7 Graph showing the effects of inhibitors on mild asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 4 hours before addition of BAL. γ = $p < 0.03$ vs ITS control * = $p < 0.05$ vs stimulation with BAL ** = $p < 0.03$ vs stimulation with BAL.

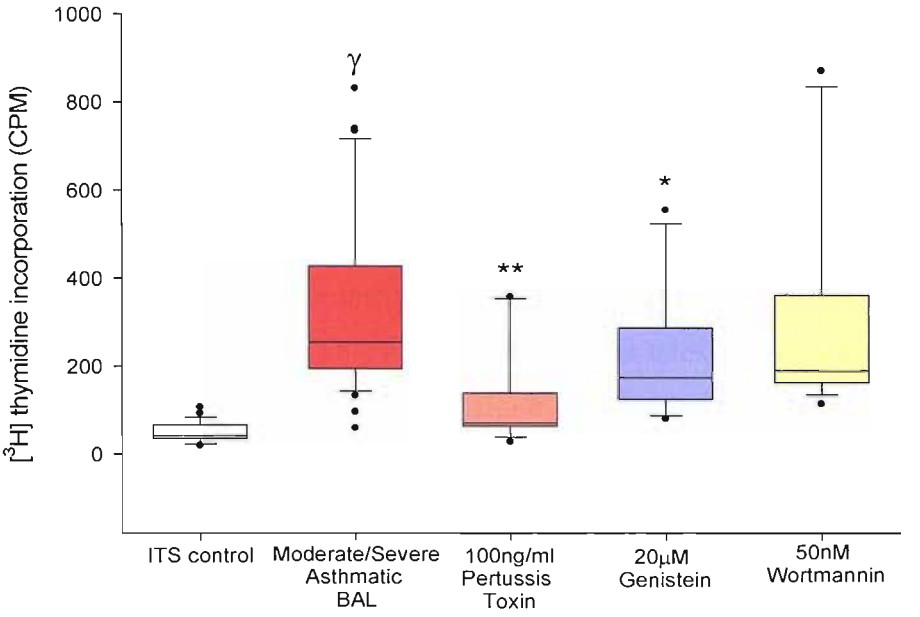


Fig 5.8 Graph showing the effects of inhibitors on moderate/severe asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 4 hours before addition of BAL. $\gamma = p < 0.03$ vs ITS control * = $p < 0.05$ vs stimulation with BAL ** = $p < 0.03$ vs stimulation with BAL.

The role of angiotensin II, bFGF and EGF in fibroblast mitogenesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL.

In figs 5.9, 5.10 and 5.11 the data show that as in figs 5.6, 5.7 and 5.8 all BAL could lead to a significant increase in [^{3}H] thymidine incorporation compared to the control ($p < 0.03$). The figure suggests that inhibition of the receptors of bFGF, EGF and angiotensin II all led to a significant decrease in fibroblast [^{3}H] thymidine incorporation compared to the uninhibited cells ($p < 0.05$). This observation was not unexpected as these factors are known mitogens, although angiotensin II alone did not lead to an increase in [^{3}H] thymidine incorporation in my earlier work (fig 5.1 and 5.2). The degree of inhibition observed in these figures is large, and this may be down to a couple of considerations. Firstly it may be that bFGF, EGF and angiotensin II all play an important role in BAL mediated fibroblast mitogenesis, either alone, or in synergy with other factors, or through receptor synergy. Secondly it may be that as the inhibitors used are not completely specific for only their receptors that the level of inhibition observed is due to the inhibition of one or more receptor or MAPK by the compound used. If future work were to be performed to continue this study it would be preferable to use neutralising antibodies against these factors in the BAL, to render them biologically inactive before addition to the cells. Having an excess of neutralising antibody present in the BAL would hopefully also limit the actions of bFGF, EGF and angiotensin II which may be subsequently produced by fibroblasts during BAL challenge, or any of these factors which may be membrane bound, and then later cleaved into the supernatant.

The above work suggested that proteins in BAL play a major role in mitogenesis so it was decided to cleave all the proteins present in BAL using proteinase K, to render them inactive and thus investigate the degree of contribution of proteins. In addition to this, and following on from the BAL fractionation work performed earlier, the roles of TNF-α and TGF-β in BAL mediated mitogenesis were also studied, by using a soluble TNF-α receptor, Etanercept, and a pan-specific TGF-β antibody to neutralise their effects in BAL.

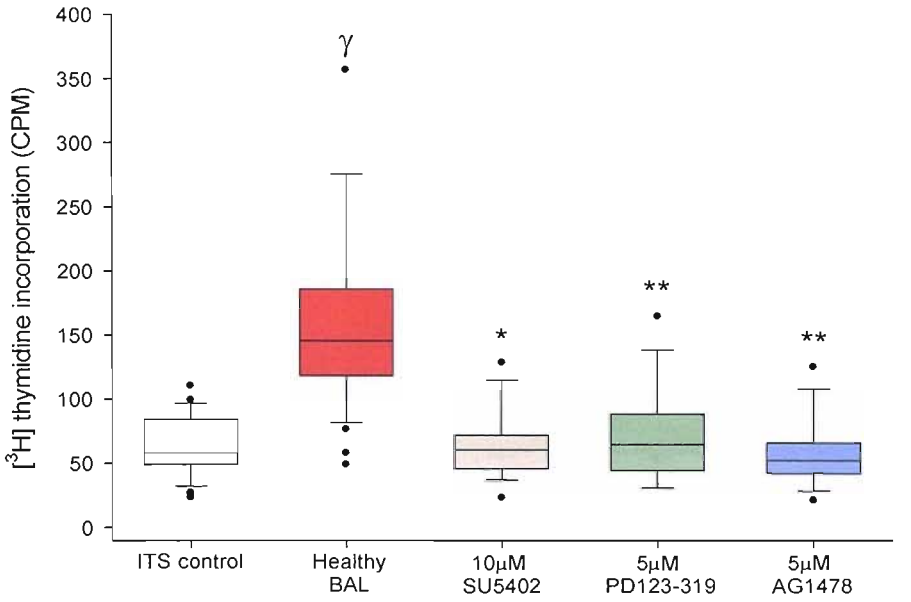


Fig 5.9 Graph showing the effects of inhibitors on healthy BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. γ = $p < 0.03$ vs ITS control * = $p < 0.05$ vs stimulation with BAL ** = $p < 0.03$ vs stimulation with BAL

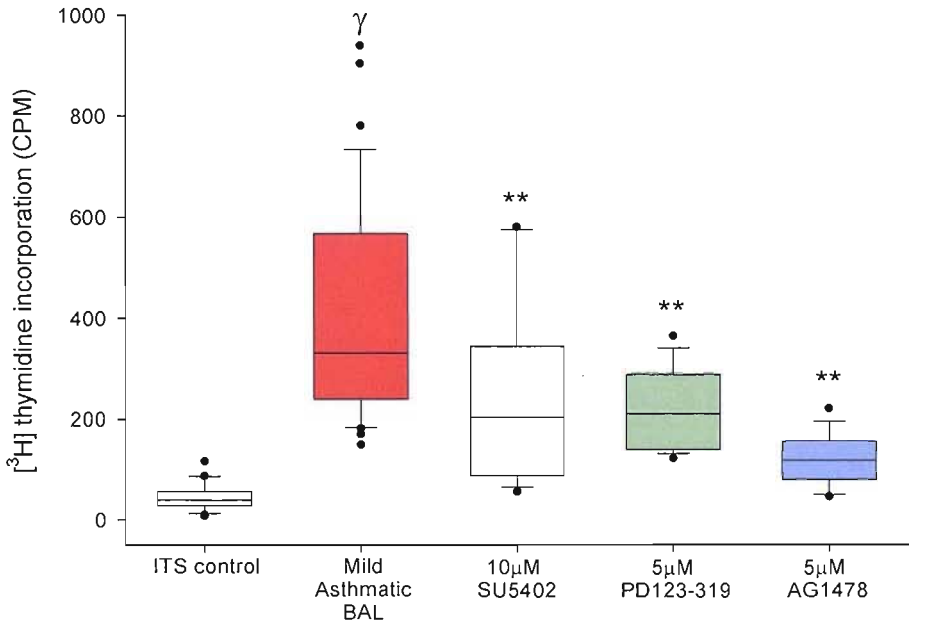


Fig 5.10 Graph showing the effects of inhibitors on mild asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs stimulation with BAL.

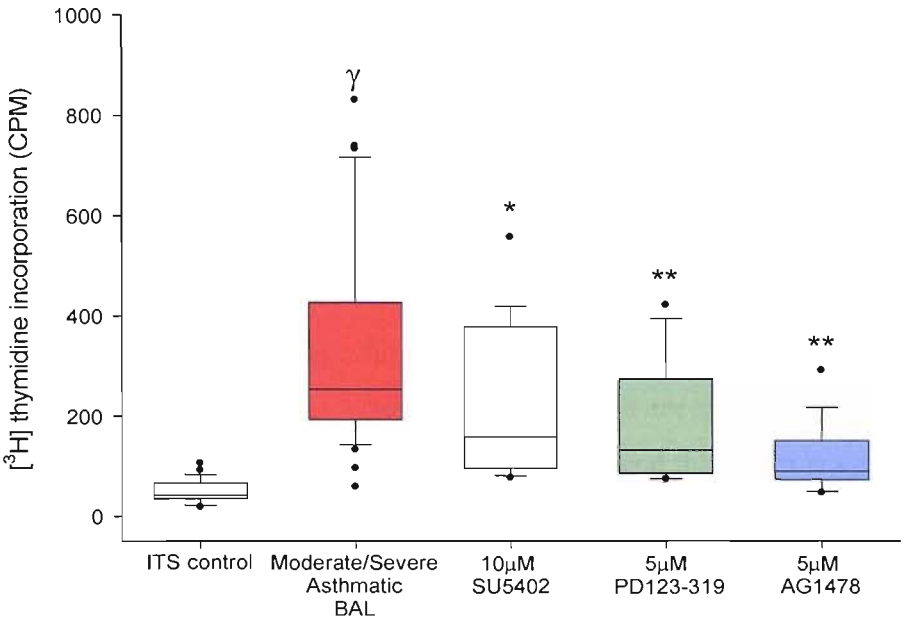


Fig 5.11 Graph showing the effects of inhibitors on moderate/severe asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. γ = $p < 0.03$ vs ITS control * = $p < 0.05$ vs stimulation with BAL ** = $p < 0.03$ vs stimulation with BAL.

The role of TNF-α and proteins in fibroblast mitogenesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL.

In figs 5.13, 5.14 and 5.15 it is shown that healthy, mild asthmatic and moderate/severe asthmatic BAL can all elicit a significant increase in fibroblasts [^{3}H] thymidine incorporation compared to the control. The data from fig 5.12 show that a range of concentrations of TNF-α is not able to elicit an increase in [^{3}H] thymidine incorporation compared to the ITS control, suggesting that when TNF-α is added alone it will not cause fibroblasts mitogenesis. However when a soluble TNF-α receptor (Etanercept) was pre-incubated with the BAL (figs 5.13, 5.14 and 5.15) it is seen that there is a significant and consistent inhibition of fibroblast [^{3}H] thymidine incorporation ($p < 0.03$). That the level of inhibition is consistent at the two doses of Etanercept indicates that even at the low dose there was an excess of antibody compared to the amount of TNF-α present in the sample. As TNF-α is unable to promote [^{3}H] thymidine incorporation alone, this raises the possibility that TNF-α is acting in synergy with other factors present in BAL to cause fibroblast mitogenesis, and that TNF-α is likely to be relatively important in this process, as its removal from the BAL leads to a significant inhibition in fibroblast [^{3}H] thymidine incorporation. The potential interactions of TNF-α with other factors will be discussed later, however as Etanercept is a soluble TNF-α receptor it is also able to bind to TNF-β and thus render it inactive. There is little work focusing on TNF-βs role in fibroblast proliferation, and no studies have shown it to be present in BAL, however, as TNF-β is produced by epithelial cells and its role in fibroblast mitogenesis has not been assessed in this study, its potential role in BAL mediated mitogenesis cannot be ignored.

Although a prominent role for TNF-α in fibroblast mitogenesis has been proposed in the above figures it is still unclear how this may be explained. To clarify these observations further work would be required to investigate how the inhibition of TNF-α is affecting the mechanics involved in BAL mediated mitogenesis, and whether this is through the interaction with other factors, by priming cells for proliferation, or by an alternate route. Nevertheless it is still noticeable that by whichever method TNF-α is influencing fibroblast [^{3}H] thymidine incorporation, that this is equally important in healthy and asthmatic BAL, and that it is unlikely that TNF-α signalling is abnormal in the asthmatic airways regulation of fibroblast proliferation.

The use of proteinase K in figs 5.13, 5.14 and 5.15 suggests that there is a prominent role for proteins in BAL, with proteinase K significantly inhibiting fibroblast [^{3}H] thymidine incorporation ($p < 0.03$), taking fibroblast mitogenesis down to baseline levels. It is likely that the use of proteinase K not only removes the mitogenic potential of proteins but also any synergy that various proteins may be having with other factors such as LPA, which is not a protein, but rather a phospholipid derivative. That proteinase K almost completely negates the mitogenic potential of all the BAL was unexpected though, as factors such as LPA are known fibroblast mitogens (van Corven E. J *et al.* 1989), and unlikely to have been affected by proteinase K, as they are not proteins. This observation may be explained, in part, by proteinase K not being inactivated before addition to the cells. Therefore the effects observed in the above figures may be due to non-specific effects of the proteinase K causing fibroblast detachment from the wells or receptor, as well protein, cleavage. Proteinase K wasn't inactivated as this requires heating to temperatures exceeding 50°C, and it was unsure how heating would have affected the BAL. To determine this the BAL could have been heated the required temperature and added to the cells along with unheated BAL, if heating the BAL had no effect then the proteinase K could be inactivated before addition to the cells in this way. Alternatively a serine protease inhibitor such as Phenylmethylsulfonyl fluoride (PMSF) or 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) could have been used to inactivate proteinase K in the BAL, but as with heating it would require the determination of their affects of BAL mediated mitogenesis before they were used. One of these inactivation steps would have to be undertaken before a definitive assessment could be made on proteins role in fibroblast proliferation.

To continue the investigation of individual factors contribution to BAL mediated mitogenesis the role of TGF-β was investigated using a pan-specific neutralising antibody.

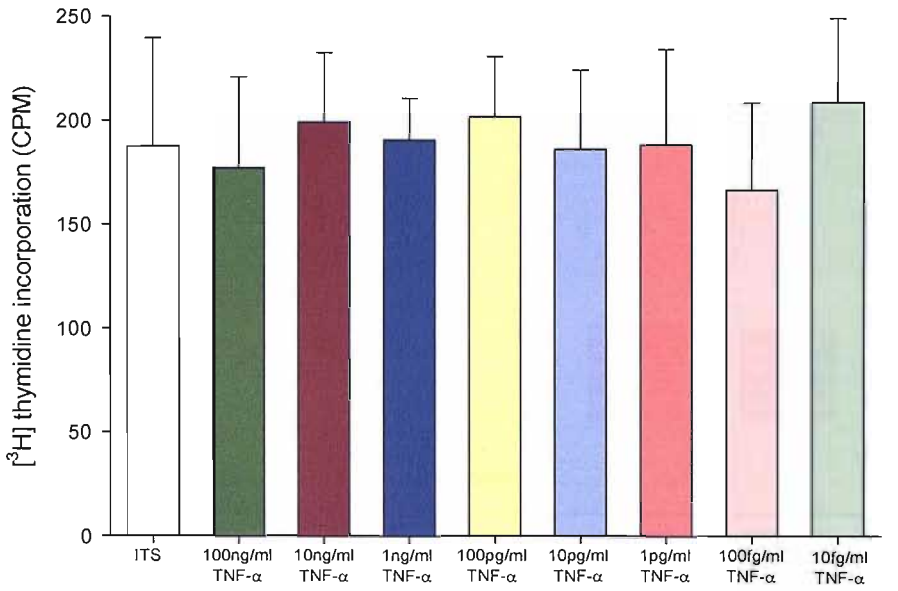


Fig 5.12 The effect of incremental TNF-α doses on primary lung fibroblasts (n = 2) assessed by the [^{3}H] thymidinine incorporation assay. Cells were seeded at 5,000 cells per well in collagen coated plates, stimuli was added for 24 hours before the addition of [^{3}H] thymidine. The data represents the mean of 2 experiments.

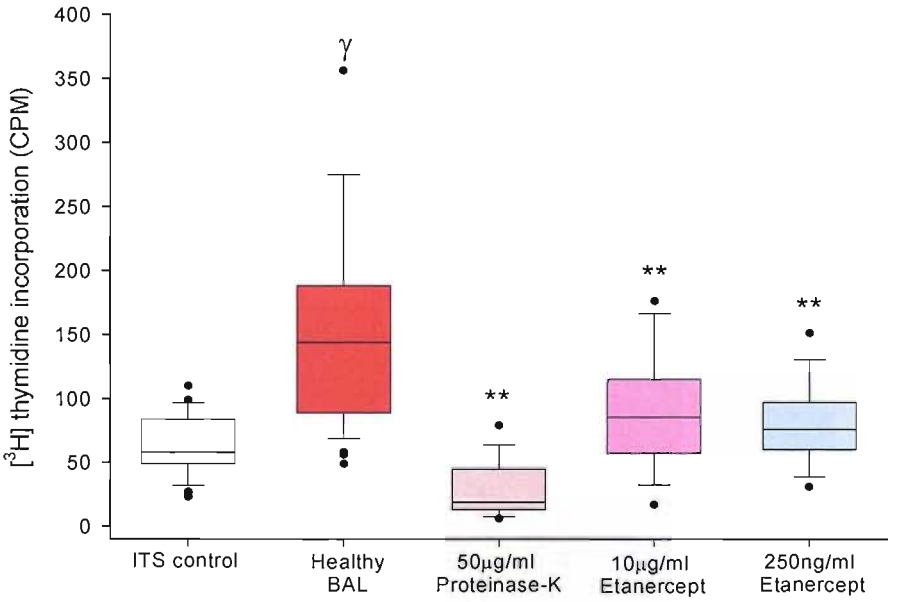


Fig 5.13 Graph showing the effects of inhibitors on healthy BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were added to the BAL 1 hour before challenge. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs stimulation with BAL.

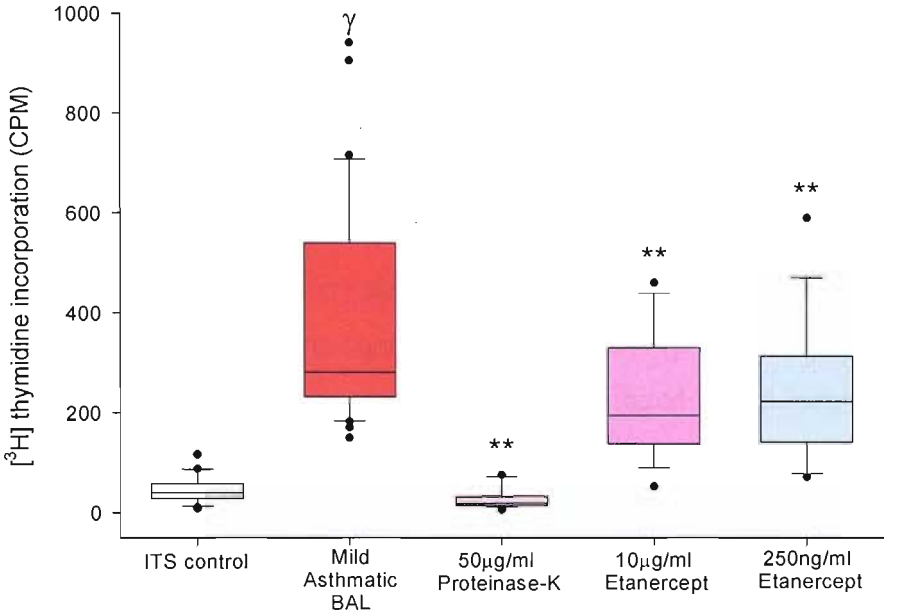


Fig 5.14 Graph showing the effects of inhibitors on mild asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were added to the BAL 1 hour before challenge. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs stimulation with BAL.

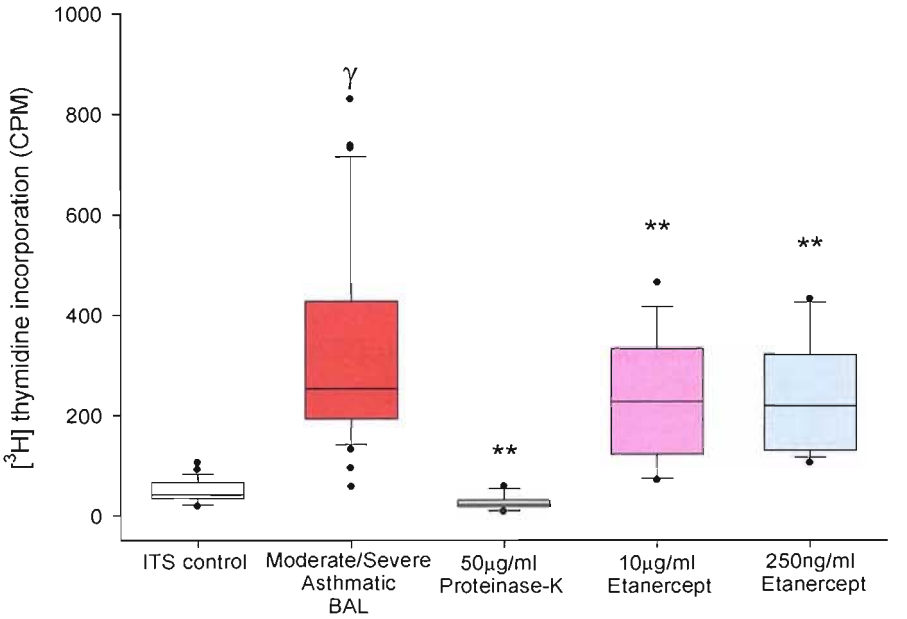


Fig 5.15 Graph showing the effects of inhibitors on moderate/severe asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were added to the BAL 1 hour before challenge. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs stimulation with BAL.

The role of TGF-β in fibroblast mitogenesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL.

In addition to the earlier work investigating the role of protein and TNF-α in BAL challenged fibroblast mitogenesis the role of TGF-β was investigated using a pan-specific neutralising antibody. Data in fig 5.18 show that healthy, mild asthmatic and moderate/severe asthmatic BAL are able to cause a significant increase in [^{3}H] thymidine incorporation from the fibroblasts compared to the control ($p < 0.008$), and this increase in [^{3}H] thymidine incorporation can be significantly inhibited by pre-incubating the BAL with the neutralising antibody for TGF-β ($p < 0.008$). The data in figs 5.16 and 5.17 show that TGF-β1 and TGF-β2 added at a range of concentrations were not able to increase [^{3}H] thymidine incorporation from fibroblasts, and this suggests that, as with TNF-α, TGF-β is likely to interact with other factors in BAL to elicit an increase, or decrese in fibroblast mitogenesis. The data in figs 5.16 and 5.17 are similar to that in fig 5.12 where fibroblasts were challenged with a range of TNF-α concentrations, in that it is unlikely that TGF-β1 or 2 are having any real affect of fibroblast [^{3}H] thymidine incorporation. The slight increases and decreases seen in [^{3}H] thymidine incorporation in these figures is likely to indicate experimental variations which are not relating to the effect of challenge. As already mentioned, if these experiments were repeated it would be likely that a similar pattern would be observed, however challenges which are showing slightly lower levels of [^{3}H] thymidine incorporation than the control in these figures may be slightly higher next time. This is not an important consideration when analysing earlier and subsequent work however, as the variations in [^{3}H] thymidine incorporation seen in figs 5.12, 5.16 and 5.17 are typically less than 50cpm, and this would have little bearing in BAL or growth factor challenged cells.

The pan-specific TGF-β neutralising antibody is specific to human TGF-β1, TGF-β2 and TGF-β3 (R&D technical document). The antibody was added to the BAL at a 1:500 dilution, giving an antibody concentration of 2μg/ml in the BAL, this concentration was determined by reading previous work by Tsang M.L. *et al.* 1995 and Giannouli C.C. *et al.* 2006. Tsang M.L. *et al.* 1995 showed that that in murine HT-2 cells the optimal antibody concentrations at which to inhibit the activity of 1-10pg/ml porcine TGF-β1 was between 1 and 10μg/ml. While Giannouli C.C. *et al.* 2006 discovered that 10μg/ml of pan-specific

TGF-β antibody could inhibit cellular proliferation bought about by challenge with 2.5ng/ml human TGF-β1 in adult skin fibroblasts. Levels of TGF-β1 in BAL have been previously measured in two separate studies, in the work by Redington A.E. *et al.* 1997 it was shown that TGF-β1 levels in atopic and asthmatic volunteers were around 5.5-8pg/ml respectively, while in the work by Batra V. *et al.* 2004 it was shown that in BAL from both healthy and asthmatic volunteers TGF-β1 levels were around 3.5pg/ml. The level of TGF-β2 in BAL from healthy and asthmatic patients has also been measured on two separate occasions (Scannell C. *et al.* 1996 and Batra V. *et al.* 2004). In the study by Scannell C. *et al.* 1996, TGF-β2 levels in BAL from asthmatic patients was shown to be around 4.9pg/ml, while in the study by Batra V. *et al.* 2004 it was shown to be around 12pg/ml, in the same study BAL from healthy patients was found to contain around 21.4pg/ml of TGF-β2. Therefore it is reasonable to assume that the concentration of antibody used in our work would be able to inhibit the activity of TGF-β1 and TGF-β2 in the BAL, as it is at a level approximately 100 times higher than either known concentration of TGF-β1 or 2. The levels of TGF-β3 have not been measured in BAL; however it is unlikely to be present at levels high enough to overcome the amount of antibody present.

The use of the pan-specific TGF-β antibody in fig 5.18 takes the [^{3}H] thymidine incorporation from the fibroblasts (1 healthy, 1 mild asthmatic and 1 severe asthmatic) down to around baseline unstimulated levels. This level of inhibition was unexpected given the previous findings in figs 5.16 and 5.17, however not unexpected when the relevant literature is taken into consideration. It has been shown that TGF-β1 can act in synergy with factors to promote fibroblast proliferation, and this will be discussed later. However, that TGF-β1 has been linked to a range of factors in regulating fibroblast proliferation helps explain how its inhibition could lead to such a large inhibition of fibroblast [^{3}H] thymidine incorporation. The pan-specific TGF-β antibody also inhibits the actions of TGF-β2 and TGF-β3 though, and work assessing their role in fibroblast proliferation is less complete. That they are inhibited by this antibody though means that their role cannot be ignored in BAL mediated mitogenesis; however it is still likely that TGF-β1 is the major regulator of fibroblast proliferation of the three.

As with the role of TNF-α it is difficult to determine exactly how the inhibition of TGF-β in the BAL is leading to the inhibition of [^{3}H] thymidine incorporation from the data in fig 5.18, however TGF-β appears to be equally important in healthy BAL mediated mitogenesis as it does in that of asthmatic BAL. It is therefore likely that TGF-β signalling is not abnormal in [^{3}H] thymidine incorporation promoted by asthmatic BAL. The further investigate of whether TGF-β and TNF-α are interacting with other factors present in the BAL could be approached in a number of ways including; assessing the proliferation of fibroblasts after challenge with individual factors of interest such as bFGF, EGF, PDGF and angiotensin II, and then in the presence of bFGF, EGF, PDGF and angiotensin II plus TGF-β 1, 2 and 3 and TNF-α, to determine whether there is any increase in proliferation. Alternatively the activation of receptors of interest such as the bFGF, EGF, PDGF and angiotensin II could be assessed after challenge with bFGF, EGF, PDGF and angiotensin II, and then after challenge with bFGF, EGF, PDGF and angiotensin II plus TNF-α and TGF-β, to investigate whether there is any up regulation of these receptors, and whether this relates to an increase in proliferation. As well as this additional work it would also be necessary to perform an isotype control using a IgG antibody, this would ensure the observed effects of the pan-specific TGF-β antibody were due to the inhibition of the activity of TGF-β, and not due to non-specific activity of the antibody.

The investigation of which MAPKs healthy and asthmatic BAL utilised was the next step in our work; there had so far been little variation between the three groups, however the search had been preliminary and superficial given the number of receptors and factors likely to be involved in fibroblast mitogenesis. It was hoped that the investigation of which MAPKs each BAL utilised would provide a more fundamental assessment of healthy and asthmatic BALs signalling routes, and illuminate any abnormalities in signalling.

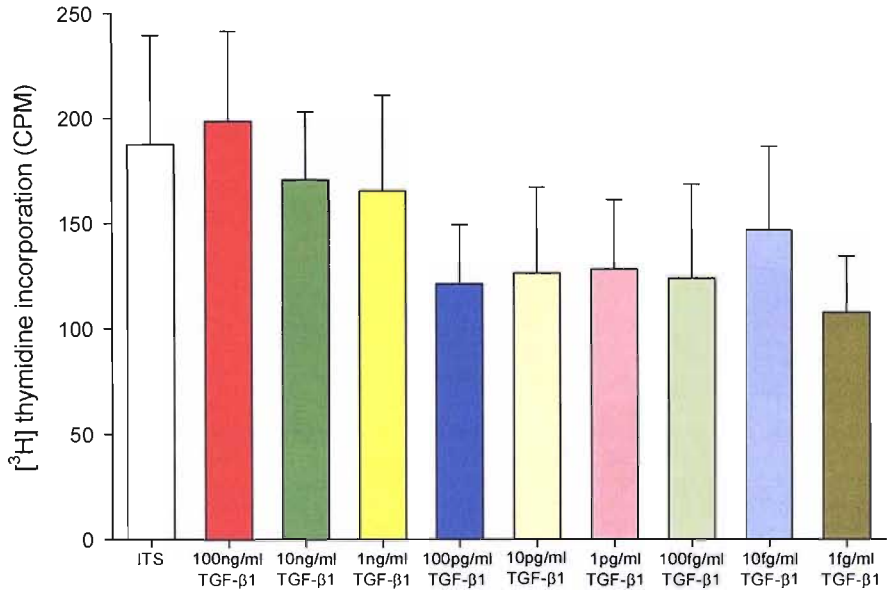


Fig 5.16 The effect of incremental TGF-β1 doses on primary lung fibroblasts (n = 2) assessed by the [^{3}H] thymidinine incorporation assay. Cells were seeded at 5,000 cells per well in collagen coated plates, stimuli was added for 24 hours before the addition of [^{3}H] thymidine. The data represents the mean of 2 experiments.

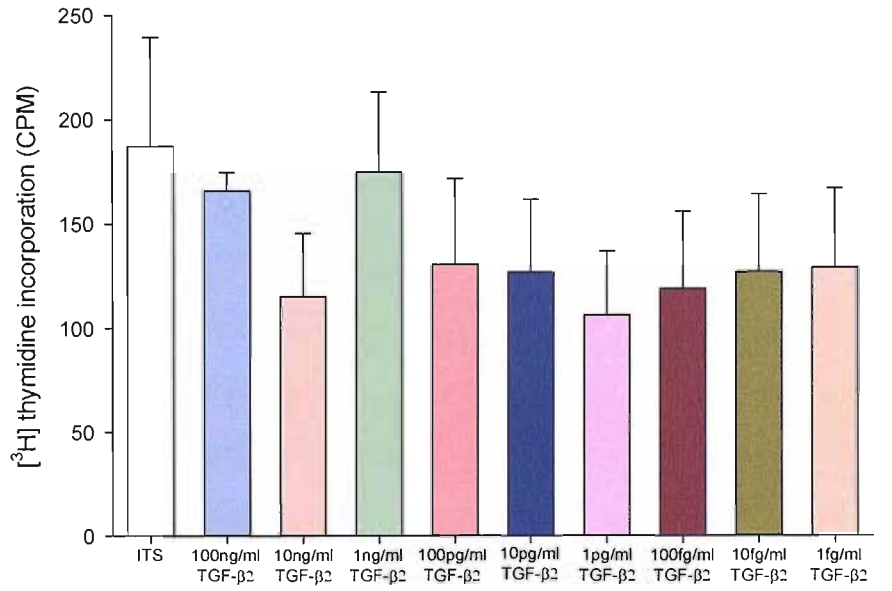


Fig 5.17 The effect of incremental TGF-β2 doses on primary lung fibroblasts (n = 2) assessed by the [^{3}H] thymidinine incorporation assay. Cells were seeded at 5,000 cells per well in collagen coated plates, stimuli was added for 24 hours before the addition of [^{3}H] thymidine. The data represents the mean of 2 experiments.

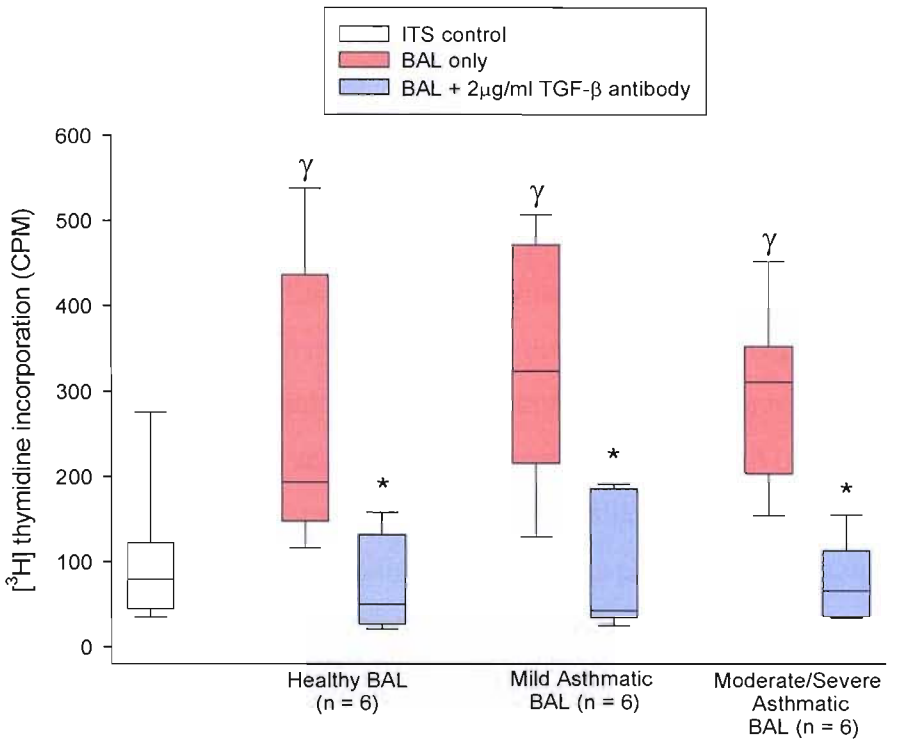


Fig 5.18 Fibroblasts from 1 healthy, 1 mild asthmatic and 1 severe asthmatic donor were challenge with BAL from healthy, mild asthmatic and moderate/ severe asthmatic volunteers in the presence or absence of 2μg/ml pan specific TGF-β antibody. Cells were seeded at 5,000 cell/well and challenged with BAL for 24 hours which had been pre-treated with the antibody for 1 hour, or left un-inhibited. $\gamma = p < 0.008$ vs ITS control * = $p < 0.008$ vs BAL only.

The role of MEK1/2, p38 and JNK in fibroblast mitogenesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL.

In figs 5.19, 5.20 and 5.21 the effect of inhibitors of p38, MEK1/2 and JNK MAPKs are investigated after fibroblasts were challenged with healthy, mild asthmatic and moderate/severe asthmatic BAL, and as with the previous figures the BAL was able to cause a significant increase in fibroblast [^{3}H] thymidine incorporation compared to the control ($p < 0.03$). The data in these figures show that inhibition of MEK1/2 with U0126 leads to a significant ($p < 0.03$) inhibition of [^{3}H] thymidine incorporation after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL, suggesting that MEK1/2 is important in fibroblast mitogenesis, and is unlikely to be abnormally activated by asthmatic BAL. This was as anticipated as MEK1/2 is positioned upstream of ERK1/2 which is involved in many mitogenic signals, in a variety of cells.

The role of the other MAPK was more variable however; the use of 10μM JNKI (JNK) and 10μM SB203580 (p38) lead to an increase in [^{3}H] thymidine incorporation, although this failed to reach statistical significance JNK and p38 MAPKs may be part of an important regulatory process involved in suppressing fibroblast proliferation to prevent a cascading in cellular division, although more work would be required to investigate this. However in fig 5.4, 10μM JNKI did lead to an increase in [^{3}H] thymidine incorporation from fibroblasts when added to the cells in the absence of challenge, although as this increase was not in the magnitude seen in the above figures, it is unlikely to be the sole explanation for this observation, but may be a contributory factor.

Conversely, data from fig 5.21 also show that when 10nM JNKI was pre-incubated with the fibroblast before addition of moderate/severe asthmatic BAL that there was a significant ($p < 0.03$) inhibition of [^{3}H] thymidine incorporation. That treating cells with 10nM JNKI before challenge with healthy and mild asthmatic BAL did not lead to a significant decrease in [^{3}H] thymidine incorporation may suggest JNK signalling is abnormally regulated by moderate/severe asthmatic BAL. However, when the median is compared between the BAL only challenged cells and the cells pre-incubated with 10nM JNKI before BAL challenge, it can be seen that there is only around a 30cpm disparity. This suggests that although the data may be significant, that in fact inhibition of JNK may

not be significantly inhibiting fibroblast mitogenesis, and that the significance observed may be due to random error.

The inhibitors were chosen for this work firstly for their selective ability to inhibit their targets, but also as they are believed not to inhibit the other targets we would be investigating. For example JNKI inhibits the phosphorylation of JNK without affecting the activation of ERK1/2 or p38, SB203580 inhibits p38 without affecting the activation of ERK or JNK, and U0126 inhibits MEK1/2 without inhibiting the phosphorylation of JNK. However, these inhibitors are only selective for their targets, and work has suggested that SB230580 can affect the activity of other MAPKs such as PKB-α, GSK3-β, ROCK II and LCK, while U0126 can affect the activity of PKB-α, p38 and ERK1 (Davies S. P. *et al.* 2000). Even so the role of JNK in moderate/severe asthmatic BAL mediated mitogenesis should be further investigated, by increasing the fibroblast numbers and by the use of a more specific inhibitor of JNK such as JNK inhibitor VIII.

That these inhibitors are not as specific as we'd have liked is not a major concern given that p38 and JNK inhibitors had little effect of fibroblast mitogenesis. Nevertheless if this work was to be extended to investigate the role of more MAPKs in BAL challenged mitogenesis then more specific MAPKs inhibitors would be required. The data from figs 5.19, 5.20 and 5.21 suggest that, as with the previous figures, there is little variation in the activation of MAPKs in mitogenic signalling initiated by healthy and asthmatic BAL. There may be a possible mis-regulation of JNK after challenge with moderate/severe asthmatic BAL, however, as already mentioned, this requires further investigation.

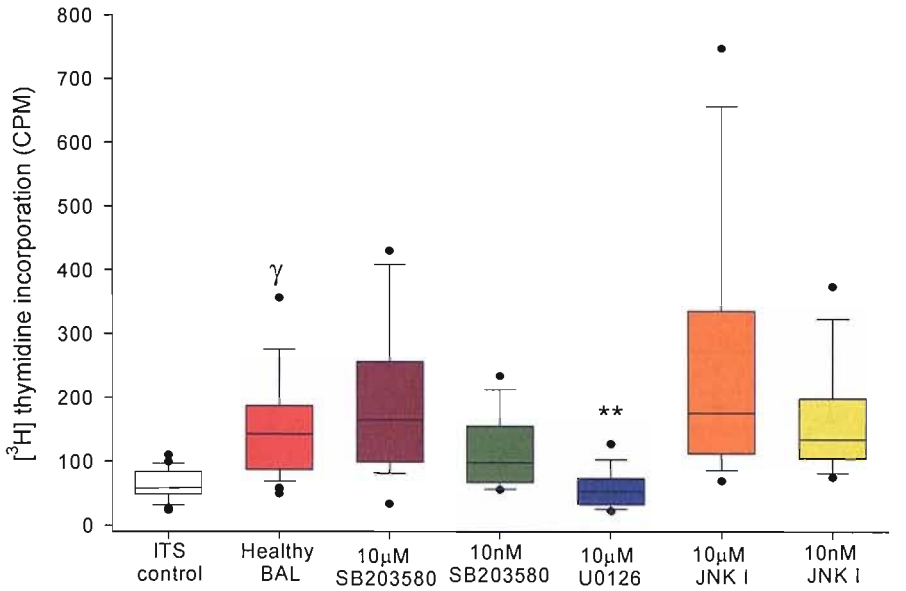


Fig 5.19 Graph showing the effects of inhibitors on healthy BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs challenge with BAL.

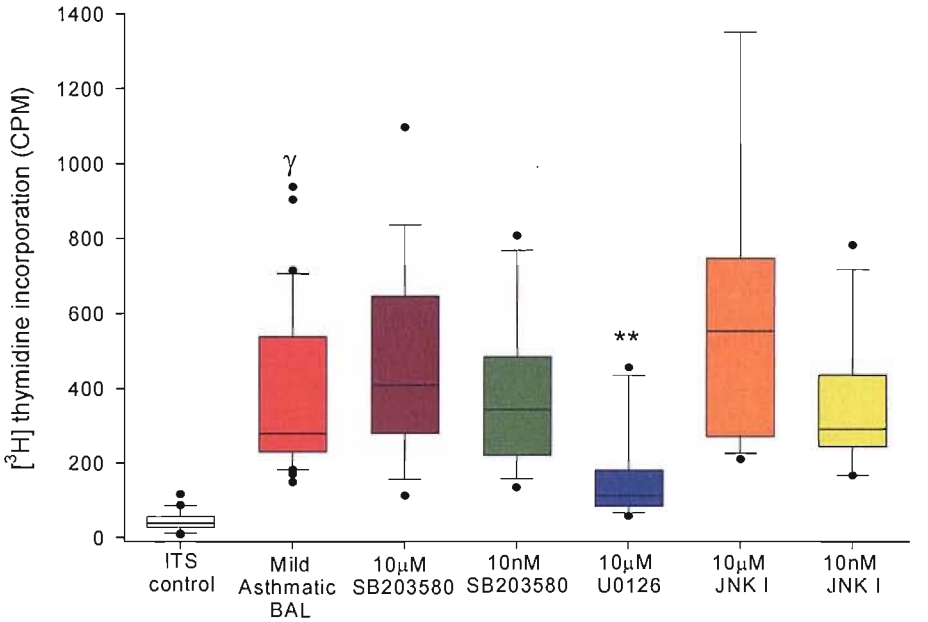


Fig 5.20 Graph showing the effects of inhibitors on mild asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. γ = $p < 0.03$ vs ITS control ** = $p < 0.03$ vs challenge with BAL.

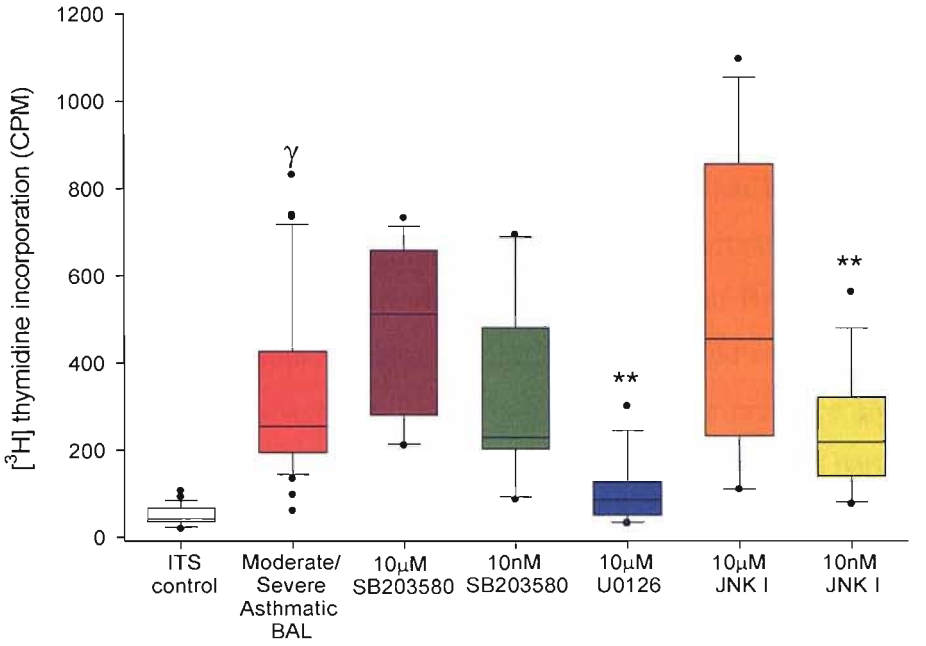


Fig 5.21 Graph showing the effects of inhibitors on moderate/severe asthmatic BAL (n = 6) mediated primary lung fibroblast (n = 6) mitogenesis, assessed after a 24 hour challenge using the [^{3}H] thymidine incorporation assay. Inhibitors were pre-incubated for 1 hour before addition of BAL. $\gamma = p < 0.03$ vs ITS control ** = $p < 0.03$ vs challenge with BAL.

Discussion

Although it was established in preceding work that it was most likely the fibroblasts themselves rather than the environment in which they reside that led to the variation in the response of severe asthmatic fibroblasts, it was important to establish which pathways, factors and intra-cellular messengers are involved in BAL induced fibroblast mitogenesis. Therefore fibroblasts grown from 2 healthy, 2 mild asthmatic and 2 severe asthmatic bronchial biopsies were challenged with BAL in the presence and absence of various inhibitors, and their data pooled. The fibroblasts chosen for this part of my study were identified as the fibroblasts which were able to best respond to BAL challenge from each group in the previous chapter. This was because, unlike in the work where direct cell counts were performed after BAL challenge (figs 4.8, 4.10 and 4.12), the response of healthy and asthmatic fibroblasts were not being compared. Therefore to provide the most comprehensive data the most responsive cells from each group were chosen, as it would be difficult to assess the effect of the various inhibitors if there was an insignificant increase in [^{3}H] thymidine incorporation after BAL challenge, when compared to the ITS control.

In an attempt to understand the mechanisms and mediators of relevance to the mitogenic activity in BAL we used pertussis toxin to inhibit G-protein coupled receptors, genistein to inhibit tyrosine kinase pathways and wortmannin to inhibit the intracellular signalling molecule PI3-K. As well as these broad spectrum inhibitors more specific inhibitors have been used to inhibit the angiotensin II receptor 2 (PD123-319), EGF receptor (AG1478), an FGFR1 inhibitor (SU54020), TGF-β family (pan-specific-TGF-β antibody), TNF-α (Etanercept), MEK1/2 (U0126), p38 (SB203580) and JNK (JNKI1). The inhibitor concentrations used in this work were determined by looking at previous literature to determine a commonly used starting concentration, and then analysing whether this was the optimum concentration for our study by the use of dose response. These data are not presented in this work, however the work from which the starting concentrations were established were; pertussis toxin (Chen Y.H. *et al.* 1994), genistein (Segain J.P. *et al.* 1996 and Phagoo S.B. *et al.* 2001), wortmannin (Ricupero D.A. *et al.* 2001a and Utsugi M. *et al.* 2003), SU5402 (Rosenthal R. *et al.* 2001), AG1478 (Tebar F. *et al.* 2002), PD123-319 (Peng H. *et al.* 2001) Etanercept (Loos T. *et al.* 2006), pan-specific-TGF-β

antibody (Yurovsky V.V. *et al.* 2003 and Lam S. *et al.* 2004), U0126 (Favata M.F. *et al.* 1998), SB203580 (Morley S.J. *et al.* 1997), JNK I (Sugisawa N. *et al.* 2004) and proteinase K (Zhang M. *et al.* 2004). The data obtained from the use of these inhibitors have indicated that G-protein coupled receptors, bFGF, EGF, angiotensin II, TNF-α, TGF-β, MEK1/2 and proteins are all important in the ability of BAL to elicit an increase in [^{3}H] thymidine incorporation. There are also be roles for tyrosine kinase receptors and PI3-K, however from these initial data it appears that they play a lesser role.

The role of the receptors, factors and MAPKs used in this study to assess fibroblast mitogenesis are well characterised inhibitors, and although they may not be as specific as other commercially available inhibitors, they are nonetheless selective for their targets, and were the best options of which we were aware at the time. Previous studies assessing fibroblast proliferation have indicated a role for:

- **G-protein coupled receptors** - (Kelvin D.J. *et al.* 1989, Huang N.N. *et al.* 1993, Alderton F. *et al.* 2001, Kim J. *et al.* 2002, Matthiesen S. *et al.* 2006 and Pierce E.M. *et al.* 2007).
- **Tyrosine kinase receptors** - (van Corven E.J. *et al.* 1993, Bennett S.A. *et al.* 1997 and Kuzumaki T. *et al.* 1998).
- **PI3-K** - (Hafizi S. *et al.* 1999, Kim G. *et al.* 2002, Kim J. *et al.* 2002 Gerasimovskaya E.V. *et al.* 2005 and Li J. *et al.* 2006).
- **bFGF** - (Chambard J.C. *et al.* 1987, Inoue Y. *et al.* 2002 and Chen Y. *et al.* 2003).
- **EGF** - (Carpenter G. *et al.* 1976, Lembach K.J. 1976, Chambard J.C. *et al.* 1987, Huang N.N. *et al.* 1993, Kim D.S. *et al.* 1998, Hetzel M. *et al.* 2005 and Motoki T. *et al.* 2005).
- **Angiotensin II** - (Neuss M. *et al.* 1994, Schuttert J.B. *et al.* 2003, Hafizi S. *et al.* 2004, He Y. *et al.* 2006 and Liu H.W. *et al.* 2007).
- **TNF-α** - (Elias J.A. 1988a, Kitasato H. *et al.* 2001, Lacey D. *et al.* 2003, Ouyang P. *et al.* 2004b, Magne D. *et al.* 2006 and Yokota K. *et al.* 2006).

- **TGF-β** - (McAnulty R.J. *et al.* 1997, Thannickal V.J. *et al.* 1998, Kim G. *et al.* 2002, Denk P.O. *et al.* 2003, Ge X.N. *et al.* 2004, Kottler U.B. *et al.* 2005 and Pelaia G. *et al.* 2007).
- **MEK1/2** - (Laine P. *et al.* 2000, Dabbagh K. *et al.* 2001, Phan T.T. *et al.* 2003 and Stockand J.D. *et al.* 2003).

There have also been suggested roles for p38 (Chou F.P. *et al.* 2002, Schiemann W.P. *et al.* 2002 and Mandal S.K. *et al.* 2005) and JNK (Chen G. *et al.* 2000, Chou F.P. *et al.* 2002 and He X.Q. *et al.* 2007) in fibroblast proliferation, however observations from this study are unclear on the role of these MAPKs in BAL challenged fibroblast proliferation. There is some suggestion that the inhibition of p38 and JNK may be leading to an increase in fibroblast [^{3}H] thymidine incorporation (figs 5.19, 5.20 and 5.21), while the inhibition of JNK may also lead to the inhibition of fibroblast mitogenesis, however both of these observations requires more investigation.

The data from our observations suggest that although a number of receptors, factors and MAPKs are implicated in BAL mediated mitogenesis, that there is little, if no, variation in the utilisation of these signalling mediators between healthy or asthmatic BAL. This does not mean that the composition of each BAL is the same though; it is likely that there is great variation in the composition of BAL from the same groups, as well as the composition of BAL from healthy and asthmatic donors. However as BAL is a complex mixture of factors, there are likely to be multiple proliferative pathways activated at the same time, which should mean that the inhibition of a receptor or MAPK could be compensated for by the other activated pathways. However this is not the case, we can see in the previous data that the inhibition of multiple receptors individually leads to a significant inhibition in fibroblast mitogenesis, and this suggests that there is either significant factor-factor and/or receptor-receptor synergy present in BAL, and that the disruption of one of these interactions leads to a disproportionate reduction in fibroblast [^{3}H] thymidine incorporation, or that the inhibitors used are inhibiting multiple targets and this is affecting the data.

When tyrosine kinase receptor activity was assessed using genistein, it was seen that there was a significant inhibition of [^{3}H] thymidine incorporation observed, however this

was not consistent, or to the degree observed when inhibiting G-protein coupled receptors (fig 5.6, 5.7 and 5.8). The importance of bFGF and EGF receptors shown earlier (figs 5.9, 5.10 and 5.11), but a lesser importance of tyrosine kinase receptors therefore seems to be at odds, as both the bFGF and EGF receptors are receptor tyrosine kinases. This observation may be due to a number of considerations, such as genistein having a weaker inhibitory effect on tyrosine kinase receptor activity than expected in this study. However, genistein is widely regarded to be a potent tyrosine kinase inhibitor, but studies have questioned its potency (Graber M. *et al.* 1992 and Vostal J. G. *et al.* 1996). It may be that the use of another broad spectrum receptor tyrosine kinase inhibitor such as SU6668 may prove to be more suitable for this work. Alternatively it may be that as SU5402, AG1478 and PD123-319 are not specific for their targets, that the significant levels of inhibition witnessed with their use may be due to the inhibition of multiple targets.

For example as SU5402 is a selective FGF-1 receptor inhibitor it will also affects the activity of aFGF which is a known fibroblast mitogen (Yu Y.L. *et al.* 1993, Saita N. *et al.* 1994 and Thannickal V.J. *et al.* 1998). SU5402 also weakly affects the activity of the PDGF receptor, also a fibroblast mitogen, and therefore to gain extra confidence from the observations using SU5402 it would be necessary to challenge fibroblasts with aFGF and PDGF in the presence and absence of SU5402 to determine the effect of SU5402 on these two mitogens. As with SU5402, it would be necessary to identify the contribution made by EGF and angiotensin II to the inhibition witnessed when using AG1478 and PD123-319, as AG1478 abolishes ERK activation induced by angiotensin II and also affects the activity of the PDGF receptor, and the specificity of PD123-319 was not determined. To achieve this neutralising antibodies could be used against bFGF, aFGF, angiotensin II, PDGF and EGF in BAL to determine the contribution of each factor to BAL mediated mitogenesis, and then these antibodies could be used in combinations to assess whether the relative inhibitory magnitude observed when using SU5402, AG1478 and PD123-319 was due to the inhibition of the individual target or a combination of targets. Alternatively we could have used siRNAs to explore the role of the various receptors by silencing their expression in the cells.

Therefore, as discussed above, the non-specific inhibitory contribution of the compounds used, could be accounting for the potentially exaggerated inhibition observed in the data in this chapter. However the synergy between different factors and receptors used could also help explain these data. Previous research by Yoshisue H. *et al.* 2007 has suggested that pertussis toxin inhibited the mitogenic effects of LTD_4 when used in synergy with EGF, bFGF and PDGF-BB, but did not inhibit EGF, bFGF or PDGF-BB mediated mitogenesis when the factors were used on their own. A finding supported by Chambard J.C. *et al.* 1987, who illustrated that pertussis toxin inhibited up to 95% of thrombin induced mitogenicity without affecting EGF or FGF induced DNA synthesis and proliferation. Therefore these observations suggest that although pertussis toxin does not affect the activity of individual factors signalling through tyrosine kinase pathways, it is able to remove the mitogenic synergy seen between factors acting through G-protein coupled receptors in concert with factors acting through tyrosine kinase pathways. This implies that the inhibitory effect seen when using pertussis toxin is likely to be due to both the inhibition of individual factors acting through G-protein coupled receptors, and also the removal of synergy with other pathways, hence this may explain the large inhibitory effect seen with the use of pertussis toxin.

This work is supported by data using genistein as an inhibitor of tyrosine kinase receptors where work by Bennett S.A. *et al.* 1997 suggested that platelet activating factor (PAF) could cause proliferation in primary human skin fibroblasts, and that this effect could be inhibited by pre-treatment with genistein. This is intriguing as PAF acts through G-protein coupled receptors, and this observation is supported by work undertaken by van Corven E.J. *et al.* 1993, who suggested that fibroblast proliferation, after stimulation with thrombin, was inhibited by pertussis toxin and genistein, thus illuminating a possible co-operation between the two signalling pathways in fibroblast proliferation. The use of pertussis toxin and genistein in conjunction has not been used to assess primary human lung fibroblast mitogenesis upon stimulation with BAL in my study; however this could be an intriguing area of future work. Although the data in this work has suggested that

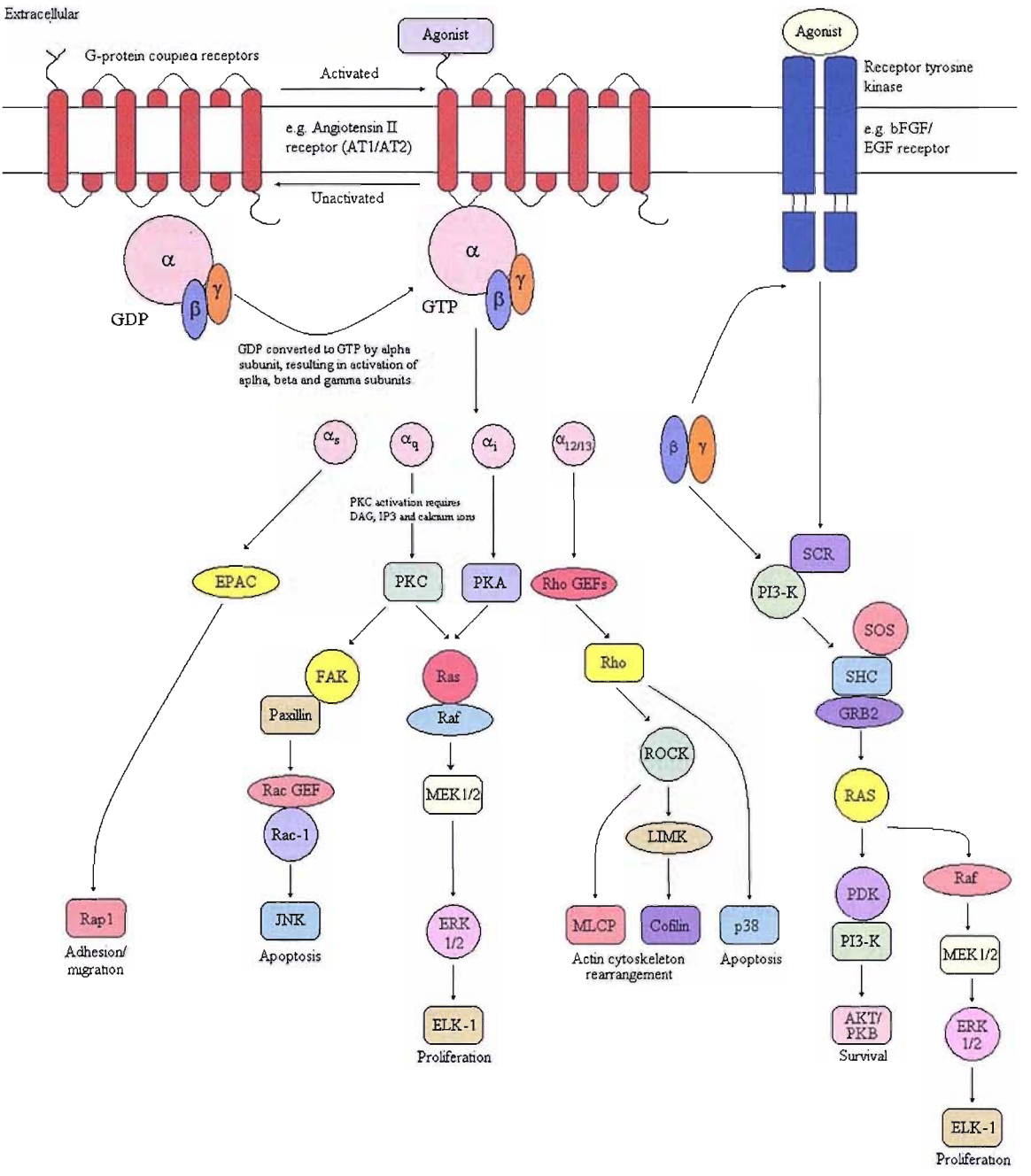


Fig 5.22 G-protein coupled receptor and tyrosine kinase signalling pathways, adapted from Sodhi A. *et al.* 2004.

receptor tyrosine kinases may not be the major signalling pathway involved in fibroblast mitogenesis, it is still likely that they are important, as indicated by the inhibition of the bFGF and EGF receptors (fig 5.9, 5.10 and 5.11), and as mentioned earlier, the use of an alternate receptor tyrosine kinase inhibitor may elicit a larger inhibitory effect. The previous work referenced above suggest that there is likely to be cross-talk between G-protein coupled and tyrosine kinase signalling pathways, and this is a potentially interesting avenue of exploration for BAL mediated fibroblast mitogenesis.

One such interaction is the mediation of G-protein coupled receptors activity by EGF receptors. It is believed that the activation of G-protein coupled receptors by factors such as angiotensin II (Eguchi S. *et al.* 1998 and Ohtsu H. *et al.* 2006), LPA (Kranenburg O. *et al.* 2001) and ET-1 (Hua H. *et al.* 2003) leads to the transactivation of the EGF receptor, resulting in the activation of the Ras/MAPK pathway (Gschwind A. et al. 2001, Ahmed I. *et al.* 2003 and Wetzker R. *et al.* 2003), and this has been shown to be important in fibroblasts (Schafer B. *et al.*2004). The transactivation of receptor tyrosine kinases is likely to play an important role in mediating mitogenic signals through G-protein coupled receptors (Daub H. *et al.* 1996, Weiss F.U. *et al.* 1997 and Herrlich A. *et al.* 1998). It is therefore possible that the use of AG1478, which leads to a large level of inhibition in figs 5.9 to 5.11, is truncating the mitogenic signal through inhibiting the transactivation of the EGF receptor by G-protein coupled receptors. One mechanism of EGF receptor transactivation by G-protein-coupled receptors is through the metalloproteinase cleavage of membrane bound EGF receptor ligands such as pro-heparin-binding-EGF (pro-HB-EGF) (Prenzel N. *et al.* 1999), amphiregulin (Schafer B. *et al.*2004 and Zhang Q. *et al.* 2006) and TGF-α (Schafer B. *et al.*2004). Metalloproteinases involved in the cleavage of these ligands which have been identified to date are ADAM9 (Izumi Y. *et al.* 1998), ADAM10 (Yan Y. *et al.* 2002), ADAM12 (Asakura M. *et al.* 2002), ADAM15 (Schafer B. *et al.*2004) and ADAM 17 (Schafer B. *et al.*2004).

Therefore in our model both pertussis toxin and AG1478s inhibitory effects may be partly due to the removal of cross-talk between G-protein coupled and EGF receptors. When cells were treated with pertussis toxin it may be that ADAM protein activity is down regulated culminating in decrease mitogenic signalling through the EGF receptor. While when fibroblasts are pre-treated with AG1478 the ADAM proteins may cleave the

membrane bound ligands, but as the activity of the EGF receptor is inhibited the propagation of mitogenic signal is also inhibited.

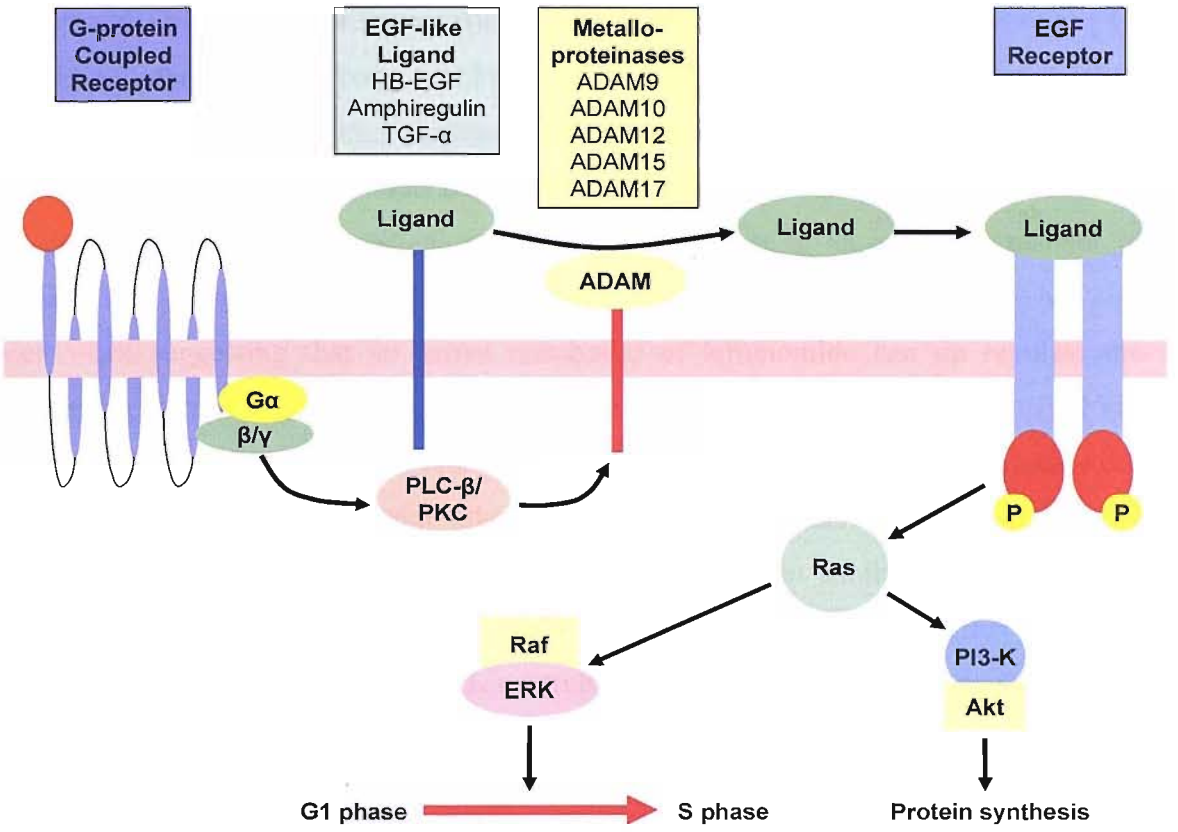


Fig 5.23 Overview of ADAM proteins role in G-protein coupled receptor cross-talk with the EGF receptor. In this interaction factors such as LPA, ET-1 and angiotensin II are able to stimulate their G-protein coupled receptor, which are then able to activate ADAM proteins through intra-cellular kinases such as PLC-β (Mifune M. *et al.* 2005) and PKC (Horiuchi K. *et al.* 2003). The activated ADAMs are then able to cleave membrane bound ligands such as HB-EGF (Prenzel N. *et al.* 1999), amphiregulin (Schafer B *et al.*2004) and TGF-α (Schafer B *et al.*2004). Once cleaved these ligands are able to stimulate EGF receptors, which may then initiate cellular events such as proliferation (Eguchi S. *et al.* 1998) and protein synthesis (Voisin L. *et al.* 2002).

As well as the complicated interactions between receptors which are likely to be involved in BAL challenged fibroblasts there is also liable to be synergy between factors present in BAL, which also contribute to the observed mitogenesis. Even though extensive analyses of the proteins present in BAL have been performed (Lindahl M. *et al.* 1999, Wattiez R. *et al.* 1999 and Wu J. *et al.* 2005), we still possess an incomplete proteome profile, and this makes the investigation of any synergy between factors difficult. Nevertheless through the observations from the data in this chapter, and previously published work, the

interactions between bFGF, EGF, TGF-β, TNF-α and angiotensin II could be investigated with regard to proteins believed to be present in BAL, and indeed with each other.

The obvious factors to begin investigating potential sources of mitogenic synergy are TGF-β and TNF-α, as these factors were shown to not elicit a significant increase in [^{3}H] thymidine incorporation from fibroblasts (figs 5.12, 5.16 and 5.17), however their inhibition lead to a significant decrease in fibroblast mitogenesis (figs 5.13, 5.14, 5.15 and 5.18). In previous work it has been shown that both TNF-α and TNF-β can cause an increase in prostaglandin production, which may then act in an autocrine manner to increase cellular proliferation (Elias J.A. 1988b and Frost A. *et al.* 1997). There has also been work suggesting that an active metabolite of leflunomide can up regulate the mitogenic effect of TNF-α in synovial fibroblasts (Magne D. *et al.* 2006), as can IL-15 in activated T cells (Rappl G. *et al.* 2001). Despite these references, there is not much work cataloguing TNF-α synergy with other factors to elicit cellular proliferation. It may therefore be that TNF-α is affecting fibroblast mitogenesis in another way, such as by priming the cells for entry into the cells cycle. To investigate whether TNF-α is interacting with other factors in BAL neutralising antibodies against a range of factors such as bFGF, EGF, TGF-β, angiotensin II and various prostaglandins, along with TNF-α, could be used in combinations to assess their contributions, and any possible interactions with TNF-α.

The documentation of TGF-βs interaction with other factors is more complete however, with TGF-β1 being shown to synergise with factors such as such as bFGF (Story M.T. *et al.* 1993, Strutz F. *et al.* 2001 and Khalil N. *et al.* 2005), EGF (Popik W. *et al.* 1991), PDGF (Dennison D.K. *et al.* 1994) and angiotensin II (Schuttert J.B. *et al.* 2003) to promote fibroblast proliferation. It has also been suggested that TGF-β1 is able to act with multiple factors to regulate fibroblast proliferation, such as bFGF and EGF (Narine K. *et al.* 2006), bFGF and PDGF (Bosse Y. *et al.* 2006) and CTGF in the presence of EGF and PDGF (Grotendorst G.F. *et al.* 2004). Interestingly in the Grotendorst G.F. *et al.* 2004 it was seen that if the levels of pro-mitogens (EGF and PDGF) fell below concentrations that supported cellular proliferation that the cells would become synthetic, if the appropriate growth factors were present, in this case IGF, and that this was also mediated by CTGF and TGF-β1. This indicates a complex synergy between factors in

BAL, and also indicates that if we begin to dilute the BAL, as suggested in the previous chapter, to investigate whether BAL was being used at a dilution at which no variation in its effects could be seen, that we may take the concentration of mitogens down to a level where we begin to promote other cellular processes.

Inhibition of bFGF, EGF and angiotensin II individually (figs 5.9, 5.10 and 5.11) show that each factor plays a role in fibroblast mitogenesis, therefore if TGF-β1 can regulate bFGF, EGF and angiotensin II mediated fibroblast proliferation, then inhibition of the actions of TGF-β1 would influence each growth factors effects, possibly leading to the large inhibitory response seen in fig 5.18 The obvious starting point for future research is to investigate whether neutralising antibodies against TGF-β1 added in combinations with antibodies against bFGF, EGF, CTGF, PDGF and angiotensin II can inhibit BAL mediated mitogenesis to the levels observed in fig 5.18, and then this may also be followed up by the investigating of whether antibodies against TGF-β2 and TGF-β3 alone can effect fibroblast mitogenesis alone, if they do then further investigation with combinations of antibodies against other factors may be of interest.

As the pan-specific TGF-β antibody also inhibited the actions of TGF-β2 and TGF-β3 it is necessary to investigate any potential roles for these mediators in BAL. There is little work supporting synergy between TGF-β2 and other growth factors in increasing cellular proliferation, with a majority of the work showing TGF-β2 to be inhibitory to cell growth. There is, however, research showing modes by which TGF-β2 may be able to affect cellular proliferation, and also interact with other factors. It has been shown that TGF-β1 and TGF-β2 inhibit IL-5 induced proliferation of the erythroleukaemia cell line (Randall L.A. *et al.* 1993), and also that suppression of human lens epithelial cell proliferation by TGF-β2 could be blocked by the addition of bFGF or hepatocyte growth factor (HGF) (Awasthi N. *et al.* 2006), suggesting that TGF-β2 can interact with factors present in BAL. In contrast to work by Awasthi N. *et al.* 2006, it has been shown that TGF-β2 can counteract the proliferative effects of bFGF in bovine corneal endothelial cells, and that this was mediated by COX-2 (Lu J. *et al.* 2006). In addition to this finding it was also suggested that the bFGF mediated proliferation could be inhibited by the TGF-β2 driven production of PGE2 and also TGF-β2 suppression of the PI3-K/Akt signalling pathway (Lu J. *et al.* 2006). There is little work suggesting a role for TGF-β3

in cellular proliferation, however TGF-β3 has been shown to increase proliferation in leiomyoma cells (Lee B.S. *et al.* 2001), however the majority of the literature show it to be inhibitory to cellular proliferation (Strife A *et al.* 1991, Boumediene K. *et al.* 2001 and Nasu K. *et al.* 2005).

The work in this chapter has suggested that G-protein coupled receptors play an important role in the proliferation of both healthy and asthmatic fibroblasts; however the role of the tyrosine kinase pathways appears to be less clear. Although the broad spectrum receptor tyrosine kinase inhibitor used in this work did not consistently inhibit fibroblast mitogenesis, that inhibitors against bFGF and EGF were significantly inhibitory suggests that there may be an important regulatory role for receptor tyrosine kinases. Whether this is achieved through transactivation between G-protein coupled receptors and the EGF receptor or through some other mechanism is an area for future exploration. Investigation of the role of individual factors suggests an important role for bFGF, EGF and angiotensin II in fibroblast mitogenesis, and that in the BAL this may be mediated by TGF-β1. There is also an important role for TNF-α, however it is unknown whether it is acting in synergy with other factors, or whether TNF-α is having another, as yet unidentified action on cells. Fibroblast mitogenesis appears to be regulated by MEK1/2 after challenge with BAL, and this is likely due to the regulation of its downstream target ERK, but with no stimulatory role for JNK or p38. There may, however, be a regulatory role for p38 and JNK in fibroblast proliferation, as their inhibition lead to an increase in [^{3}H] thymidine incorporation. The data indicate that there is little variation in the signalling pathways by which healthy and asthmatic BAL elicit their effects.

Chapter 6:

Fibroblast protein and mRNA changes after BAL challenge in the presence and absence of inhibitors, a comparison between healthy and asthmatic cells.

Introduction

The increased deposition of ECM proteins is a well characterised event within the asthmatic airway and one in which fibroblasts are believed to be heavily involved, as it is these cells which are the primary source of ECM proteins within the airway. However it is still unclear whether it is the fibroblasts themselves, or the environment in which they reside, which is responsible for the increased ECM deposition observed in the asthmatic airway.

To investigate this healthy and asthmatic fibroblasts were grown from bronchial biopsies and challenged with BAL from healthy and asthmatic volunteers, with their ability to synthesise collagen III mRNA (a characteristic protein found in thickened lamina propria in the lower airways of asthmatics), CTGF mRNA (a protein believed to be involved in TGF-β mediated collagen production in fibroblasts, Grotendorst G.R. *et al.* 2004) and IL-8 mRNA (a cytokine involved in inflammatory cell recruitment, a characteristic contributory factor to airway wall thickening in asthma), assessed by the TaqMan RT-PCR assay.

It is likely that factors within BAL fluid are able to exert multiple effects relevant to the pathogenesis of these structural changes, acting not only as fibroblast mitogens but also able to initiate an increase in the synthesis of ECM proteins. This is supported with work by Burgess J.K. *et al.* 2003 who showed that TGF-β could contribute to the up regulation of ECM proteins within the airway wall of asthmatic volunteers, with TGF-β being shown to be raised in the BAL of asthmatic patients (Redington A.E. *et al.* 1997a). It has also been suggested by Tremblay G.M. *et al.* 1995 that activated fibroblasts are likely to contribute to different ECM components in the asthmatic airway. Recently observations have been made which support this, with studies showing that collagen I synthesis can be up regulated by TGF-β1 released from eosinophils, and that the level of collagen I synthesis correlated to a decrease in $\%FEV_1$ (Nomura A. *et al.* 2002), and also that mice instilled with TGF-β exhibited increased sub-epithelial collagen deposition (Kenyon N.J. *et al.* 2003). It has also been shown that collagen III and α-SMA synthesis can be up regulated in human lung fibroblasts after challenge with IL-4 and TGF-β (Bergeron C. *et al.* 2003 and Batra V. *et al.* 2004).

However it is unlikely that it is only airway fibroblasts that contribute to ECM deposition in asthma, as *in vitro* studies by Johnson P.R. *et al.* 2000 and Johnson P.R. *et al.* 2006 have shown that the ASM may also contribute to the deposition of ECM, a finding which has been supported by subsequent studies (P.R. *et al.* 2000, Johnson P.R. *et al.* 2006 and Burgess J.K *et al.* 2003).

The use of RT-PCR to examine the change in fibroblast gene expression for ECM products and inflammatory markers post-stimulation allows comparisons to be drawn between asthmatic and healthy fibroblasts and BAL.

Methods

Real-time PCR analysis using the TaqMan system

The TaqMan protocol described in the methods section was used to analyse the production of CTGF, collagen III, and IL-8 mRNA after stimulation with 10ng/ml TNF-α, 1ng/ml TGF-β1, healthy, mild asthmatic and moderate/severe asthmatic BAL. TNF-α and TGF-β1 were used at what were believed to be physiological levels, determined by the extrapolation from known concentrations of TGF-β and TNF-α found in BAL by previous work (Redington A.E. *et al.* 1997a and Howarth P.H. *et al.* 2005), and by taking into account the dilution of factors which occurs when collecting BAL. Initially the challenge was added over a 24 hour period at 1, 2, 4, 6, 12 and 24 hours, to determine the optimum time-points at which to assess CTGF, collagen III, and IL-8 mRNA production. Subsequent work was performed using the 1 and 4 hour time-points, the BAL was added at a 1:3 dilution on the plate, and for the unstimulated control 1% ITS media was used. After challenge the supernatant was removed and stored at -80°C, the cells were lysed using Tri-reagent, the fibroblasts used were primary cells grown from biopsy from either healthy, mild asthmatic or severe asthmatic donors. Lines represent the mean and standard error while box-and-whisker plots show boxes that extend from the 25th percentile to the 75th percentile, with a horizontal line representing the median; the whiskers extend down to the 10th percentile and up to the 90th percentile, with each outlier shown as an individual point outside the plots.

ELISA measurement of IL-8 production

The levels of IL-8 protein were measured in the fibroblast supernatants collected at 1 and 4 hours after challenge with BAL, 10ng/ml TNF-α and 1ng/ml TGF-β1. The ELISA method was as described in the methods section. The IL-8 levels in the supernatants were quantified by construction of a typical standard curve using known concentrations of IL-8. The IL-8 protein already present in the BAL used for challenge (table 2.2) was subtracted from the supernatant IL-8 protein measurement in BAL treated samples before analysis; the IL-8 protein production was also corrected for baseline IL-8 protein levels when relative IL-8 protein production from fibroblasts were compared. Bars represent the mean and standard error.

Investigation of mRNA production after incubation with inhibitors before challenge

As in the [^{3}H] thymidine incorporation assays pertussis toxin and genistein were used to investigate the role of G-protein coupled and tyrosine kinase receptors respectively. These inhibitors were incubated with the cells (1 healthy, 1 mild asthmatic and 1 severe asthmatic fibroblast line) for 4 hours before addition of BAL, TGF-β1 or TNF-α. The roles of TGF-β and TNF-α were respectively investigated via the use of a pan-specific TGF-β antibody and Etanercept, a soluble TNF-α receptor. Inhibitors of intra-cellular messengers were also used including wortmannin (PI3-K), U0126 (MEK1/2) and SB203580 (p38). These inhibitors were incubated for 1 hour either with the cells or with the BAL, TGF-β1&2 or TNF-α, before addition to the fibroblasts, at concentrations dependent upon previous experimental data and publications. The BAL was either added individually or combined as pooled BAL from the individual groups. The groups were defined as healthy, mild asthmatic or moderate/severe asthmatic and the BAL was added at a 1:3 dilution. Bars represent the mean and standard error.

Analysis of MAPK phosphorylation levels in fibroblasts after challenge with BAL

Fibroblasts from 6 healthy, 6 mild asthmatic and 6 severe asthmatic volunteers were pooled into 3 separate culture flasks, grown to confluence and then serum deprived with 1% ITS media, after which the cells were challenged with BAL from 6 moderate/severe asthmatic subjects for 30 minutes. After challenge the fibroblasts were lysed and the total

protein content measured using the Bradford assay. Once this was established, around 130-150μg/ml of protein was added to each membrane. Membranes were visualised on x-ray film and also pixel density determined using the BioRad Versa Doc imaging system model 400.

Statistics

The Wilcoxon signed rank test for paired samples was used to investigate differences in mRNA and IL-8 production from stimulated samples compared to the control, and also the effect of inhibitors on the responses to challenge with BAL. The Mann-Whitney test was used to compare the effect of different BAL and the response of different fibroblasts to challenge, $p < 0.05$ indicates significance.

Aims

1. To assess the effect of recovered BAL on fibroblast mRNA expression for genes involved in the remodelling process (CTGF and collagen III) and the inflammatory process (IL-8).
2. To compare the responses in asthma to that from healthy controls
3. To assess the role of different pathways, factors and intra-cellular signalling proteins in collagen III mRNA synthesis.
4. Measure the levels of secreted IL-8 protein in supernatant from fibroblasts challenged with BAL, 10ng/ml TNF-α and 1ng/ml TGF-β1.
5. Assess the phosphorylation state of MAPKs in fibroblasts after challenge with BAL.

Results

Investigation of collagen III, CTGF and IL-8 mRNA expression over a 24 hour period after BAL challenge.

The data in fig 6.1 show the expression of collagen III, CTGF and IL-8 mRNA from healthy (n = 1) and mild asthmatic (n = 1) fibroblasts after challenge with healthy (n = 7), mild asthmatic (n = 7) and moderate/severe asthmatic (n = 7) BAL at various time-points over a 24 hour period. It was important to determine when the expression of these genes would be at their uppermost as it would be very difficult to perform 24 hour analyses of gene expression on large sample groups, due to the labour intensive and expensive procedure of RNA extraction, and subsequent reverse transcription into cDNA. It was therefore more practical to assess mRNA expression at two time-points at which the expression would be at its highest. The choice of the time to investigate the relative expression of these genes was therefore important, as mRNA is degraded rapidly after its production, with a half life typically below 1 hour, therefore it would be easy to miss its optimal expression, making comparisons between healthy and asthmatic fibroblasts difficult, and potentially inaccurate.

From the data in fig 6.1 it can be observed that when the expression of collagen III mRNA expression was assessed in healthy fibroblasts, that healthy BAL induced a significantly greater increase in collagen III mRNA expression than mild asthmatic BAL at 4 hours ($p < 0.05$), and than moderate/severe asthmatic BAL at 1 ($p < 0.001$), 4, 6 and 12 hours ($p < 0.05$). Mild asthmatic BAL caused significantly greater collagen III mRNA expression than moderate/severe asthmatic BAL at 1, 6 and 12 hours ($p < 0.05$). In mild asthmatic fibroblasts moderate/severe asthmatic BAL was able to cause significantly greater collagen III mRNA expression than healthy and mild asthmatic BAL at 1, 2, 4, 6, 12 and 24 hours ($p < 0.05$).

The expression of CTGF mRNA from healthy fibroblasts was significantly higher after challenge with healthy BAL than mild asthmatic BAL at 2 and 4 hours ($p < 0.05$), and healthy BAL could cause significantly greater CTGF mRNA expression at 2 hours than could moderate/severe asthmatic BAL ($p < 0.05$). When the expression of CTGF mRNA was assessed from mild asthmatic fibroblasts it was observed that challenge with healthy BAL elicited significantly more CTGF mRNA than did challenge with mild asthmatic BAL at 1 and 4 hours ($p < 0.001$) and 6 and 12 hours ($p < 0.05$), and that challenge with

healthy BAL also lead to the production of significantly more CTGF mRNA than challenge with moderate/severe asthmatic BAL at 1, 2, 6 and 12 hours ($p < 0.05$) and at 4 hours ($p < 0.001$). When mild asthmatic fibroblasts were challenged with mild asthmatic BAL they were able to produce significantly more CTGF mRNA than after challenge with moderate/severe asthmatic BAL at 2 and 4 hours ($p < 0.05$), but this observation was reversed at 24 hours ($p < 0.05$).

When IL-8 mRNA expression was assessed in healthy and mild asthmatic fibroblasts it was shown that after challenge with healthy BAL that healthy fibroblasts are able to produce significantly more IL-8 mRNA than after challenge with mild asthmatic BAL at 1, 2, 6 ($p < 0.001$) and 4 hours ($p < 0.05$). When mild asthmatic fibroblasts were challenged with the same BAL it was observed that healthy BAL was able to cause a significantly greater increase in IL-8 mRNA expression than mild asthmatic BAL at 1 ($p < 0.05$), 2, 4, 12 and 24 hours ($p < 0.001$), and also than moderate/severe asthmatic BAL at 1, 4, 12, 24 ($p < 0.05$) and 2 hours ($p < 0.001$). It was also observed that mild asthmatic BAL was able to increase IL-8 mRNA expression when compared to moderate/severe asthmatic BAL at 1 hour ($p < 0.05$).

From the data in fig 6.1 it was decided that in future work the mRNA expression from the fibroblasts would be analysed at 1 and 4 hours; as between 1 and 4 hours a majority of the collagen III and CTGF mRNA expression was being observed, and at the 4 hour time point IL-8 mRNA expression was also high. The use of these time-points should allow useful comparisons between the relative expression of CTGF, collagen III and IL-8 mRNA to be made between healthy, mild asthmatic and severe asthmatic fibroblasts, although the time point of the greatest expression of these genes has not been determined in severe asthmatic fibroblasts. This may prove useful, however, as if there is any abnormal up regulation of these genes from severe asthmatic volunteers then it will be being compared to the maximal production of these genes from healthy and mild asthmatic fibroblasts, with the largest level of gene expression from severe asthmatic fibroblasts potentially being at another time point. To compare the expression of collagen III, CTGF and IL-8 mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts, cells were challenged with TGF-β1, TNF-α and BAL from healthy, mild asthmatic and moderate/severe asthmatic volunteers, for 1 and 4 hours.

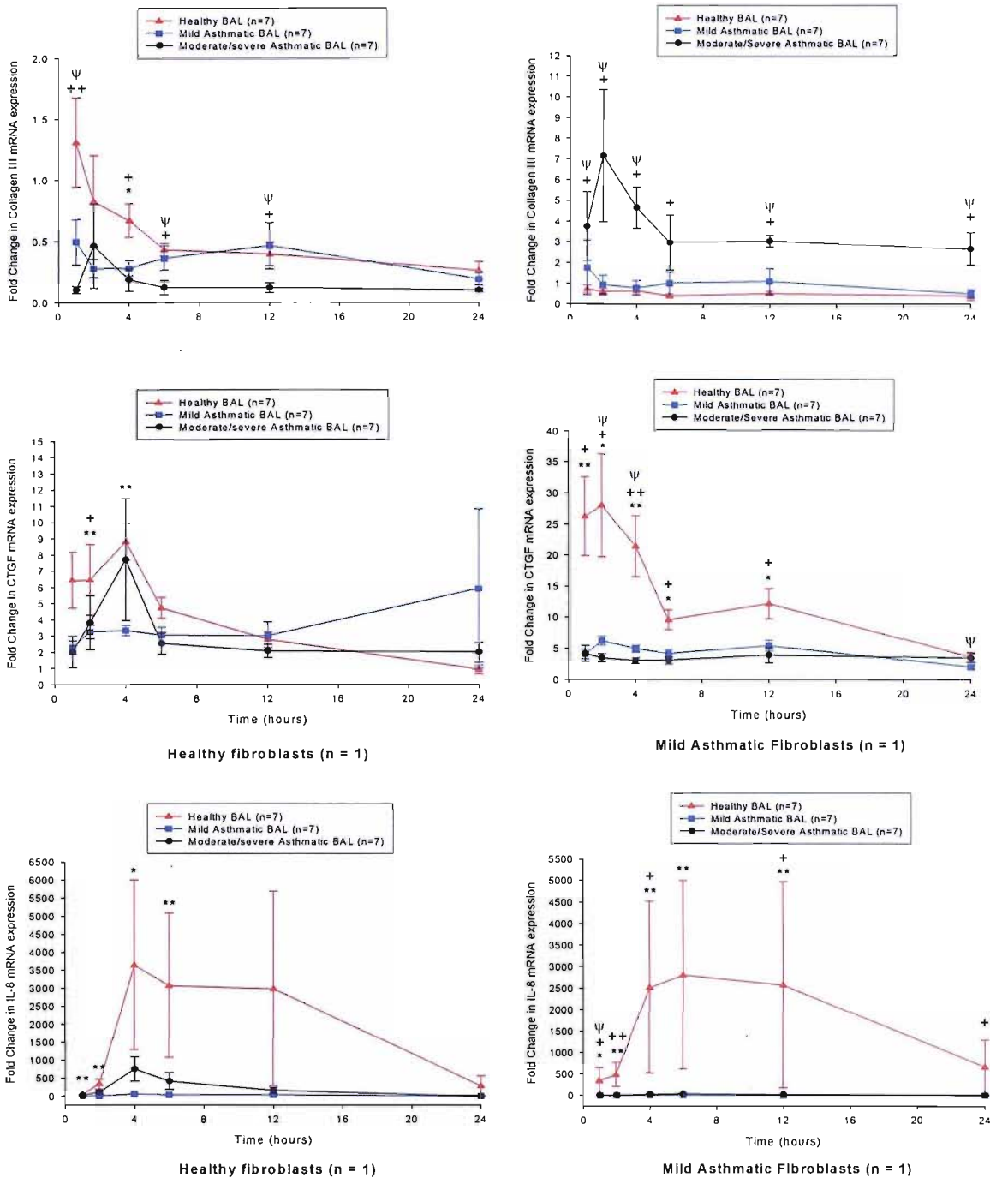


Fig 6.1 Fibroblasts from 1 mild asthmatic or 1 healthy donor were seeded at 50,000 cells per well in non-collagen coated 12 well plates. Cells were left to seed for 48 hours before being serum deprived for 24 hours. Fibroblasts were then challenged with BAL from healthy (n = 7), mild asthmatic (n = 7) or moderate/severe asthmatic (n = 7) volunteers for 1, 2, 4, 6, 12 or 24 hours. The expression of collagen III, CTGF and IL-8 mRNA was then quantified using the TaqMan RT-PCR assay.
* = $p < 0.05$ Healthy BAL vs Mild asthmatic BAL, ** = $p < 0.001$ Healthy BAL vs Mild asthmatic BAL.
+ = $p < 0.05$ Healthy BAL vs Moderate/Severe Asthmatic BAL, ++ = $p < 0.001$ Healthy BAL vs Moderate/Severe Asthmatic BAL.
ψ = $p < 0.05$ Mild Asthmatic BAL vs Moderate/Severe Asthmatic BAL and ψψ = $p < 0.001$ Mild Asthmatic BAL vs Moderate/Severe Asthmatic BAL.

The expression of collagen III mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts at 1 and 4 hours after BAL challenge.

Data in figs 6.2 to 6.4 show the production of collagen III mRNA at 1 and 4 hours from 6 healthy, 6 mild and 6 severe asthmatic fibroblast primary cultures, after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α and healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) BAL.

The data in figs 6.2 and 6.4 show that at 1 hour 1ng/ml TGF-β1, and moderate/severe asthmatic BAL could cause a significant increase in healthy and severe asthmatic fibroblast collagen III mRNA synthesis, when compared to the control ($p < 0.01$). Mild asthmatic BAL could also lead to a significant increase in collagen III mRNA from healthy fibroblasts at 1 hour ($p < 0.01$). Data in fig 6.2 also show that 10ng/ml TNF-α lead to a significant increase in collagen III mRNA synthesis from healthy fibroblasts at 1 hour ($p < 0.01$). The data in these figures also show that moderate/severe asthmatic BAL could cause a significantly greater increase in collagen III mRNA synthesis, than could healthy BAL from both healthy and severe asthmatic fibroblasts at 1 hour ($p < 0.02$).

When collagen III mRNA synthesis was assessed at 4 hours in figs 6.2 and 6.4 healthy fibroblasts were able to produce significantly more collagen III mRNA after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy, mild asthmatic and moderate/severe asthmatic BAL ($p < 0.05$) when compared to the control, however severe asthmatic fibroblasts only significantly increased their collagen III mRNA synthesis after challenge with 1ng/ml TGF-β1 ($p < 0.01$), there was no significant variation in collagen III mRNA synthesis between the BAL challenges.

In fig 6.3 where collagen III mRNA synthesis was assessed from mild asthmatic fibroblasts it was observed that only a 1 hour challenge with mild asthmatic BAL could lead to a significant increase in collagen III mRNA synthesis, compared to the control ($p < 0.05$), and the data suggested that their was little variation in collagen III mRNA synthesis between the BAL challenges.

That TGF-β1 and TNF-α had similar effects on collagen III mRNA synthesis in these figures (fig 6.2 to 6.4) is interesting as TGF-β1 is classically believed to promote cellular collagen synthesis, and was used as a positive control for collagen III mRNA synthesis in this work. These data are limited in that only one concentration of TGF-β1 and TNF-α

were used, therefore to determine whether TNF-α possesses similar potency as TGF-β1, with regard to collagen III mRNA synthesis, a range of concentrations of each would need to be used.

The data in these figures suggest that fibroblasts from healthy and severe asthmatic volunteers are able to increase their production of collagen III mRNA after challenge with TGF-β1, TNF-α and BAL, and that BAL from moderate/severe asthmatic volunteers is able to elicit a significantly greater induction of collagen III mRNA than is healthy BAL at 1 hour (fig 6.2 and 6.4). It also shows that collagen III mRNA synthesis from fibroblasts is not ubiquitous in the time of its synthesis, with production still being significantly induced by challenge in healthy fibroblasts at 4 hours, but to a lesser extent from severe asthmatic fibroblasts. It is difficult to comment on whether this is due to an altered regulation of collagen III mRNA synthesis from severe asthmatic cells from observations in this work, as the expression of collagen III mRNA was not determined over a 24 hour period from severe asthmatic fibroblasts. However it does suggest some degree of mis-regulation of severe asthmatic fibroblasts response to factors present in BAL, and whether this manifests as the cells progressing into other cellular activities after the initial synthesis of collagen III mRNA is an area for future investigation. It is also interesting that collagen III mRNA synthesis from mild asthmatic fibroblasts was not consistently significantly induced by BAL challenge in fig 6.3, as the data in fig 6.1 had suggested that mild asthmatic fibroblasts were producing larger levels of collagen III mRNA than were healthy fibroblasts. This observation is probably due to the fact that in fig 6.1 only 1 healthy and 1 mild asthmatic fibroblast line were used, and were therefore not necessarily representative of the group. Nevertheless that collagen III mRNA synthesis from mild asthmatic fibroblasts is not as inducible after BAL challenge as it is in healthy or severe asthmatic fibroblasts, either simply suggests that mild asthmatic fibroblasts do not produce large quantities of collagen III mRNA after BAL challenge, or that mild asthmatic fibroblasts have a large basal ability to synthesise collagen III mRNA, and that this is why little effect of challenge is witnessed. To analyse this a direct ELISA could be used to quantify the amount of collagen III protein deposited or coated onto the plate from unchallenged and BAL challenged healthy, mild asthmatic and severe asthmatic fibroblasts.

The median fold change in collagen III mRNA expression is quite low in the above figures, around 2-3 fold. That this increase is low is likely to represent both collagen III not being a greatly inducible gene, and also the low numbers of myofibroblasts present in the cultures. As the fibroblasts in the cultures are unlikely to have transformed into myofibroblasts at the time-points used, and myofibroblasts are liable to be the cells synthesising collagen III this observation is unsurprising. If a later time point, 48 hours for example, were used to assess collagen III mRNA expression greater levels may be observed, and this would probably be due to more myofibroblasts being present. Nevertheless the aim of this study was to compare the initial responses of these cells, and the time-points used achieve this. Extending the study to later time-points where more collagen III mRNA may be present would allow greater confidence in our statistical analysis to be drawn.

Another consideration from the data in these figures is the variability in collagen III mRNA expression, as represented by the 10^{th}, 25^{th}, 75^{th} and 90^{th} interquartile ranges. This is a consequence of using primary cultures of cells along with BAL obtained from volunteers. The variability of the data means that the confidence we can have in the observations is reduced, however by using 6 fibroblast lines from each group the variability was addressed to the best of our ability. To minimise the affect of the variability this study would need to be extended to include greater numbers of fibroblasts. Once the investigation of the relative abilities of the different BAL fluids to elicit collagen III mRNA expression from the fibroblasts was performed, the above data was used to compare the production of collagen III mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts.

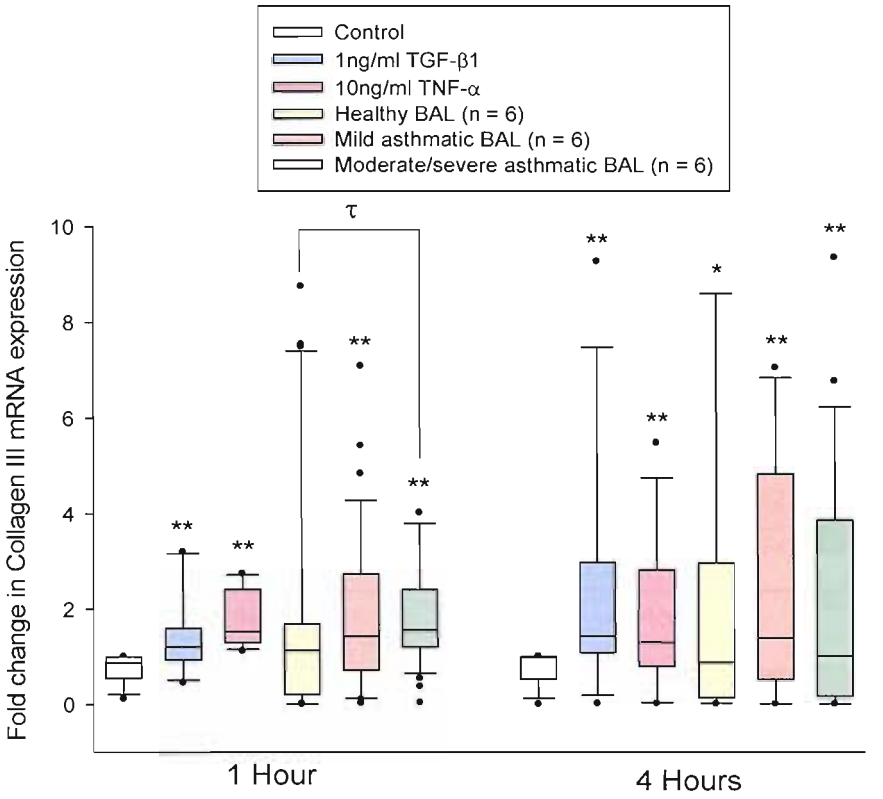


Fig 6.2 Collagen III mRNA expression from healthy fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control τ = $p < 0.02$

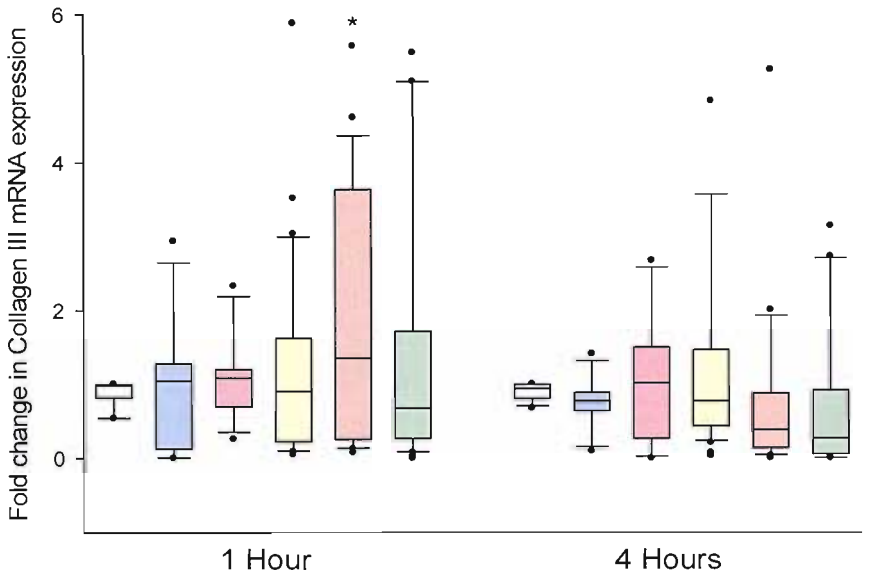


Fig 6.3 Collagen III mRNA expression from mild asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control τ = $p < 0.02$

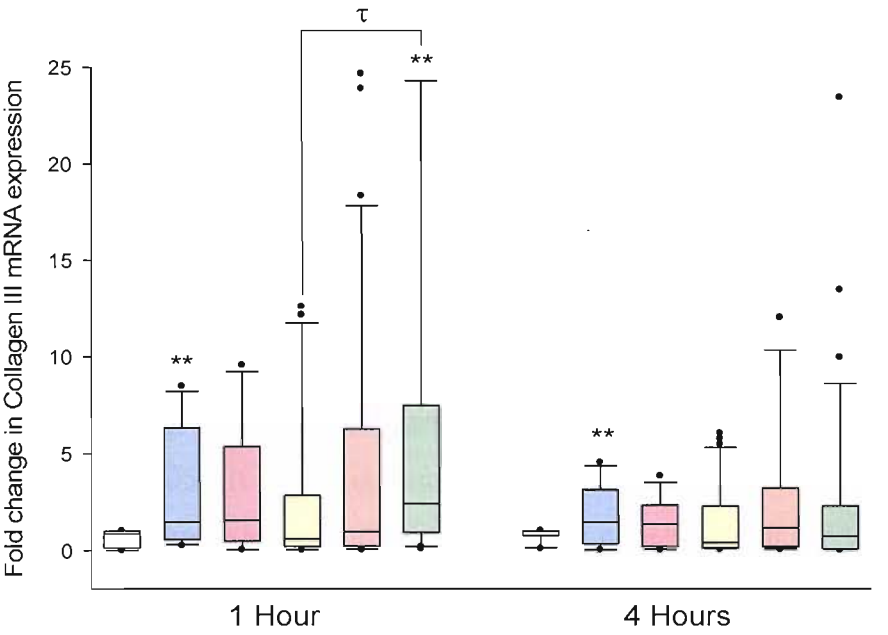


Fig 6.4 Collagen III mRNA expression from severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control τ = $p < 0.02$

Comparison of collagen III mRNA expression from healthy, mild asthmatic and severe asthmatic fibroblasts.

In fig 6.5, 6.6 and 6.7 where the expression of collagen III mRNA from healthy and asthmatic fibroblasts is compared it can be observed in fig 6.5 that after a 1 hour challenge with moderate/severe asthmatic BAL, there was a significant increase in collagen III mRNA production from healthy and severe asthmatic fibroblasts, compared to mild asthmatic fibroblasts ($p < 0.005$), and also a significant increase in collagen III mRNA expression from severe asthmatic fibroblasts when compared to healthy fibroblasts ($p < 0.05$). It is also shown in fig 6.6 that after a 4 hour challenge with mild asthmatic BAL there was a significant increase in collagen III mRNA production from healthy and severe asthmatic fibroblasts compared to mild asthmatic fibroblasts ($p < 0.005$ and $p < 0.05$ respectively), and also a significant increase in collagen III mRNA expression from healthy fibroblasts compared to mild asthmatic fibroblasts after challenge with moderate/severe asthmatic BAL ($p < 0.05$). The data in fig 6.7 show there is little significant variation in collagen III mRNA synthesis between the fibroblasts after challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α, with there only being a significant variation seen in TNF-α and TGF-β1 challenged healthy fibroblasts when compared to mild asthmatic fibroblasts ($p < 0.01$) at 1 and 4 hours respectively.

From these data there appears to be a trend indicating that fibroblasts from severe asthmatic donors posses an increased ability to synthesise collagen III mRNA than do healthy or mild asthmatic fibroblasts, but that this was only observed at 1 hour, with the expression at 4 hours from healthy and severe asthmatic fibroblasts being more equal. Collagen III mRNA synthesis from mild asthmatic fibroblasts was consistently low, at both 1 and 4 hours. The increased ability to synthesise collagen III mRNA exhibited by severe asthmatic fibroblasts at 1 hour was only significant compared to healthy and mild asthmatic fibroblasts after challenge with moderate/severe asthmatic BAL. The lack of consistent significance though may be partly explained by the collagen III mRNA synthesis at 1 hour from severe asthmatic volunteers being more variable than that from healthy or mild asthmatic volunteers, and this is represented by the inter-quartile ranges and the outliers in the box plots in both fig 6.5 and 6.6. As the data was more variable there would be more overlapping data points, and this leads to a lack of significance. The

variability of the data is a consequence of using primary cultures of cells, as even though by increasing the number of donor samples in each group to six you are getting a better representation of each group, there is still always going to be variability present. To counter this the numbers of fibroblasts used would have to be increased, however this was not possible given the time frame and sample numbers available to us.

The data from 6.7 showed that although TGF-β1 is believed to be a major factor driving collagen production from fibroblasts, that its effects alone at 1 hour could not account for the levels of collagen III mRNA synthesis observed from severe asthmatic fibroblasts at 1 hour in fig 6.5. In fig 6.6 The median fold change in collagen III mRNA expression elicited by TGF-β1 alone at 1 hour in fig 6.7 was around 1.5, whereas in fig 6.5 where severe asthmatic fibroblasts were challenged with moderate/severe asthmatic BAL there was around a 3-4 fold increase in collagen III mRNA expression. This suggests that although TGF-β may be accounting for a proportion of the collagen III mRNA expression from fibroblasts that it is likely that there are significant contributions from other factors present in BAL. It is also likely that the factors which are contributing to this increase in collagen III mRNA synthesis are acting in synergy with TGF-β, and this will be discussed later in the chapter. The data in fig 6.7 also suggest that TNF-α is able to increase the expression of collagen III mRNA synthesis, albeit to a slightly lower magnitude than that observed from TGF-β challenged cells.

That the data shown above suggest that severe asthmatic fibroblasts are able to produce significantly more collagen III mRNA that could healthy or mild asthmatic fibroblasts after challenge with moderate/severe asthmatic BAL, but that this was not completely mediated by TGF-β, alluded to the involvement of other factors in this observation. A factor believed to interact with TGF-β to mediated collagen synthesis from fibroblasts is CTGF (Grotendorst G.R. *et al.* 2004), therefore the expression of CTGF mRNA was assessed in the same set of challenged cells used previously to assess collagen III mRNA expression.

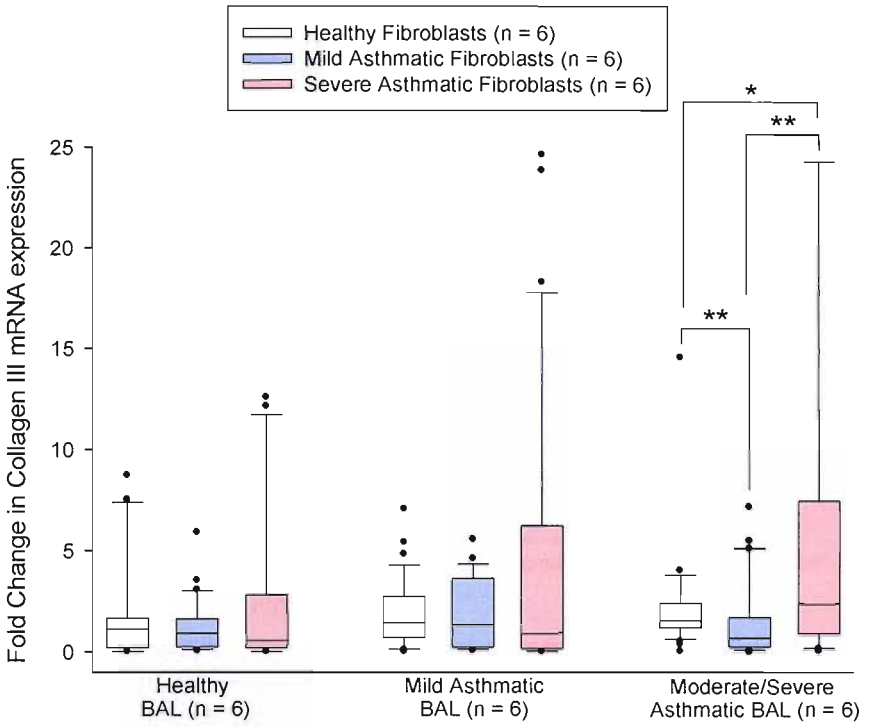


Fig 6.5 Collagen III mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 hour * = $p < 0.05$ ** = $p < 0.005$

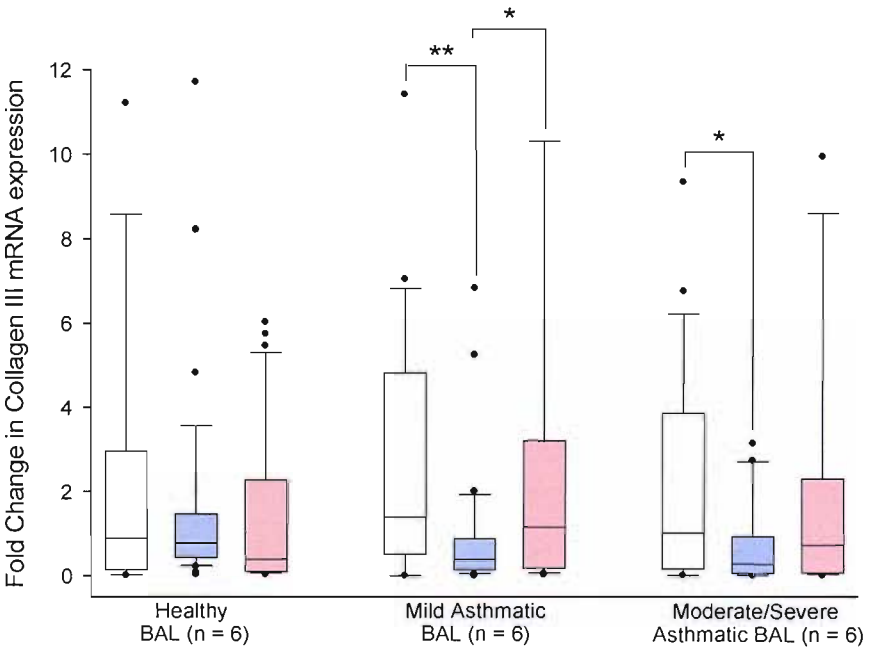


Fig 6.6 Collagen III mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 4 hours * = $p < 0.05$ ** = $p < 0.005$

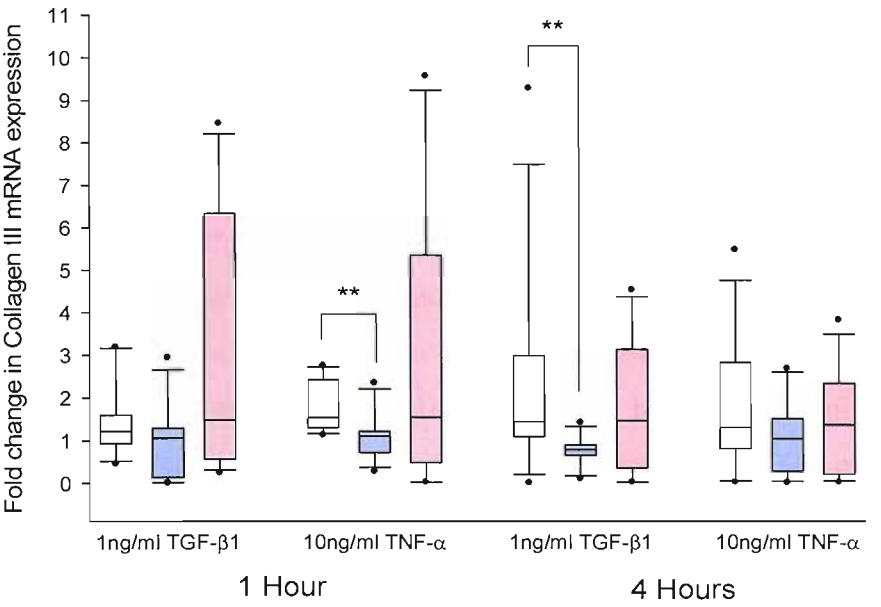


Fig 6.7 Comparrison of collagen III mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. ** = $p < 0.01$

The expression of CTGF mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts at 1 and 4 hours after BAL challenge.

As it is believed that CTGF is able regulated both collagen synthesis and proliferation in fibroblasts through its interactions with TGF-β, IGF-2 and EGF (Grotendorst G.R. *et al.* 2004), it expression after challenge with 1ng/ml TGF-β, 10ng/ml TNF-α, healthy (n = 6), mild asthmatic (n = 6) (n = 6) and moderate/severe asthmatic (n = 6) BAL was assessed from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts .

In figs 6.8 to 6.10 it can be seen that 1ng/ml TGF-β1, healthy BAL, mild asthmatic BAL and moderate/severe asthmatic BAL were able to increase CTGF mRNA expression compared to the control at 1 hour ($p < 0.05$) and 4 hours ($p < 0.01$) from healthy, mild asthmatic and severe asthmatic fibroblasts; TNF-α was also able to elicit a significant increase in CTGF mRNA expression from all fibroblasts after 1 hour ($p < 0.01$), but only in severe asthmatic fibroblasts after 4 hours ($p < 0.05$). The data in these figures also show that after a 4 hour challenge TGF-β1 was able to significantly increase CTGF mRNA expression when compared challenge to with TNF-α ($p < 0.02$) in all groups, which was as expected as TGF-β1 is a potent inducer of CTGF synthesis, and was a positive control for CTGF mRNA expression in this work.

As CTGF is a factor involved in a variety of cellular processes it is unsurprising that its expression in cells is more inducible than collagen III after BAL challenge. This is represented by the larger fold change increases observed in figs 6.8 to 6.10 compared to in figs 6.2 to 6.4. It is noticeable that, unlike the collagen III mRNA expression, mild asthmatic fibroblasts synthesise around the same amounts of CTGF mRNA as do severe asthmatic fibroblasts. This is not unexpected because, as already mentioned, CTGF is involved in cellular proliferation as well as collagen synthesis, therefore it may be that the CTGF synthesised by mild asthmatic fibroblasts is utilised in cellular events such as proliferation, while the CTGF synthesised by severe asthmatic fibroblasts is utilised in collagen synthesis. This could be facilitated by the transformation of fibroblasts into myofibroblasts, as if severe asthmatic fibroblasts are rapidly transforming into this more synthetic phenotype, then they will respond to factors present in the BAL in an altered manner, than would the mild asthmatic fibroblasts, which may not be transformed. The work by (Grotendorst G.R. *et al.* 2004) did suggest that CTGF can interact with EGF to

facilitate fibroblast proliferation, while CTGF may also synergise with IGF-2 to induce collagen synthesis from myofibroblasts. This would help explain both the lack of proliferation observed in BAL challenged severe asthmatic fibroblasts (figs 4.7 to 4.12), and also their increased ability to synthesis collagen III mRNA (figs 6.5). However, if CTGF were heavily involved in TGF-β/IGF-2 mediated collagen III mRNA synthesis then it would be expected that CTGF mRNA expression would be significantly raised compared to the healthy and mild asthmatic fibroblasts, and this is the focus of the next figures.

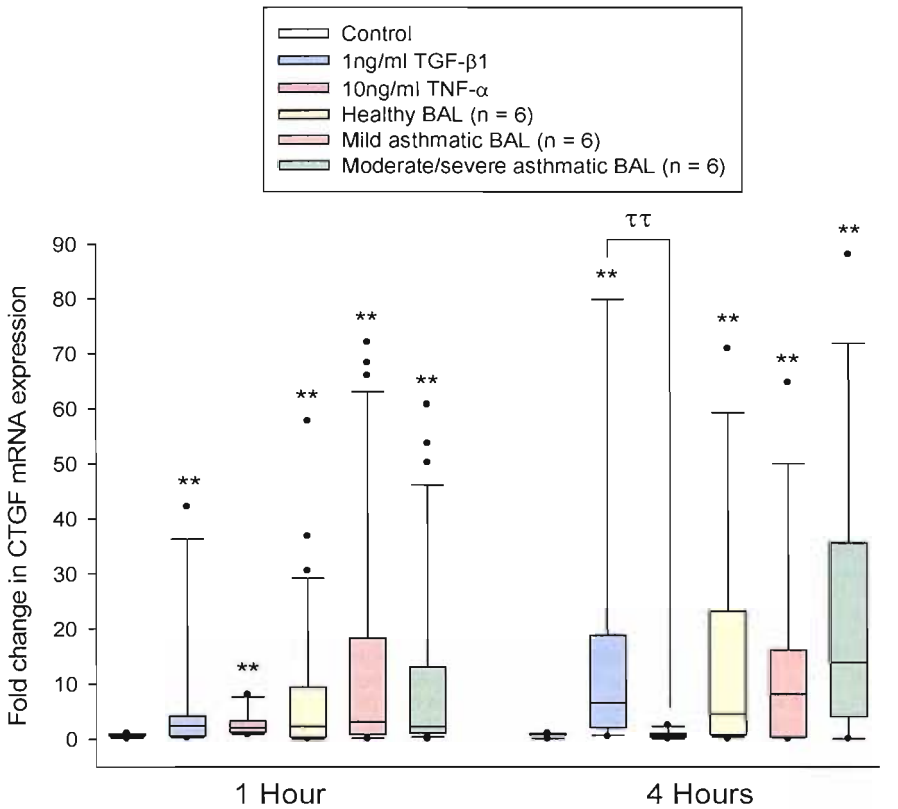


Fig 6.8 CTGF mRNA expression from healthy fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control ττ = $p < 0.0001$

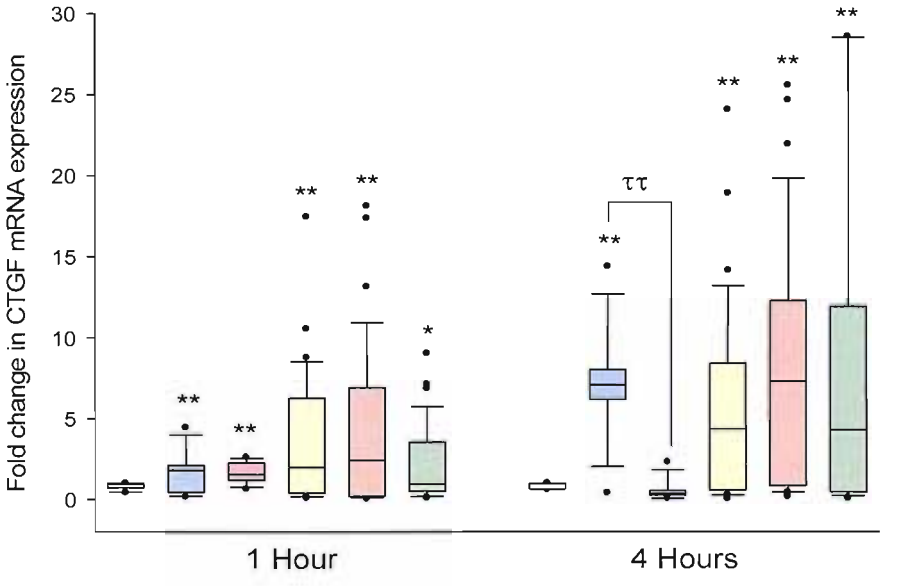


Fig 6.9 CTGF mRNA expression from mild asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control ττ = $p < 0.0001$

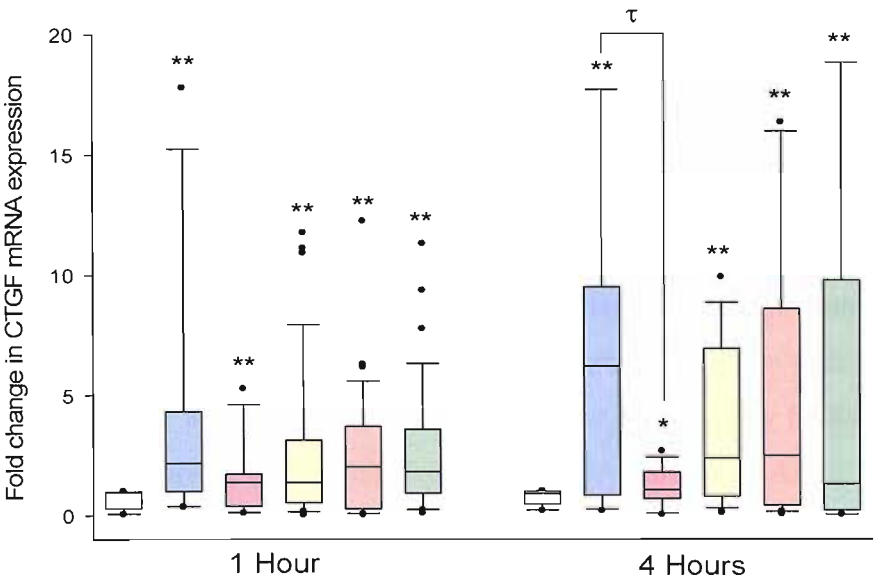


Fig 6.10 CTGF mRNA expression from severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control τ = $p < 0.02$

Comparison of CTGF mRNA expression from healthy, mild asthmatic and severe asthmatic fibroblasts.

When the comparison of CTGF mRNA expression from healthy, mild asthmatic and severe asthmatic fibroblasts is assessed in fig 6.11 to 6.13 it can be observed that healthy fibroblasts consistently synthesise greater levels of CTGF mRNA than do mild or severe asthmatic fibroblasts, with CTGF mRNA expression being significantly higher from healthy fibroblast than mild asthmatic fibroblasts after challenge with moderate/severe asthmatic BAL at 1 and 4 hours ($p < 0.05$), and also significantly higher than that from severe asthmatic fibroblasts after challenge with mild asthmatic BAL at 1 and 4 hours ($p < 0.05$) and moderate/severe asthmatic BAL after a 4 hour challenge ($p < 0.005$). The data also show that mild asthmatic fibroblasts produce significantly more CTGF mRNA than severe asthmatic fibroblasts after a 4 hour challenge with mild asthmatic BAL ($p < 0.05$). However there was no significant variation in CTGF mRNA expression from the fibroblasts after challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α (fig 6.13)

That healthy fibroblasts synthesise more CTGF than do severe asthmatic fibroblasts seems to suggest that there is little correlation between the amount of CTGF mRNA produced and the expression collagen III mRNA, with healthy fibroblasts synthesising more CTGF mRNA than severe asthmatic fibroblasts, and severe asthmatic fibroblasts synthesising more collagen III mRNA.

Although it is likely that CTGF is involved with TGF-β in regulating collagen III mRNA synthesis from fibroblasts, it is unlikely that this is the pathway by which the abnormal synthesis of collagen III mRNA is being regulated in moderate/severe asthmatic BAL challenged severe asthmatic fibroblasts. However the increased synthesis of CTGF mRNA from healthy fibroblasts is interesting, as fibroblasts from healthy donors had a proliferative potential equal to that of mild asthmatic fibroblasts (figs 4.7 to 4.12), but also these cells tended to produce more collagen III mRNA than did mild asthmatic fibroblasts (fig 6.5). Therefore maybe CTGF is contributing to the regulation of both collagen synthesis and proliferation in healthy fibroblasts, proliferation in mild asthmatic fibroblasts, and collagen synthesis in severe asthmatic fibroblasts. To investigate this neutralising antibodies to CTGF could be added to the BAL, and then the ability of fibroblasts to proliferate and synthesise collagen III mRNA assessed. Although it is

unlikely that CTGF is regulating abnormal collagen III mRNA expression from severe asthmatic fibroblasts, its role in fibroblast proliferation and collagen synthesis is still an area which requires clarification.

Following on from this work it was decided to investigate any possible role for fibroblasts in the recruitment of inflammatory cells to the asthmatic airway in response to airway insult. Interleukin-8 is a potent pro-inflammatory chemokine, involved in inflammatory cell recruitment in asthma. To investigate whether fibroblasts from severe asthmatics were producing more IL-8 and perpetuating the inflammatory response in asthma, the levels of IL-8 mRNA and protein were measured in healthy, mild asthmatic and severe asthmatic fibroblasts, after BAL, TGF-β and TNF-α challenge.

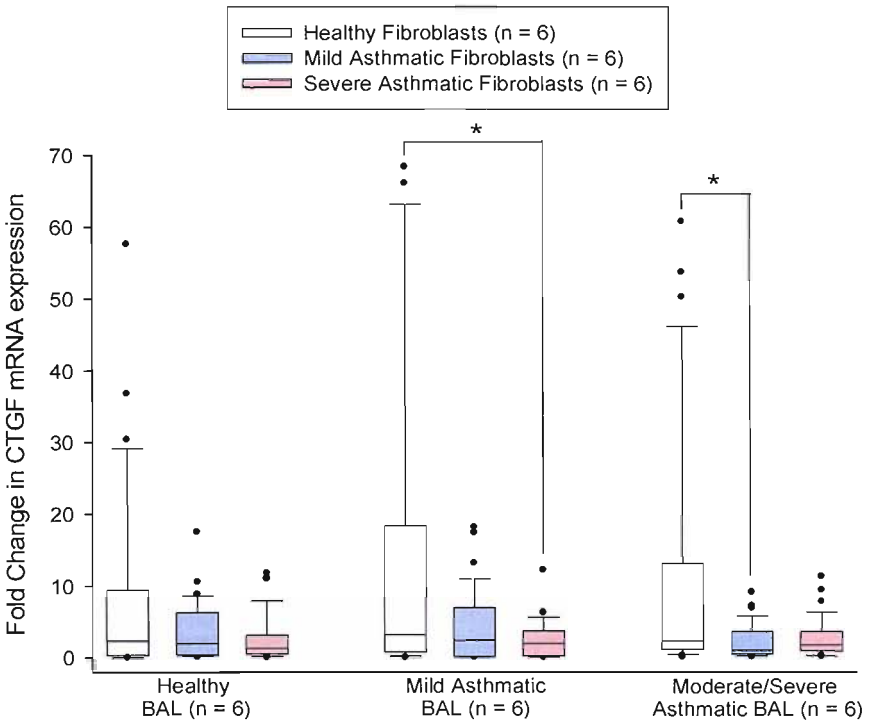


Fig 6.11 CTGF mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 hour * = $p < 0.05$ vs control

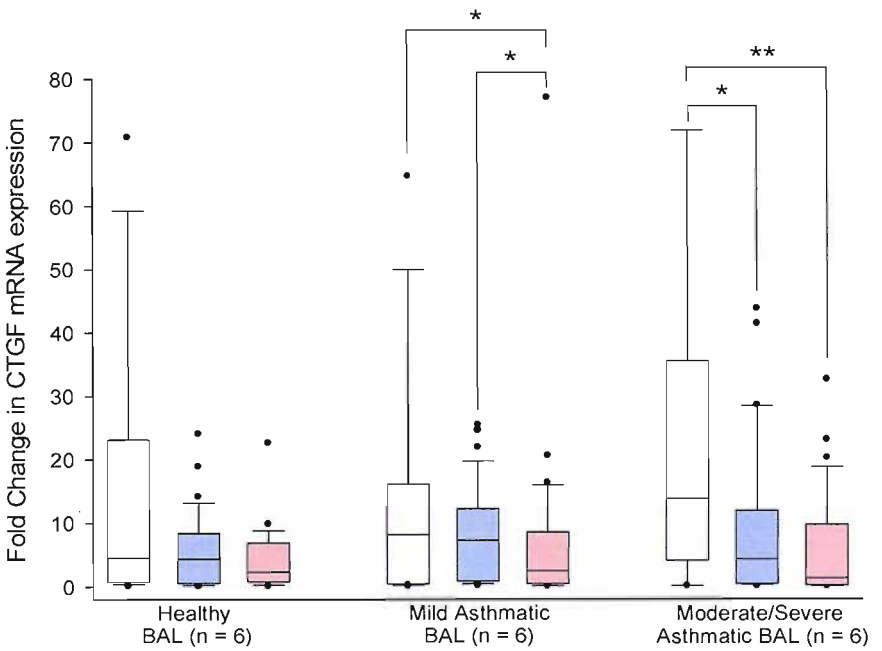


Fig 6.12 CTGF mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 4 hours * = $p < 0.05$ ** = $p < 0.005$

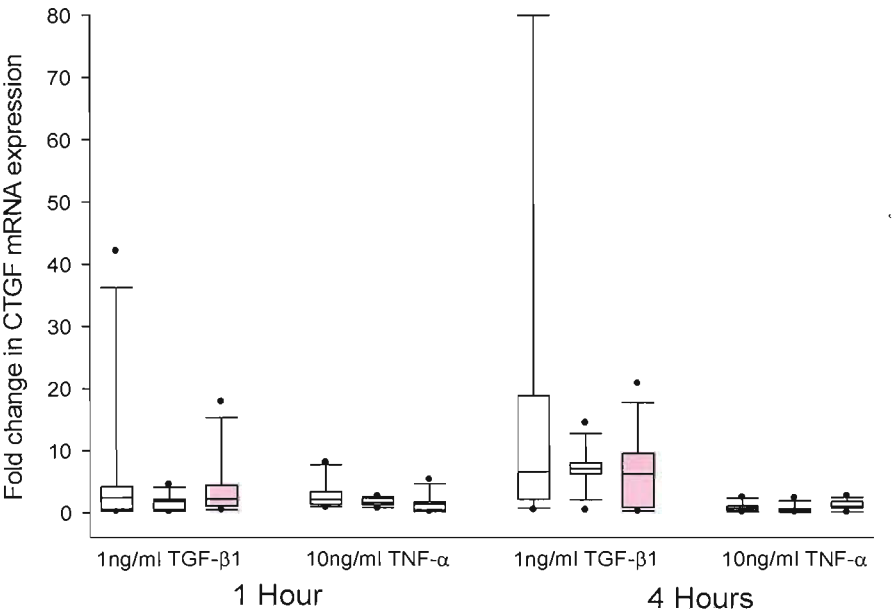


Fig 6.13 CTGF mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours.

The expression of IL-8 mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts at 1 and 4 hours after BAL challenge.

The data in figs 6.14 to 6.16 show that IL-8 mRNA expression was able to be up regulated compared to the control in healthy, mild asthmatic and severe asthmatic fibroblasts by 10ng/ml TNF-α, healthy, mild asthmatic and moderate/severe asthmatic BAL at 1 and 4 hours ($p < 0.05$). The data also show that 1ng/ml TGF-β1 was able to increase IL-8 mRNA expression compared to the control in mild asthmatic fibroblasts at 1 and 4 hours ($p < 0.05$) and in severe asthmatic fibroblasts after a 4 hour challenge ($p < 0.01$) (figs 6.15 and 6.16). There is no significant variation on the different BALs effect on IL-8 mRNA expression from the fibroblasts, however 10ng/ml TNF-α is able to consistently cause a significant increase in IL-8 mRNA expression from healthy, mild asthmatic and severe asthmatic fibroblasts compared to challenge with 1ng/ml TGF-β1 at 1 and 4 hours ($p < 0.0001$). That TNF-α is able to significantly increase IL-8 mRNA expression to a greater degree than TGF-β1 was expected, as TNF-α is a potent inducer of IL-8 expression, and was used as a positive control for IL-8 mRNA expression in this work.

The data in these figures show that although the BAL is able to significantly increase IL-8 mRNA expression that there is no significant variation in the amount of IL-8 mRNA expression induced by these various BAL fluids; that this variation is not significant indicates that either the balance of stimulatory and inhibitory factors for IL-8 mRNA synthesis in each group of BAL is similar, or that, as with the mitogenesis work, the BAL is being used at a dilution causing the maximal amount of IL-8 mRNA expression from fibroblasts, meaning no variation is observed. The latter is unlikely, however, as in the previous figures a significant variation in the amount of collagen III and CTGF mRNA was observed (fig 6.2 and 6.13). Nevertheless, a range of BAL dilutions could still be used to analyse their effects on collagen III, CTGF and IL-8 mRNA expression. Using the data from figs 6.14 to 6.16 the expression of IL-8 mRNA was compared between healthy and asthmatic fibroblasts at 1 and 4 hours.

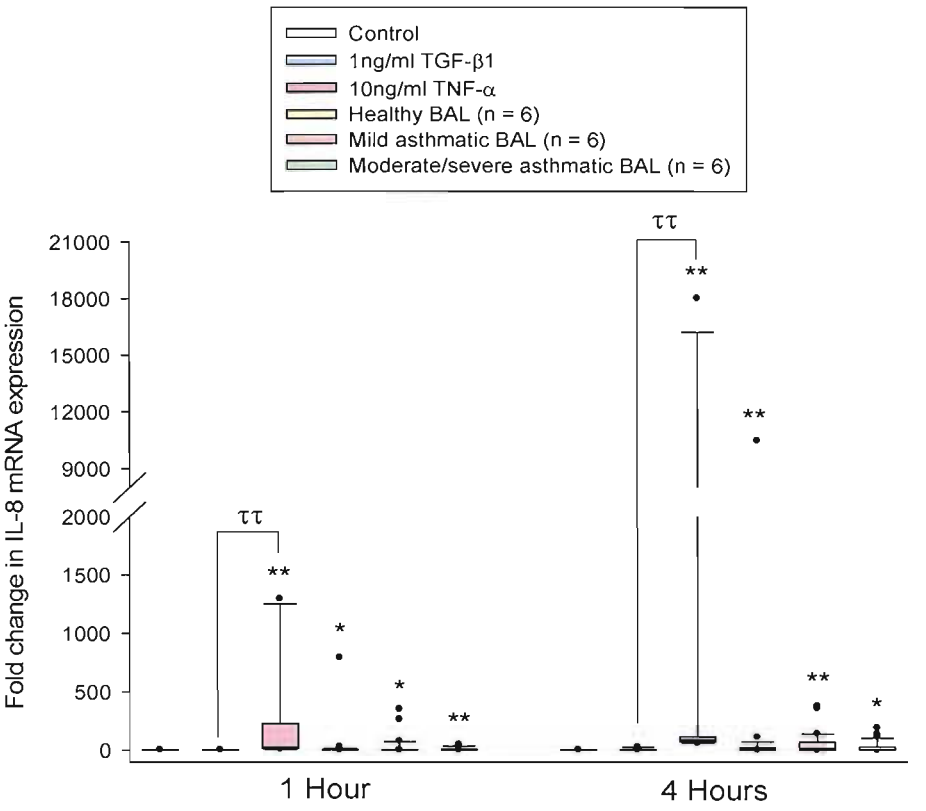


Fig 6.14 IL-8 mRNA expression from healthy fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6).Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control ττ = $p < 0.0001$

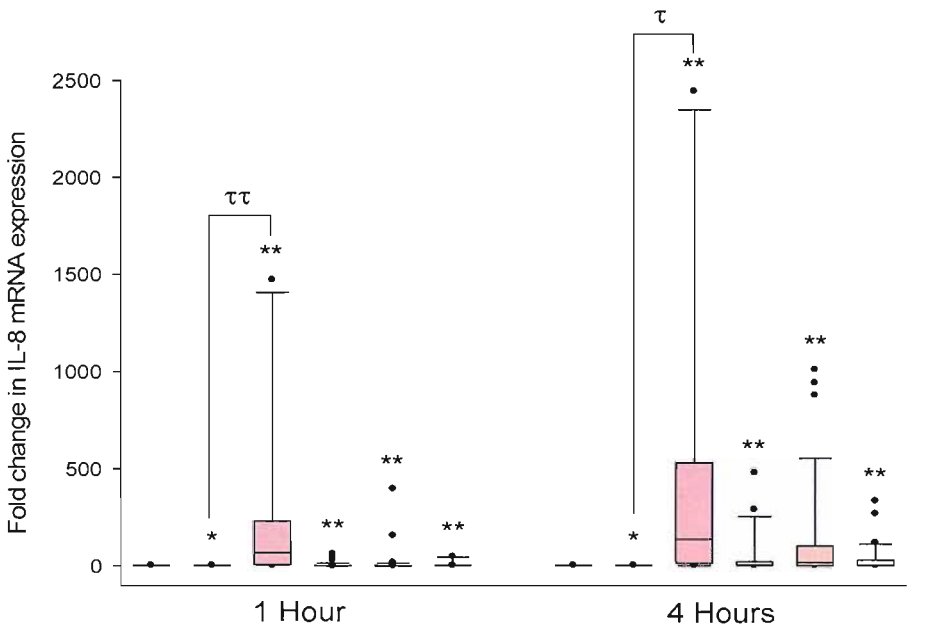


Fig 6.15 IL-8 mRNA expression from mild asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6).Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.05$ vs control ** = $p < 0.01$ vs control τ = $p < 0.02$ ττ = $p < 0.0001$

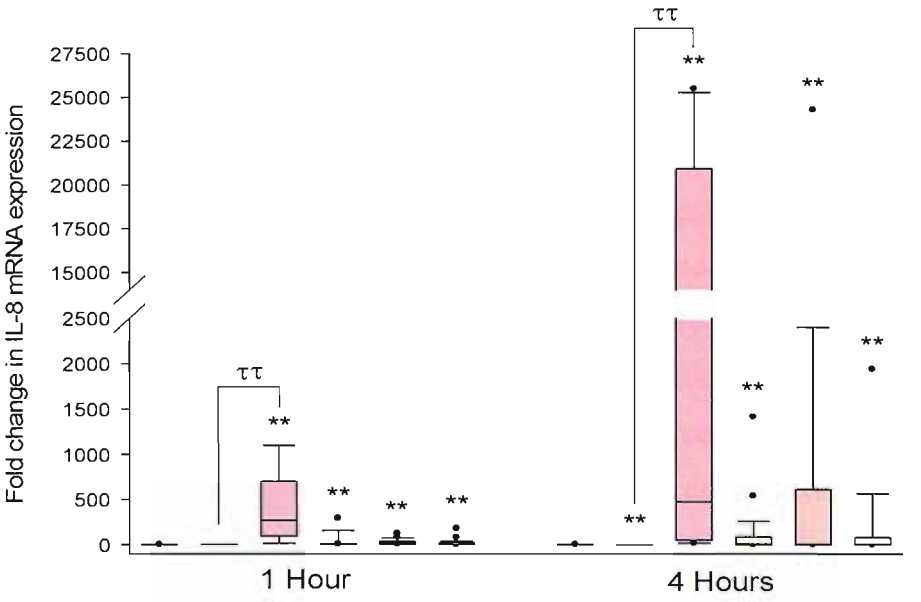


Fig 6.16 IL-8 mRNA expression from severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. ** = $p < 0.01$ vs control ττ = $p < 0.0001$

Comparison of IL-8 mRNA expression from healthy, mild asthmatic and severe asthmatic fibroblasts.

Data in fig 6.17 and 6.18 show that there is little significant variation in IL-8 mRNA production from healthy and asthmatic severe asthmatic fibroblast, although severe asthmatic fibroblasts are able to produce significantly more IL-8 mRNA than mild asthmatic fibroblasts after challenge with mild asthmatic BAL after 1 hour ($p < 0.05$). Although these data are significant it is unlikely that severe asthmatic fibroblasts possess an overall greater ability to synthesise IL-8 mRNA than do mild asthmatic fibroblasts.

It is also noticeable that there is no significant variation in IL-8 mRNA synthesis from healthy and asthmatic fibroblasts after challenge with 10ng/ml TNF-α and 1ng/ml TGF-β1 (fig 6.19). This is interesting as after a 4 hour challenge with 10ng/ml TNF-α there is a lot of variability in the amount of IL-8 mRNA synthesised by severe asthmatic and healthy fibroblasts, as represented by the large inter-quartile ranges in each box. This again highlights the inducibility of the IL-8 gene, and also helps illustrate the difficulty in obtaining statistically significant values in smaller data sets, as there is invariably a lot of overlap between the healthy and asthmatic values. When considering the inconsistency of the data it is necessary to acknowledge that this may be caused by varying qualities of RNA. Although the quality of the RNA was assessed periodically, not every sample was tested, as such it cannot be confirmed that all RNA was of equal quality. If this study were to be extended a more comprehensive analysis could be performed, and then more certain conclusions drawn. However, from this work, it is unlikely that the fibroblasts in severe asthma are contributing to the influx of inflammatory cells into the airways through the abnormally high expression of IL-8.

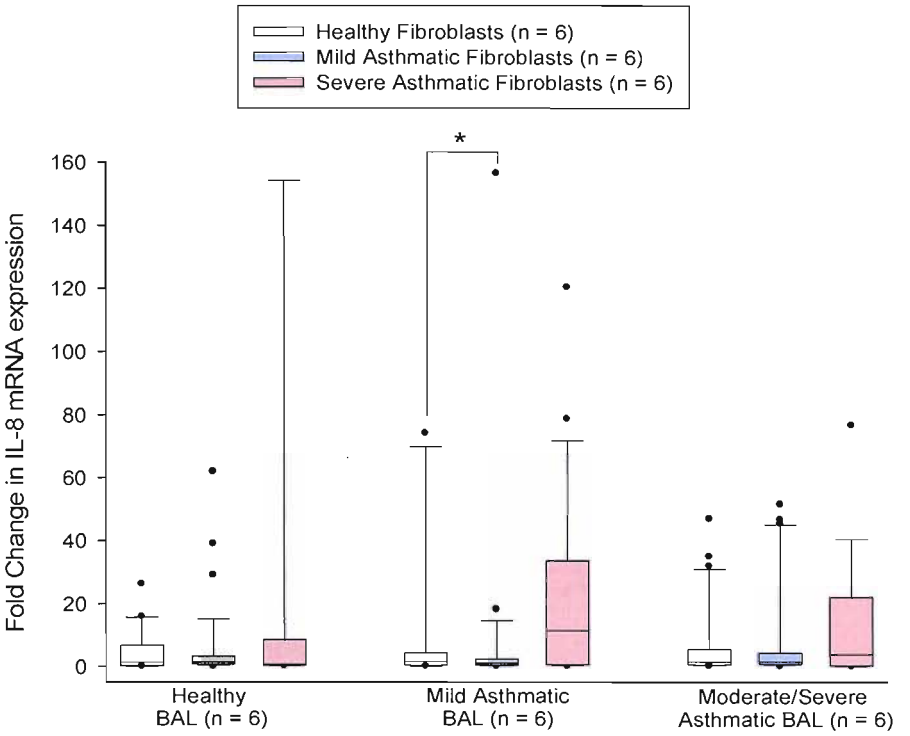


Fig 6.17 IL-8 mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 1 hour * = $p < 0.05$ vs control

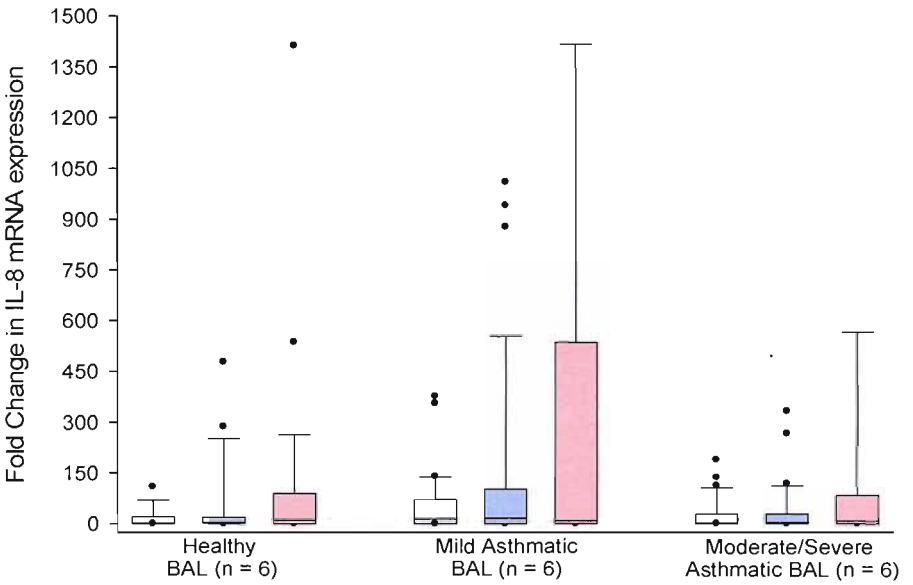


Fig 6.18 IL-8 mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 4 hours.

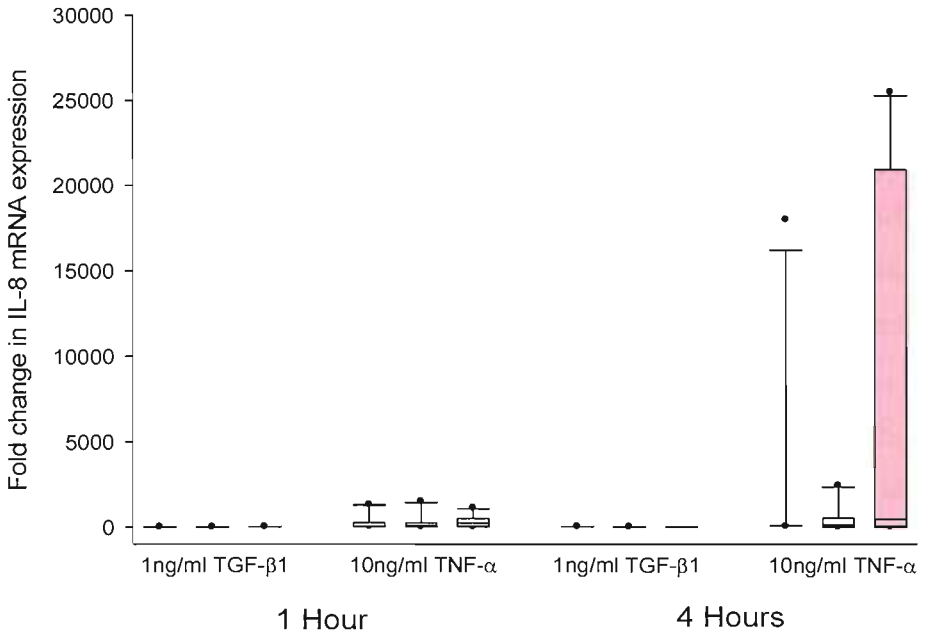


Fig 6.19 Comparison of IL-8 mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic fibroblasts (n = 6) after challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. ** = $p < 0.01$

Baseline expression of collagen III, CTGF and IL-8 mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts.

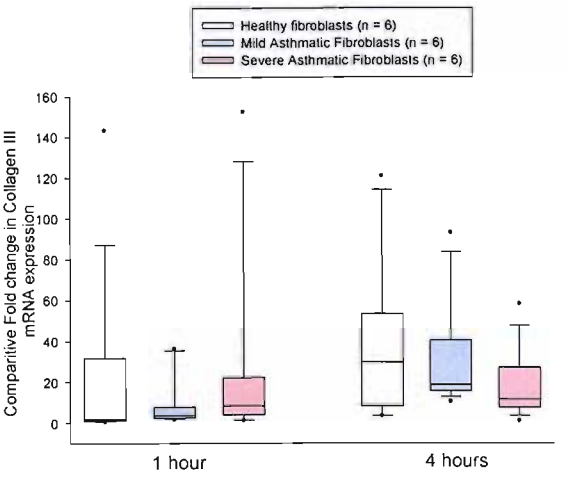
Fig 6.20 shows the expression of collagen III, CTGF and IL-8 mRNA from unstimulated healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts. The Ct values from the healthy, mild asthmatic and severe asthmatic fibroblast unstimulated control samples were pooled together for each gene. The highest Ct value from all 3 cell lines was then used to compare Ct values from all the other 17 values for each gene, and this was then represented as a comparative fold change. There was little variation in collagen III and CTGF mRNA expression between the 3 cell types, however significant difference was seen when comparing IL-8 mRNA synthesis from healthy and severe asthmatic fibroblasts at 1 hour ($p < 0.05$), and mild asthmatic and severe asthmatic fibroblasts at 4 hours ($p < 0.05$), with healthy and mild asthmatic fibroblasts having the larger comparative fold changes.

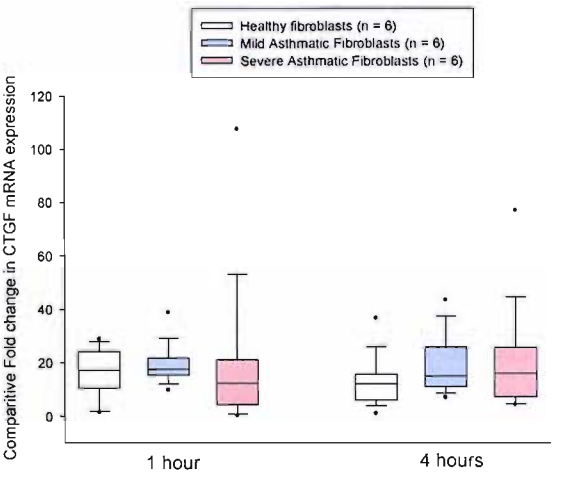
This data in fig 6.20 showing the baseline expression of collagen III mRNA is interesting for a number of reasons, firstly it suggests that the baseline expression of collagen III mRNA from the healthy and asthmatic fibroblasts is similar, and therefore in figs 6.2 to 6.7 the increase in collagen III mRNA is likely to be due to the effect of challenge, and not due to the fibroblasts synthesising more collagen III mRNA while resting. However the data also show that at 4 hours the baseline amount of collagen III mRNA being synthesised has risen in the healthy and mild asthmatic fibroblasts, but remained stable from the severe asthmatic fibroblasts. This shouldn't be the case as the cells were kept in serum free conditions for 24 hours previously, and the addition of an extra 500µl of serum free media at time of challenge shouldn't cause an increase in collagen III mRNA synthesis.

The data assessing the baseline expression of CTGF mRNA show that there is little variation between the groups, and therefore the increase in CTGF mRNA expression observed after challenge is likely to be due to the fibroblasts responding to challenge.

The baseline expression of IL-8 appears to be lower in severe asthmatic fibroblasts than in healthy or mild asthmatic fibroblasts, and this is significant at 1 hour compared to healthy fibroblasts ($p < 0.05$) and mild asthmatic fibroblasts at 4 hours ($p < 0.05$). That the baseline expression of IL-8 mRNA from severe asthmatic fibroblasts is significantly

lower that in mild asthmatic fibroblasts, but not healthy fibroblasts, even though the median value is higher in healthy fibroblasts plots than the mild asthmatic fibroblasts again highlights the variability in IL-8 mRNA synthesis. If this study were to be extended, to include larger numbers of fibroblasts, then it is likely that the baseline expression of IL-8 mRNA from healthy fibroblasts would be significantly higher than that of severe asthmatic fibroblasts at 4 hours. That severe asthmatic fibroblasts have a significantly lower baseline ability to synthesise IL-8 mRNA than do healthy or mild asthmatic fibroblasts suggests they may be responding to challenge in an up regulated manner in figs 6.15 and 6.16, but as their baseline production of IL-8 mRNA is low, that this does not translate into a significant increase in IL-8 mRNA expression after challenge. To investigate this IL-8 ELISAs were performed to determine the amount of IL-8 protein being synthesised and secreted into the supernatant by healthy and asthmatic fibroblasts, after a 1 and 4 hour challenge with BAL, TGF-β1 and TNF-α. The supernatant was collected from the cells used for the work in figs 6.2 to 6.20.





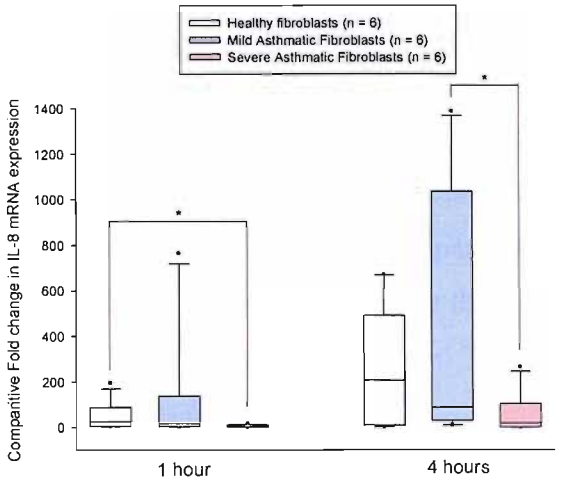


Fig 6.20 Comparison of baseline collagen III, CTGF and IL-8 mRNA expression from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts at 1 and 4 hours. Fibroblasts were seeded at 50,000 cells/well, baseline was calculated by taking the highest control Ct value from all 18 cell lines, and comparing all other values to this. * = $p < 0.05$.

Measurement of IL-8 protein levels in the supernatants of BAL challenged healthy, mild asthmatic and severe asthmatic fibroblasts at 1 and 4 hours.

The levels of IL-8 protein in supernatants collected after challenging healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts with 1ng/ml TGF-β1, 10ng/ml TNF-α, healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) BAL for 1 and 4 hours was assessed in figs 6.21 to 6.23.

The data in these figures show that after a 1 hour challenge with 1ng/ml TGF-β1, 10ng/ml TNF-α and BAL that there was no significant increase in IL-8 protein production from the fibroblasts, which isn't surprising as at 1 hour there was little IL-8 mRNA expression. However after a 4 hour challenge 10ng/ml TNF-α was able to significantly increase the production of IL-8 protein from healthy, mild asthmatic and severe asthmatic fibroblasts compared to unstimulated cells ($p < 0.05$). This was logical as it was shown earlier (figs 6.14, 6.15 and 6.16) that at 4 hours 10ng/ml TNF-α was able to significantly increase the expression of IL-8 mRNA from healthy, mild asthmatic and severe asthmatic fibroblasts compared to the control. However the data also show that healthy BAL was able to increase the expression of IL-8 from mild asthmatic fibroblasts ($p < 0.05$) (fig 6.22) as was 1ng/ml TGF-β1 in healthy and severe asthmatic fibroblasts ($p < 0.005$) (figs 6.11 and 6.23).

There was no significant increase in IL-8 protein production from the fibroblasts after challenge with healthy and asthmatic BAL, however these are early time-points and it is difficult to determine whether this is representative of the effect of BAL on fibroblast IL-8 protein production. However that these data suggest that fibroblasts are able to synthesise and secrete IL-8 protein into the supernatant at 4 hours allows the tentative comparison of IL-8 protein production from healthy and asthmatic fibroblasts.

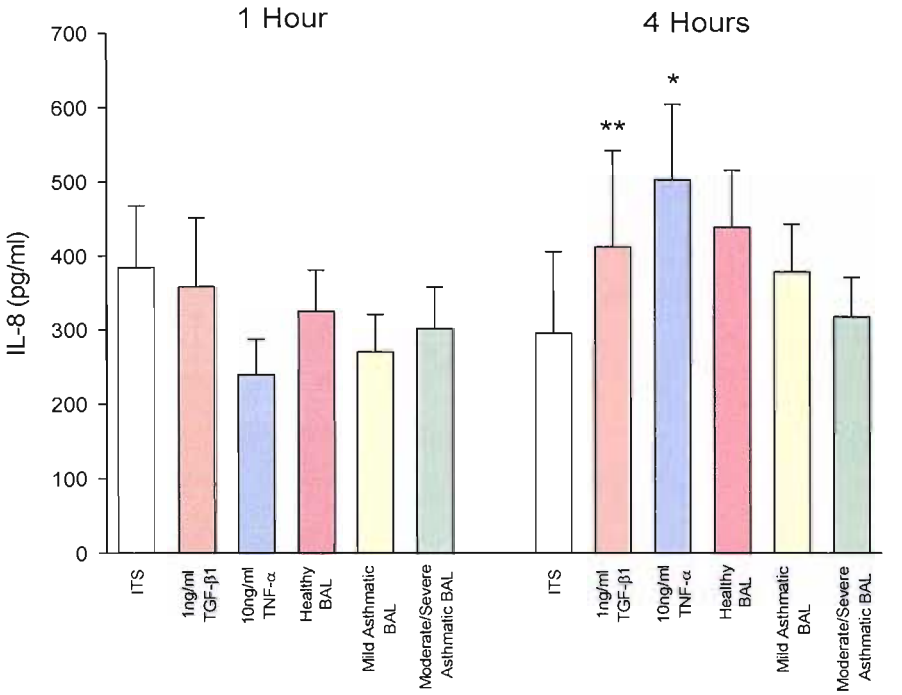


Fig 6.21 IL-8 production from healthy primary lung fibroblasts (n = 6), challenged with healhy BAL (n = 6), mild asthmatic BAL (n = 6), moderate/severe asthmatic BAL (n = 6), 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.03$ ** = $p < 0.005$ both vs ITS control

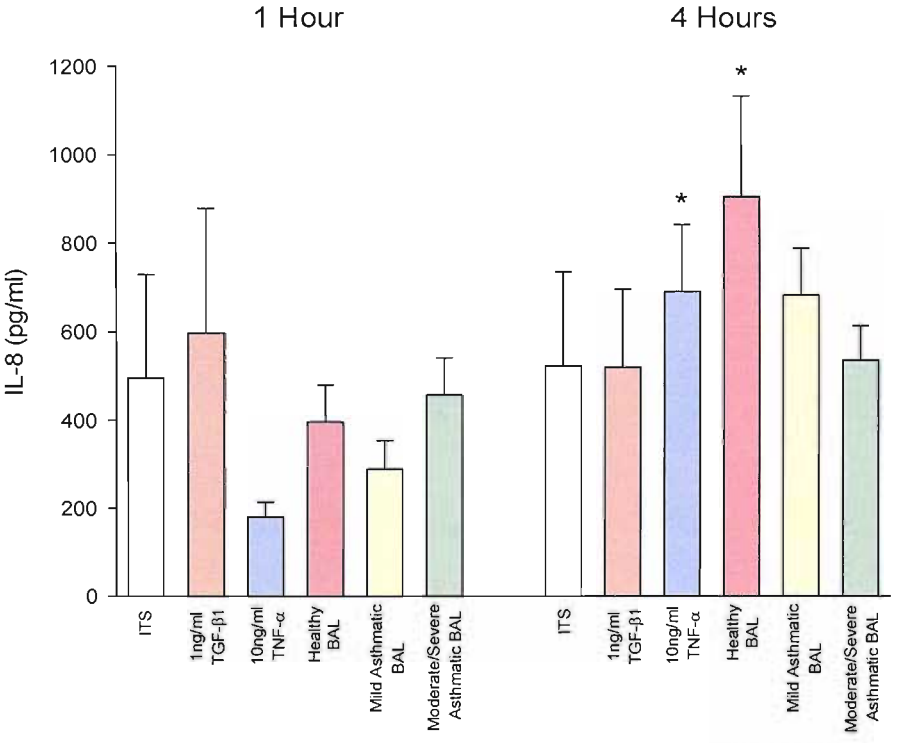


Fig 6.22 IL-8 production from mild asthmatic primary lung fibroblasts (n = 6), challenged with healhy BAL (n = 6), mild asthmatic BAL (n = 6), moderate/ severe asthmatic BAL (n = 6), 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. * = $p < 0.03$ ** = $p < 0.005$ both vs ITS control

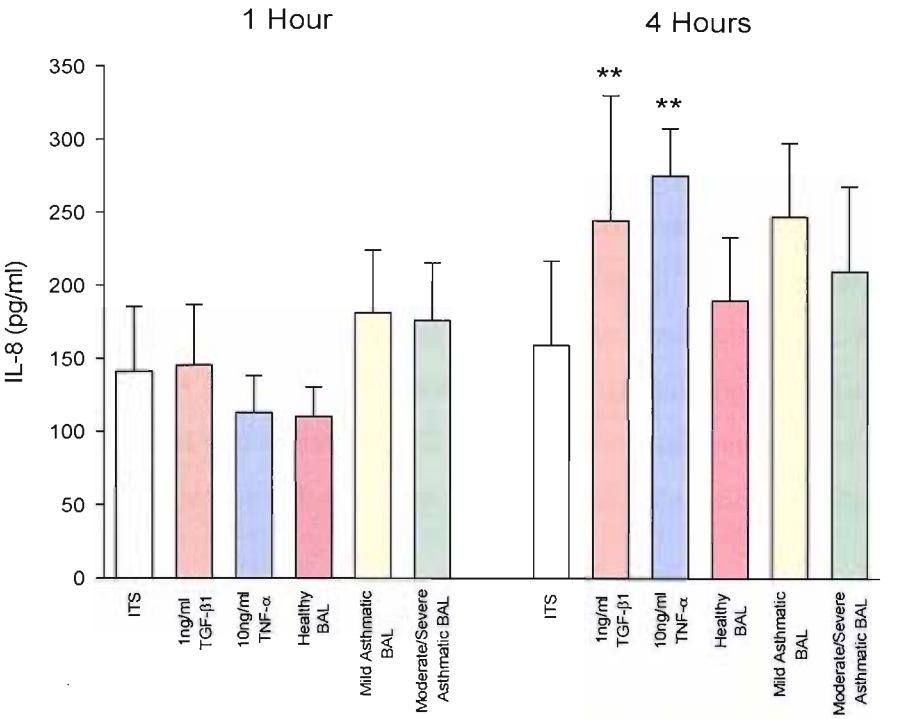


Fig 6.23 IL-8 production from severe asthmatic primary lung fibroblasts (n = 6), challenged with healhy BAL (n = 6), mild asthmatic BAL (n = 6), moderate/ severe asthmatic BAL (n = 6), 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and challenged for 1 or 4 hours. ** = $p < 0.005$ both vs ITS control.

Comparison of IL-8 protein production from healthy, mild asthmatic and severe asthmatic fibroblasts.

The baseline comparison of IL-8 protein production from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts is shown in fig 6.24, while the comparison of IL-8 protein production from healthy and asthmatic fibroblasts after a 4 hour challenge with healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) BAL is shown in figs 6.25, and the production of IL-8 protein after a 4 hour challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α is shown in fig 6.26.

The data in fig 6.24 show that fibroblasts from severe asthmatic donors possess a lower baseline ability to produce IL-8 protein than do mild asthmatic or healthy fibroblasts at 1 and 4 hours.

There was no significant increase in IL-8 protein present in the supernatant of BAL challenged fibroblasts at 1 hour challenge once the data had been corrected for baseline IL-8 protein production at 1 hour. This fits in with data in figs 6.21 and 6.23 where IL-8 protein production was not increased compared to the unstimulated control.

In fig 6.25 IL-8 protein production is shown after a 4 hour challenge with BAL with the baseline IL-8 protein production at 4 hours subtracted. The data suggest that mild asthmatic fibroblasts are able to produce significantly more IL-8 protein than healthy and severe asthmatic fibroblasts after challenge with mild asthmatic BAL ($p < 0.01$) and also significantly greater levels of IL-8 protein than severe asthmatic fibroblasts after challenge with healthy and moderate/severe asthmatic BAL ($p < 0.01$). There is little literature suggesting that mild asthmatic fibroblasts are to produce increased levels of IL-8 protein, and the data in this study are preliminary. However further work comparing the ability of healthy and asthmatic fibroblasts to produce IL-8 protein at later time-points may allude to a degree of abnormal behaviour from mild asthmatic fibroblasts. It is worth noting, however, that the data assessing IL-8 mRNA expression (fig 6.17 to 6.18) do not suggest that mild asthmatic fibroblasts possess an increased ability to synthesise IL-8 mRNA at 1 and 4 hours, when compared to healthy or severe asthmatic fibroblasts. Therefore along with investigating IL-8 protein production at later time-points it may also be interesting to couple this with assessing IL-8 mRNA synthesis from healthy and asthmatic fibroblasts at later time-points.

In fig 6.26 the data show that after a 4 hour challenge with 1ng/ml TGF-β1 there was no significant variation in the amount of IL-8 protein produced by healthy, mild asthmatic and severe asthmatic fibroblasts. However after a 4 hour challenge with TNF-α mild asthmatic fibroblasts were able to produce significantly more IL-8 protein than severe asthmatic cells ($p < 0.01$). As with BAL challenge, once the data was corrected for the baseline IL-8 protein production at 1 hour, there was an insufficient increase in IL-8 protein production from the fibroblasts to undergo analysis after a 1 hour challenge with TGF-β1 and TNF-α.

The data in figures 6.24 to 6.26 show that fibroblasts from severe asthmatics produce less IL-8 protein both at baseline and after challenge than do healthy or mild asthmatic fibroblasts. This is likely to be due, at least in part, to the decreased baseline ability to synthesis IL-8 mRNA observed in fig 6.20; however this may also be due to severe asthmatic fibroblasts possessing a reduced mitogenic potential, as eluded to earlier (figs 4.7 to 4.14). If these fibroblasts are not increasing their cell number to the same degree as the healthy or mild asthmatic fibroblasts, then as they left to seed for 48 hours there may be more healthy and mild asthmatic fibroblasts present in the wells than there are severe asthmatic fibroblasts prior to challenge. This would affect the amount of IL-8 protein present in the supernatant even at baseline levels, and this may be the explanation for the observations in figs 6.24 to 6.26. To better quantify this fibroblasts from healthy and asthmatic fibroblasts would need to be counted prior to and after challenge, but also subjected to a longer challenge with BAL, and then the production of IL-8 protein could be compared more accurately between healthy and asthmatic fibroblasts, along with the affects of healthy and asthmatic BAL. The data also show that mild asthmatic fibroblasts are synthesising the largest amount of IL-8 protein of the three fibroblast lines, and this may indicate some abnormal behaviour from these cells. To clarify this observation IL-8 protein production would need to be measured at a later time-points.

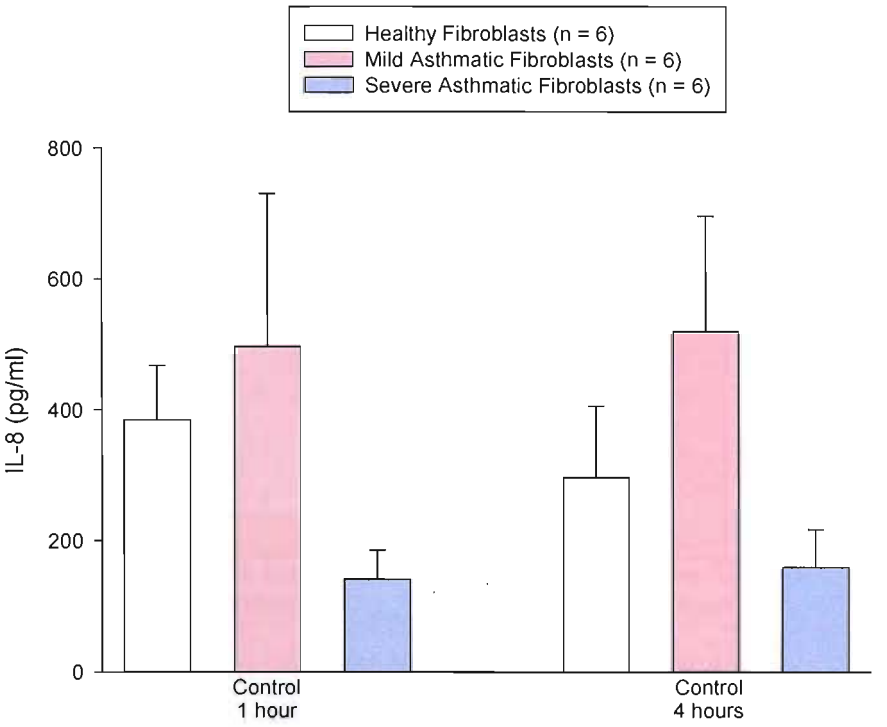


Fig 6.24 Baseline comparisson of IL-8 protein production from primary lung fibroblasts in the absence of challenge. Fibroblasts were seeded at 50,000 cells/well and serum deprived with 1% ITS media for 24 hours, after which an additional 500µl of 1% ITS media was added for 1 or 4 hours.

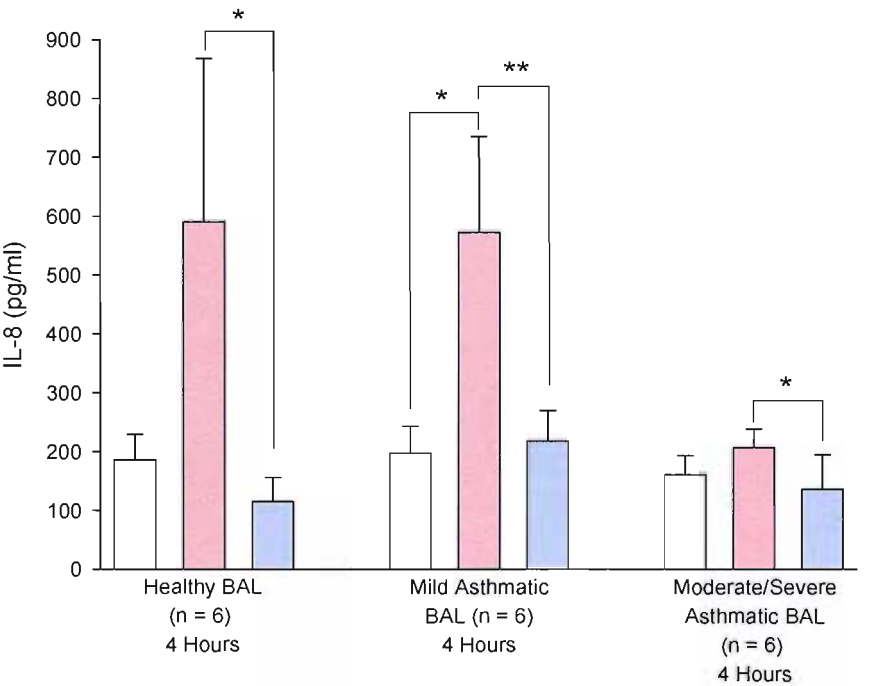


Fig 6.25 IL-8 production from primary lung fibroblasts, challenged with healthy BAL (n = 6), mild asthmatic BAL (n = 6) and moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and challenged for 4 hours. * = $p < 0.01$ ** = $p < 0.001$

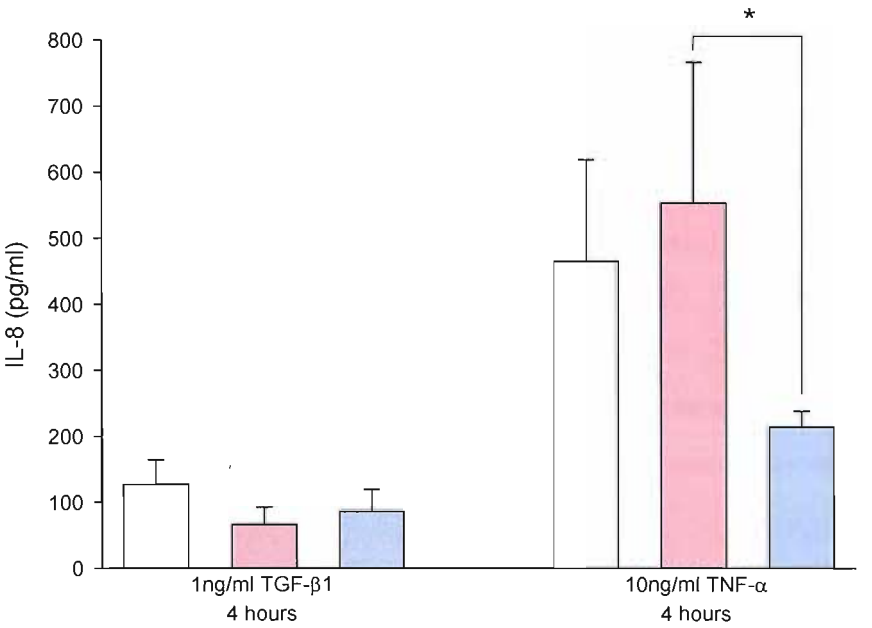


Fig 6.26. IL-8 production from primary lung fibroblasts after a 4 hour challenge with 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well. * = $p < 0.01$

Investigation of the affect of various inhibitors on collagen III mRNA expression.

To investigate the increased expression of collagen III mRNA exhibited by severe asthmatic fibroblasts (fig 6.5), and to try and dissect out any mis-regulated pathways involved in this, inhibitors were used against pathways and intra-cellular kinases. These included G-protein coupled receptors (pertussis toxin), tyrosine kinase pathways (genistein), PI3-K (wortmannin), TNF-α (Etanercept), TGF-β (pan-specific TGF-β neutralising antibody), p38 (SB230580) and MEK1/2 (U0126). Before this work could be performed though it was necessary to ensure the inhibitors would not lead to an increased expression of collagen III mRNA. The cytotoxicity of these inhibitors had already been determined (fig 5.5).

The data in fig 6.27 show the effect of the inhibitors to be used in subsequent work on the expression of collagen III mRNA from 1 mild asthmatic fibroblast. The data indicates that although the inhibitors do cause some variation in the amount of mRNA expressed, that at its maximum this only accounts for around a 2 fold increase. This is unlikely to adversely affect future data, as the fold changes in these genes observed previously in this chapter have been significantly higher than 2 fold increases. Although it is difficult to determine a decrease in the expression of mRNA when comparing it to an unstimulated control, it does appear that U0126 is affecting the expression of collagen III mRNA, therefore analysis of subsequent data may have to bear this in mind.

Once the effect of inhibitors on collagen III mRNA synthesis had been determined the affect of the pan-specific TGF-β antibody and Etanercept on TGF-β1, TGF-β2 and TNF-α mediated collagen III mRNA synthesis were determined.

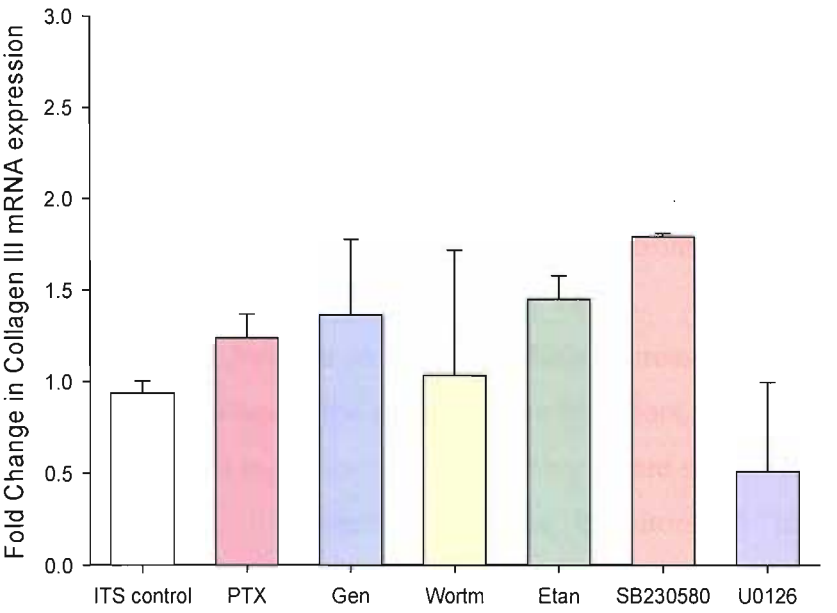


Fig 6.27 Fibroblasts from 1 mild asthmatic donor were seeded at 50,000 cells/well and left to seed for 48 hours before being serum deprived for 24 hours. After serum deprivation the cells were incubated with the inhibitors to be used in the subsequent work for 4 hours in the absence of challenge. The expression of collagen III mRNA was assessed using the TaqMan RT-PCR assay.

Investigating the selectivity of a pan-specific TGF-β antibody and Etanercept, with regard to suppressing collagen III mRNA expression.

Fig 6.28 shows collagen III mRNA expression from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts challenged with 1ng/ml TGF-β1, 1ng/ml TGF-β2 and 10ng/ml TNF-α in the presence or absence of 1:500 pan-specific TGF-β neutralising antibody and 10μg/ml Etanercept. The data from this work show that no challenge could elicit a large increase in collagen III mRNA.

This data is disappointing because as there was little increase in collagen III mRNA synthesis it was difficult to assess the actions of the inhibitors. It would have been ideal to repeat these experiments to ensure that the inhibitors were selective, and then also to conduct investigations on the specificity of the inhibitors by using non-specific challenges, such as TGF-β1 and Etanercept, and TNF-α and the pan-specific TGF-β antibody. However these repeats and additional experiments were unable to be performed due to time and funding constraints. Even so work by other researchers has indicated that these inhibitors are selective for their targets; however it is still accepted that without proving the selectivity of the inhibitor in these assays, that the conclusions drawn from any data utilising these inhibitor will be preliminary. If the work were to be extended in the future, by increasing the numbers of fibroblasts and inhibitors used, then the initial step would require these considerations to be addressed.

As well as the determination of the selectivity of Etanercept and the pan-specific TGF-β antibody it would also have been prudent to ensure that pertussis toxin and genistein were selective in these assays, by using specific and non-specific challenges, and this is an area which would also require attention if this work were to be extended.

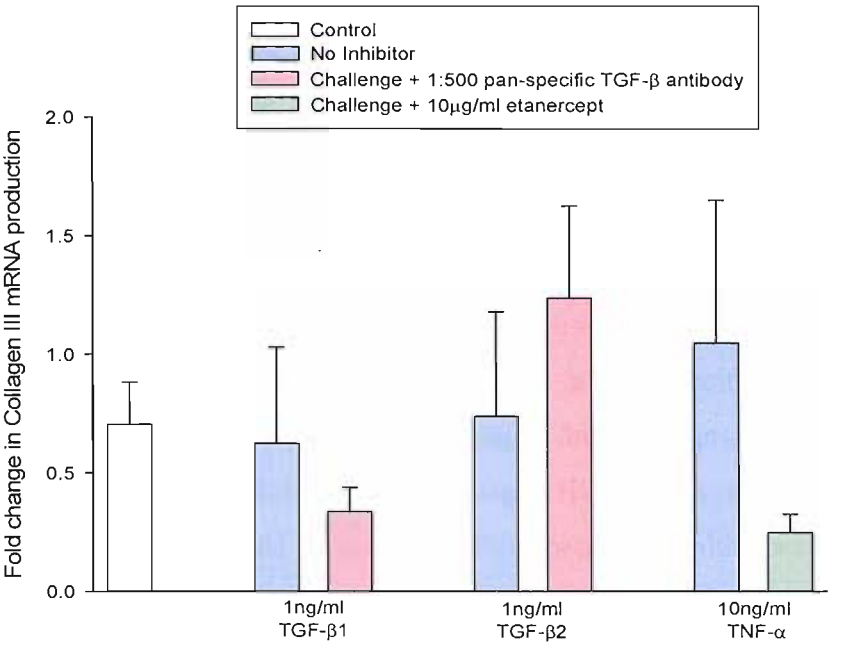


Fig 6.28 Collagen III mRNA production from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts after challenge with 1ng/ml TGF-β1, 1ng/ml TGF-β1 and 10ng/ml TNF-α. Fibroblasts were seeded at 50,000 cells/well and either challenged with stimuli alone or in the presence of 1:500 pan-specific TGF-β antibody or 10μg/ml Etanercept.

Investigating the signalling pathways involved in collagen III mRNA synthesis after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL from 1 healthy, 1 mild asthmatic and 1 severe asthmatic fibroblast line.

In figs 6.29 to 6.31 collagen III mRNA expression from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts was assessed after challenge with healthy (n = 6), mild asthmatic (n = 6) and moderate/severe asthmatic (n = 6) BAL, in the presence or absence of inhibitors. The inhibitors used were pertussis toxin, genistein, wortmannin, Etanercept, U0126, SB203580 and a pan-specific TGF-β neutralising antibody. The fibroblasts were pooled as the aim of this initial investigation was to assess the signalling routes by which BAL elicits collagen III mRNA synthesis from fibroblasts, as data in fig 6.5 had alluded to some variation between healthy and asthmatic BAL. Although there were differences between the fibroblasts in regard to their collagen III mRNA synthetic ability, this is an area for future investigation and one that, although would have been interesting, we could not pursue due to time constraints.

It can be seen from these figures that only moderate/severe asthmatic BAL caused a significant increase in collagen III mRNA expression compared to the control ($p < 0.05$). That the data also show that challenge with healthy and mild asthmatic BAL lead to no increase in collagen III mRNA expression from the fibroblasts, and therefore the actions of the inhibitors in these instances is unclear. However after challenge with moderate/severe asthmatic BAL pre-treatment of the cells with wortmannin ($p < 0.03$), Etanercept ($p < 0.03$), SB203580 ($p < 0.03$), pertussis toxin ($p < 0.05$) and U0126 ($p < 0.05$) all lead to a significant inhibition of collagen III mRNA expression.

That only moderate/severe asthmatic BAL lead to a significant increase in collagen III mRNA expression means that the determination of the effect of inhibitors on healthy and mild asthmatic BAL mediated collagen III mRNA synthesis is difficult. This is because if the challenge is not causing an increase in the production of collagen III mRNA, then the actions of the inhibitor in suppressing, or increasing this synthesis will be indeterminable. These experiments would need to be repeated in order to investigate the role of these pathways, factors and intra-cellular kinases in healthy and mild asthmatic mediated collagen III mRNA synthesis. If this work were to be repeated, then a comparison could be made between the signalling involved in collagen III mRNA synthesis after challenge

with healthy and asthmatic BAL, however given this data this comparison is not possible. To extend this work it would be interesting to increase the numbers of fibroblasts in each group, so that a comparison could be made between the fibroblasts, as well as the BAL. It may also be interesting to assess the effect of the inhibitors on collagen III mRNA synthesis at later time-points, where there may be more myofibroblasts present.

The data from these figures again indicates that moderate/severe asthmatic BAL has the greatest potential to induce the expression of collagen III mRNA from fibroblasts of the three BAL fluids, and that this is mediated, in part, by G-protein coupled receptors, PI3-K, TNF-α, p38 and MEK1/2. However it is also likely that tyrosine kinase receptors and TGF-β1 are involved in collagen III mRNA synthesis, as the inhibition of their actions lead to a large decrease in collagen III mRNA synthesis, which would likely become significant if this study were to be extended. That inhibition was observed when inhibiting a range of targets suggests that the mediation of collagen III mRNA synthesis involves the interactions of multiple factors and pathways, as with the mitogenesis work previously, and these will be discussed later.

Given that moderate/severe asthmatic BAL was inducing fibroblasts from severe asthmatics to synthesise increased levels of collagen III mRNA (fig 6.5), but also appeared to have a general increased ability to increase the expression of collagen III mRNA (fig 6.2, 6.4 and 6.31), it was decided to analyse the phosphorylation status of a variety of MAPKs after a 30 minute challenge with moderate/severe asthmatic BAL in healthy, mild asthmatic and severe asthmatic BAL. It was decided to choose 30 minutes as at 1 hour collagen III mRNA expression was up regulated, so at 30 minutes MAPK phosphorylation associated with this expression should be raised.

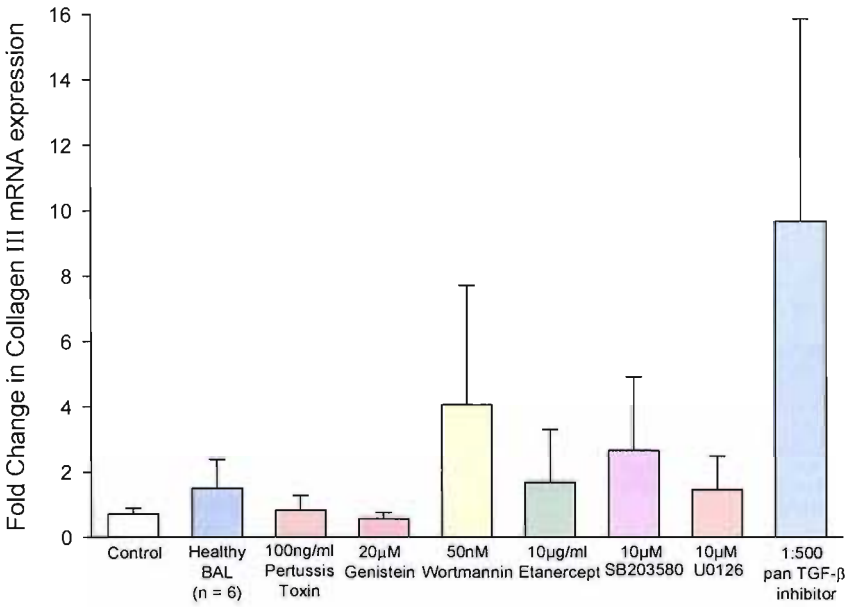


Fig 6.29 Collagen III mRNA production from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts after challenge with healthy BAL (n = 6). Fibroblasts were seeded at 5,000 cells/well and pre-incubated with inhibitors for either 1 or 4 hours, before addition of BAL for 1 hour.

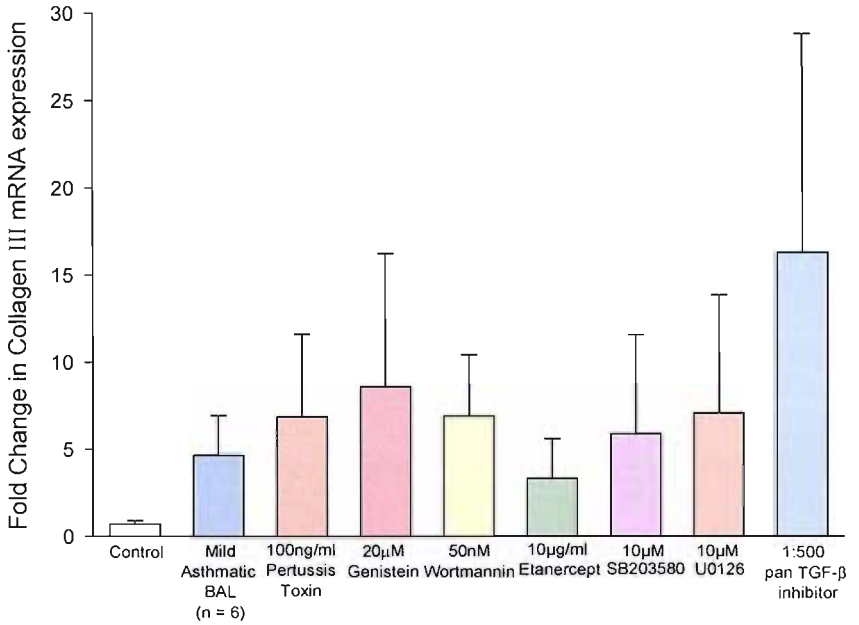


Fig 6.30 Collagen III mRNA production from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts after challenge with mild asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and pre-treated with inhibitors for 1 or 4 hours before a 1 hour challenge with BAL.

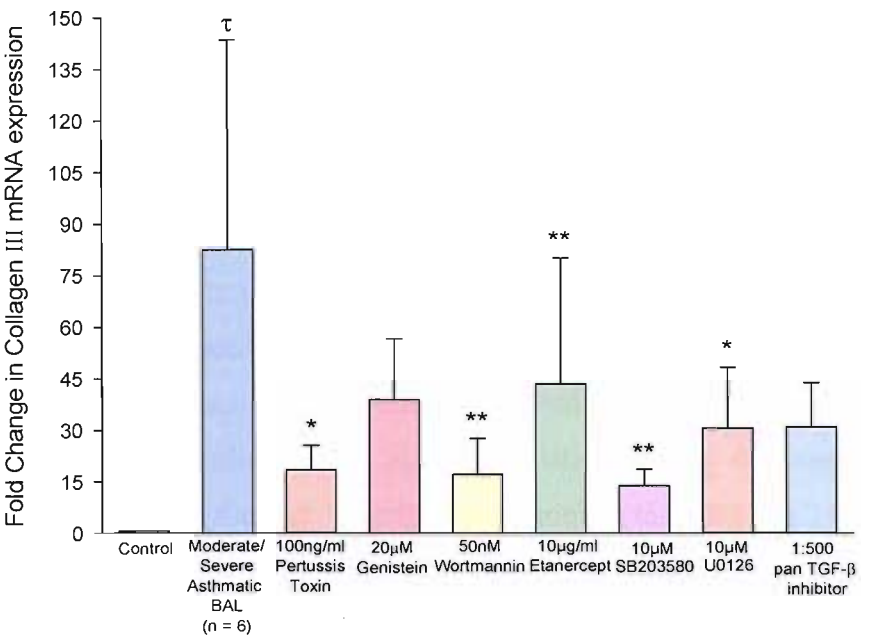


Fig 6.31 Collagen III mRNA production from healthy (n = 1), mild asthmatic (n = 1) and severe asthmatic (n = 1) fibroblasts after challenge with moderate/severe asthmatic BAL (n = 6). Fibroblasts were seeded at 50,000 cells/well and pre-treated with inhibitors for 1 or 4 hours before a 1 hour challenge with BAL.
$\tau = p < 0.05$ vs control * = $p < 0.05$ vs BAL ** = $p < 0.03$ vs BAL

Determination of MAPKs activated after challenge with moderate/severe asthmatic BAL in healthy, mild asthmatic and severe asthmatic fibroblasts.

The data in fig 6.32 show a photographic representation of the phosopho-MAPK array membrane, visualised using chemiluminescent reagents on x-ray film. The spots represent phosphorylated intra-cellular MAPKs and signify their activation, with the degree of activation, or number of activated MAPKs within the cells being represented by the intensity of the spot. The figure shows the films for healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) fibroblasts challenged with moderate/severe asthmatic (n = 6) BAL at 30 minutes, and also a template depicting the position of each set of capture antibodies on the film, with the information in table 6.1 (pg 233) identifying these antibodies. Each array contains negative controls to ensure there is no non-specific binding; these control antibodies are situated in row E, and it can be seen that in all 3 arrays there are no spots visualised. The arrays also contain 6 positive controls in positions A1, A2, A21, A22, F1 and F2, and it can be seen in all 3 arrays that the positive controls are strongly visualised. The arrays show that there are spots visualised at positions B3, B4, B9, B10, B13, B14, B15, B16, C3, C4, C9, C10, C13, C14, D15 and D16 in all 3 arrays. These spots represent phosphorylated ERK1 (B3, B4), pan JNK (B7, B8), p38γ (B9, B10), RSK1 (B13, B14), GSK-3α/β (B15, B16), ERK2 (C3, C4), p38α (C9, C10), RSK2 (C13, C14) and HSP27 (D15, D16). The spots representing the highest level of activation are ERK1, ERK2, p38γ and GSK-3α/β, and of these spots ERK1 and ERK2 are the most intense in all 3 arrays.

As the activation of the MAPKs was not assessed in unstimulated conditions, to give baseline observations, it is not possible to determine whether the phosphorylation of the above MAPKs is due to the response of the fibroblasts to the moderate/severe asthmatic BAL, or whether the level of phosphorylation observed is present in resting cells. To determine whether the activation of the MAPKs observed in fig 6.32 were due to BAL challenge, the phospho-MAPK arrays would need to be performed in unchallenged healthy, mild asthmatic and severe asthmatic fibroblasts initially. It would also be necessary to determine whether the activation observed in fig 6.32 was due to the challenge with moderate/severe asthmatic BAL; and to achieve this arrays would need to be performed on fibroblasts challenged with healthy and mild asthmatic BAL.

Although it isn't possible to determine whether moderate/severe asthmatic BAL is eliciting the activation of the MAPKs mentioned previously, the role of the activated MAPKs observed in fig 6.32 in cellular activity can be discussed.

ERK1 and ERK2 are typically involved in proliferative responses; however they have also been implicated in the regulation of other cellular responses such as collagen synthesis (Reunanen N. *et al.* 2000 and Tang M. *et al.* 2007). As well as the activation of ERK1 and ERK2 there is also activation of p38γ and GSK-3α/β. As p38γ is primarily involved in stress responses it may be that its activation is alluding to this, however p38γ has also been shown to be involved in cellular differentiation (Lechner C *et al.* 1996). Glycogen synthase kinase-3 α/β is a mediator of many cellular processes including proliferation, protein synthesis, responses to DNA damage and differentiation. However GSK-3α/β is also a downstream target of Akt (Frederick T.J. *et al.* 2007), and therefore may be a marker of Akt phosphorylation shown in the mild asthmatic and severe asthmatic arrays at positions B7, B17, B18 (Akt1), B19, B20 (Akt2) and C19, C20 (pan Akt), although this may be unlikely as the Akt spots are not seen on the healthy array. These Akt1, Akt2 and pan Akt spots are most intense on the severe asthmatic array, which is interesting as Akt has been shown to help regulate fibroblast differentiation into a contractile phenotype (Shi-Wen X. *et al.* 2004) and also regulate collagen I-III synthesis from fibroblasts (Lim I.J. *et al.* 2003). There is also a weak activation of the pan JNK (B7, B8) spots in the mild asthmatic and severe asthmatic arrays; JNK is primarily involved in the regulation of apoptosis, differentiation and proliferation, however as the spots are weakly visualised its involvement in any signalling in asthmatic fibroblasts would require further quantification. The inhibition of JNK in previous figures (figs 5.19, 5.20 and 5.21) did indicate slight variation in the response of fibroblasts to asthmatic BAL compared to healthy BAL, with regards to mitogenesis, however as already mentioned this observation required further investigation. Nevertheless it may be interesting to analyse the role of JNK in the synthesis of collagen III mRNA, and this could be achieved by pre-treating the fibroblasts with an inhibitor of JNK such as JNK inhibitor VIII, before challenge with BAL.

It appears from this preliminary study that Akt may be mis-regulated in asthmatic fibroblasts; however whether this is due to the challenge with moderate/severe asthmatic

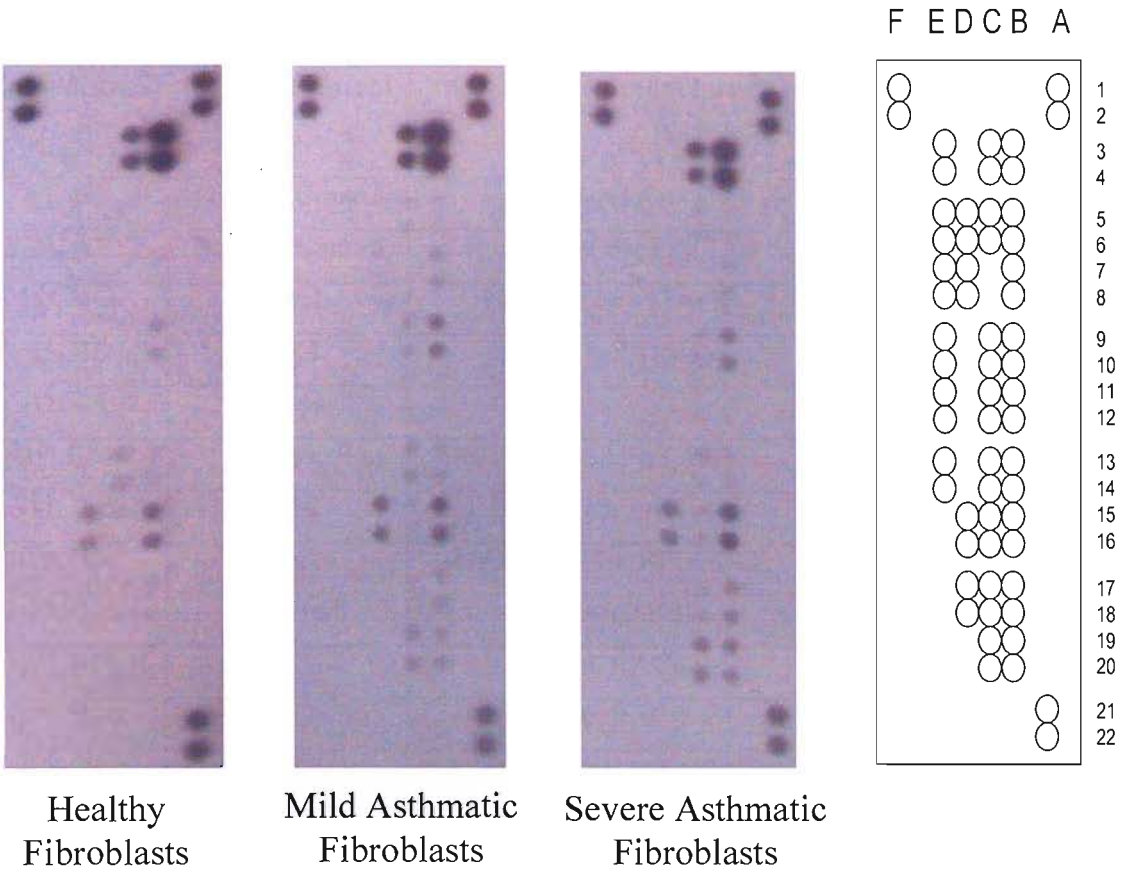


Fig 6.32 MAPK phosphorylation assay performed on fibroblast from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) subjects were pooled and challenged with moderate/severe asthmatic BAL (n = 6) for 30 minutes. The membranes were exposed to chemiluminescent reagents for 1 minute then the spots visualised on x ray film for 5 minutes.

Co-ordinate	Target/Control	Alternate Nomenclature	Phosphorylation Site Detected
A1, A2	Positive Control	Control (+)	-
A21, A22	Positive Control	Control (+)	-
B3, B4	ERK1	MAPK3, p44 MAPK	T202/Y204
B5, B6	JNK1	MAPK8, SAPK1γ	T183/Y184
B7, B8	JNK pan	-	T183/Y185, T221/Y223
B9, B10	p38γ	MAPK12, SAPK3, ERK6	T183/Y185
B11, B12	p38δ	MAPK13, SAPK4	T180/Y182
B13, B14	RSK1	MAPKAPK1α, RPS6KA1	S380
B15, B16	GSK-3α/β	GSK3A/GSK3B	S21/S9
B17, B18	Akt1	PKBα, RACα	S473
B19, B20	Akt2	PKBβ, RACβ	S474
C3, C4	ERK2	MAPK1, p42 MAPK	T185/Y187
C5, C6	JNK2	MAPK9, SAPK1α	T183/Y184
C9, C10	p38α	MAPK14, SAPK2A, CSBP1	T180/Y182
C11, C12	p38β	MAPK11, SAPK2β, p38-2	T180/Y182
C13, C14	RSK2	ISPK-1, RPS6KA3	S386
C15,C16	GSK-3β	GSK3B	S9
C17, C18	Akt3	PKBγ, RACγ	S472
C19, C20	Akt pan	-	S473, S474, S472
D5, D6	JNK3	MAPK10, SAPK1β	T221/Y223
D7, D8	MSK2	RSKβ, RPS6KA4	S360
D15, D16	HSP27	HSPB1, SRP27	S78/S82
D17, D18	p70 S6 Kinase	S6K1, p70α, RPS6KB1	T421/S424
E3, E4	Rabbit IgG	Control (-)	-
E5, E6	Mouse IgG_1	Control (-)	-
E7, E8	Mouse IgG_{2A}	Control (-)	-
E9, E10	Mouse IgG_{2B}	Control (-)	-
E11, E12	Goat IgG	Control (-)	-
E13, E14	PBS	Control (-)	-
F1, F2	Positive Control	Control (+)	-

Table 6.1 Phospho-MAPK array co-ordinates

Quantification of spots visualised on the phospho-MAPK array.

The data in fig 6.33 show a densiteometric analysis of the arrays seen in fig 6.32, which allows the relative density of each spot to be determined for comparative purposes. The spots which were intense enough to be measured were the positive controls, ERK1, ERK2, HSP27, p38γ and GSK-3α/β on all three arrays and Akt1, Akt2 and pan Akt on the severe asthmatic array. The spots which were too weak to be visualised on in this analyses were RSK1, p38α and RSK2 from all three arrays, pan JNK from the mild and severe asthmatic arrays, and Akt1, Akt2 and pan Akt on the mild asthmatic array.

These densities are plotted in figs 6.34 and 6.35 along with measurements from the positive controls.

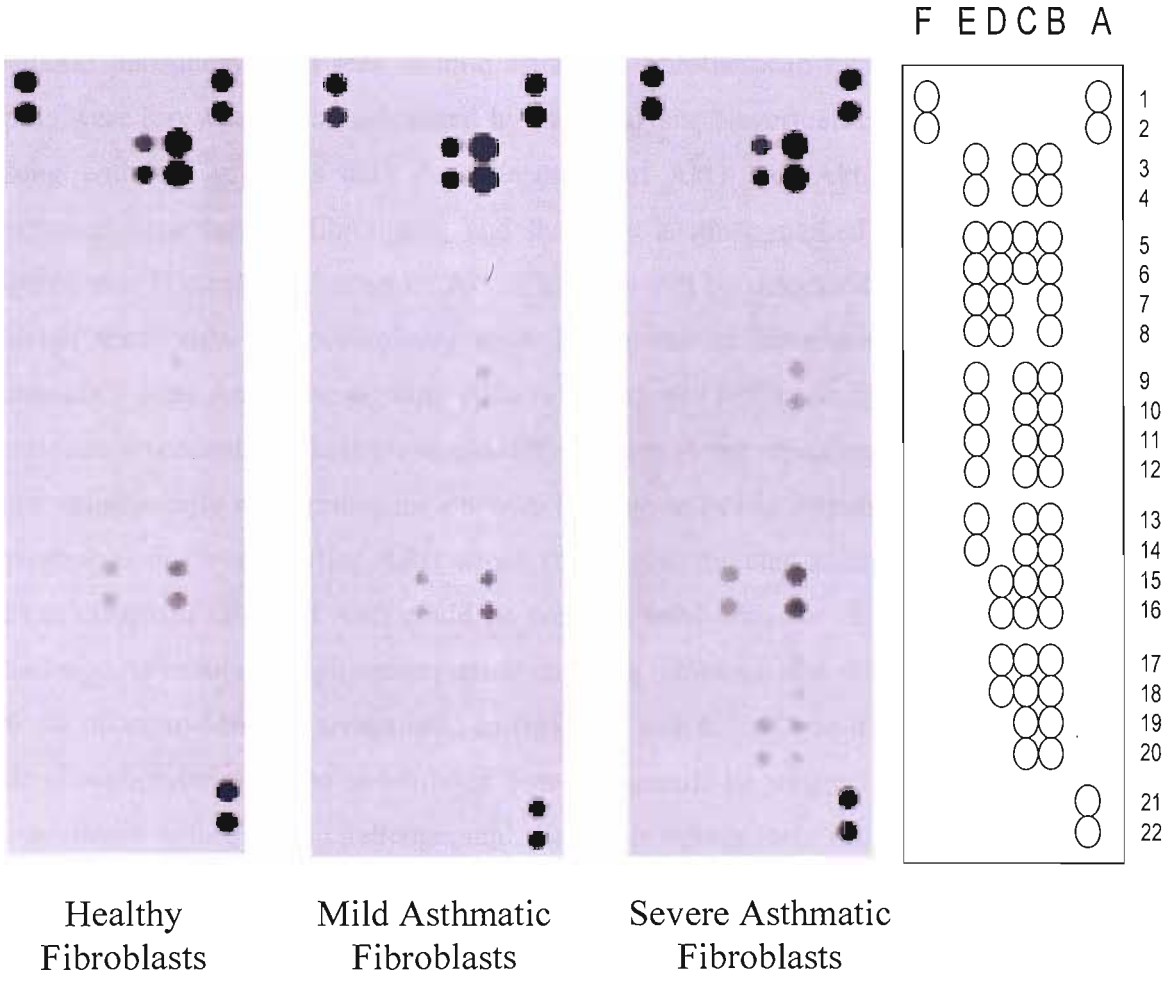


Fig 6.33 MAPK phosphorylation assay performed on fibroblast from healthy (n = 6), mild asthmatic (n = 6) and severe asthmatic (n = 6) subjects were pooled and challenged with moderate/severe asthmatic BAL (n = 6) for 30 minutes. The spot density was measured using the BioRad Versa Doc imaging system, model 400 at 1 minute time-points over a 10 minute period.

There was insufficient data to perform statistical analysis, however it can be seen in fig 6.34 that ERK1 phosphorylation is higher in mild asthmatic fibroblasts than in healthy or severe asthmatic fibroblasts however this may not be due to challenge with moderate/severe asthmatic BAL. There was little difference seen in ERK1 activation between healthy and severe asthmatic fibroblasts. It is also shown in fig 6.34 that there is little variation in the phosphorylation of ERK2, HSP27 and p38γ between the three fibroblast groups.

In fig 6.35 it can be seen that GSK-α/β phosphorylation is slightly higher in asthmatic fibroblasts than in healthy fibroblasts, and that this was most pronounced in severe asthmatic fibroblasts. Although Akt1, Akt2 and pan Akt phosphorylation was only able to be quantified on the severe asthmatic fibroblasts array, there was weak Akt1, Akt2 and pan Akt phosphorylation seen in mild asthmatic fibroblasts in fig 6.32, however, these spots were too weak to be calculated by this analysis. Nevertheless the data in fig 6.35 along with the previous data does suggest that Akt1 and Akt2 phosphorylation is increased in asthmatic fibroblasts, and that this is most marked in severe asthmatic fibroblasts. The potential roles of Akt in asthma will be discussed later, however even though these data are preliminary they do present an interesting target for further research. Future work investigating Akts role in severe asthmatic fibroblasts response to moderate/severe asthmatic BAL would initially require the repeating of these assays, and then subsequently performing the control, healthy and mild asthmatic BAL challenged membranes discussed earlier. After which compounds directed against both Akt and PI3-K (an upstream target of Akt) could be pre-incubated with the fibroblasts before BAL challenge, to ensure Akt phosphorylation could be inhibited, and this could be visualised on the phospho-MAPKs arrays used in figs 6.32 and 6.33. Once it was established that Akt phosphorylation could be inhibited fibroblasts could be pre-treated with Akt and PI3-K inhibitors before BAL challenge, and then their mitogenesis and collagen III mRNA synthesis investigated. This would help determine whether Akt was involved in the abnormal proliferation and collagen III mRNA synthesis observed previously in this work.

Although measuring the spot intensity in this manner is not ideal, in that not all spots could be measured, it would be a useful technique to utilise for extension of this work

mentioned earlier. If the arrays were performed in duplicate, then it is possible to generate an x-ray film and also quantify the spots at the same time. Although this would be prone to experimental error, it would also provide a 'hard copy' of the array, as well as an array from which the spots could be quantified. As a large excess of supernatant is produced when lysing the cells after challenge, the experimental variation introduced by this method should be kept to a minimum, with the major sources of disparity being the membranes themselves, and pipetting error. Although it is unlikely that the duplicated membranes produced in this manner would be identical, they should be comparable.

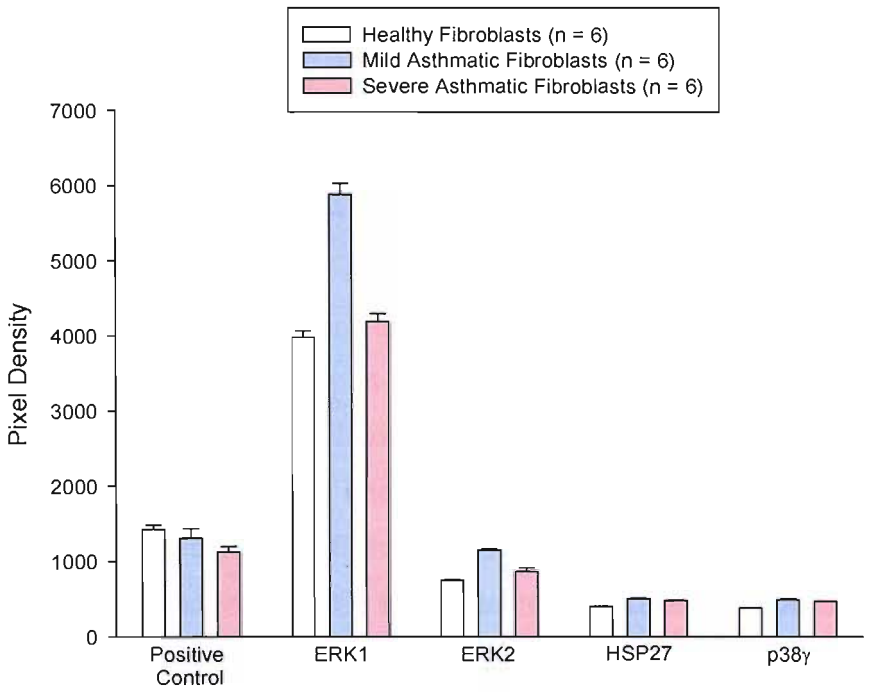


Fig 6.34 Primary cultures of human lung firoblasts were challenged with moderate/severe asthmatic BAL (n = 6) for 30 minutes. Array signals were analysed using the BioRad Versa Doc imaging system, model 400 to give pixel densities. Pixel density represents the level of activation for each MAPK.

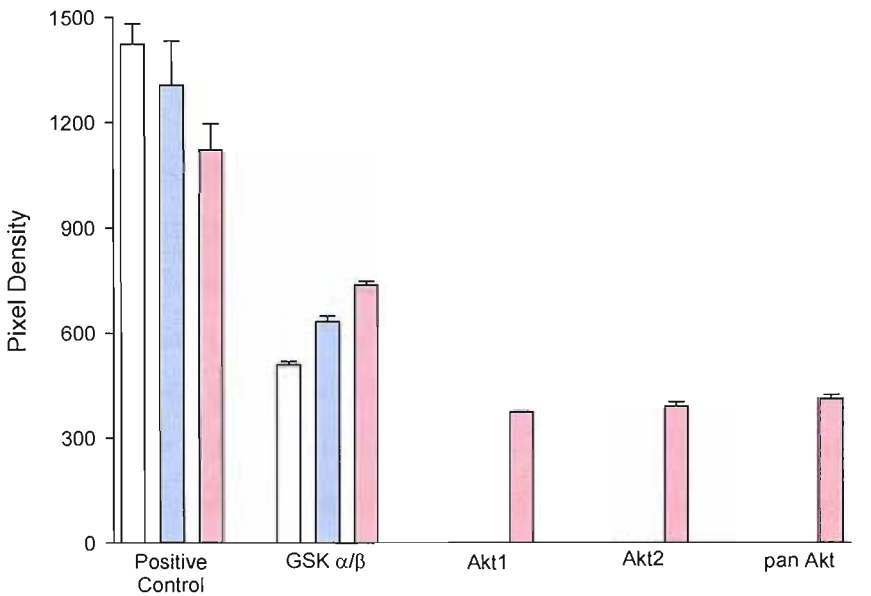


Fig 6.35 Primary cultures of human lung firoblasts were challenged with moderate/severe asthmatic BAL (n = 6) for 30 minutes. Array signals were analysed using the BioRad Versa Doc imaging system, model 400 to give pixel densities. Pixel density represents the level of activation for each MAPK.

Discussion

TaqMan RT-PCR has proven to be a useful tool in the quest to understand intra-cellular events which occur not only in healthy cells, but also in disease states. The role of Helicobacter pylori (H. pylori) in gastric ulcers was investigated by Takahashi M. *et al.* 2000 using the TaqMan RT-PCR assay. During this study it was shown that human gastric fibroblasts produce prostaglandins and HGF in response to the presence of H. pylori, and that this may be part of the body's defence reaction to H. pylori toxicity. It was also shown was that cyclooxygenase-2 (COX-2) plays an important role in chronic H. pylori infection. Work by Pitetti R.D. *et al.* 2003 showed that the TaqMan RT-PCR assay allows the rapid identification of patients with primary Epstein-Barr virus (EBV) infection, and those with EBV infectious mononucleosis, while Sofi I.M. *et al.* 2003 showed the potential for the early detection of smallpox virus infections using the TaqMan assay. Rao K.M. *et al.* 2005 showed that lung fibroblasts are a significant source of IL-6 and MCP-1 in the lung using the SYBR Green RT-PCR technique.

The observations in this chapter suggest that although fibroblasts from severe asthmatics possess a similar baseline potential to synthesise collagen III mRNA as do healthy and mild asthmatic fibroblasts, that when they are exposed to BAL from moderate/severe asthmatic donors for 1 hour these cells are able to produce significantly more collagen III mRNA than can healthy or mild asthmatic fibroblasts. This work fits in with an investigation by Dubé J. *et al.* 1998 who showed that fibroblasts from asthmatic volunteers possessed a similar baseline potential to synthesise pro-collagen I and III as did healthy controls, and also a study by Lewis C.C. *et al.* 2005 who showed that fibroblasts from severe asthmatic bronchial biopsies had an increased ability to synthesise pro-collagen I than did mild asthmatic or healthy fibroblasts. The increased presence of collagen III mRNA in our work suggests that the cells will go on to produce collagen III protein, however it is accepted that not all collagen III mRNA will definitely precede protein synthesis, as some mRNA may be degraded before protein synthesis occurs. Previous work by Fine A. *et al.* 1990 has, however, shown that the increase in collagen I mRNA was associated with a marked increase in the production of collagen I protein, although this may not necessarily apply to collagen III.

It is also interesting to note that in the work by Dubé J. *et al.* 1998 the asthmatic fibroblasts possessed a significantly decrease ability to proliferate with cell passage than healthy controls, supporting an observation made earlier in this work (figs 4.7 to 4.12) regarding the mitogenic potential of severe asthmatic fibroblasts. Although these studies support the observations made in my work, they focused on the actions of individual factors (PDGF-BB and TGF-β), whereas the work in this study has utilised a basic model of the asthmatic environment. The use of this model was hoped to emulate a representation of the local environment to which fibroblasts may be exposed, and thus help to understand whether the environment in which the fibroblasts are residing *in vivo* is affecting their behaviour. And from the work in figs 6.2 to 6.5 it does appear the milieu in which the fibroblasts reside does indeed affect their behaviour, with data in figs 6.2 and 6.4 showing that both healthy and severe asthmatic fibroblasts synthesise more collagen III mRNA after challenge with moderate/severe asthmatic BAL than after challenge with healthy BAL. However along with the contribution of the local environment, it also appears that the fibroblasts in severe asthma are responding to factors in moderate/severe asthmatic BAL in an altered manner than are fibroblasts from healthy and mild asthmatic donors. This can be seen in fig 6.5 where severe asthmatic fibroblasts produce significantly more collagen III mRNA than do healthy or mild asthmatic fibroblasts.

Collagen deposition in the sub-epithelial basement membrane is a characteristic observation in the asthmatic airway (Roche W.R. *et al.* 1989 and Brewster C.E. *et al.* 1990), with myofibroblast numbers correlating to the thickness of this sub-epithelial basement (Brewster C.E. *et al.* 1990), and the fibroblasts used in this study are likely to have had to undergo transformation into myofibroblasts in order to be able to synthesise collagen III mRNA. Therefore the starting levels of myofibroblasts/smooth muscle in the populations of severe asthmatic and mild asthmatic fibroblast cultures being higher than that of the healthy fibroblasts is a consideration which requires addressing at this point. Smooth muscle has been shown to be able to synthesise collagen (Johnson P.R. *et al.* 2000, Johnson P.R. 2001 and Johnson P.R. *et al.* 2006) and airway smooth muscle from asthmatics has been shown to be able to produce greater levels of CTGF than healthy smooth muscle (Burgess J.K *et al.* 2003). Therefore if the starting level of

myofibroblasts/smooth muscle was responsible for the amount of collagen III mRNA being observed after BAL challenge in fig 6.5, then it would be expected that healthy fibroblasts would produce less collagen III mRNA than would mild asthmatic fibroblasts, which in turn would produce less collagen III mRNA than severe asthmatic fibroblasts. This is because when fibroblasts were stained for α-SMA and vimentin it was found that healthy fibroblasts had less α-SMA positive cells than did mild asthmatic fibroblasts, which in turn had less α-SMA positive cells than did severe asthmatic fibroblast. However in fig 6.5 healthy fibroblasts produce more collagen III mRNA than do mild asthmatic fibroblasts. This is not to say that the starting level of myofibroblasts/smooth muscle is not having some affect on the amount of collagen III mRNA being synthesised by severe asthmatic fibroblasts, however it would appear that there is no direct correlation between the amount of collagen III mRNA being produced and the number of myofibroblasts/smooth muscle present in the starting culture. This is probably due to a majority of the populations of the cultures used being of fibroblast origin, and therefore suggests that the effect observed after BAL challenge is due to the behaviour of the fibroblasts in the culture.

Although work by Grotendorst G.R. *et al.* 2004 suggested that fibroblasts are able to rapidly increase their expression of α-SMA mRNA, typically at 1 hour, it is unlikely that this is representative of actual fibroblast to myofibroblast differentiation. The rapid expression of α-SMA mRNA may however, allude to the fibroblasts responding to a challenge in a manner which may subsequently lead to their differentiation at a later time point. If this study were to be extended, and the collagen III mRNA expression observed at a later time point after BAL challenge, then there may be higher numbers of myofibroblasts present, able to synthesise greater levels of collagen III mRNA. As myofibroblasts are believed to possess a decreased proliferative potential than are fibroblasts this may explain the data shown previously in figs 4.7 to 4.12. To determine whether the differentiation into myofibroblasts can explain the decreased mitogenic potential the amount of α-SMA positively stained cells in the cultures of fibroblasts could be measured at various times over a 48 hour period after BAL challenge to determine whether the number of positively stained cells differs between the groups.

That the severe asthmatic fibroblasts are able to synthesise greater levels of collagen III mRNA, but possess a decreased ability to proliferate suggests that they may be in a myofibroblastic phenotype. There is evidence of Akt being able to regulate (Shi-Wen X. *et al.* 2004 and Winbanks C.E. *et al.* 2007) and then maintain (Ricupero D.A. *et al.* 2001b) the myofibroblast phenotype, which may explain why there is Akt phosphorylation observed in fig 6.32.

The Akt family, or PKB, are serine/threonine kinases, of which there are 3 members, Akt1 (PKBα), Akt2 (PKBβ/c) and Akt3 (PKBγ/c). Protein Kinase B was first identified in by Jones P.F. *et al.* 1991, and was termed Akt after its identification as a retroviral oncogene (Bellacosa A. *et al.* 1991).

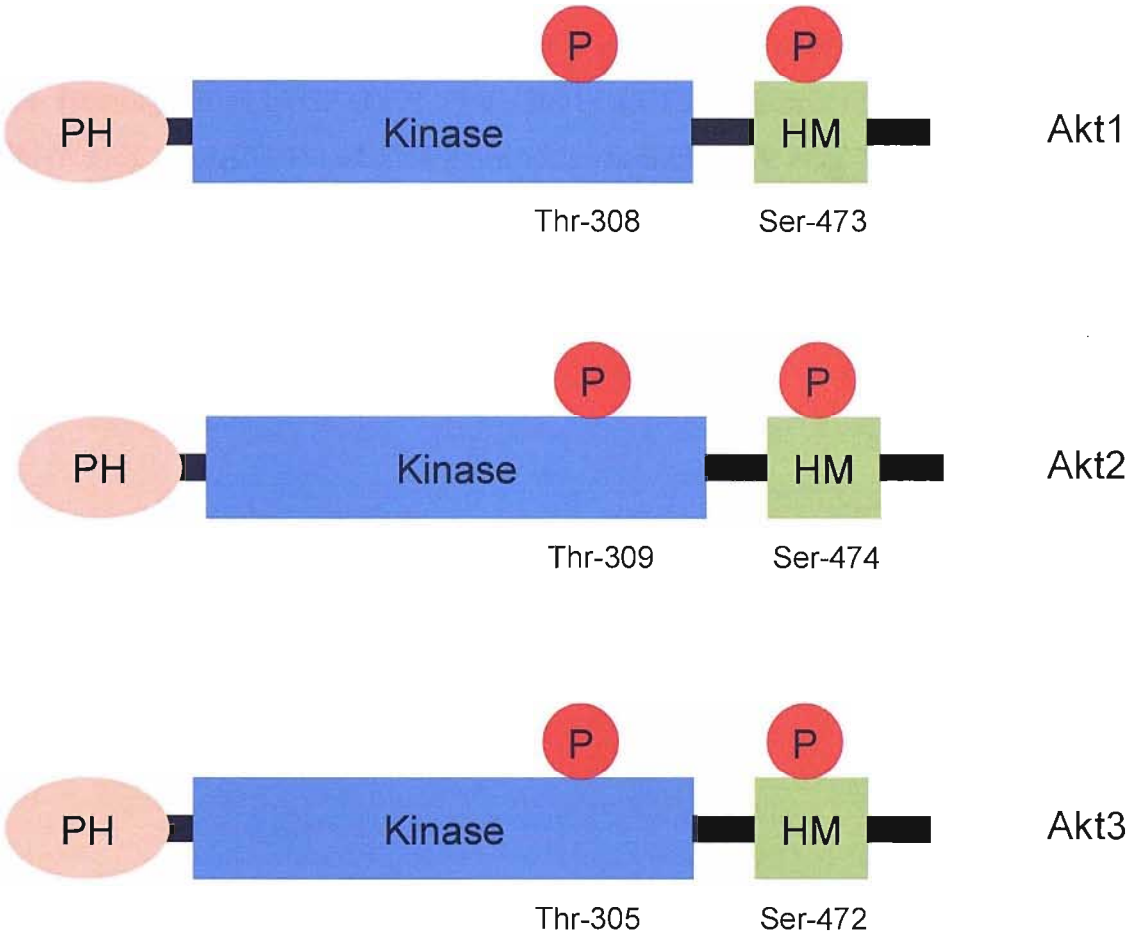


Fig 6.36 Domain structure of Akt isoforms. Each isoform contains a PH (pleckstrin homology) domain, which acts as a phosphoinositide-binding module, a catalytic kinase domain, and a hydrophobic motif (HM) which is located at the carboxyl-terminal, adjacent to the kinase domain. The phosphorylation sites for each isoform are also indicated (P).

Akt is able to regulate a variety of cellular responses, including apoptosis (Burgering B.M. *et al.* 2003), cell survival (Datta S.R. *et al.* 1997), glycogen synthesis (Burgering B.M. *et al.* 1995 and Ueki K. *et al.* 1998), glucose uptake (Kohn A.D. *et al.* 1996), cell cycle progression (Rossig L. *et al.* 2001, Liang J. *et al.* 2002 and Lynch D.K. *et al.* 2002), cardiovascular homeostasis (Dimmeler S. *et al.* 1999, Fulton D. *et al.* 1999 and Michell B.J. *et al.* 1999) and protein synthesis (Kitamura T. *et al.* 1998 and Ueki K. *et al.* 1998). Phosphorylation of Akt is primarily by PI3-K (Burgering B.M. *et al.* 1995 and Kohn A.D. *et al.* 1995), which is a downstream regulator of many signalling pathways including tyrosine kinase receptors, G-protein coupled receptors, integrins and various cytokine receptors.

It has been shown that the regulation of collagen synthesis can be mediated through Akt (Li X. *et al.* 2001, Lim I.J. *et al.* 2003 and Winbanks C.E. *et al.* 2007), after challenge with factors such as bFGF (Li X. *et al.* 2001), EGF (Wennström S. *et al.* 1999), ET-1 (Shi-Wen X. *et al.* 2004) and TGF-β1 (Rodríguez-Barbero A. *et al.* 2006). Therefore Akt may be able to both mediate fibroblast to myofibroblast differentiation, as well as subsequently maintaining the myofibroblast phenotype and promoting collagen synthesis.

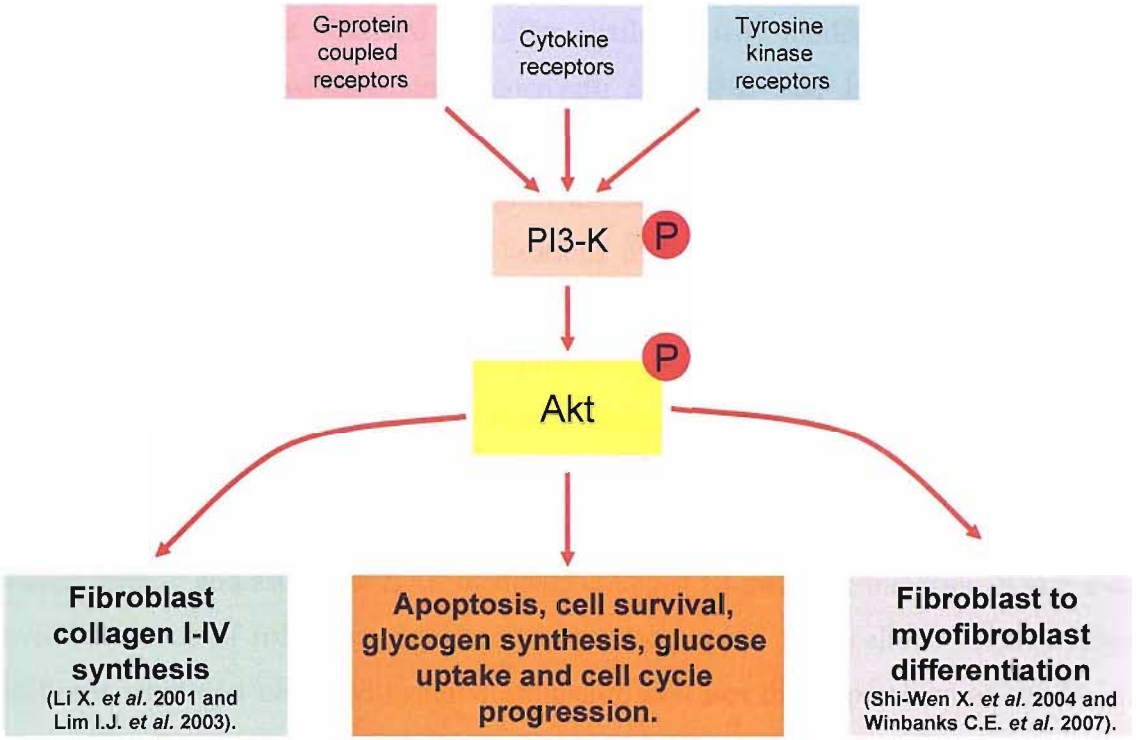


Fig 6.37 Representation of Akts ability to regulate cellular events.

The involvement of Akt in the regulation of severe asthmatic fibroblast behaviour is an area which, as already mentioned, requires further investigation. The data from fig 6.32 requires repeating in addition to performing the controls and other challenges mentioned earlier. However if the data show that Akt activation is up regulated in severe asthmatic fibroblasts, and that this is most prominent after challenge with moderate/severe asthmatic BAL, then the subsequent use of cell permeable inhibitors against Akt phosphorylation could illuminate a potentially important role for Akt activation in the increased synthesis of collagen III mRNA from severe asthmatic fibroblasts, and thus the pathogenesis of asthma. Also if the decreased proliferative potential observed from severe asthmatic fibroblasts previously in this study is due to their differentiation into myofibroblasts, and if Akt phosphorylation is responsible for this differentiation and subsequent maintenance of the myofibroblast phenotype, then its inhibition may also increase the ability of the severe asthmatic cultures to proliferate.

When the effects of various inhibitors on collagen III mRNA expression were analysed in figs 6.29 to 6.31 it was observed that after challenge with moderate/severe asthmatic BAL pertussis toxin, wortmannin, Etanercept, SB203580 and U0126 were able to significantly inhibit fibroblast collagen III mRNA expression. The pan-specific TGF-β antibody and genistein did not cause a significant decrease in collagen mRNA expression, although as numbers were low the implications of these experiments are limited. This investigation was a preliminary one as only 3 cell lines (1 healthy, 1 mild asthmatic and 1 severe asthmatic) were used in total at a 1 hour time point, however the data does indicate that the regulation of collagen III mRNA expression is orchestrated through a variety of mediators.

Although the data from these assays was not able to be used to draw comparisons between healthy and asthmatic BAL mediated collagen III mRNA expression, it still did provide evidence of roles for certain pathways, factors and intra-cellular kinases. The significant inhibition observed using wortmannin indicates the importance of PI3-K in collagen III mRNA expression. The role of PI3-K in collagen production from fibroblasts has been suggested by other researchers (Ivarsson M. *et al.* 1998, Lim I. J. *et al.* 2003 and Jinnin M. *et al.* 2004). The inhibition of PI3-K activity will likely prevent the phosphorylation of its downstream targets, which include Akt, and this may be the method by which collagen III mRNA expression is truncated in this study. Alternatively it has been shown that the stability of collagen mRNA is dependent on PI3-K activation (Ricupero D.A. *et al.* 2001a and Asano Y. *et al.* 2004), and this may account for the decreased levels of collagen III mRNA observed in the above work. Regardless of the manner by which PI3-K mediates collagen III mRNA production, further investigation of its role is required.

The inhibitory actions of U0126 and SB203580 in fig 6.31 are also supported by findings from previous researchers, with ERK1/2, a downstream target of MEK1/2, being shown to regulate collagen III mRNA synthesis (Reunanen N. *et al.* 2000, Atamas S.P. *et al.* 2003, Lim I.J. *et al.* 2003, Papakrivopoulou J. *et al.* 2004, Nagai Y. *et al.* 2005, Tourkina E. *et al.* 2005, Hu Y.B. *et al.* 2006, Liu X. *et al.* 2006, Mimura Y. *et al.* 2006 and Tang M. *et al.* 2007). It has also been shown that p38 may regulate collagen production from

fibroblasts (Reunanen N. *et al.* 2000, Sato M. *et al.* 2002, Daian T. *et al.* 2003, Ihn H. *et al.* 2005 and Meyer-Ter-Vehn T. *et al.* 2006).

Factors acting through ERK1/2 to regulate collagen production include pulmonary and activation regulated chemokine (PARC) (Atamas S.P. *et al.* 2003), aldosterone (Nagai Y. *et al.* 2005), TGF-β1 (Hu Y.B. *et al.* 2006) and TGF-β1 plus cAMP (Liu X. *et al.* 2006), while p38 mediates the actions IGF-1 plus TGF-β1 (Daian T. *et al.* 2003) and TGF-β1 alone (Sato M. *et al.* 2002, Ihn H. *et al.* 2005 and Hu Y.B. *et al.* 2006), it was also shown by Papakrivopoulou J. *et al.* 2004 that mechanical stress was able to increase collagen expression through p38 activation.

These findings support work in my study, which suggest a role for p38 and ERK1/2 in collagen III mRNA expression from primary airway fibroblasts after challenge with moderate/severe asthmatic BAL. As with ERK1/2 it is likely that TGF-β1 is the most prominent factor involved in p38 activation, however as shown above other factors may play a role. Although a comprehensive analysis of intra-cellular MAPK has not been performed, the data suggest that collagen III mRNA expression is controlled by multiple pathways, arising from various stimuli. This was not unexpected as BAL is a soup of factors, however it does pose problems when trying to assess any differences between asthmatic and healthy subjects, and it may well be that this is too narrow an approach to solve such a wide and extensive puzzle. With the increasing efficiency of DNA micro-arrays, where a whole genome can be assessed and the expression of thousands of genes quantified in stimulated and unstimulated cells, there is a more suited tool for future work in this area.

From previous research it was known that both TGF-β1 and TNF-α could lead to an increase in collagen production from fibroblasts (Elias J.A. *et al.* 1990, Fine A. *et al.* 1990 and Redlich C.A. *et al.* 1995); however it was believed from previous work that TGF-β1 would be a more potent stimulus than TNF-α. Although the data in this work suggest that the effects of the combination of TGF-β1 and TNF-α on collagen III mRNA expression could account for a large proportion of the stimulatory effect of the various BAL, it is unlikely that these two factors together are solely responsible for this. It is more likely that these two factors play a significant role in a network of contributory signals, as there are at least 1592 proteins which have been identified already in asthmatic

BAL (Wu J. *et al.* 2005), and the subsequent work evaluating the influence of pathway inhibitors has indicated that several different signalling pathways have relevance.

The data in fig 6.31 suggest a role for G-protein coupled receptors in collagen III mRNA expression from primary lung fibroblasts, a finding which supports the idea that TGF-β1 and TNF-α are not solely responsible for collagen III mRNA expression from fibroblasts in this study, as it is believed that these two mediators act through independent pathways. This is supported in work by McAnulty R.J. *et al.* 1995 who showed that TGF-β1 mediated pro-collagen (hydroxyproline) synthesis in human foetal fibroblasts was not inhibited by pertussis toxin, in fact the study shows that the addition of pertussis toxin to fibroblasts treated with TGF-β1 lead to an increase in pro-collagen production.

It had been shown in earlier figures that in this study 10ng/ml TNF-α was able to stimulate a significant increase in collagen III mRNA expression from primary cultures of fibroblasts, when this is compared to unstimulated samples (figs 6.2 and 6.4). The increase in collagen III mRNA expression seen in my study correlate well with findings in previous studies which found a 2-4 fold increase in collagen production after TNF-α challenge (Elias J.A. *et al.* 1990 and Weiner F.R. *et al.* 1990) This finding taken in conjunction with the inhibition in collagen III mRNA expression witnessed when challenging fibroblasts with moderate/severe asthmatic BAL treated with Etanercept (fig 6.31), suggest an important role for TNF-α in BAL mediated collagen III mRNA expression. The concentration of TNF-α used in our work is not too disparate from the levels likely to be found in the lung. In work by Howarth P.H. *et al.* 2005 TNF-α levels were found to be higher in the BAL of severe asthmatics (100-2000fg/ml) than in healthy donors (60-400fg/ml). It is believed that the surface lining fluid volume is around 0.1ml, and during BAL collection 120ml of saline is added to the lungs, giving a 1200 fold dilution. This suggests that the surface concentration of TNF-α is around 120-2400pg/ml (0.12-2.4ng/ml) in severe asthmatic lungs, and 72-480pg/ml (0.072-0.48ng/ml) in healthy volunteers. Although the levels used in our study are still 4 times higher than the largest amount of TNF-α measured in the lungs, there is no knowledge of how much TNF-α is lost or degraded during BAL recovery and also during the assay to quantify TNF-α levels in the BAL.

The actions of TNF-α in our model may be via a direct interaction with cell surface receptors; however it may also be via interactions with other factors, or through interactions with other signalling pathways. It has been shown that TNF-α is able to increase in collagen production when added in combination with IL-1α, a process thought to be controlled by PGE2 levels (Mauviel A. *et al.* 1991). It has also been shown that TNF-α is able to interact with the TGF-β pathway (Yamane K. *et al.* 2003), and this interaction may play a role in collagen synthesis, however whether it is stimulatory or inhibitory is unknown. There is likely to be many other factors in BAL which TNF-α may interact with, which may explain the level of inhibition seen when Etanercept was used, this is worth mentioning as when TNF-α was used alone, a 2-8 fold increase in collagen III mRNA expression was seen, with this in mind Etanercept caused a disproportionate decrease in collagen III mRNA expression when fibroblasts were challenged with moderate/severe asthmatic BAL. Dissecting out the factors in concert, or the pathways influenced by TNF-α may spread light on a useful area of BAL controlled fibroblast behaviour.

That an increased ability to synthesise CTGF mRNA did not correlate with an increase ability to synthesise collagen III mRNA (figs 6.5 and 6.11) suggests that although previous research has suggested that TGF-β induced production of CTGF is able to increase collagen synthesis (Duncan M.R. *et al.* 1999 and Blalock T.D. *et al.* 2003), that it is unlikely that TGF-β/CTGF is responsible for the abnormal increase in collagen III mRNA synthesis observed from severe asthmatic fibroblasts. It is also unlikely that the infiltration of inflammatory cells into the asthmatic airway after insult is due to abnormal synthesis of IL-8 from the residing fibroblasts, as the IL-8 mRNA expression observed from severe asthmatic fibroblasts is not consistently higher than from mild asthmatic or healthy fibroblasts. It was noticeable, however, that the baseline levels of IL-8 mRNA expression from severe asthmatic fibroblasts was lower than that from healthy or mild asthmatic fibroblasts, and this translated into a lower ability to synthesise IL-8 protein (figs 6.20, 6.24, 6.25 and 6.26). From these figures it was also noticed that mild asthmatic fibroblasts were able to synthesise more IL-8 protein than healthy or severe asthmatic fibroblasts. This may suggest that mild asthmatic fibroblasts are more focused on synthesizing factors such as IL-8 rather than ECM proteins, indicated by their decreased

ability to synthesise collagen III mRNA compared to healthy and severe asthmatic fibroblasts (fig 6.5).

As mentioned, future work comparing healthy and asthmatic fibroblasts and BAL would be better served by taking a more broad approach. This is because, as suggested by this work, BAL, and fibroblasts response to the factors present in this BAL is a complex one, and is unlikely to be determined by the use of blocking antibodies and inhibitors against individual factors and pathways. The use of whole genome micro-arrays would allow the assessment of a wide range of genes, whose activation could be back tracked to indicate which factors and pathways are involved in their activation. This is a more logical route by which to proceed with future analyses.

Nevertheless, this work has indicated that it is likely to be both the fibroblasts themselves, along with the environment of the asthmatic lung which is responsible for the increased deposition of collagen within the sub-epithelial basement membrane. This collagen production is mediated by a variety of factors; however there may be a prominent role for PI3-K/Akt activation, although this will require clarification.

Chapter 7:

General Discussion

Introduction

The findings from this study have suggested that there are fundamental differences between asthmatic and healthy fibroblasts, which manifest themselves as an altered response to challenge, and which may be accompanied by differential basal activity. These responses have been quantified as a decreased ability to incorporate [^{3}H] thymidine after challenge, which is an indicator of cellular mitogenic potential, and also an increased ability to produce collagen III mRNA, a protein associated with airway remodelling in asthma (Chu H.W. *et al.* 1998). Although the decreased mitogenic potential was uniform and independent of challenge, the ability to produce collagen III mRNA was dependent on the cells being challenged with moderate/severe asthmatic BAL. This suggests that although fibroblasts from severe asthmatics have a decreased ability to proliferate, that in order to produce more collagen III mRNA the cells must be in an environment representative of more severe asthma.

The response of fibroblasts to different challenge was explored using inhibitors of different pathways and factors, and these studies proposed a prominent role for G-protein coupled receptors, bFGF, EGF, angiotensin II, TNF-α, PI3-K and MEK1/2 in fibroblast mitogenesis, and also G-protein coupled receptors, TNF-α, PI3-K, MEK1/2 and p38 in collagen III mRNA expression after challenge with moderate/severe asthmatic BAL. It is unsurprising that such homology exists between two distinct cellular reactions to stimuli as these pathways, factors and intra-cellular proteins are important regulatory signals for a variety of cellular behaviours. However, work in this study has suggested a possible role for Akt in regulating altered fibroblast behaviour in asthma.

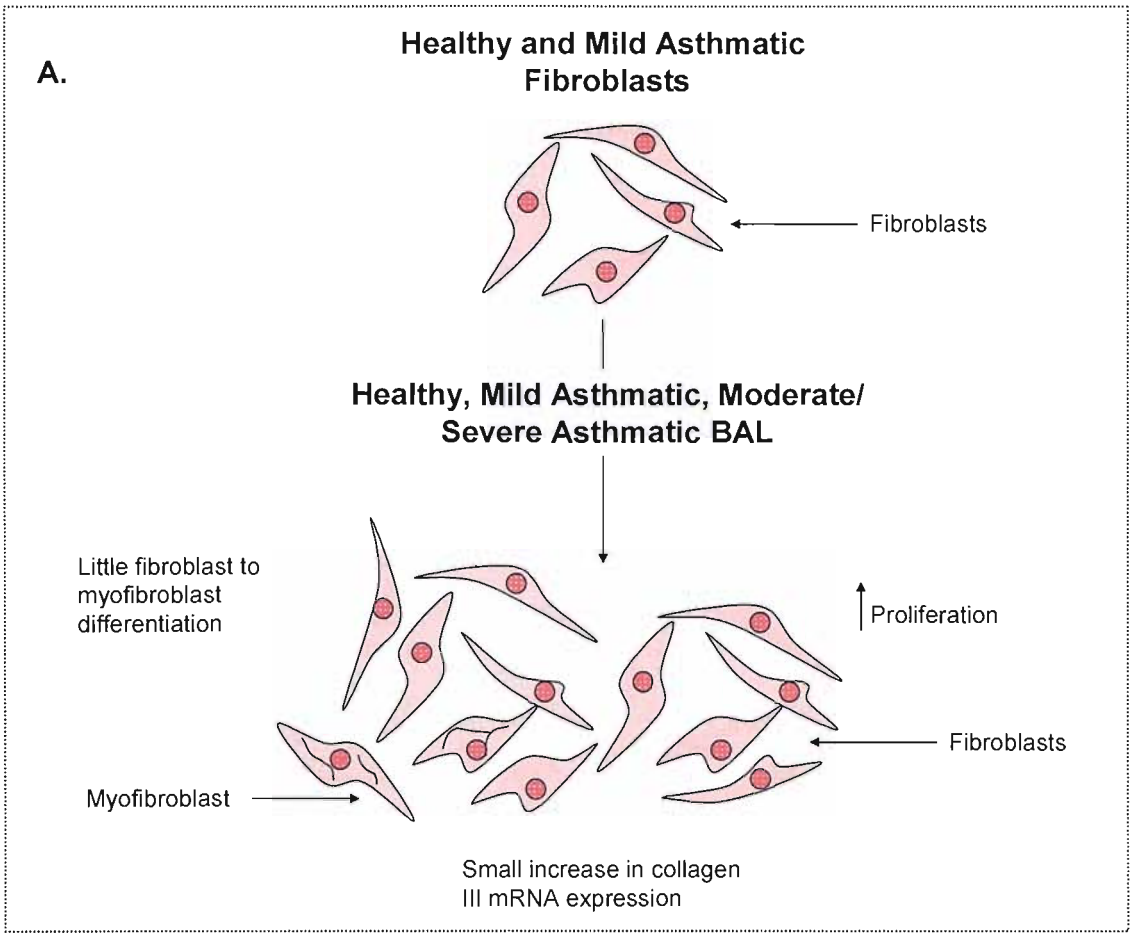
The discussion will examine further possible explanations for:

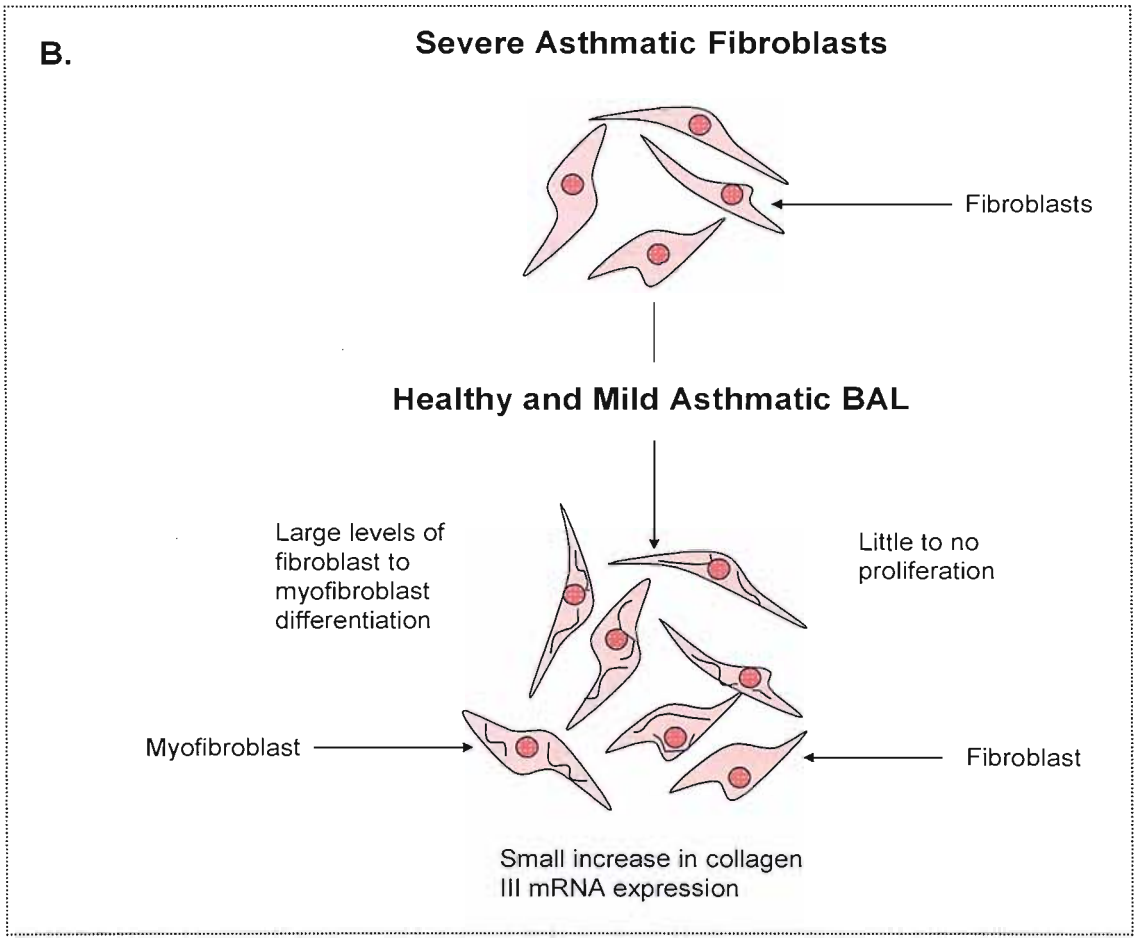
- Altered fibroblast behaviour in severe asthma
- Potential factors in BAL able to mediate asthma
- Akts involvement in asthma
- Factors utilising the PI3-K/Akt pathway
- Future Work

Fibroblast behaviour after BAL challenge

The ability of BAL to cause significant increases thymidine incorporation (figs 5.1 to 5.6) in healthy and mild asthmatic fibroblasts, but to a lesser extent in severe asthmatic fibroblasts, suggests these cells have an altered cellular response to stimuli, which may manifest itself as differentiation into a more synthetic phenotype. It was shown by Grotendorst G.R. *et al* 2004 that fibroblasts expressed higher levels of α-SMA when collagen levels were elevated, suggesting cells were in a myofibroblastic phenotype, and these increased levels of α-SMA were accompanied by a decrease ability to incorporate [^{3}H] thymidine. This finding may be how fibroblasts from severe asthma are responding to challenge with BAL, with cells undergoing myofibroblast differentiation more readily than healthier cells.

When similar work was performed in healthy primary human lung fibroblasts by Batra V. *et al.* 2004, it was seen that there was dissociation between myofibroblast transition and collagen III synthesis. It was found in this work that only after challenge with IL-4 was there a positive correlation between collagen III synthesis and myofibroblast transition; when cells were challenged with TGF-β1 and TGF-β2 there was fibroblast differentiation into myofibroblasts, but with little increase in collagen III synthesis. This work raised the possibility that myofibroblast transition may not necessarily be accompanied by increased collagen synthesis, indicating that collagen synthesis may be regulated by other growth factors and cytokines in BAL. This work also fits in with my findings as it was only after challenge with moderate/severe asthmatic BAL that a significant increase in collagen III mRNA expression was seen in severe asthmatic fibroblasts compared to healthier cells, however there was a decreased ability to incorporate [^{3}H] thymidine after challenge with all BAL.


A.
Healthy and Mild Asthmatic Fibroblasts
Fibroblasts
Healthy, Mild Asthmatic, Moderate/ Severe Asthmatic BAL
Little fibroblast to myofibroblast differentiation
Proliferation
Fibroblasts
Myofibroblast
Small increase in collagen III mRNA expression


B.
Severe Asthmatic Fibroblasts
Fibroblasts
Healthy and Mild Asthmatic BAL
Large levels of fibroblast to myofibroblast differentiation
Little to no proliferation
Myofibroblast
Fibroblast
Small increase in collagen III mRNA expression

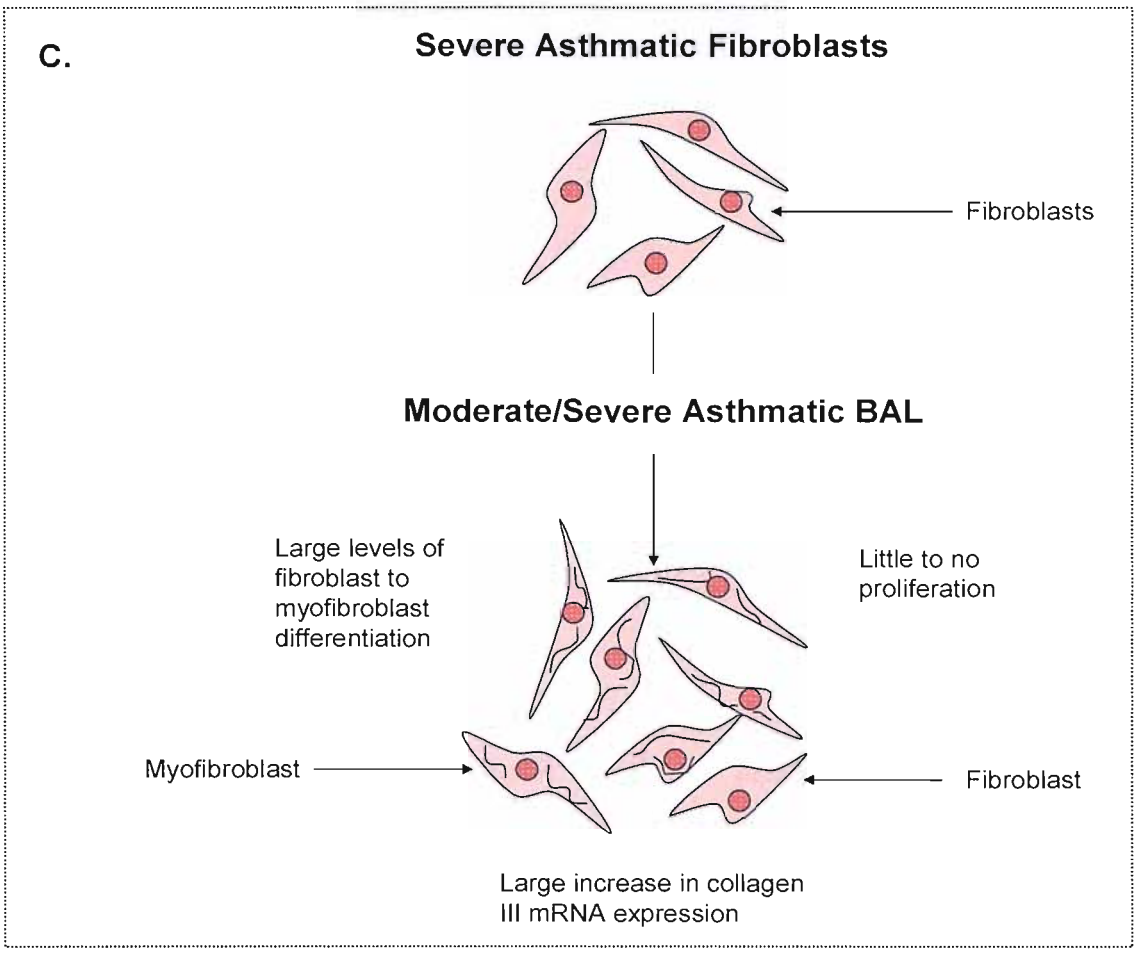


Fig 7.1 Proposed fibroblast behaviour after BAL challenge, A. represents healthy and mild asthmatic fibroblast responses to challenge with all BAL. B. shows the response of severe asthmatic fibroblasts to challenge with healthy and mild asthmatic BAL, while C. shows severe asthmatic fibroblast response to challenge with moderate/severe asthmatic BAL.

Differentiation into a myofibroblast phenotype was not assessed in my study after challenge with BAL; therefore it is difficult to determine whether the increased collagen synthesis, and decreased mitogenic potential observed in severe asthmatic fibroblasts, can be attributed solely to this phenotypic switch. However the decreased mitogenic potential seen from severe asthmatic volunteers suggests that these cells undergo phenotypic differentiation into myofibroblasts more rapidly, or that this signal is initiated in an alternate manner, than in healthier cells. The investigation of MAPK-phosphorylation after challenge with moderate/severe asthmatic BAL showed that there may be altered activation of Akt1, Akt2 and also possibly Akt3 in mild and severe asthmatic fibroblasts as indicated by visualisation of the pan Akt spot (fig 6.32), which may be a marker of fibroblast to myofibroblast differentiation. Although the alternate phosphorylation of Akt has proposed disparity in signal transduction in

asthmatic fibroblasts, it is likely not to be the only mis-regulated intra-cellular protein; however it does provide a starting point for further investigation. Therefore this observation, along with previous research, will be the basis for further discussion of signal transduction in this chapter.

Receptors

Broncho-alveolar lavage is a complicated soup of factors, so when cells are challenged with BAL it is likely that there is activation of multiple receptors, accompanied by activated intra-cellular pathways. Inhibitors were used to dissect out pathways involved in fibroblasts response to BAL, and this alluded to the potential involvement of G-protein coupled and tyrosine kinase receptors.

G-protein coupled receptors

G-protein-coupled receptors are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices, with with the ligands of G-protein coupled receptors typically bind. Examples of G-protein coupled receptors include the thrombin, angiotensin II, PAF, $\beta_{1/2}$ adrenergic, chemokine and $M_{1/2/3/4}$ muscarinic receptors (Bunemann M. *et al.* 1999).

Upon binding of agonist, G-protein-coupled receptors undergo a conformational change which then allows coupling to heterotrimeric guanine regulatory proteins (G-proteins). Heterotrimeric G proteins are made up of an α subunit, which contains the guanine nucleotide binding site, and the β and γ subunits which form a tightly bound dimer. The guanosine'5 diphosphate (GDP) bound form of the G protein is inactive, while the guanosine'5 triphosphate (GTP) bound form is active. The activated heterotrimeric complex dissociates into the α subunit (which consists of four subunit types) and the $\beta\gamma$ dimer which are free to interact with and regulate various cellular responses. Each of the four α subunits, α_s, α_q, α_i, α_o and $\alpha_{12/13}$ and the $\beta\gamma$ dimer interact with different areas of the cell, for example the α_q subunit promotes the activation of phosphatidylinositol specific phospholipases, which generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). These elevate intracellular Ca^{2+}concentrations and induce the activation of several protein kinases, including protein kinase-C/A (PKC/PKA) (Sodhi A. *et al.* 2004). The actions of G-protein coupled receptors depend on which α subunit is attached to the transmembrane receptor in the inactive

state, for example the α_s subunit regulates cellular adhesion and migration. Cellular proliferation is regulated through the α_q, α_i, $\beta\gamma$ dimer and ERK1/2 intra-cellular messengers.

Pertussis toxin is a specific inhibitor of G-protein coupled receptors, and prevents the $G_{i/o}$ protein heterotrimers from interacting with the transmembrane receptor, thus blocking their coupling and activation. The blocking of this activation prevents the recruitment and activation of second messengers such as PKC, PKA and PI3-K, thus inhibiting proliferation, apoptosis, cell survival, adhesion etc.

Stimulation of G-protein coupled receptors is likely to be a major route by which fibroblasts response to stimuli is mediated. It has been shown that inhibition of G-protein coupled receptors is able to significantly reduce collagen III mRNA synthesis (fig 6.31) and [^{3}H] thymidine incorporation (figs 5.6, 5.7 and 5.8), however it is unclear exactly which factors are responsible for these observations.

Factors which may act though G-protein coupled receptors to cause fibroblast proliferation include lysophosphatidic acid (LPA) (van Corven E.J. *et al.* 1993 and Chen J. *et al.* 2006), platelet activating factor (PAF) (Roth M. *et al.* 1996 and Bennett S.A. *et al.* 1997), trypsin (Akers I.A. *et al.* 2000 and Reed C.E. *et al.* 2004), tryptase (Akers I.A. *et al.* 2000 and Reed C.E. *et al.* 2004), adenosine (Volpini R. *et al.* 2003), thrombin (Dik W.A. *et al.* 2003 and Massi D. *et al.* 2005), ET-1 (Turner N.A. *et al.* 2004 and Solini A. *et al.* 2005) and bradykinin (Vancheri C. *et al.* 2005).

While factors able to stimulate collagen production through G-protein coupled receptors include LTC4 (Phan S.H. *et al.* 1988), LTD4 (Phan S.H. *et al.* 1988 and Asakura T. *et al.* 2004), ET1 (Guarda E. *et al.* 1993, Tian X. *et al.* 2002, Hafizi S. *et al.* 2004b, Horstmeyer A. *et al.* 2005 and Nishida T. *et al.* 2006), ET-3 (Guarda E. *et al.* 1993), thrombin (Sundqvist G. *et al.* 1995, Chambers R.C. *et al.* 1998 and Hewitson T.D. *et al.* 2005), MCP-1 (Gharaee-Kermani M. *et al.* 1996 and Hogaboam C.M. *et al.* 1999), angiotensin II (Kawaguchi H. *et al.* 1996, Hafizi S. *et al.* 1998, Lijnen P.J. *et al.* 2001, Min L.J. *et al.* 2004 and An S.J. *et al.* 2006), PARC (Atamas S.P. *et al.* 2003), bradykinin (Vancheri C. *et al.* 2005), factor Xa (Blanc-Brude O.P. *et al.* 2005), eotaxin (Puxeddu I. *et al.* 2006), LPA (Chen J. *et al.* 2006) and urotensin II (Dai H.Y. *et al.* 2007). There is also evidence that adenosine may act through G-protein coupled receptors to inhibit fibroblast collagen production (Dubey R.K. *et al.* 1998).

Previous work has proposed that ET-1 may act through its G-protein coupled receptor ETA to control fibroblast to myofibroblast differentiation (Shi-Wen X. *et al.* 2004), and also that MCP-1 activation of its receptor, CCR2, is required for TGF-β induced myofibroblast differentiation through TGF-βRII and Smad3 (Gharaee-Kermani M. *et al.* 2003). Mediators, such as LTD4 (Asakura T. *et al.* 2004), can act through G-protein coupled receptors to increase collagen production in cells which have already been transformed into myofibroblasts by TGF-β, whilst, eotaxin (Puxeddu I. *et al.* 2006) can increase collagen production and cellular proliferation in cells without causing myofibroblast differentiation.

The transformation of fibroblasts to myofibroblasts by ET-1 is dependent on PI3-K/Akt activation (Shi-Wen X. *et al.* 2004), which therefore proposes a route by which Akt may be contributing to increased fibroblast to myofibroblast differentiation which may occur in severe asthmatic cells. The subsequent exaggerated stimulation of collagen III mRNA expression may be due to the presence of eotaxin or LTD4 in moderate/severe asthmatic BAL, which activates signalling cascades with which Akt may interact, or which may stimulate other mis-regulated pathways in severe asthmatic cells. The abundance of factors that may influence fibroblast behaviour through G-protein coupled receptors indicates that they are likely to be vital in fibroblasts response to BAL challenge. An important role for angiotensin II was shown in fibroblast proliferation in figs 5.9, 5.10 and 5.11, and it is likely that angiotensin II is also important in collagen synthesis. However in severe asthmatic fibroblasts it may be that ET-1 and MCP-1 are regulating the switch to a myofibroblastic phenotype after BAL challenge, and therefore ultimately determining cellular behaviour. To determine their importance ET-1 and MCP-1 could be used alone or in combination to challenge severe asthmatic fibroblasts. After challenge the fibroblasts could be characterised to evaluate their phenotype and also the levels of phosphorylated Akt1, 2 and 3 measured. It may also be interesting to adding blocking antibodies for ET-1 and MCP-1 to BAL and then assess fibroblast mitogenesis and collagen III mRNA production, along with Akt phosphorylation, to determine their role in BAL challenge.

It is also probable that interactions between G-protein coupled receptors and tyrosine kinase receptors is important in regulating fibroblasts behaviour in asthma, although this has not been assessed in this study. Stimulation of the EGF receptor by G-protein coupled receptor activation of ADAMs is liable to be important in BAL mediated

mitogenesis, and future work may investigate this by using cocktails of G-protein coupled and EGF receptor inhibitors.

Tyrosine kinase receptors

Protein tyrosine kinases are critical components of signalling pathways that control cell proliferation and differentiation (Mohammadi M. *et al.* 1997). Tyrosine kinases are enzymes which facilitate the transfer a phosphate group to a tyrosine residue in a protein. Factors which act on tyrosine kinase receptors are generally growth hormones and factors that promote cell division such as insulin, IGF, bFGF, PDGF and EGF. The tyrosine kinase receptor comprises an extra-cellular domain which binds an agonist, an intra-cellular tyrosine kinase domain and a C terminal tyrosine residue. Activation of the kinase is achieved by a ligand binding to the extracellular domain, which induces dimerisation and activation of the receptors. The activated receptors are able to subsequently utlisie intra-cellular kinases such as growth-factor receptor bound protein 2 (GRB2) and PI3-K, leading to e.g. proliferation via the ERK-1/2 pathway or cell survival via the Akt pathway.

Genistein is a broad spectrum tyrosine kinase inhibitor (Akiyama T. *et al.* 1987). The use of genistein to investigate synergy between tyrosine kinase pathways and G-protein coupled receptors was performed by van Corven E.J. *et al.* 1993, and it was found that G-protein coupled receptors may regulate activation of Ras by LPA and thrombin, possibly through an intermediary tyrosine kinase, and that this pathway may participate in mitogenic signalling. This finding was supported by Ohba T. *et al.* 1994 who demonstrated that the mitogenic effect of thrombin is associated with an increase in the expression of the ligand PDGF-AA and up-regulation of PDGFα-receptor. These findings coupled with the work showing that ET-1 (Hua H. *et al.* 2003) and angiotensin II (Eguchi S. *et al.* 1998 and Ohtsu H. *et al.* 2006) are able to stimulate their G-protein coupled receptors to subsequently activate the EGF receptor through ADAMs, suggest there is much cross-talk between the two receptor groups, and that this is an area for future investigation in BAL challenged fibroblasts.

Factors known to elicit fibroblast proliferation through tyrosine kinase receptors include; EGF (Jetten A.M. *et al.* 1982 and Purdom S. *et al.* 2005), PDGF (Benito M. *et al.* 1993 and Zheng X.Y. *et al.* 1998), IGF (Pietrzkowski Z. *et al.* 1992 and Zadeh S.M. *et al.* 1997), VEGF (Gerber H.P. *et al.* 1998) and bFGF (Strutz F. *et al.* 2000 and Wang X.P. *et al.* 2004).

The role of tyrosine kinase receptors in collagen production has also been assessed with factors such as EGF (Colige A. *et al.* 1988), insulin (Goldstein R.H. *et al.* 1989), IGF-1 (Goldstein R.H. *et al.* 1989, Gillery P. *et al.* 1992, Bird J.L. *et al.* 1994, Bird J.L. *et al.* 1995, Simmons J.G. *et al.* 2002, Chetty A. *et al.* 2006, Harrison C.A. *et al.* 2006 and Izumi K *et al.* 2006), PDGF-BB (Ivarsson M. *et al.* 1998), bFGF (Harrison C.A. *et al.* 2006) and butyrate (Karna E. *et al.* 2006) being shown to increase collagen production in fibroblasts. However aFGF has been shown to decrease collagen production in fibroblasts (Becerril C. *et al.* 1999). There is also evidence suggesting NGF can act through its tyrosine kinase receptor to cause fibroblast differentiation into myofibroblasts in conjunction with TGF-β (Micera A. *et al.* 2005). Other findings of interest indicate that IGF-1 may play a role in perpetuating the transformation of fibroblasts into myofibroblasts when in conjunction with TGF-β (Simmons J.G. *et al.* 2002).

PI3-K

Phosphatidylinositol 3-kinase can be activated through both G-protein coupled receptors and tyrosine kinase receptors, as a downstream an intra-cellular messenger. PI3-K is a heterodimeric complex consisting of an 85kDa regulatory subunit, p85, and a 110-kDa catalytic subunit, p110 (Roche S. *et al.* 1994). Within the cell PI3-K can mediate both G-protein coupled receptor activity via the βγ subunit (Kim J. *et al* 2002) and tyrosine kinase activity via the GBR2/SHC complex (Rameh L.E. *et al.* 1999). Wortmannin is a potent and specific PI3-K inhibitor (Powis G. *et al.* 1994). As PI3-K is an intra-cellular messenger it is not directly in contact with pro-fibrotic factors, however its role in proliferation is well characterised after cellular stimulation with factors such as PDGF (Grammer T.C. *et al.* 1997), VEGF (Gerber H.P. *et al.* 1997), bFGF (Wang X.P. *et al.* 2004), insulin (Jiang Z.Y. *et al.* 2005) and EGF (Kim J. *et al.* 2002).

Phosphatidylinositol 3-kinase has also been shown to mediate collagen synthesis in fibroblasts by acting downstream after challenge with factors including PDGF-BB (Ivarsson M. *et al.* 1998), TGF-β1 (Ricupero D.A. *et al.* 2001a and Martínez-Salgado C. *et al.* 2006), insulin (Tokudome T. *et al.* 2004) and IL-13 (Jinnin M. *et al.* 2004). There has also been work indicating that collagen synthesis initiated by IGF-1 is mediated by PI3-K and ERK in hepatic stellate cells (Svegliati-Baroni G. *et al.* 1999).

The multi-functional role of PI3-K indicates its importance in cellular responses to stimuli, however little work has been published showing its involvement in asthma. The data in fig 6.32 showing the activation of Akt in asthmatic fibroblasts may elude to a possible role for PI3-K/Akt activation in asthma.

A role for Akt in altered fibroblast behaviour in asthma

Challenge with moderate/severe asthmatic BAL lead to severe asthmatic fibroblasts producing significantly more collagen III mRNA than healthier cells. This observation proposes the presence of factors in moderate/severe asthmatic BAL which play a role in propagating the signal to increase collagen III mRNA synthesis in severe asthmatic fibroblasts, that are not present in BAL from healthier volunteers, and that the transcriptional regulation of collagen III mRNA is regulated by differing pathways in severe asthmatic cells compared to healthier cells.

One route by which fibroblasts may differentiate into myofibroblasts is through PI3-K phosphorylation of Akt (Shi-Wen X. *et al.* 2004); with recent work by Winbanks C.E. *et al.* 2007 showing that Akt was able to regulate this process through its ability to phosphorylate mTOR. It has also been proposed that the maintenance of the myofibroblast phenotype is dependent on continual Akt phosphorylation (Ricupero D.A. *et al.* 2001b). This transformation is likely to involve TGF-β, however the abnormal response observed in severe asthmatic cells indicates the involvement of additional routes. Candidates for this include ET-1, MCP-1, IGF-1 and NGF, with both ET-1 (Gawlik R. *et al.* 2006) and MCP-1 (Jahnz-Rózyk K. *et al.* 2000) being shown to be present in increased levels in BAL from asthmatic volunteers compared to BAL from healthy candidates. As myofibroblasts are believed to possess a decreased ability to proliferate and an increased ability to synthesise collagen, it may be that the presence of phosphorylated Akt seen in severe asthmatic fibroblasts in fig 6.32 correlates to a switch to a myofibroblastic phenotype. Akt phosphorylation was only assessed after challenge with moderate/severe asthmatic BAL; therefore it is unknown whether Akt plays a role in severe asthmatic fibroblasts response to challenge with healthy or mild asthmatic BAL. However as it is likely that fibroblasts from severe asthmatic are transforming into myofibroblasts after BAL challenge, it is plausible that Akt may play a role in this independent of challenge, through factors ubiquitous in BAL from all groups.

In fig 6.32 it is noticeable that Akt phosphorylation was seen in mild asthmatic fibroblasts, albeit to a lesser degree than in severe asthmatic fibroblasts, however the responses of the fibroblasts were markedly different. This suggests that other pathways may be involved in both fibroblast differentiation and collagen synthesis. For example the differentiation signal may be initiated by TGF-β through Smad3 (Gu L. *et al.* 2007) or JNK (Hashimoto S. *et al.* 2001b), however in severe asthma the presence of more phosphorylated Akt, bought about through stimulation by alternate factors, may play a role in perpetuating the signal to a greater degree than in mild asthmatic cells. Akt may also be able to influence collagen production through a variety of routes; including phosphorylation of downstream MAPK to directly lead to collagen production, interaction with other pathways, such as the MEK/ERK pathway through intermediary MAPK such as Pak1 (Fan S. *et al.* 2001 and Yuan Z.Q. *et al.* 2005), to exaggerate the collagen synthesis signal, or through stabilisation of collagen III mRNA, which would ultimately lead to increased levels of collagen III protein. Any of these routes could lead to the increased levels of collagen synthesis seen when severe asthmatic fibroblasts respond to factors present in moderate/severe asthmatic BAL, however interaction of phosphorylated Akt with other pathways may best explain my observations.

Although the data in fig 6.32 requires further clarification it may be that Akt phosphorylation in severe asthmatic fibroblasts occurs after challenge with BAL, through factors ubiquitous in BAL from all groups, and this contributes to abnormal differentiation into myofibroblasts. When fibroblasts from severe asthmatics are challenged with BAL from moderate/severe asthmatic volunteers though the presence of factors unique to this BAL lead to the activation of alternate pathways. These pathways contribute to enhanced collagen synthesis, and may or may not affect the level of fibroblast differentiation. These alternate pathways may be able to interact with Akt exaggerating the level of collagen production. Nevertheless it is also possible that the presence of phosphorylated Akt in severe asthmatic fibroblasts is not related to challenge with moderate/severe asthmatic BAL, and is related to neither the increased collagen III mRNA synthesis nor myofibroblast transition.

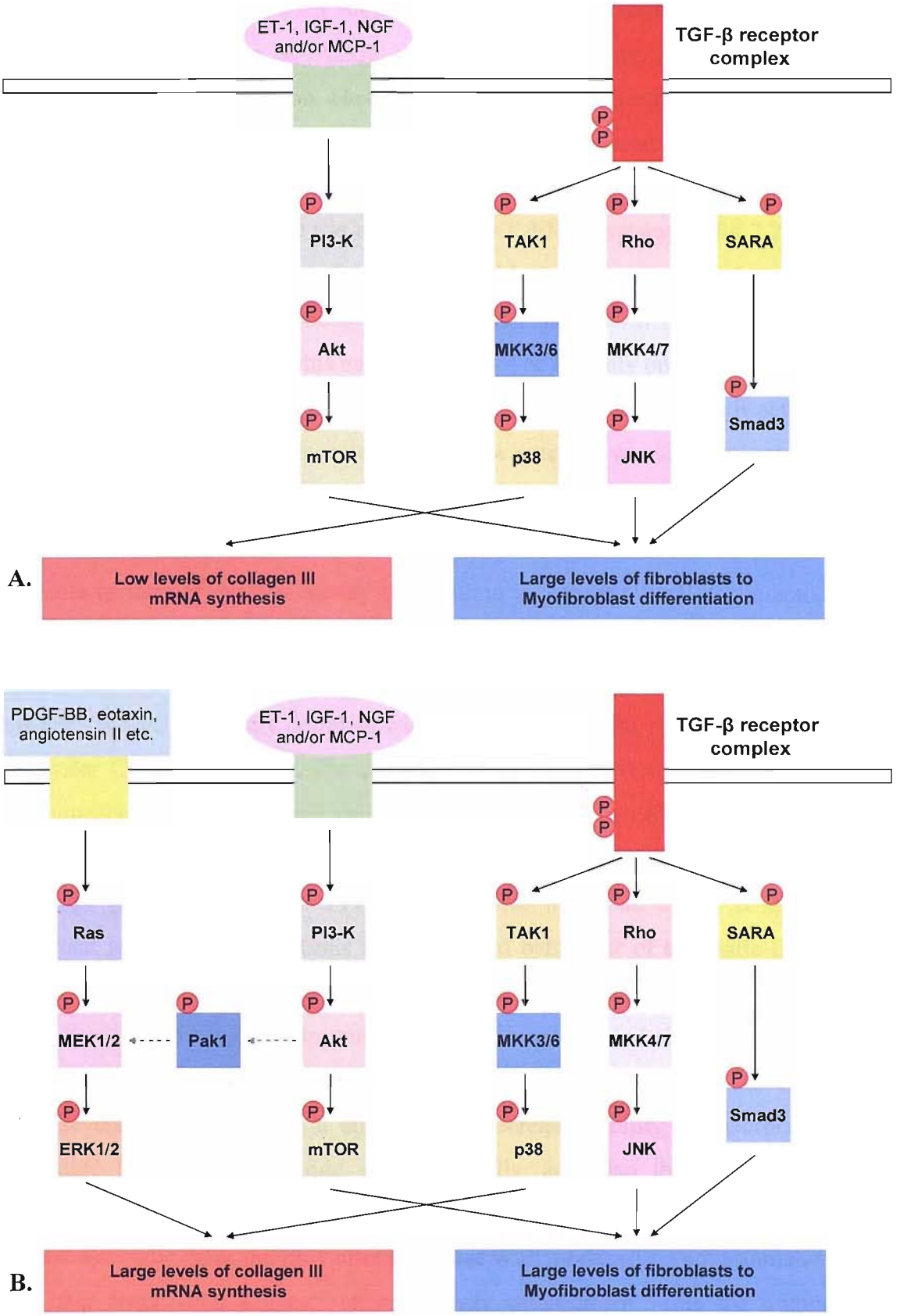


Fig 7.2 Proposed signalling events occurring within severe asthmatic fibroblasts after challenge with healthy and mild asthmatic BAL (A) or moderate/severe asthmatic BAL (B). Black lines represent likely cellular events as reported by other researchers, grey dotted lines represent proposed signalling events. The role of Pak1 in activating MEK has been proposed by Charvet C. *et al.* 2002, with Fan S. *et al.* 2001 and Yuan Z.Q. *et al.* 2005 showing Akt can phosphorylate Pak1.

In the above figure it has been suggested that PDGF-BB, angiotensin II and eotaxin may be responsible for activation of an altered pathway, ultimately leading to increased collagen production. However as mentioned earlier there are many factors which may act through G-protein coupled and tyrosine kinase receptors to modulate this effect including thrombin, PARC, bradykinin, ET-3, factor Xa, urotensin II, bFGF, LTD4 and LTC4. The use of the Ras/MEK/ERK pathway in fig 7.2 as a route to increase collagen synthesis after challenge with moderate/severe asthmatic BAL is a speculation, based on previous research which has shown its ability to increase collagen synthesis; however this pathway may not be the only one responsible. Any of the factors listed above may play a role in collagen synthesis, through activation of different intra-cellular pathways; nevertheless the outcome would be the comparable.

Summary

The role of the fibroblast in airway remodelling in asthma is well documented, with hyperplasia of differentiated fibroblasts and increased ECM deposition two distinct hallmarks of airway remodelling. The number of myofibroblasts present in the airway has been shown to directly correlate with the thickness of the reticular collagen layer (Brewster C.E. *et al.* 1990). The thickness of the basement membrane has clinical consequences for the asthmatic patient such as decrease in FEV_1 (Boulet L.P. *et al.* 1997) and increased airway hyperresponsiveness as measured by methacholine challenge (Boulet L.P. *et al.* 1997 and Milanese M. *et al.* 2001). It is therefore important to understand the basis for the altered behaviour of asthmatic fibroblasts, and determine whether it is due to the cells themselves or their environment.

From work in this study it appears that both fibroblasts and their environment are altered in asthma, it was seen in work assessing fibroblast mitogenesis that fibroblasts from severe asthma possess a significantly decreased ability to incorporate [^{3}H] thymidine that healthy or mild asthmatic cells, and also that severe asthmatic fibroblasts were able to produce significantly more collagen III mRNA than healthy or mild asthmatic cells, but only after challenge with moderate/severe asthmatic BAL. The later observation suggests altered airway environment while the mitogenesis work suggests altered fibroblast behaviour, most likely manifesting as differentiation into myofibroblasts. Although the *in vitro* findings from my work suggest fibroblasts from severe asthma possess a decreased ability to proliferate than healthier cells, we

are unable to postulate on whether this relates to fibroblast numbers *in vivo*, as no measurements of airway fibroblasts have be undertaken in this study. Nevertheless the are numerous other ways in which fibroblast/myofibroblast numbers may be increased within the asthmatic airway, these include their maintenance in a myofibroblastic phenotype by intra-cellular proteins such as Akt (Ricupero D.A. *et al.* 2001b) which would prevent myofibroblast numbers decreasing after wound repair is complete. In addition to this fibrocytes may be able to infiltrate into the lung and subsequently differentiate into myofibroblasts (Schmidt M. *et al.* 2003), or there may be differentiation of epithelial cells into myofibroblasts through epithelial–mesenchymal transition (EMT). Epithelial–mesenchymal transition is a form of metaplasia where fully differentiated epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells, often fibroblasts and myofibroblasts, under the influence of factors such as EGF, bFGF, HGF and TGF-β1 (Savagner P. *et al.* 2001, Kalluri R. *et al.* 2003 and Zavadil J. *et al.* 2005). One mode by which EMT may be initiated is via damage to epithelial cells (Iwano M. *et al.* 2002 and Saika S. *et al.* 2004), a process which is known to occur in asthma after environmental insults such as allergen exposure (Holgate S.T. *et al* 2003). In the study by Schmidt M. *et al.* 2003 it was shown following allergen exposure, circulating fibrocytes were recruited to the lung, and that under the influence of TGF-β or ET-1 fibrocytes down-regulated their CD34 expression, up-regulated α-SMA, and differentiated into myofibroblasts. Therefore increased myofibroblast numbers in asthma may occur though the gradual proliferation of myofibroblasts, with their phenotype maintained through continual Akt activation, and through the infiltration and differentiation of fibrocytes and epithelial cells, acting to amplifying their numbers.

The increased deposition of collagen in asthmatic airways is likely to be due to the release of factors not present in the lung of healthier patients. This proposal is supported by Hastie A.T. *et al.* 2002, who showed that mediators produced from asthmatic epithelial cells, but not healthy cells were able to stimulate collagen III production from lung myofibroblasts. Broncho-alveolar lavage from all groups was able to stimulate collagen III mRNA expression in my study, therefore there are likely to be other mediators present in BAL able to stimulate collagen synthesis, released from cells, such as infiltrating inflammatory cells, endothelial cells or from fibroblasts themselves. In a study on idiopathic pulmonary fibrosis it was shown that PDGF and IGF-1 proteins were localized exclusively in alveolar macrophages, mononuclear

phagocytes, fibroblasts, alveolar Type II cells, vascular endothelial cells, and vascular smooth-muscle cells (Homma S. *et al.* 1995). The increased ability of moderate/severe asthmatic BAL to increase collagen III mRNA expression from healthy and severe asthmatic fibroblasts compared to challenge with healthy BAL, and also from severe asthmatic fibroblasts compared to healthy fibroblasts indicates that the environment of the lung in more severe asthma is capable of stimulating increased levels of collagen synthesis, but also that fibroblasts from severe asthma are predisposed to synthesise greater levels of collagen than healthier cells.

This study has proposed that fibroblasts from severe asthma possess a decreased mitogenic potential after BAL challenge, possibly due to a switch to a myofibroblastic phenotype, with an increased ability to synthesise collagen III mRNA when in a severe asthmatic environment. The regulation of these two processes may be controlled through the altered activation of Akt, caused by fibroblasts from severe asthma responding to factors present in BAL differently than healthier cells.

Future Work

Initially it would be interesting to determine whether BAL challenge leads to fibroblast to myofibroblast differentiation, and this could be achieved through characterisation of fibroblasts by staining for vimentin and α-SMA after challenge. This could confirm whether the decreased mitogenic potential of severe asthmatic fibroblasts is due to this phenotypic switch. Another area for investigation is Akts role in asthma, the use of the MAPK-phosphorylation kit to assess the activation of MAPKs in healthy, mild asthmatic and severe asthmatic fibroblasts at baseline and also after challenge with healthy, mild asthmatic and moderate/severe asthmatic BAL would allow a greater understanding of intra-cellular events occurring in these cells. This would also allow the conformation of my initial observation, and may also show whether Akt is involved in severe asthmatic fibroblasts response to all BAL challenge.

The complex nature of BAL means that investigation of individual factors or pathways role in fibroblast behaviour is difficult, therefore to understand fibroblast response to BAL it may be beneficial to examine cellular response on a whole genome level, using DNA micro-arrays. Although this would give a vast amount of information, it would also allow the in depth comparison of fibroblast responses to

different BAL fluids, and also the comparison of different fibroblast responses to the same BAL, providing insight into pathways through which these responses may be mediated.

To further understand the responses of different fibroblasts it is also necessary to increase the numbers of fibroblasts and BAL used from each group, this may help increase the levels of significance seen in the mRNA expression work, as some of these observations can be variable. Increasing these numbers would also provide more data from areas of research such as direct cell counting, to provide data to compare with observations from the mitogenesis work.

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