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The effects of transforming growth factor beta on asthmatic and non-asthmatic fibroblasts: Their role in the epithelial-mesenchymal trophic unit

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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RESPIRATORY CELL AND MOLECULAR BIOLOGY

Doctor of Philosophy

The effects of transforming growth factor beta on asthmatic and non-asthmatic fibroblasts: Their role in the epithelial-mesenchymal trophic unit

by Nveed Chaudhary

The aim of this study was to investigate the hypothesis that asthmatic fibroblasts exhibit hyperproliferation due to the production of autocrine growth factors. Although the cytoskeletal and cytocontractile immunoreactivity observed in non-asthmatic and asthmatic fibroblasts is identical, asthmatic fibroblasts exhibit an ability to proliferate in serum free medium, whereas non-asthmatic fibroblasts remain quiescent. Endothelin (ET)-1, connective tissue growth factor (CTGF) and transforming growth factor (TGF)β1, were investigated as candidate autocrine growth factors. ET-1 and CTGF expression was significantly higher in asthmatic fibroblasts, but were observed to have little or no role in proliferation but roles in differentiation to myofibroblasts. TGFβ1 gene expression was equal in non-asthmatic and asthmatic fibroblasts however protein levels in conditioned medium of asthmatic fibroblasts was significantly lower suggesting autocrine use. Specific neutralisation of TGFβ1 resulted in an inhibition of the hyperproliferation. Cultured medium from asthmatic fibroblasts resulted in increased mitogenesis of NR6/HER fibroblasts, which was significantly blocked with AG1478 and anti-EGFR antibodies, suggesting EGFR ligands may also contribute to the hyperproliferation. Addition of AG1478 and anti-EGFR antibodies also inhibited hyperproliferation of asthmatic fibroblasts. HB-EGF gene expression was seen to be higher in asthmatic fibroblasts, which was significantly reduced by specific neutralization of autocrine TGFβ1. These observations suggested that autocrine TGFβ1 could result in activation of EGFR through expression of HB-EGF, which may be responsible for driving the hyperproliferation. These observations may lead to novel therapeutic targets, which may dampen down the remodeling response in asthma.

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APPENDIX E - Investigating Gene expression in primary asthmatic and non-asthmatic fibroblasts

Publications arisen as a result of these studies

1. *N Chaudhary, A Richter, JE Collins, WR Roche, DE Davies and ST Holgate.* Phenotypic comparison of asthmatic and nonasthmatic fibroblasts. Am J Resp Crit Care Med. Vol. 163, No. 5 A473 (2001)

2. *N Chaudhary, A Richter, W R Roche, R M Powell, L M Hamilton*, S T Holgate and D E Davies.* The role of autocrine growth factor in the proliferation of asthmatic (myo)fibroblasts. Am J Respir Crit Care Med. Vol. 165, No.8 A78 (2002)

3. *N I Chaudhary, A Richter, R M Powell, W R Roche, A.Young*, L.Hamilton*, S T Holgate, D E Davies* . Connective Tissue Growth Factor is Up regulated and Controls Myofibroblast Differentiation In Asthmatic Fibroblasts. *In press* (Am J Respir Crit Care Med. Abstract)

4. *N I Chaudhary, A Richter, A.Young*, L.Hamilton*, ST Holgate, DE Davies.* Hyperproliferation of Asthmatic Fibroblasts is Mediated by an HB-EGF/ EGF Receptor Autocrine Loop. *In press* (Am J Respir Crit Care Med. Abstract)

5. *N I Chaudhary, A Richter, R M Powell, W R Roche, A.Young*, L.Hamilton*, S T Holgate, D E Davies* . Connective Tissue Growth Factor is Up regulated and Controls Myofibroblast Differentiation In Asthmatic Fibroblasts. **SUBMITTED** (FASEB J)

* *AstraZeneca Charnwood, Discovery Bioscience, Loughborough.*

List of Abbreviations

α-SMA	α smooth muscle actin
16HBE	16 human bronchial epithelial cell line
^{3}H Thymidine	Tritiated thymidine
A	Adenine
A_{630}	Absorbance at 630nm
ADAM (12,33)	A disintegrin and a metalloproteinase
aFGF	Acidic fibroblast growth factor
AP-1	Activating protein -1
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic Acid
bFGF	Basic fibroblast growth factor
BHR	Bronchial Hyperresponsiveness
BLAST	Basic local alignment search tool
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
C	Cytosine
CCN	**C**TGF/fisp12, **c**ef/10/cyr61 and **n**ov
cDNA	Copy deoxyribonucleic acid
COX	Cyclooxygenase
CPM	Counts per minute
CTGF	Connective tissue growth factor
DAB	Diaminobenzidine
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleoside triphosphate

DPM	Disintegrations per minute
DPX	P-xylene-bis-pyridium bromide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMTU	Epithelial-mesenchymal trophic unit
ErK	Extracellular signal regulated kinase
ET-1	Endothelin-1
ETRA / ETRB	Endothelin receptor A / receptor B
FBS	Foetal bovine serum
FEV_1	Forced expiratory volume in one second
FITC	Fluorescein
FuDR	5'fluoro-2'deoxyuridine
G	Guanine
GINA	Global initiative for asthma
GM-CSF	Granulocyte macrophage stimulating factor
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HB-EGF	Heparin binding epidermal growth factor
HBSS	Hank's balanced salt solution
HDM	House dust mite
HRCT	High resolution computer tomography
HRP	Horse radish peroxidase
ICAM-1	Intracellular adhesion molecule - 1
IFNγ	Interferon γ

Ig	Immunoglobulin
IGF	Insulin–like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IPF	Interstitial pulmonary fibrosis
JNK	c-Jun-NH_2-terminal kinase
LPA	Lysophosphatidic acid
LT	Leukotrienes
LTBP	Latent TGFβ binding protein
MAPK	Mitogen activated protein kinase
MBP	Major granular basic protein
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
NFA	Near fatal asthma
NGF	Nerve growth factor
NHLIB	National centre for biotechnology information
NRK	Normal rat kidney
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC_{20}	Provocative concentration of agonist causing 20% reduction in FEV_1
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PI	Propidium Iodide
PKC	Protein kinase C

PMA	phorbol 12-myristate 13-acetate
QIP	Quench index parameter
RANTES	Regulated upon activation normal T-cell expressed and secreted
RBM	Reticular basement membrane
RER	Rough endoplasmic reticulum
RT	Reverse transcriptase
SAPK	Stress-activated protein kinase
SARA	Smad anchor for receptor activation
SCF	Stem cell factor
SDS	Sodium dodecylsulphate
SMAD	Sma- and Mad- related proteins
SRS-A	Slow releasing substance of anaphylaxis
TβRI / TβRII	TGFβ receptor I / TGFβ receptor II
Tak-1	TGFβ activated kinase
TBST	Tris-buffered saline with Tween™
TCA	Tricholoroacetic acid
TE	Tris-EDTA
TEMED	N,N,N,N',N'-tetramethylenediamine
TGFβ	Transforming growth factor β
TIE	TGFβ inhibitory element
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor
tSIS	Transformed spectral index of the sample
TSP-1	Thrombospondin 1
V cells	Vimentin positive fibroblasts
VAD cells	Fibroblasts co-expressing Vimentin, Desmin and α-SMA
VCAM-1	Vascular cell adhesion molecules-1

VA	Fibroblasts co-expressing Vimentin and α-SMA
VD cells	Fibroblasts co-expressing Vimentin and Desmin
VEGF	Vascular endothelial growth factor
VLA	Very late antigen

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There are many times during a PhD when things just don't work, or the result is the opposite of the one that was expected. Inevitably, it is these times when you come to appreciate the help and support of the people around you.

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CHAPTER ONE
Introduction

1.1 Asthma- an overview

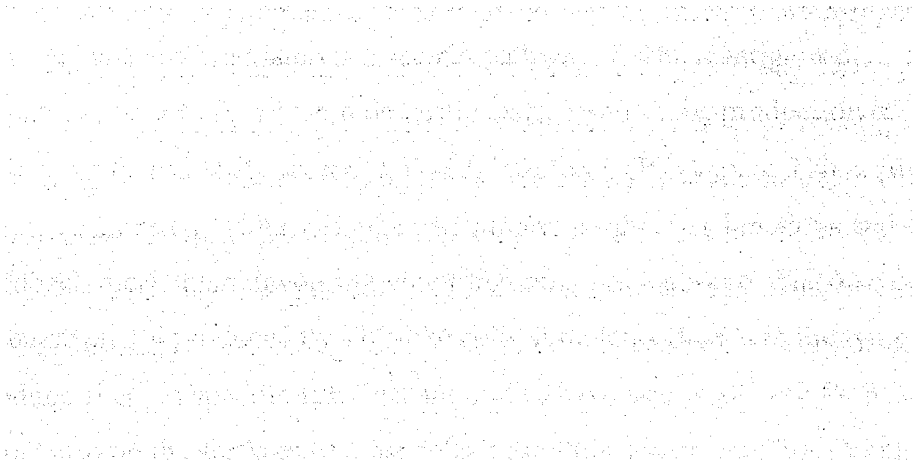
Asthma is an inflammatory condition of the conducting airways, in which bronchoconstriction is the major physiological abnormality. The official definition of asthma, as defined by the Global Strategy For Asthma Management and Prevention Report, clearly describes the symptoms experienced by asthmatic patients: "...in susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and/or early morning." (Busse *et al.*,2002).

Although our understanding of the molecular mechanisms underlying the disease has been, and continues to be, enhanced since the mid 1960s, the number of children admitted to hospital for asthma in western countries has increased markedly.

The central dogma surrounding the molecular basis of asthma has been that it is a chronic inflammatory disease usually in response to sensitising allergen. The peak prevalence occurs early in life and is frequently associated with the presence of atopy (seasonal combination of asthma, eczema and hayfever). Atopic individuals are those who produce specific immunoglobulin E (IgE) antibody in response to allergen exposure. The view placing inflammation as the sole component of bronchial asthma is now altering with the realisation that remodelling of the airways is also an important component of the disease. Remodelling refers to structural changes including extensive damage to the surface epithelium, prominent homogeneous thickening of the reticular basement membrane and hyperplasia of airway smooth muscle, as well as infiltration by inflammatory cells into the respiratory mucosa.

Essentially, the aim of therapy is to help patients lead as normal a life as possible. Since acute attacks occur most commonly at night, this involves allowing patients to gain a full night's sleep and for others, the participation in sports and exercise. Further aims include the prevention and a reduction in the number of severe attacks. In most cases, treatment with inhaled β_2 adrenoceptor agonists and low dosed inhaled corticosteroids can help alleviate symptoms and prevent acute attacks, but probably does little to the underlying remodelling response.

Bronchial asthma has been defined as 'the development of reversible, obstructive airway disease', however it has become apparent that an irreversible component plays an important role in the chronic state. In a study to determine the long-term effects of asthma, Lange et al. (1998) conducted a 15-year follow-up study of ventilatory function in 17,506 Danish adult subjects of which 1095 had asthma. It was concluded that people who identified themselves as having asthma had substantially greater declines in forced expiratory volume in one second (FEV_1) over time than those who did not. These findings were in line with current opinions, which indicate that asthma is a chronic inflammatory disease in which ongoing tissue injury and repair may result in irreversible fibrotic changes in the airways (Holgate 1999). Damage to the epithelium (the effective barrier between the airway and the environment) is suggested to initiate these permanent structural changes. The repeated damage to the epithelium and the repeated repair (inflammation-repair cycle) is thought to augment the remodelling process (reviewed in Holgate 1998). Damage can be due to inflammatory mediators acting on the epithelium but there is an increasing wealth of evidence suggesting that environmental pollutants, viruses and genetic susceptibility are also important factors. Thus the paradigm underlying asthma seems to be altering. No longer is inflammation solely thought to be responsible for the disease. The remodelling leads to irreversible changes, rendering asthma as a chronic problem, which worsens over time.



1.2 Airway inflammation in asthma

1.2.1 The IgE response and development of the inflammatory cascade

Atopy is an important risk factor for asthma. Atopic individuals are those who produce IgE specifically against commonly encountered allergens e.g. pollen, dust mites, etc. Usually, this is referred to as allergy.

In allergic asthma, professional antigen-presenting cells are present in large numbers in the epithelium and submucosa of the asthmatic airways (figure 1.1). Derived from CD34 precursors, these cells play a key part in the uptake of allergens and subsequent T-cell presentation (Holgate 1997). The dendritic cells differentiate in the presence of stem cell factor (SCF) and granulocyte macrophage colony stimulating factor (GM-CSF) and express CD1a MHC class II and progressively lose their ability to secrete interleukin (IL)-12. Under the influence of IL-4 and tumour necrosis factor (TNF)α, the high affinity FCERI and FCERII receptors for IgE are expressed on the surface of the dendritic cells, which increases their antigen capturing and processing capacity (Tunon-De-Lara *et al.,* 1996).

After capturing antigen, dendritic cells migrate to local lymph nodes and present selected peptides in the groove of their MHC class II molecules to the T-cell receptor (Holgate 1997). In genetically susceptible individuals, antigen presentation to naïve Th0 CD4 T cells leads to their commitment to Th2-like T cells. These cells secrete cytokines encoded on the IL-4 gene cluster located on the long arm of chromosome 5 (Wilkinson *et al.,* 1996). These cytokines include IL-4, IL-5, IL-6, GM-CSF and IL-13 (Wilkinson *et al.,* 1996). T cells may also differentiate via a second pathway in which antigens derived from micro-organisms along with IL-12 from dendritic cells, result in the production of T cells, with a Th1-like profile, primarily secreting IL-12, interferon (IFN)-γ and TNFα (Robinson *et al.,* 1992). IL-10 secreted by the Th2-like cell suppresses the Th1 functions and results in a positive feedback mechanism favouring the preferential production of Th2-like cells.

Interleukin (IL)-4 produced by Th2-like cells stimulates B cells in the lymphoid tissues to produce allergen specific IgE. IgE antibodies have strong affinity for FCεRI receptors found also on the surfaces of mast cells, basophils and eosinophils (Yamaguchi *et al.,* 1999). Cross-linkage of cell membrane bound IgE, via allergen molecules, initiates a number of intracellular signalling events, mediated by tyrosine kinase and protein kinase C (Leitges *et al.,* 2002). As a result, granules containing pre-formed mediators and precursor

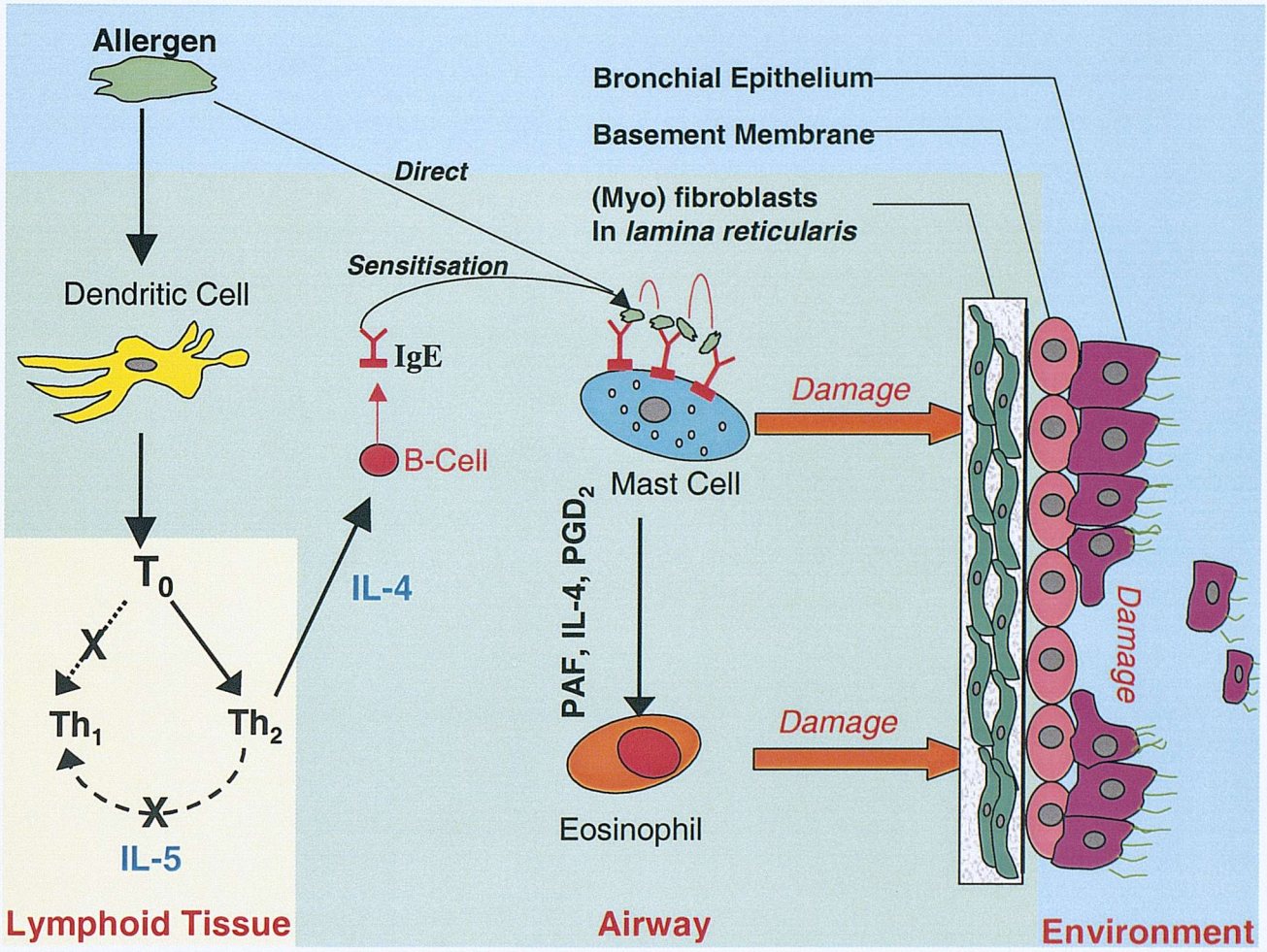


Figure 1.1 The Inflammatory cascade in bronchial asthma. Asthmatic subjects inhale allergen to which they are sensitised. This is picked up by specialised immune cells (dendritic cells) in the airway which present the allergen to naïve T-cells. Antigen presented in this way results in the preferential production of Th_2 helper cells, which secrete an array of chemical mediators favouring production of more Th_2 cells and fewer Th_1 cells. One of these mediators (IL-4) stimulates B cells to secrete IgE type antibodies against the allergen. IgE binding to mast cells, results in their release of mediators that cause acute narrowing of the airways, as well as other molecules that attract inflammatory cells that release cationic proteins, which damage the epithelium. Mast cell activation can also occur via allergen binding directly to IgE receptors, resulting in a more acute response (direct).

molecules for the generation of prostaglandins (PGs) or leukotrienes (LTs) are released by exocytosis.

The activation of mast cells produces bronchoconstriction, due to the actions of histamine, PGD_2 and the slow reacting substances of anaphylaxis (SRS-A) which have now been identified as an array of leukotrienes, namely LTC_4,LTD_4 and LTE_4 (reviewed in O'Byrne 1997). These mediators also increase microvascular permeability, enhance airway smooth muscle responsiveness and more importantly initiate neutrophil and eosinophil recruitment, which are responsible for the later phase of an asthmatic response.

An asthmatic attack is biphasic. The initial response is acute bronchoconstriction, caused by mast cell degranulation as outlined above. The late-phase reaction is normally separated from the acute phase by several hours. Both phases are thought to be the result of direct inflammatory attack to the airway wall. The later phase is attributed to eosinophils, which are regulated by IL-5, IL-13 and GM-CSF secreted by the activated mast cells (Galli and Costa 1995).

1.2.2 Recruitment of inflammatory cells into the asthmatic airway

In the late phase of asthma, inflammatory cells are actively recruited into the airways in response to chemoattractant stimuli released mainly by mast cells in the early phase. Inflammatory cells are released by the bone marrow into the circulation, and are able to enter the inflamed airways via adhesive interactions with microvascular endothelial cells (figure 1.2) (Bousquet *et al.,* 2000).

Histamine and leukotrienes secreted by mast cells promote the transport of an adhesion molecule, P selectin, from structures known as Weibel Palade bodies to the endothelial surface (reviewed in Springer 1990). P selectins are then able to interact with Sialyl-Lewis and other lectins on the surface of the endothelial wall.

The mast cell is also a source of TNFα, which, within 2-3 hours of its release, results in endothelial expression of a number of adhesion molecules, namely E-selectin, intercellular adhesion molecule I (ICAM-I) and vascular cell adhesion molecule I (VCAM-I) (reviewed in Holgate 1997). Heterodimeric integrins, found on rolling leukocytes, interact with VCAM-I and ICAM-I to produce firm adhesion and eventual transendothelial migration. Very late antigen 4 (VLA-4) is up-regulated on eosinophils and interacts with VCAM-I.

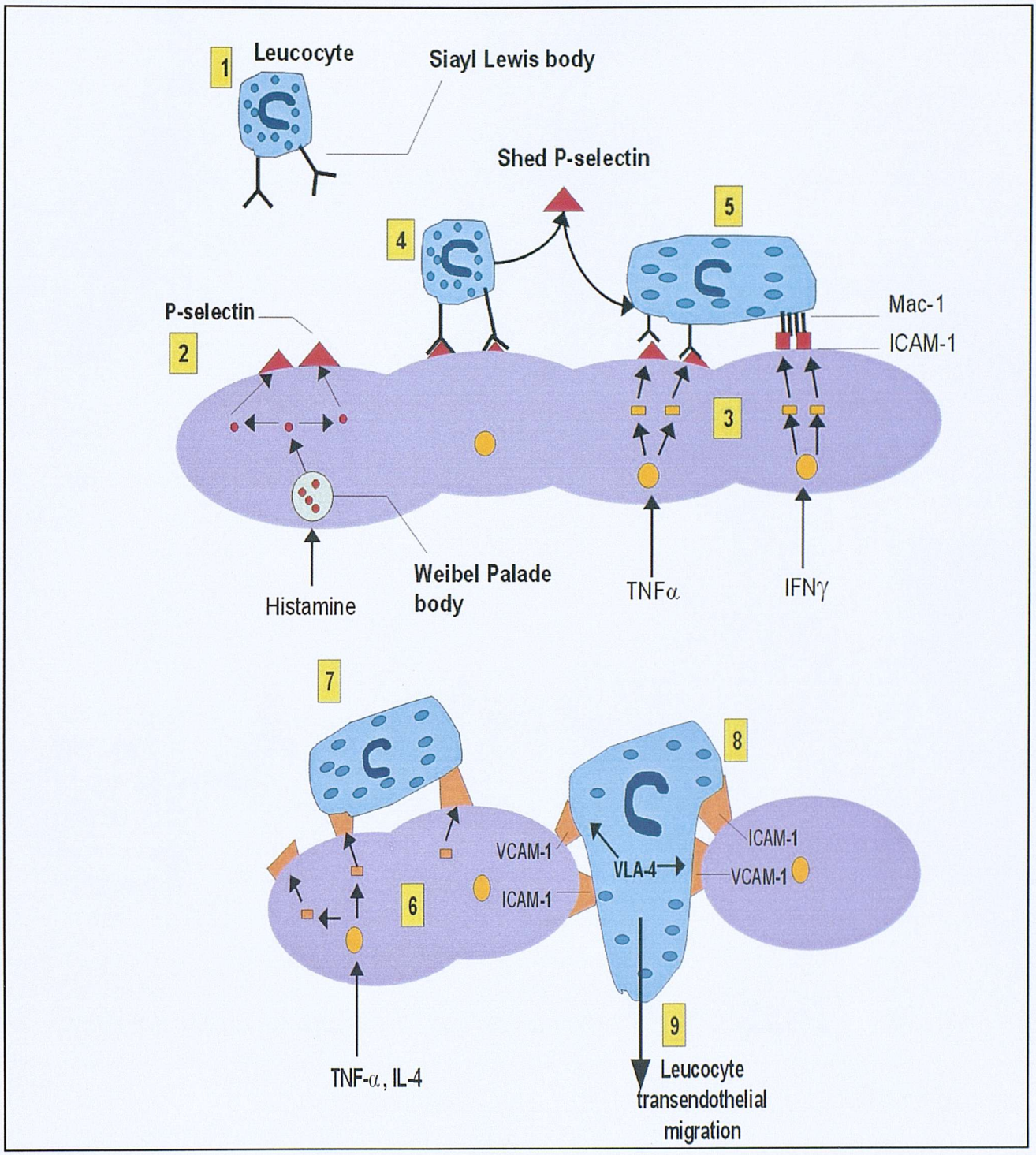


Figure 1.2 Transendothelial migration of inflammatory cells. Leucocytes (1) have Siayl-Lewis bodies, which bind onto P-selectin (2) molecules on the endothelial cells. P-selectins, and ICAM-1 are expressed in response to Histamine, TNFα and IFNγ stimulation (3). The leucocytes bind to P-selectin (4). Interaction of Mac-1 molecules with ICAM-1 molecules on the endothelial surface results in the characteristic rolling of the leucocytes (5). TNFα stimulation results in further expression of ICAM-1 and VCAM-1 molecules (6) to which the leucocytes bind firmly (7). Transendothelial migration then occurs via the interaction of VCAM-1 on the endothelial cells and VLA-4 in the leucocytes (8).

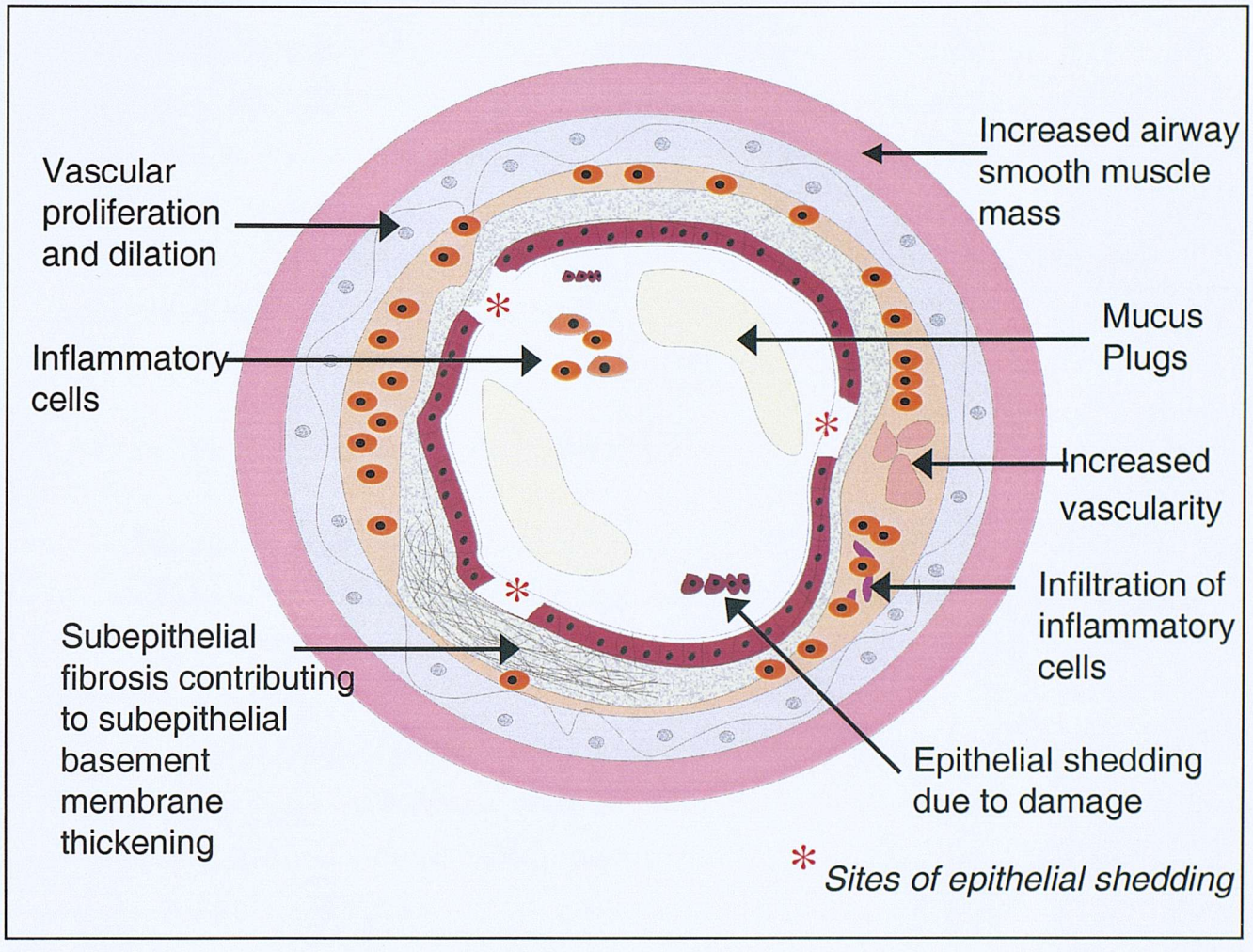


Figure 1.3 The characteristics of a remodelled airway

VLA-4 is not present on neutrophils, hence accounting for the increased recruitment of eosinophils into the asthmatic airway (eosinophilia) (reviewed in Holgate 1997).

Cytokine action on inflammatory cells is also important in the priming and activation prior to recruitment. Such cytokines include IL-5 and GM-CSF which greatly enhance eosinophil recruitment and terminal maturation (Lopez, AF *et al.* 1988). Chemokines, such as RANTES (**R**egulated upon **A**ctivation, **N**ormal **T**-cell **E**xpressed and **S**ecreted) and eotaxin, have also been shown to act on T-cells and eosinophils to enhance recruitment and possibly activation. IL-16 and RANTES are lymphocyte chemoattractant factors and are found in the broncho-alveolar lavage fluid of antigen-challenged asthmatics, and are thought to participate in the recruitment process (Cruikshank *et al.,* 1995).

1.2.3 Inflammatory damage to the epithelium

Structural damage to the bronchial epithelium is characteristic of asthma and has been proposed to be driven by inflammatory cells and their mediators causing the shedding of columnar epithelium, probably via the loosening of adhesive contacts with the basal cell surface (Holgate *et al.,* 2000).

The eosinophil is the principal inflammatory cell, which has the potential to elicit damage to the bronchial epithelium in asthma. Eosinophils secrete three main groups of proteins which are toxic to the epithelium: major granular basic protein, eosinophil cationic protein and eosinophil derived neurotoxin (Frigas and Gleich 1986).

The major granular basic protein (MBP) is highly cationic, and studies have shown that it is present in the airways of patients that have died of asthma, even when no eosinophils can be detected (Frigas and Gleich 1986). Eosinophil cationic protein is also found in the bronchial mucosa of asthmatic airways. Both of these proteins are able to elicit profound damage to the epithelium, resulting in cell death. It has been postulated that these eosinophil-derived proteins are responsible for the observed epithelial cell exfoliation in the asthmatic airway, and that these proteins may substantially alter the function of epithelial cells and perpetuate the asthmatic state (Woltmann *et al.,*1999).

Mast cells also release factors that are able to cause direct damage to the epithelium. Mast cells, found even in the mildest forms of asthma, appear to be more degranulated and active than in non-asthmatics. Their main secreted mediators, histamine tryptase and leukotrienes, are believed to alter airway epithelial function (Pesci *et al.,* 1993).

1.3 Airway Remodelling in Bronchial Asthma

The vast majority of asthmatic patients exhibit complete reversibility of long-standing abnormal spirometric measurements following treatment with β_2 agonists. However, some patients with asthma are seen to have residual airway obstruction. This irreversible component has been thought to be due to airway remodelling (Lange *et al.*, 1998).

Airway remodelling refers to the development of specific structural changes in the airway wall in asthma (figure 1.3). In recent times evidence has accumulated suggesting that structural tissue cells such as epithelial cells, fibroblasts and smooth muscle cells also play important effector roles through the release of a variety of mediators, cytokines and growth factors (reviewed in Chung and Barnes 1999).

1.3.1 Pathological changes in asthmatic airway remodelling - Damage to the bronchial epithelium

Epithelial damage and phenotypic changes are characteristic features of asthma, exhibiting sloughing of columnar cells and goblet cell hyperplasia with increased mucus production and airway plugging. The nature of this epithelial damage caused by inflammatory (see section 1.2.3) or direct environmental damage predominantly due to pollutants (e.g. diesel particles) or viruses, is a distinctive feature of asthma (Monefort *et al.*, 1993).

Studies by Hackel and co-workers (1999) have shown that the epidermal growth factor receptor (EGFR) serves a central role as a primary regulator of epithelial function and may have an influence in epithelium integrity. A study by Puddicombe demonstrated autocrine activation of the EGFR in epithelial cell repair mechanisms (Puddicombe *et al.* 2000). A damaged 16HBE epithelial cell monolayer repaired itself in the absence of growth factors. This repair was accelerated by addition of exogenous EGF, but could be appreciably impeded by the use of a selective EGF receptor tyrosine kinase inhibitor e.g. tyrophostin AG1478 in the absence of exogenous ligand. In the same studies, bronchial biopsy specimens from asthmatic airways showed substantial EGFR expression in areas of local epithelial damage, including the apical surface, which normally does not have EGF

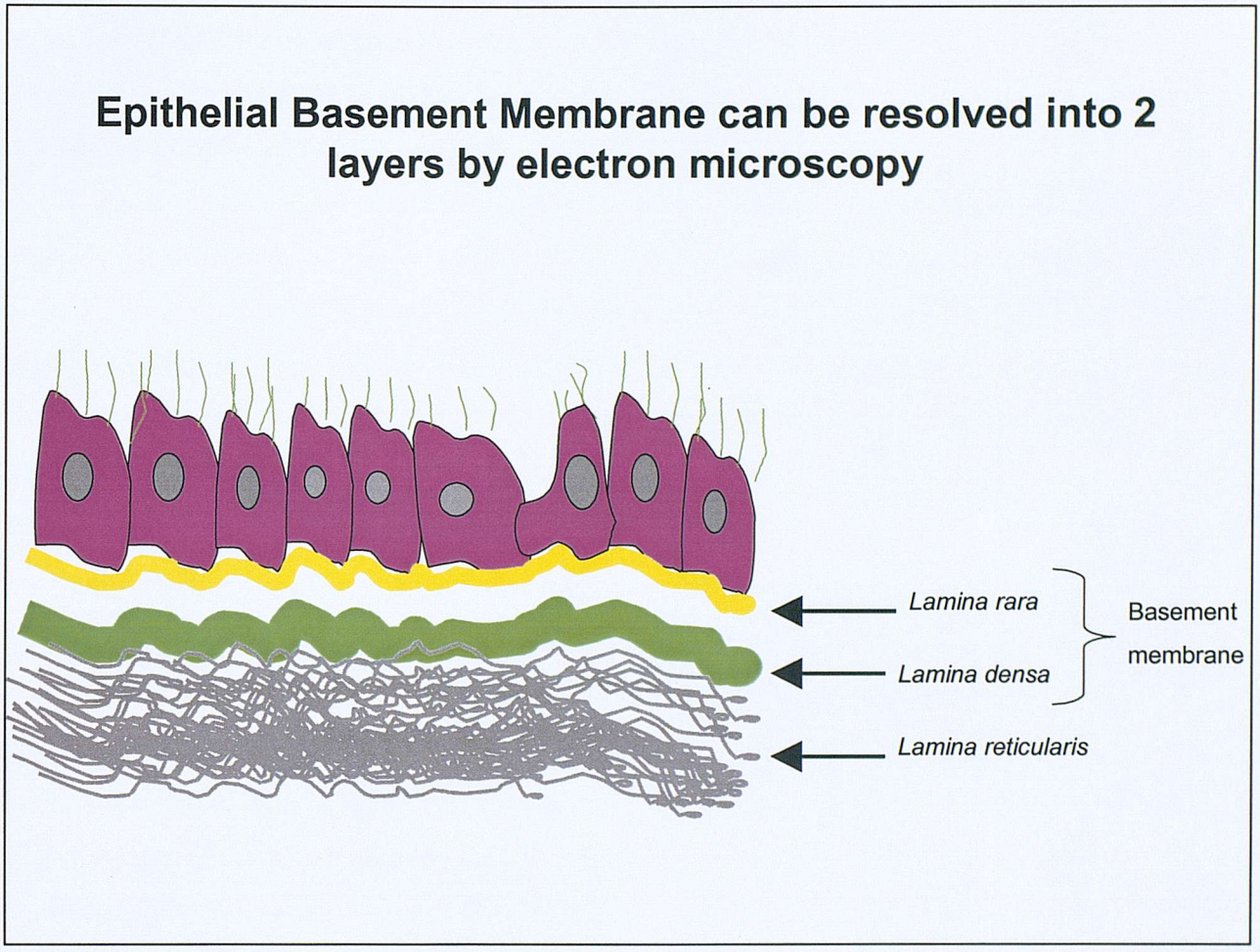


Figure 1.4 Sub-Epithelial basement membrane thickening. The observed fibrosis observed in chronic asthma is not found in the basement membrane as originally believed. The true basement membrane can be resolved into two layers, the *lamina rara* and the *lamina densa*. The fibrosis in fact is confined to the *lamina reticularis*, an area below the basement membrane.

receptors. The up-regulation of EGFR in response to injury is thought to allow increased exposure to ligand and hence hasten epithelial repair (Puddicombe *et al.* 2000).

It has also been observed that in response to mechanical damage and oxidant stress, the number of EGFRs on airway epithelial cells, is markedly increased. The immunoreactivity of EGFR in the nasal epithelium has been seen to be increased in response to *in vivo* exposure to ozone (Harkema *et al.* 1997).

Whereas in the normal epithelium EGFR is up-regulated in response to stress, in asthma a disease-related overexpression of EGFR both in damaged and intact epithelium has been observed. EGFR immunoreactivity is observed throughout the whole asthmatic bronchial epithelium. There are two possible explanations for this overexpression, either the damage to the epithelium is widespread in asthma or there is a failure to down regulate EGFR expression after restitution, with the consequence that the epithelium remains in the 'repair' phenotype.

In other diseases, where EGFR expression is upregulated i.e. psoriasis and carcinomas, the up regulation is accompanied by hyperplasia driven by an EGFR autocrine loop, however in asthma, there is no evidence to suggest that the epithelium is hyperproliferative. Demoly and co-workers (1994) have shown that in asthmatic biopsy specimens where extensive epithelial shedding was observed, the levels of proliferating cell nuclear antigen (PCNA) were low in the basal epithelial cells. On exposure to allergen, however, epithelial proliferation was observed. This suggests that although the EGFR is upregulated, and held in a repair phenotype, there is some suppression of epithelial cell proliferation. Transforming growth factor β counteracts the proliferative response mediated by EGF (Moses *et al.* 1991a). EGF and TGFβ signalling pathways, with regards to epithelial proliferation, both converge on the Sma- and Mad- related (SMAD) proteins (Moses *et al.* 1991b).

Redington and co-workers (1997) have shown that TGFβ1 levels are elevated in the bronchoalveolar lavage fluid of asthmatics. This ligand imbalance may account for the lack of epithelial cell proliferative response to damage in asthma.

1.3.2 Airway wall thickening

Asthma airways display many structural alterations, all of which have the potential to contribute to an overall increase in airway wall thickness. There have been reports

indicating that asthma fatalities have airways with significantly thickened walls (James *et al.* 1989). A recent investigation by Awadh and co-workers (1998) used high resolution computerised tomographic scanning (HRCT) to measure airway wall thickness at the segmental and sub segmental levels in 40 non-smoking asthmatic patients and 14 non-smoking control subjects. The subjects were categorised into patients who had experienced a near fatal attack of asthma (NFA), those with moderate asthma and those with mild asthma. The results of the HRCT scans showed that all patient groups had greater airway wall thickness than normal subjects. In addition, airway wall thickness positively correlated with disease severity. The sites of thickening also varied with asthma severity. Patients with mild asthma presented with thickening in both large cartilaginous and small membranous bronchi whereas the thickening in patients with NFA occurred predominantly in the smaller airways. Okazawa and co-workers (1996) conducted similar studies on airway wall thickening using HRCT scanning. They showed that the smaller airways of asthma patients were significantly thickened compared with normal controls. In contrast, Boulet and co-workers (1995) found no difference in the T/D (thickness / diameter) ratio of the *bronchus intermedius* between asthmatic and normal controls, however, no smaller airways were studied by this group.

Wiggs and co-workers (1992) have shown with computer modelling that the thickening of the airway walls is sufficient to explain the exaggerated challenge response and loss of the plateau in the challenge dose-response curve in patients with asthma.

There is evidence to suggest that remodelling can occur independently from inflammation. Warner and co-workers (2000) showed that in children as young as 18 months, histological remodelling changes are visible, with no correlation between subepithelial fibrotic thickening and the duration of symptoms. Recently, Payne and co-workers (2003) have published similar studies reporting that thickening of the reticular basement membrane (RBM) was present in children with difficult asthma to a similar extent to that seen in adults with asthma. These observations lead to the suggestion that the immunopathological features of airway inflammation and remodelling in asthma are fully developed at an extremely early stage in the evolution of the disease.

1.3.3 The thickened airway wall and increased smooth muscle mass

Smooth muscle is present in both the central and peripheral airways and studies have shown that airway smooth muscle forms part of a greater proportion of the airway wall in smaller peripheral airways rather than the larger central airways. The first indication of an increased smooth muscle mass in asthma was published in 1922 (Huber 1922). This study investigated the cross sections of small and large airways of 6 asthma fatalities and 7 non-respiratory related fatalities. Haematoxylin and eosin staining of these samples indicated that the thickness of the smooth muscle layer in the asthmatic samples was markedly thicker in the smaller airways than in the larger airways i.e. those with an external diameter greater than 2mm. It has subsequently been reported that two patterns of smooth muscle thickening are evident in the airways of patients with severe recurrent asthma. The first one is type I, in which smooth muscle hypertrophy is present only in the large airways. Type II indicates smooth muscle hypertrophy in both large and small airways (Ebina *et al.*, 1993).

Ebina and co-workers (1993) have used extensive computer imaging techniques to classify 5 non-asthmatic and 10 asthmatic patients in terms of their type of airway smooth muscle thickening. Tracings of individual smooth muscle cells from up to 200 serial airway sections were integrated to create a three-dimensional model of the airway. They reported that the smooth muscle density increased towards the periphery of the bronchial tree, whereas the mean volume of a single smooth muscle cell decreased. They determined that there was a reduced number of smooth muscle cells in the smaller airway of type II asthmatics, but a greater single cell mean volume, indicating smooth muscle cell hypertrophy. The number of smooth muscle cells per unit length was greater in both types compared to normal control patients, suggesting smooth muscle hyperplasia.

The increased smooth muscle layer is thought to contribute to airway narrowing and bronchial hyperresponsiveness. It is thought that the thickened smooth muscle results in exaggerated shortening response to spasmogens and bronchoconstrictor stimuli.

Many studies have indicated that stimulation of smooth muscle growth occurs via mitogenic agents such as polypeptide growth factors, inflammatory mediators and cytokines (Walker *et al.*, 1998; Hirst 2000). However, relatively little is known about the cellular and molecular mechanisms that contribute to the increase in muscle content of the airway wall in asthma.

1.3.4 The thickened airway wall and extracellular matrix remodelling

The thickened airway wall seen in asthma has also been attributed to an increased deposition of extracellular matrix (ECM proteins) especially in the sub-epithelial basement membrane (Molina *et al.* 1977; Cutz *et al.* 1978).

1.3.4.1 The Extracellular Matrix

The ECM has, in the past, been considered to be an inert filler substance between the functioning cells of an organ (Roche 2000). Now, however, it is regarded by a number of researchers as a variable and dynamic element of the tissues that contributes to both structure and function (Streuli 1999). The ECM is made up of molecules from a wide variety of biological proteins, of which the most prominent ones are the collagens. Different collagens have characteristic functions and distribution patterns in the ECM. Fibrillar collagens such as collagen I and collagen III provide tensile strength in tissues, whereas non-fibrillar collagens, e.g. collagen IV, form open mesh works in basement membranes (Timpl 1996). Elastins are another group of proteins in the ECM that contribute to the mechanical deformability and recoil of the tissues (reviewed in Midwood and Schwarzbauer 2002). Other proteins, which contribute to the flexible protein networks, include the laminins, fibronectin, tenascin and entactin, all of which are thought to be binding sites for other matrix molecules found on epithelial and mesenchymal cells. As a result, complex meshworks can be formed by the aggregation of multiple species of extracellular matrix molecules in the assembly of specific zones in the ECM such as the basement membrane (Timpl 1996).

Proteoglycans of the ECM have protein backbones and side chains composed of covalently linked disaccharides which bear carboxyl and sulphate groups, resulting in a net electrical charge of the ECM. The charged ECM is a major determinant of its affinity for ions and water, and allows function as an important as a storage mechanism for non-matrix proteins, such as cytokines and growth factors (Streuli 1999). Studies have shown that many of the critical protein mediators of allergic inflammation and tissue remodelling can be detected in the ECM (Roche 2000). An example of this are the heparin sulphates which are found beneath the bronchial epithelium and are known to bind fibroblast-growth factor 2 (FGF-2). Decorin (a proteoglycan) binds transforming growth factor (TGF)β in the underlying connective tissue (Reddington *et al.*1998).

Tenascins are a large family of oligomeric ECM glycoproteins with limited expression in adult human tissues, but widespread expression during embryogenesis (Erickson *et al.* 1993). There is also abundant re-expression during wound healing (Chiquet-Ehrisman *et al.* 1986). Studies by Laitinen and co-workers (1997) showed that there is increased immunoreactivity against tenascin in airway basement membrane of asthmatics. This re-expression in wound healing and in asthma suggests that during airway inflammation and repair, mechanisms normally involved in embryogenesis, are recapitulated (Raghow *et al.* 1994; Mackie *et al.* 1988). Tenascin may modulate cell adhesion and allow cell migration in the presence of other highly adhesive glycoproteins, such as fibronectin.

From the description above, it follows that any event that may alter the composition or structure of the ECM, can have profound effects on cellular differentiation, either by altered cell signalling pathways or by an alteration in the growth factors and cytokines available to the cells.

1.3.4.2 Proteolytic release of growth factors as a result of extracellular matrix remodelling

A classical example of an ECM sequestered growth factor is insulin-like growth factor (IGF). When bound to the ECM, the IGF binding protein (IGFBP)-5 has lower affinity for IGF-1, thus potentiating the cellular response to IGF-1 (Jones *et al.* 1993). Latent TGFβ binding protein (LTBP) is a structural ECM protein that provides a storage vehicle for TGFβ within the matrix (Dallas *et al.* 1995). Both the IGFBPs and LTBPs can be released through proteolytic cleavage by serine proteases (Parker *et al.* 1995; Imai *et al.* 1997), illustrating a mechanism whereby remodelling changes in the ECM are able to trigger alterations in cellular phenotype through growth factor release.

1.3.4.3 Regulation of the extracellular matrix composition

A large number of enzymes are involved in the remodelling of the ECM, including serine proteases and matrix metalloproteinases (MMPs), all of which act as broad-spectrum proteases for ECM degradation events, which occur during ECM remodelling (Streuli *et al.* 1999). They cleave specific ECM proteins to produce subtle changes in the structure of the matrix, therefore interfering with stored growth factors or cell signalling receptors.

MMPs are a family of zinc- and calcium-dependent endopeptidases that degrade various components of the ECM and have crucial roles in cell development, migration, infiltration, tissue remodelling and wound healing (Mackie *et al.* 1988; O'Conner *et al.* 1994) Most MMPs are secreted from cells as zymogens and require cleavage of their amino-terminal pro-domains in order to become activated. In order for MMP proteolysis not to cause widespread damage to the ECM, their activation is orchestrated close to the cell surface (Werb Z *et al.* 1997). MMPs are regulated by a family of proteins known as tissue inhibitors of metalloproteinases (TIMPs). The co-ordinated production of MMPs and their TIMPs in a range of development and remodelling situations, suggests that TIMPs play a significant role in the regulation of MMP activation *in vivo* (Hoshino *et al.* 1998; Matrisian 1990). To date four TIMPs have been reported (TIMP1-4), but most investigations have been carried out on TIMP-1 and TIMP-2, both of which inhibit all active forms of MMPs (Hoshino *et al.*1998).

In order to determine how these enzymes may contribute to tissue remodelling Hoshino and co-workers (Hoshino *et al.* 1998) investigated whether the degree of subepithelial thickening in patients with asthma correlated with levels of MMP-9 and TIMP-1 by use of immunohistochemistry. They found that MMP-9 and TIMP-1 were both co-localised to epithelial cells, submucosal inflammatory cells and the ECM. It was also observed that in all locations, both TIMP-1 and MMP-9 were expressed at significantly higher levels in asthmatics when compared to controls. They also observed a weak but significant correlation between the thickness of collagen III and submucosal expression of TIMP-1. The TIMPs are able to preserve tissue integrity by maintaining a dynamic equilibrium between the MMPs and the ECM. In oestoarthiritis and cystic fibrosis a slight imbalance in the equilibrium between TIMP and MMP concentrations can result in tissue remodelling (Hoshino *et al.* 1998; Dean *et al.* 1989; Delacourt *et al.* 1995). The results by Hoshino and co-workers (Hoshino *et al.* 1998) may represent a mechanism by which allergic inflammation can be propagated and exacerbated. The source of MMP-9 in allergic asthma seems to come not only from the epithelium, but also from eosinophils (Ohno *et al.* 1997) and from macrophages (Mautino *et al.* 1997).

1.3.4.4 The subepithelial basement membrane

Roche and co-workers (Roche 1989) carried out ultrastructural analysis of asthmatic bronchial biopsies (figure 1.4) and found that the *lamina rara* and the *lamina densa* of the true basement membrane were of normal appearance in asthmatic sections.

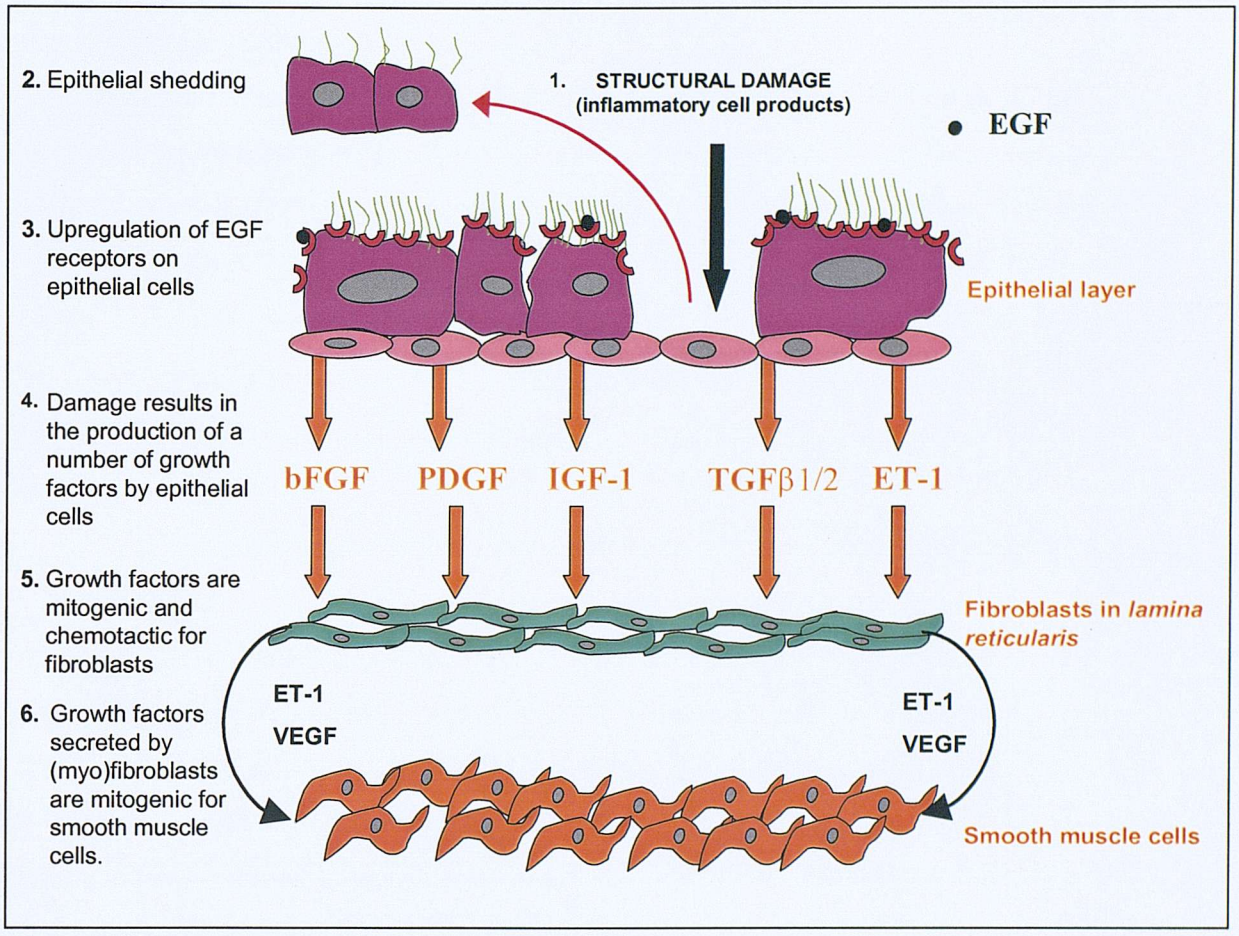


Figure 1.5 The factors secreted by the damaged epithelium. Damage occurs to the epithelium, either directly via environmental pollutants, viruses etc. or as a secondary consequence of the inflammatory response to environmental stimuli (1). When damage occurs, epithelial cells are shed (2) leaving areas of denuded epithelium. The epithelium adopts a repair phenotype, and this is characterised by an up regulation of EGF receptor expression (3). The damaged epithelium secretes a number of growth factors (4) which activate the fibroblasts in the underlying mesenchyme (5). When activated, the fibroblasts themselves secrete growth factors which are mitogenic for smooth muscle cells (6).

Beneath the true basement membrane in control subjects was a loose array of collagen fibrils forming a normal reticular layer. However, in asthmatic patients, this was replaced by a dense network of collagen fibrils.

Subsequent immunohistochemical analysis revealed that the thickening of the *lamina reticularis* was due to interstitial collagens, confirming the observations made by Jeffery and co-workers (Jeffery *et al.* 1989). Collagens III and V, and to a lesser extent collagen I, contributed to the thickened *lamina reticularis*. However, the epithelial basement membrane components, collagen IV and laminin, were absent from the *lamina reticularis* although they were present in the true basement membrane. This observation implied that the *lamina reticularis* was composed primarily of mesenchymal connective tissue.

Hoshino and co-workers (1998) investigated correlations between the thickening of the *lamina reticularis* (although the distinction between *lamina reticularis* and true subepithelial basement membrane was not made) with the duration of the disease and airway responsiveness. In patients with asthma they found no significant correlation between the subepithelial thickening and the duration of the disease. However, they found a close correlation between the thickness of the *lamina reticularis* baseline FEV_1 and the threshold of airway responsiveness, suggesting that it may have an effect on airflow.

1.3.4.5 The use of corticosteroids to reduce airway remodelling

The reported observations on the effects of inhaled corticosteroids on the thickened *lamina reticularis* have been controversial. Lundgren and co-workers (1988) reported that long-term steroid inhalation reduced the inflammatory cell infiltration, but the thickness of the *lamina reticularis* was not altered. Jeffery and co-workers (1989) compared the *lamina reticularis* thickness before and after treatment with the inhaled corticosteroid budesonide and noted no difference regardless of treatment time. However, in a study by Laitinen and co-workers (1994) there was a significant decrease in the amount of tenascin found in budesonide-treated patients. Trigg and co-workers (1994) have reported that the thickness of type III collagen deposition in the bronchial *lamina reticularis* was also reduced in patients that inhaled budesonide. The reduction of *lamina reticularis* thickness in some studies indicates that inhaled budesonide may have the potential to reduce long-term fibrosis in the airways of asthmatic patients.

1.3.4.6 Myofibroblasts cause subepithelial fibrosis

The apparent thickening of the subepithelial membrane is analogous to the gastrointestinal condition *collagenous colitis*. It has many analogies to asthma, including eosinophilia of the mucosa and collagen deposition beneath the colonic epithelial basement membrane due predominantly to collagen III. Specialised types of fibroblastic cells 'pericryptal fibroblasts', have been described in the gastrointestinal tract of patients with *collagenous colitis* and are thought to be responsible for the fibrosis beneath the colonic epithelium. Ultrastructurally, Hwang and co-workers (1986) showed that these cells have increased numbers of synthetic organelles and contractile apparatus. They also have thick and thin filaments and possess numerous polyribosomes throughout their cytoplasm, all of which characteristically classify these cells as myofibroblasts. It has been shown that myofibroblasts form constitutive populations in the connective tissue of the lung and gastrointestinal tracts. Classically, they are found in association with wound healing and fibrotic diseases. Myofibroblasts are thought to be derived from fibroblasts normally resident at the site of injury (Gabbiani *et al.,* 1971). The mode of induction of this phenotype *in vivo* is unclear, and is further discussed in section 1.4.5.

A combination of *in vivo* studies demonstrating the proximity of myofibroblasts to areas of excess collagen deposition (Roche 1991) and *in vitro* studies demonstrating increased collagen production by these cells relative to fibroblasts, lead to the suggestion that myofibroblasts were important contributors to increased matrix synthesis. This was directly demonstrated using combined *in situ* hybridisation (to assess pro-collagen I gene expression) and immunohistochemistry (to demonstrate α-smooth muscle actin expression) in the same lung tissue section. The observations that α-smooth muscle actin expression coincided with pro-collagen gene expression, provided evidence that myofibroblasts were responsible for the increased collagen gene expression in bleomycin-induced pulmonary fibrosis (Zhang *et al.,* 1994; Zhang *et al.,* 1996). Similar observations have been made in hepatic fibrosis (Yang *et al.,* 2003).

1.4 Intertwining inflammation and remodelling - The epithelial-mesenchymal trophic unit

The local production of cytokines by epithelial and mesenchymal cells have been shown to mediate bi-directional growth control during lung development, repair and inflammation.

The concept of the epithelial-mesenchymal trophic unit (EMTU) can be used to explain how damage to the epithelium can translate into the permanent structural changes of remodelling as discussed above. In a study by Zhang and co-workers (1999), the interactions between epithelial cells and myofibroblasts were investigated by use of an *in vitro* co-culture model, in which a monolayer of human bronchial epithelial cells were grown on a collagen gel seeded with human myofibroblasts. Damage to the epithelial monolayer via chemical (polyarginine as a surrogate for eosinophil basic protein) or mechanical damage, led to an enhanced proliferation and increased collagen gene expression in the myofibroblasts. It was found that the damaged epithelium secreted a number of cytokines, namely basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, platelet derived growth factor (PDGF)-BB, TGFβ2 and endothelin (ET)-1. Each growth factor exhibited different kinetics of release, suggesting phenotypic changes occurring within the epithelial cells during repair. This study was the first to provide direct evidence that injured and repairing bronchial cells are able to regulate the remodelling process through an increased production of proliferative and profibrogenic growth factors (figure 1.5). Puddicombe and co-workers (2000) investigated the effect of blocking EGFR-mediated bronchial epithelial repair on epithelial-mesenchymal interactions. They also found that this increased the release of profibrogenic growth factors. When cultured media from these experiments were applied to myofibroblasts, collagen gene expression was enhanced.

As mentioned above, TGFβ2 is released by the damaged bronchial epithelium. It has been observed that TGFβ1 and TGFβ2 have the ability to stimulate fibroblasts, altering their phenotype to that of myofibroblasts (Foo *et al.,* 1992). This is indicated by an increase in the expression of the cytoskeletal protein, α-smooth muscle actin (this phenomenon is further discussed in 1.4.4). It is therefore unclear, whether the fibroblastic cells underlying the epithelium are exhibiting proliferation and increased ability to produce ECM components before, or after, phenotypic transformation by the released TGFβ.

he concept of an EMTU is not exclusive to asthma. A very similar pathogenetic mechanism has been proposed for the fibrosis observed in the liver (reviewed in Iredale 1997). Liver fibrosis is also characterised by an accumulation of interstitial collagens and other matrix components. Fibrosis in the liver is accompanied by activation of the hepatic stellate cell, which results in its phenotypic transformation into a cell with myofibroblast properties. Iredale and co-workers (1997) have proposed that once the hepatic stellate cell is activated, the matrix changes that occur would result in an amplification of the activated state, and the initial injury.

Richter and co-workers (2001) published data to show that TGFβ2 has the ability to stimulate the release of ET-1 and vascular endothelial growth factor (VEGF) from asthmatic (myo)fibroblasts. ET-1 and VEGF are both very potent mitogenic stimuli for smooth muscle cells. As discussed in section 1.3.3, increased smooth muscle mass, increased microcirculation and nervous innervation are all characteristic of airway remodelling in asthma. Therefore as well as the (myo)fibroblasts responding to epithelial damage by directly secreting ECM components, it seems evident that, through the release of growth factors, (myo)fibroblasts are further regulating the remodelling response.

These observations help build up the model of an epithelial-mesenchymal trophic unit in asthma, whereby the damaged epithelium releases growth factors that can activate fibroblasts to myofibroblasts. These fibroblasts may be able to contribute to the increased smooth muscle mass either directly, or via growth factors that act on cells deeper in the airway wall. Lung (myo)fibroblasts are emerging as key effector cells, transducing signals from the damaged epithelium (via inflammation and / or direct damage) into direct fibrotic and remodelling responses via the action of TGFβ2 alone or in combination with other growth factors that may be secreted in response to epithelial damage.

1.4.1 Transforming Growth Factor β

The transforming growth factor β family is comprised of multifunctional cytokines which are able to modulate a wide range of cellular activities. Their actions are varied and highly dependent on the cells on which they act. For example, they have been shown to have both proliferative and inhibitory effects on different cells (Chatani *et al.*, 1995).

It has been shown that the human genome contains 28 genes that encode members of this family, including TGFβ isoforms and bone morphogenetic proteins (BMPs)

(Massague 1998). Members of the TGFβ superfamily share amino acid homology and very similar mRNA processing. All member peptides have conserved cysteine residues, which are required for receptor interactions (Heldin *et al.,* 1997).

Multiple forms of TGFβ have been identified and termed TGFβ1,2,3,4 and 5. TGFβ1 is a protein of 25kDa, consisting of two identical subunit chains joined covalently by disulphide bonds; it is found in abundance in many tissues including bone, platelets, kidney and the lungs (Cheifetz *et al.,*1987). Like TGFβ1, TGFβ2 is a 24kDa dimer of two identical β2 chains derived from a much larger (412-amino acid) precursor. TGFβ2 exhibits 70% homology with TGFβ1, with maintained amino acid homology of nine critical cysteine residues required for receptor interactions (Cheifetz *et al.,*1987). Both TGFβ1 and TGFβ2 have 20-30 amino acid signal peptide domains involved in transcellular secretions. It is accepted that both TGFβ1 and TGFβ2 have the same overall spectrum of actions, however there are some exceptions to this generalisation (TGFβ2 was shown to be much less potent that TGFβ1 at inhibiting DNA synthesis in aortic endothelial cells (Danielpour *et al.,*1989). The relative proportions of types of TGFβ released by different cell types can vary considerably, however, most of the TGFβ activity triggered by cells can be accounted for by TGFβ1 and TGFβ2 (Derynck *et al.,* 1988).

TGFβ and related factors activate receptors at the cell surface and transduce signals to target genes. Some of the genes encode immediate effectors of ultimate cellular responses, e.g. cell cycle regulators that mediate antiproliferative responses or extracellular matrix components that mediate cell adhesion, positioning and movement (Roberts *et al.,* 1988).

1.4.2 Transforming growth factor β in human disease

Virtually every cell in the body produces isoforms of TGFβ and has receptors for it. TGFβ can regulate many processes, such as the proliferation and differentiation of cells, embryonic development, wound healing and angiogenesis (reviewed in Lyons and Moses 1990). Increases or decreases in the production of TGFβ have been linked to numerous disease states, including atherosclerosis and fibrotic disease of the kidney, liver and lung. Mutations in the genes for TGFβ, its receptors or intracellular signalling molecules associated with TGFβ are also important in disease pathogenesis.

TGFβ is an immune system suppressor, and it induces the production of extracellular matrix components. As a consequence of these two properties, it has become apparent that TGFβ plays an important role in wound healing and tissue repair (Cheifetz *et al.*,1987). However, although necessary for tissue repair, overproduction of TGFβ can result in excessive deposition of scar tissue and therefore fibrosis. The expression of TGFβ mRNA and the production of TGFβ are increased in patients with hepatitis C virus infection, alcohol-induced hepatic fibrosis, iodiopathic or bleomycin-induced pulmonary fibrosis, systemic sclerosis, myleofibrosis, proliferative vitreoretinapathy, Crohn's disease and eosinophilia in myalgia syndrome (Blobe *et al.*, 2000).

1.4.3 Transforming growth factor β in fibrosis

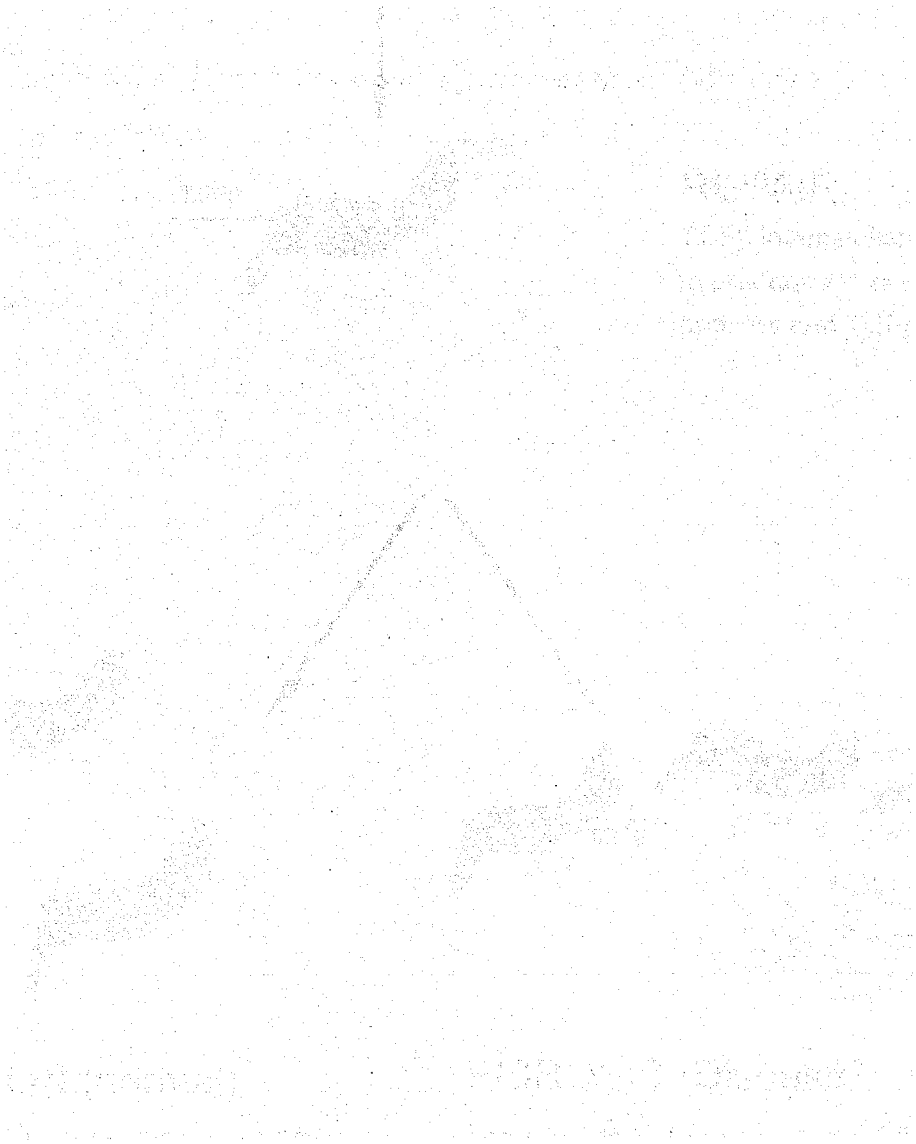
Although TGFβ has been implicated in regulating repair and regeneration following tissue injury, it has also become evident that excessive production may be responsible for the tissue damage caused by scarring in many serious diseases (see figure 1.6).

The role of TGFβ in scarring has been the focus of much research (Huang *et al.*, 2002). Foetal skin heals without scarring and only after birth does healing of a wound generate scar tissue (Choi *et al.* 1996). It has been shown that there is a correlation between the lack of scarring in foetal skin and the greatly reduced or absent TGFβ response to wounding of the skin. No TGFβ could be detected in the tissue surrounding the foetal wound, which is in sharp contrast to the tissue surrounding a wound in neonatal and older skin, where high levels of TGFβ are found in the surrounding skin (Soo *et al.*, 2000).

TGFβ is strongly chemotactic for many types of cells including smooth muscle cells, and also stimulates matrix production, both of which may be important in maintaining a scarring phenotype.

There have been a number of experiments that have shown that the specific inhibition of TGFβ1 can lead to an attenuation of fibrosis. One example of such an investigation is the use of a tetracycline-regulated TGFβ1 expression transgenic mouse model. In their study Ueberham and co-workers (2003) were able to control the plasma levels of TGFβ1 by the addition or removal of doxycycline hydrochloride to the drinking water of the mice. By applying a cyclic induction-deinduction of the deoxycycline protocol, the deleterious effects of the high plasma levels of TGFβ1 were overcome. Futhermore, in advanced stages, fibrogenesis was stopped by switching off TGF-beta1

production and reversal of fibrosis was shown by (immuno)histochemistry within 6 to 21 days (Ueberham *et al.,* 2003). In another study, inhibition of TGFβ1 was achieved by inhibiting TGF-beta binding with a soluble TGF-beta type II receptor (STR) construct, administered intraperitoneally. In the 4-week study, type I collagen mRNA expression was reduced by up to 60% (Yata *et al.,* 2002).



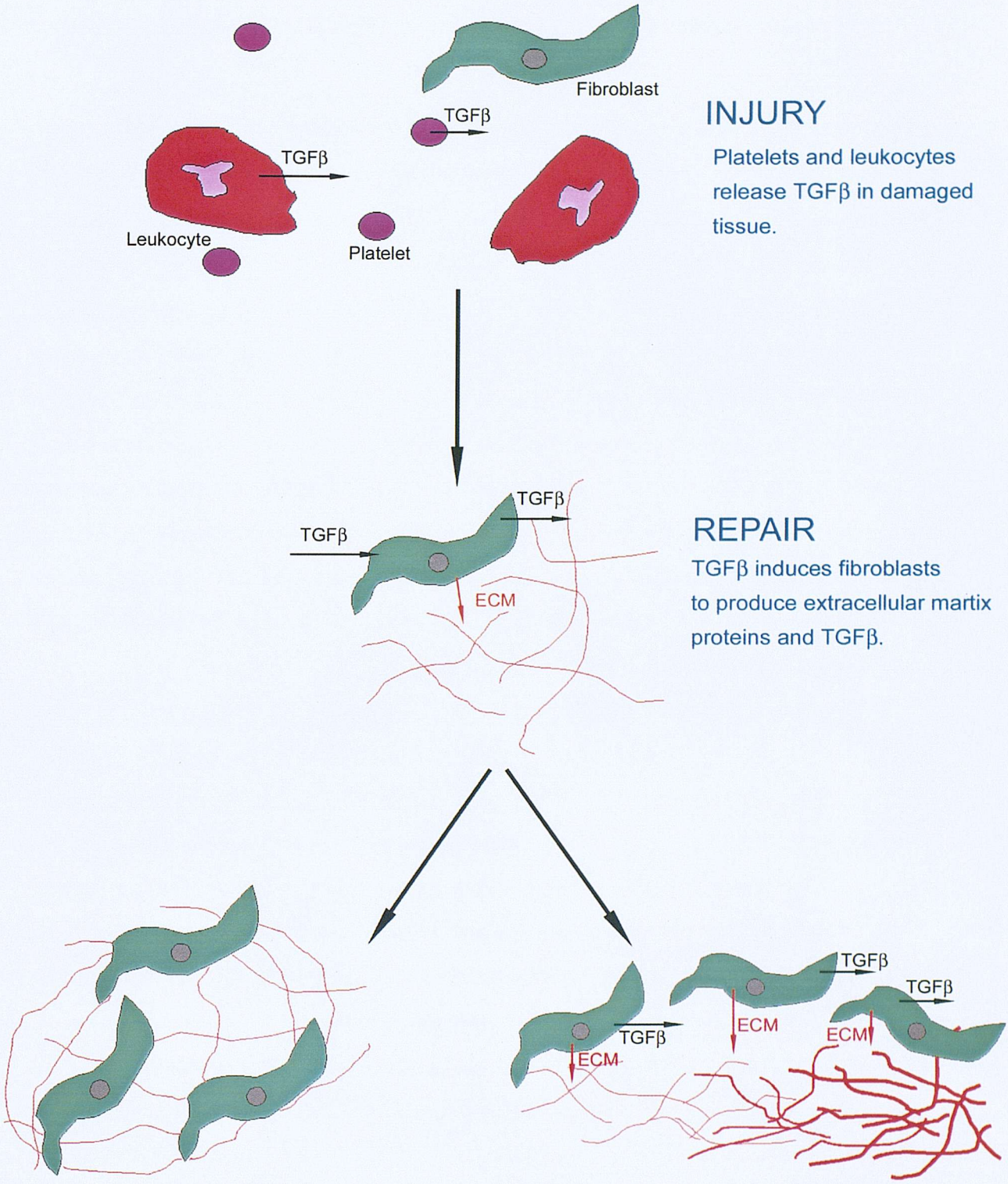


Figure 1.6 Tissue injury can result in resolution of the damage or fibrosis

1.4.4 Transforming growth factor β in airway fibrosis and asthmatic airway remodelling

There are strong correlations to suggest that TGFβ overproduction is a contributing factor in lung fibrosis. A study by Broekelmann and co-workers (1991) showed that TGFβ expression was greatly increased in patients with iodiopathic fibrosis. The increased TGFβ expression was co-localised with sites of abnormal extracellular matrix. Bleomycin-induced pulmonary fibrosis is also associated with increased TGFβ expression (Denholm and Rollins 1993). More recent studies have shown the importance of TGFβ in fibrotic airways conditions. Recent animal studies showed that expression levels of several TGF-beta-inducible genes were dramatically increased as early as 2 days after the induction of injury in a model of acute respiratory distress syndrome (ARDS; reviewed in Dhainaut *et al.,* 2003). In an integrin alpha(v)beta(6) (which activates latent TGFβ to active TGFβ in the lungs) knockout mouse model, bleomycin-injury failed to induce pulmonary oedema typically observed (reviewed in Dhainaut *et al.,* 2003).

Elevated levels of TGFβ1 have been found in the broncho-alveolar lavage fluid (BALF) of asthmatic patients in comparison with control subjects (Redington *et airwayal.,*1997).Increased expression of TGFβ1 has also been significantly correlated with two markers of remodelling i.e. thickness of subepithelial basement membrane, and number of fibroblasts, in both asthma and chronic bronchitis (Redington *et al.,* 1998). TGFβ1 is also produced by macrophages and eosinophils which are recruited to the airway in an asthmatic response. Vignola and co-workers (1996) have shown that alveolar macrophages isolated from chronic bronchitic subjects released significantly larger amounts of TGFβ than those from normal subjects.

The increased expression of TGFβ by epithelial cells, fibroblasts, macrophages and eosinophils in chronically inflamed bronchial tissue suggests that these cells contribute to remodelling in asthma. They are probably involved in a dynamic cellular network based on intercellular communication between structural cells and cells recruited from the blood. Similar intercellular communications have already been highlighted in other forms of pulmonary fibrosis (Li *et al.,* 2002).

1.4.5 TGFβ interactions with fibroblastic cells results in the induction of CTGF

There is a wealth of evidence to suggest that TGFβ may have important roles in regulating the synthetic properties of fibroblasts. Studies by Grotendorst and co-workers (1996) have shown that the connective tissue growth factor (CTGF) gene is strongly induced by TGFβ, but not by other growth factors including EGF, PDGF, bFGF and IGFs. It has also been shown that TGFβ causes a prolonged activation of CTGF gene expression, which lasts for 24-36 hours after addition of TGFβ to normal rat kidney (NRK) fibroblasts (Frazier *et al.,* 1996). TGFβ stimulation of the ECM via expression of CTGF has been observed in human cardiac and foreskin fibroblast. Many of the effects of TGFβ in fibroblasts, particularly those relating to increased proliferation and increased ECM production, are thought to be mediated by CTGF. In cardiac fibrosis, Chen and co-workers (2000) showed that the activity of a CTGF promoter-based construct, correlated with endogenous CTGF expression, which suggested that TGFβ promoted CTGF expression by activating its promoter. An up-regulation of CTGF coincided with an increase in fibronectin, collagen type I and plasminogen activator inhibitor-1 production. In asthma (as in cardiac fibrosis) levels of fibronectin, collagen I and III are increased in the *lamina reticularis*.

In a number of diseases, fibroblasts have shown an altered response to TGFβ. For example, in atherosclerotic plaques (areas of fibrous tissue) (Clark *et al.,* 2001) and in colorectal cancer (Iacopetta *et al.,* 1998), somatic mutations have been reported within a microsatellite poly A tract in the coding region of the type II TGFβ receptor gene. In colorectal cancer this results in the loss of sensitivity to the growth inhibitory effects of TGFβ in the tumour cells. Similarly, Clark and co-workers (2001) proposed that the mutation accounted for the clonal expansion of vascular smooth muscle cells observed in atherosclerotic plaques, through the loss of the growth inhibitory effect of TGFβ. Primary pulmonary hypertension is another disease, in which there is a mutation in the BMPR2 (a TGFβ superfamily receptor) that results in an altered response to TGFβ (Lane *et al.,* 2000).

1.4.6 TGF β induces a number of cytoskeletal changes in fibroblasts

Fibroblasts are cells of mesenchymal origin, which are largely responsible for the production and regulation of the extracellular matrix (ECM). The ECM is a complex,

highly organised network of molecules that provides structure and support for tissues and cells. *In vivo*, fibroblastic stromal cells are responsible for the production of most connective tissue components, including the various collagen molecules. It has been shown that fibroblasts when in culture, will synthesise different types of collagen according to their site of origin (reviewed in Gabianni 1996).

The varied function and origin-dependent collagen production of fibroblasts, point towards phenotypic heterogeneity. Phenotypic diversity was first suggested due to the morphological differences observed between cells analysed *in vivo* and *in vitro* (Eyden 2001). *In vivo*, they have large amounts of rough endoplasmic reticulum (rER), Golgi apparatus, many mitochondria and intermediate filaments. When in culture, they appear flattened and polarised. They possess stress fibres and are interconnected by gap junctions, whereas *in vivo* they do not contact one another.

Phenotypic modulations *in vivo* have been reported and confirmed by ultrastructural analysis (Eyden 2001). In wound repair and granulation tissue, fibroblastic cells, which exhibit biological and morphological properties somewhere between those of fibroblasts and smooth muscle cells, have been identified. These 'intermediate' cell types are referred to as myofibroblasts.

All fibroblastic cells are generally thought to express vimentin, although some fibroblasts from specific areas have been found to express desmin (e.g. fibroblastic cells from the uterine mucosa, lymphatic organs and intestinal mucosa) (reviewed in Sappino *et al.,* 1990). However, the presence of vimentin does not distinguish fibroblasts from other mesenchymal cells or from such cells as monocytes, macrophages and lymphocytes. Desmin is a protein thought to be specific to smooth muscle, but not associated with all muscle cells (Sappino *et al.,* 1990). Studies of the actin isoforms in stromal cells has led to the identification of specific differences between cell types (Sappino *et al.* 1990). The primary amino acid sequence of actin has remained highly conserved, however, biochemical heterogenity has been observed in the 17 amino-terminal amino acids of actin from different tissues. Initially, three actin isoforms were described, α,β and γ, but further analysis characterised three additional isoforms of α-actin, one specific to striated muscle, one specific to cardiac muscle and one specific to smooth muscle cells (α-smooth muscle actin, α-SMA) (Sappino *et al.,* 1990). This then allowed the combined use of anti-vimentin, anti-desmin and anti-α-SMA antibodies to characterise cells according to muscular or fibroblastic origin.

Table 1.1 (adapted from Sappino *et al.*, 1990) indicates classical cytoskeletal and cytocontractile markers for mesenchymal and muscular differentiation. The use of a combination of markers can be used to assess the extent of muscular differentiation in fibroblastic cells associated with diseased states, and to analyse which stimuli may modulate fibroblastic phenotype plasticity. Studies by Skalli and co-workers (1986), investigated the cytoskeletal characterisation of stromal cells present in a variety of human and experimental soft tissue specimens known to contain fibroblasts. Their results yielded the presence of four main phenotypes:

i) those expressing vimentin (V cells)

ii) those co-expressing vimentin and desmin (VD cells)

iii) those co-expressing vimentin and α-SMA (VA cells)

iv) those co-expressing vimentin, desmin and α-SMA (VAD cells)

The presence of smooth muscle markers in fibroblastic cells indicates that these cells are in fact myofibroblasts.

	Fibroblast cells	Myofibroblast cells	Smooth muscle cells
Vimentin	+	+ (*)	-
Desmin	-	- (*)	+
Smooth muscle myosin	-	+	+
Skeletal muscle myosin	-	-	-
Cardiac myosin	-	-	-
α-smooth muscle actin	-	+	+
γ-smooth muscle actin	-	- / +	+
α-skeletal actin	-	-	-

(*) Present (vimentin) in all vascular smooth muscle cells and absent (desmin) in some vascular smooth muscle cells

Table 1.1: Classical cytoskeletal markers of mesenchymal cells

Myofibroblasts are found in a wide variety of normal tissues and pathological conditions. In bronchial asthma, Brewster and co-workers (1990) identified and quantified the number of myofibroblastic cells beneath the bronchial epithelium in bronchial biopises from normal and asthmatic subjects. For that they used a monoclonal antibody raised to colonic pericryptal cells, which also bound to vascular smooth muscle cells found in the plexus beneath the bronchial epithelium. Using a combination of anti-α-SMA antibodies and the monoclonal anti-colonic pericryptal myofibroblast antibody, PR2D3, on serial sections, the number of myofibroblasts present was determined. It was reported that myofibroblast number was increased in atopic asthma and correlated positively with the depth of collagen beneath the bronchial epithelium.

Many cytokines have been implicated in the induction of the myofibroblastic phenotype, however, TGFβ is by large considered to be the direct inducer of the differential phenotype. Studies by Desmoulière and co-workers (1993) have shown that TGFβ1 is capable of up-regulating α-SMA in (myo)fibroblasts. Zhang and co-workers (1994) have shown that, in pulmonary fibrosis, collagen production by these cells is upregulated by TGFβ1. α-SMA-expressing myofibroblasts are important cells in wound healing, where they generate granulation tissue contraction during wound closure (Gabbiani *et al.,* 1972). In fibrotic disease, however, they produce excess collagen, which is deleterious.

There are three isoforms of TGFβ, TGFβ1, TGFβ2 and TGFβ3, which exist in mammals and share a 70-80% homology. Gabbiani (1996) has shown that TGFβ2 is able to induce α-SMA in myofibroblasts (as is TGFβ1) both *in vivo* and *in vitro*. They found that *in vivo* TGFβ3 acts as a negative regulator of the myofibroblast phenotype but *in vitro* it too induced α-SMA protein. In light of the evidence suggesting different *in vivo* and *in vitro* activities of TGFβ on myofibroblasts, it has been hypothesised that α-SMA expression is highly dependent on the microenvironment surrounding the cell. *In vivo*, this transpires to be due to a major influence from extracellular matrix components (Boudreau and Bissell 1998). It is of course possible that the *in vitro* conditions may alter the TGFβ receptor repertoire and hence the resultant cellular response.

1.4.6.1 Fibroblast, myofibroblast and smooth muscle plasticity

There is evidence to support that there is plasticity between fibroblasts, myofibroblasts and smooth muscle cells, suggesting that this may be an important factor to

consider in diseased states such as asthma (figure 1.7). There is currently much debate surrounding the origin of the increased smooth muscle mass in bronchial asthma. Evidence from other disease models, such as the obstructed urinary bladder (Buoro *et al.,* 1993; Roelof *et al.,*1998) suggests that fibroblasts, myofibroblasts and smooth muscle cells follow a single lineage on a linear pathway and that the factor causing the transformation from myofibroblasts to smooth muscle cells is TGFβ, the same factor that activates fibroblasts to become myofibroblasts (figure 1.7).

A study by Hirst and co-workers (1996) proposed that smooth muscle cells can have a phenotype which is plastic between a contractile and synthetic phenotype. They proposed that sparsely seeded contractile smooth muscle cells in culture undergo a number of spontaneous changes in phenotype over a period of about a week, depending on species, to become synthetic smooth muscle cells. The synthetic phenotype is characterised by increased mitogenic activity and the expression of intracellular organelles associated with synthesis, such as Golgi cisternae, ribosomes and rough endoplasmic reticulum (reviewed in Eyden 2001).

Accompanying these changes is a decreased immunoreactivity for smooth muscle specific contractile proteins. Campbell and co-workers (1988) postulated that the function of the contractile phenotype, is to constrict and maintain tension, whereas the function of more synthetic cells is dedicated to the synthesis and deposition of extracellular matrix components, as well as production of paracrine and autocrine growth-promoting factors.

The characteristics of myofibroblasts are virtually undistinguishable from the synthetic smooth muscle phenotype. Myofibroblasts also show increased expression of intracellular organelles associated with synthesis. Indeed, the increase in production of collagen and other ECM components by myofibroblasts (compared to fibroblasts and smooth muscle cells of the contractile phenotype) are testament to this.

1.4.6.2 Mechanism of TGFβ mediated transformation

The mechanism of TGFβ mediated transformation is summarised in figure 1.8. Hashimoto and co-workers (2001) have investigated the intracellular signalling that regulates the induction the myofibroblast phenotype in human lung fibroblasts. Many extracellular stimuli elicit specific biological responses through the activation of mitogen-activated protein kinases (MAPK) (Hashimoto *et al.,* 2001; Davis 1994) hence Hashimoto and co-workers (2001) investigated the potential role of MAPK in TGFβ mediated

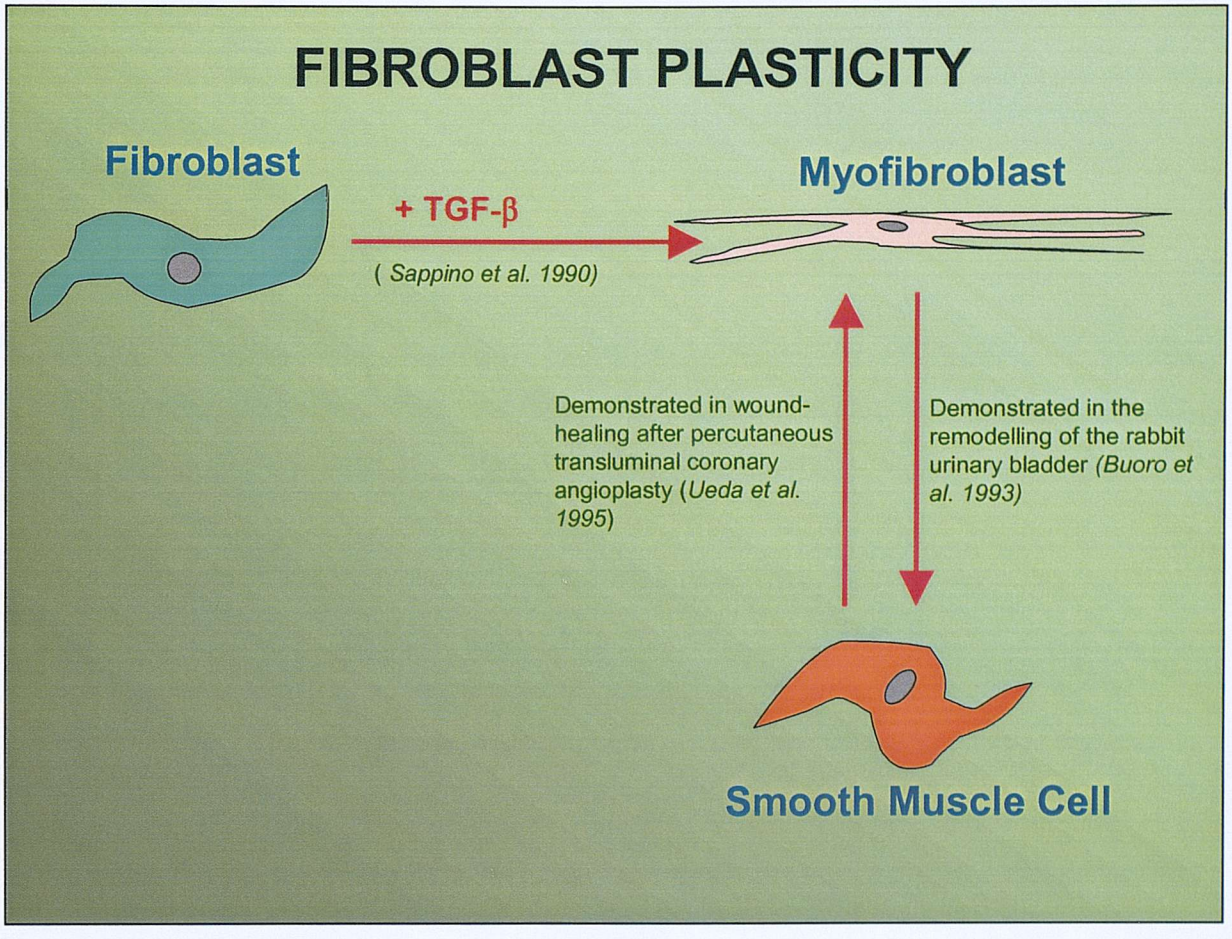


Figure 1.7 The Phenotypic plasticity of fibroblasts, myofibroblasts and smooth muscle cells. TGFβ is known convert fibroblasts to myofibroblasts. It has been shown that sustained exposure to TGFβ can further convert myofibroblasts into smooth muscle cells. Smooth muscle cells also have the potential to de-differentiate into myofibroblasts.

transformation of fibroblasts. Three subgroups of MAPK pathways have been identified in mammals:

i) extracellular signal regulated kinase (ErK)
ii) p38 mitogen-activated protein kinase (p38) MAPK
iii) c-Jun-NH_2-terminal kinase (JNK)

ErK is activated by mitogenic stimuli and plays a central role in cell proliferation and differentiation. Both p38 MAPK and JNK are activated by environmental insult, and often result in the expression of cytokines (Hoshimoto *et al.,* 2001; Raingeaud *et al.,*1995). Hoshimoto and co-workers (2001) attempted to determine if any of the MAPK superfamily was involved in the transformation of fibroblasts to myofibroblasts in response to TGFβ. They found that TGFβ1 caused increases in JNK, p38 MAPK and ErK phosphorylation and activity. In an attempt to determine which one of these pathways was crucial for the expression of α-SMA, they used a series of inhibitors specific for each of the individual pathways; CEP-1347 (a specific inhibitor of the JNK-mediated signalling pathway), SB203580 (a specific inhibitor of the P38 MAPK pathway) and PD98059 (a specific inhibitor of MAPK-1 (MEK-1) which is upstream of ErK).

CEP-1347 resulted in the attenuation of the TGFβ1-induced differentiation in a dose-dependent manner. This suggested that the C-Jun-NH_2 terminal kinase pathway regulates the phenotypic modulation of fibroblasts by TGFβ.

1.4.6.3 The activation of latent TGFβ and its implications in the phenotypic conversion of fibroblasts

As detailed in section 1.4.1, TGFβ is produced as a larger protein which must be processed to be biologically active. In addition, a variety of proteins bind TGFβ to regulate its activity. Studies by Ribeiro and colleagues (1999) have shown that latent TGFβ interacts with thromobospondin-1 (TSP-1) to produce activated TGFβ (Schulz-Cherry *et al.,*1993).

Morishima and co-workers (2001) studied the induction of the myofibroblast and fibrogenesis by airway epithelial shedding. In order to investigate the mechanism by which TGFβ affects the epithelial-mesenchymal trophic unit, they used an *in vitro* co-culture system, in which guinea-pig tracheal epithelial cells and fibroblasts were cultured on and beneath an amnion membrane. Culturing epithelial cells on an air-liquid interface resulted

in the differentiation of pseudo-stratified epithelial cells, almost identical to those seen in the *in vivo* trachea (Noguchi *et al.*,1995). The epithelial cells were then mechanically scraped to mimic epithelial shedding as seen in an asthmatic response. They observed that 4 days after injury, epithelial cells migrated and differentiated into cuboidal appearances and then returned to their original shapes by 8 days post-injury. It was found that myofibroblast induction occurred at the same time as the epithelial cell differentiation on day 4. The myofibroblast induction was inhibited by blocking of TGFβ1 and by blocking of TSP-1, which implied that activation of TGFβ1 by TSP-1 was required for the myofibroblast induction. It was observed that in response to epithelial injury, TGFβ1 immunoreactivity was reduced in the amnion membrane, but TGFβ1 mRNA expression in the epithelial cells and fibroblasts remained unchanged. In this study, expression of TGFβ2 mRNA was not investigated in damaged epithelium, however protein levels of TGFβ2 have been found to increase in response to epithelial damage of human airway epithelial cells (Zhang *et al.*, 1999). The fact that TGFβ1 immunoreactivity was reduced in the amnion membrane, suggested that TGFβ1 supplied by the extracellular matrix could result in fibroblast to myofibroblast transdifferentiation. They also observed that procollagen type I and III mRNA were up-regulated accompanying myofibroblast induction, and that there was deposition of collagen around the myofibroblast. This is in line with unpublished studies by Zhang and colleagues (personal communication), where procollagen I and III mRNA were up-regulated in fibroblasts in response to TGFβ2 in a dose-dependent fashion. Morishima and co-workers (2001) found that epithelial injury stimulates TGFβ1 release and its activation from the extracellular matrix via TSP-1 production. They proposed a hypothetical model for myofibroblast differentiation in response to airway epithelial shedding via the actions of TGFβ1 (figure 1.8). They hypothesised that epithelial shedding in the airway results in proteolytic cleavage of latent TGFβ binding protein (LTBP) which causes release of latent TGFβ1 from the amnion membrane. The increase in TGFβ in response to epithelial shedding (Zhang *et al.*, 1999) resulted in an up-regulation of TSP-1 on the surface of the fibroblast. Morishima and colleagues (2001) showed that the TSP-1 mRNA was expressed in fibroblasts before epithelial shedding and that it was up-regulated on day 4 after injury.

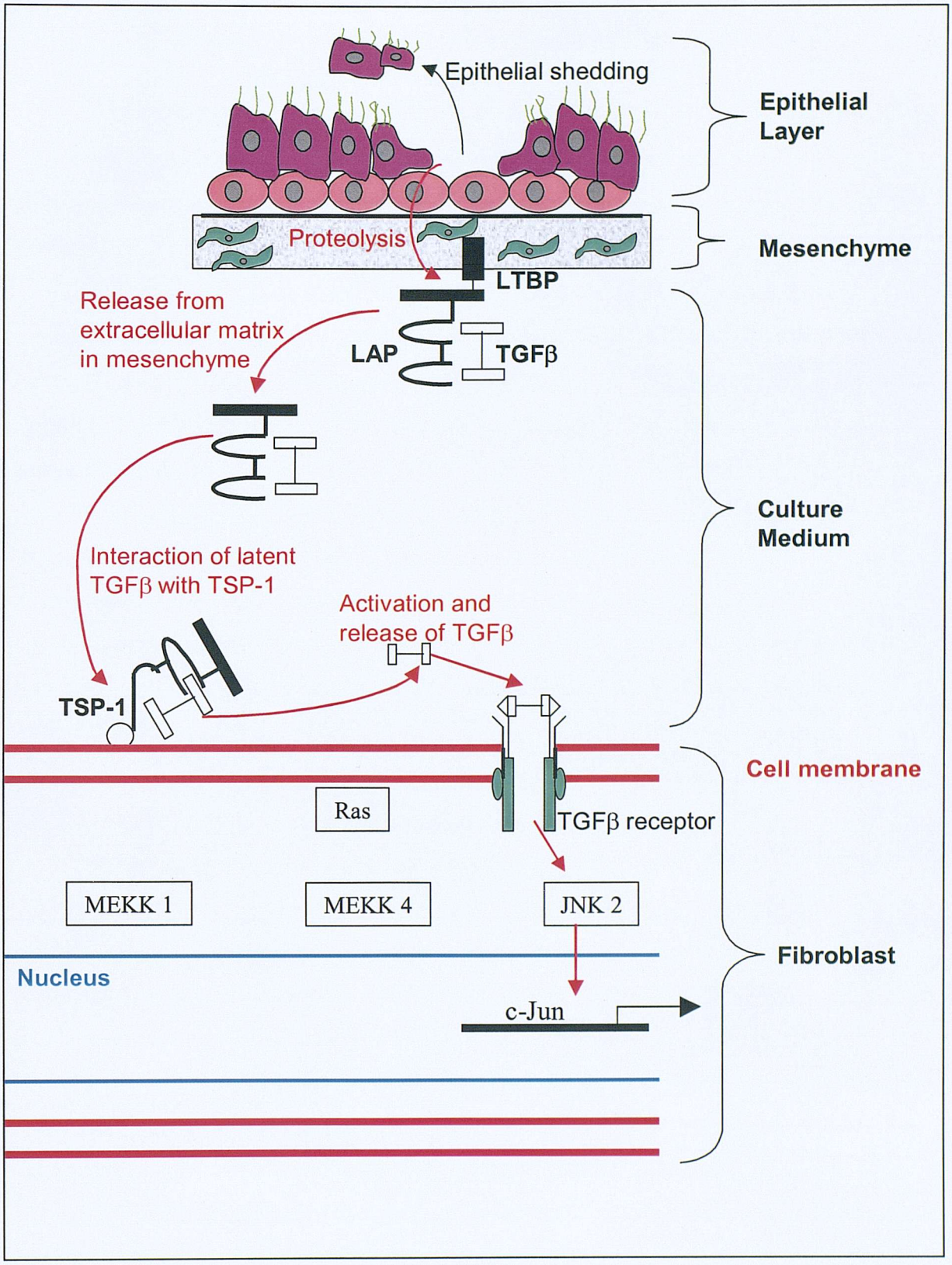


Figure 1.8 Activation of TGFβ and phenotypic transformation of Fibroblasts into myofibroblasts. Epithelial shedding in results in the proteolytic cleavage of latent TGFβ binding protein (LTBP), which results in the release of latent TGFβ from the mesenchyme. Thrombospondin-1 is also up regulated by epithelial shedding and an increase in TGFβ concentration. TSP-1 then activates latent TGFβ on the surface of the fibroblast, which then bind to TGFβ receptors. Binding result in the activation of Ras, which then phosphorylates c-JunN-terminal kinase, resulting in the expression of c-Jun, which is then responsible for transcription of α smooth muscle actin.

1.4.7 The regulation of collagen gene expression in (myo)fibroblasts by TGFβ

TGFβ increases procollagen synthesis via an increase in mRNA and decrease in the proportion of procollagen degraded intracellularly. The effect of TGFβ on collagen synthesis is known to be via the binding of transcription factors to binding elements in the promoter region of procollagen genes.

McAnulty and co-workers (1995) have shown that TGFβ induces procollagen production, and that addition of pertussis toxin (which blocks G_i and G_0 protein activation) increased the procollagen production in response to basal and TGFβ stimulated procollagen synthesis. This suggested that TGFβ-involved procollagen synthesis is not mediated by receptors coupled to pertussis toxin-sensitive G-protein. From these observations, they reasoned that the increase in procollagen production on treatment with pertussis toxin may be due to a blocking of a TGFβ-induced inhibitory pathway of procollagen metabolism. They postulated that such a pathway may involve prostaglandins (PGs) since it had previously been shown that PGE_2 potently inhibited procollagen metabolism (Goldstein and Polgar 1982). In support of this, other studies (Jackson *et al.,* 1993) showed that TGFβ1 stimulates cyclooxygenase (COX) 1 (but not COX2) gene expression and production of PGE_2 in lung fibroblasts. An increase in PGE_2 has been shown to limit the TGFβ1-induced stimulation of collagen production (McAnulty *et al.,* 1995).

To investigate the potential inhibitory effect of PGE_2, indomethacin (a specific COX-1 inhibitor) was used to inhibit PGE_2 production (Keerthisingam *et al.,* 2001). Addition of indomethacin resulted in stimulation of procollagen production, which suggested that under basal conditions PGE_2 is produced. Pertussis toxin behaved in a similar manner to indomethacin. Addition of both indomethacin and pertussis toxin did not produce additive effects, suggesting both inhibitors worked by acting on the same pathway.

However PGE_2 is not the only molecule that has been found to antagonise the pro-fibrotic effects of TGFβ. Another classical example if interferon (IFN)-γ, which has been the focus of much investigation as a potential anti-fibrotic agent. Investigators have shown that IFN- has the ability to inhibit the myofibroblastic phenotype of rat palatal fibroblasts, which is normally induced by TGFβ1 (Yokozeki *et al.*, 1999).

Studies by Yuan an co-workers (1999) investigated the modulation of procollagen I α1 gene expression by recombinant IFN-γ. The showed that IFN-γ stimulated the rapid accumulation of interferon regulated factor (IRF)-1 mRNA followed by a delayed and

dose-dependent inhibition of procollagen I α1 mRNA in human skin fibroblasts. More recently, Yamanaka and colleagues (2003) investigated the affect of IFN-γ on protein production of extracellular matrix components in cultured human subconjunctival fibroblasts alone or those stimulated by exogenous TGFβ1. They reported that IFN-γ upregulated Smad-7 and hence blocked the pro-fibrotic actions of TGFβ1.

Studies by a number of researchers have demonstrated that (myo)fibroblasts can no longer be considered to be merely structural cells capable of making ECM. It is now known that these cells are able to secrete a whole host of cytokines, which are able to affect both inflammation and remodelling. Clearly, through the secretion of a number of mediators, fibroblasts also have the potential to auto-regulate.

1.5 How TGFβ induces its effects

1.5.1 TGFβ Signalling Receptors

TGFβ molecules signal through a family of transmembrane protein serine/threonine kinases referred to as the TGFβ receptor family. The TGFβ receptor family is sub divided into two further types, type I and type II receptors, which are transmembrane glycoproteins approximately 55kDa and 70kDa in size respectively (reviewed in Matthews and Vale 1991).

The extracellular domain of type I and type II receptors is N-glycosylated and contains 10 or more cysteines, which are thought to determine the general folding of this region (Wells *et al.*,1997). Three of these cysteines form a characteristic cluster near the transmembrane sequence. An important amino acid, ser213, in this region of the type II receptor is phoshorylated by the receptor kinase in a ligand-dependent manner and is crucial for signalling activity (Luo and Lodish 1997). Ser165 in the juxtamembrane region of type I receptors is phosphorylated by TGFβ receptor type 2 (TβRII) also in a ligand dependent manner, and has been reported to be important in selectively modulating the intensity of the different TGFβ responses (Souchelnytskyi *et al.*,1996).

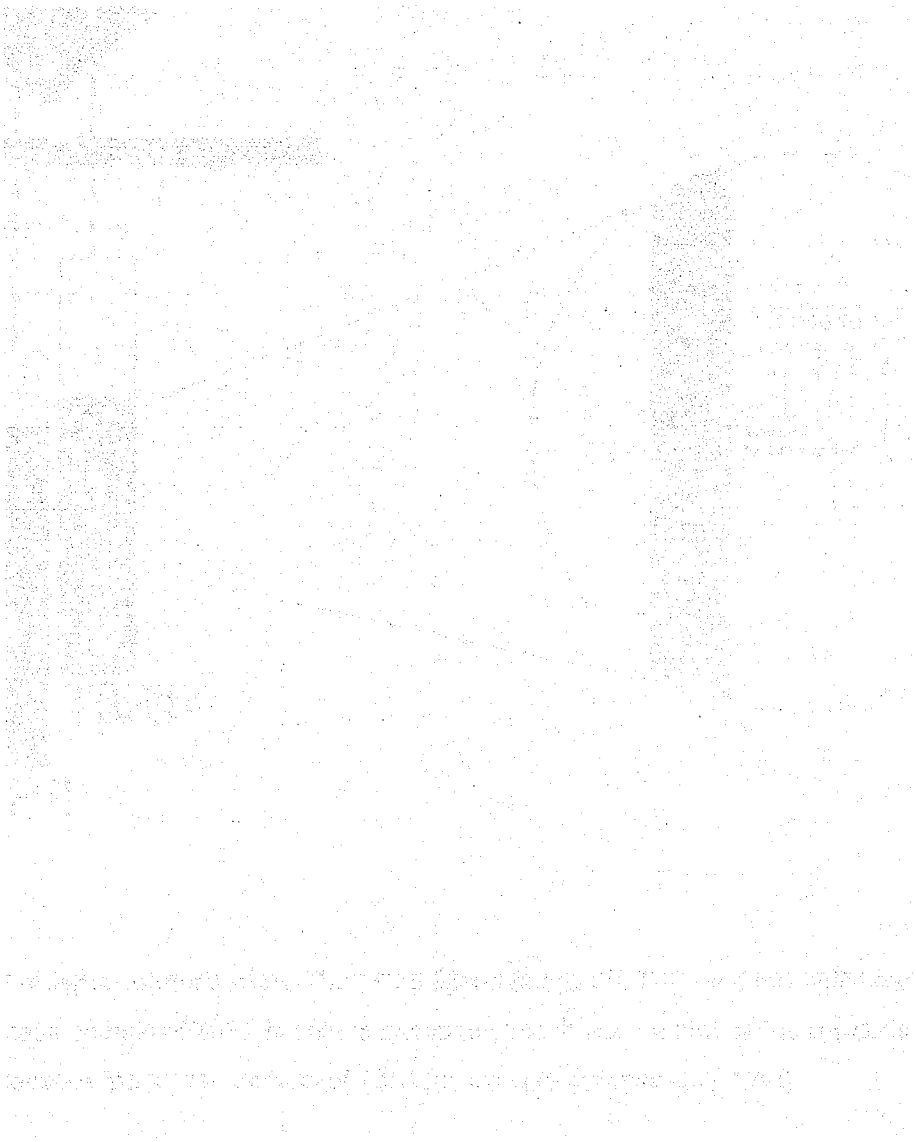
Type I receptors have a highly conserved 30 amino acid region immediately before the protein kinase domain. This region has a characteristic SGSGSG sequence and hence is termed the GS domain. For the activation of receptor signalling, it is necessary for ligand induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of TβRI by the TβRII to occur (Wiesser *et al.*, 1995).

The kinase domain in TGFβRI and TGFβRII is typical of a serine/threonine protein kinase domain. TGFβRI molecules have shown the ability to phosphorylate their substrate (SMAD proteins) on the serine residues. However TGβFRII molecules phosphorylate themselves and serine/threonine residues on TGFβRI (reviewed in Luo and Lodish 1997).

1.5.2 Binding of TGFβ ligand to signalling receptors

TGFβ activates signalling by binding to and bringing pairs of TGFβRI and TGFβRII receptors into close proximity (see figure 1.9). Two models of ligand binding have been observed (Franzen *et al.*, 1993). The first is known as sequential binding, and

involves direct binding of the ligand to TGFβRII and then subsequent interaction of this complex with the TGFβRI, which is recruited to form a receptor-ligand complex



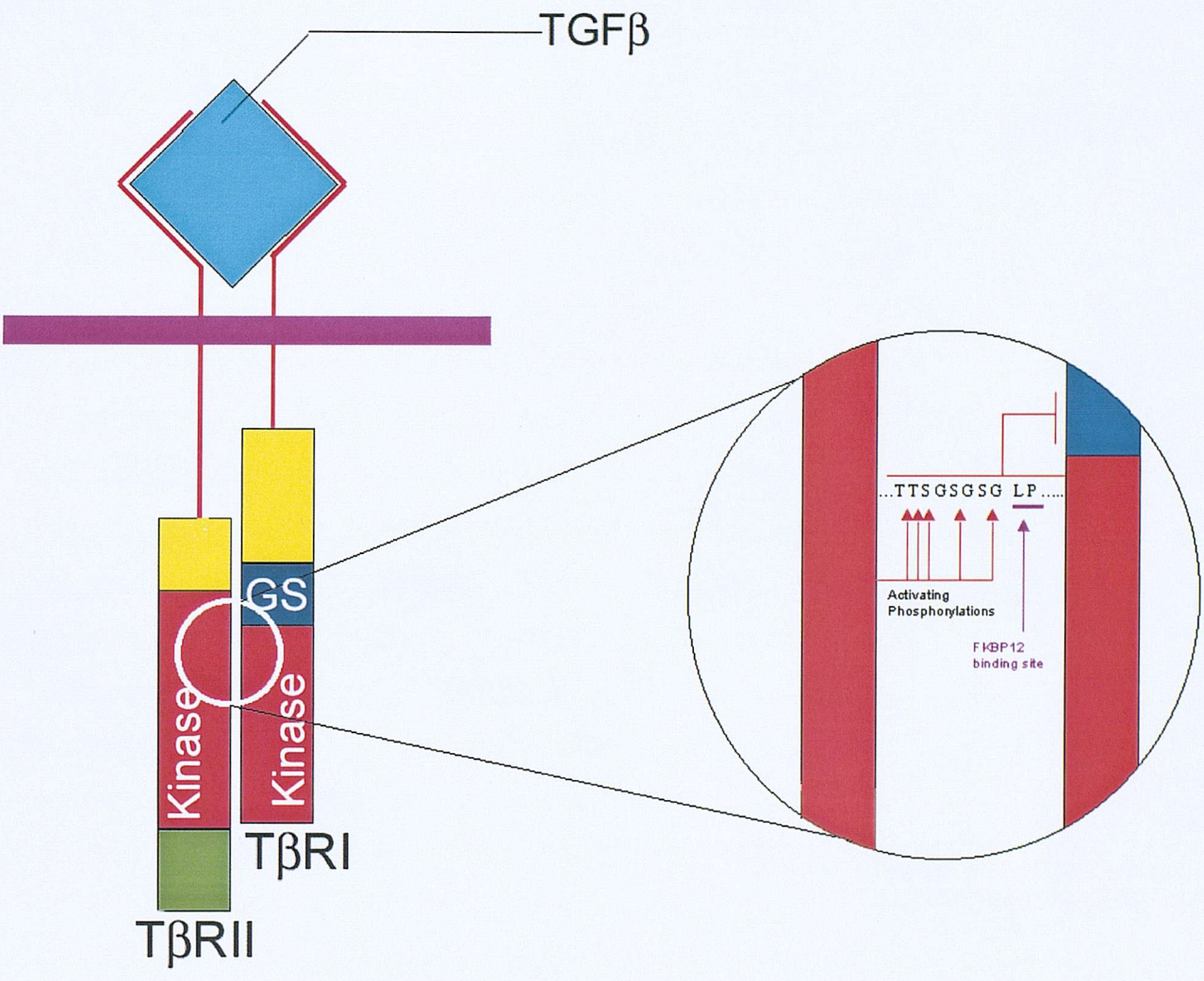


Figure 1.9 TGFβ receptor dimerization. The TGFβ ligand brings the TβRI and the TβRII receptors in close proximity. The kinase domain of TβRII is able to phosphorylate threonine and serine residues found in the GS domain, which precedes the kinase domain of TβRI *(adapted from Massague 1998).*

(Massague 1998). Type I receptors can only recognise ligand that is bound to type II receptor, but not free ligand. The second model, termed the cooperative binding model, is more typical of BMP receptors. It involves TGFβRI and TGFβRII molecules that bind ligand with high affinity when expressed together, but low affinity when expressed separately (Souchelnytskyi *et al.,* 1996).

As well as the two signalling receptors, type III receptors have also been identified and correspond to one of two related proteins, betaglycan or endoglin (Cheiftez *et al.* 1988). The evidence seems to suggest that TGFβRIII molecules do not have signalling functions, but regulate TGFβ access to the signalling receptors (Lastres *et al.,* 1996).

TGFβ binding activity has been demonstrated in separate N-terminal and C-terminal domains of the extracellular region of betaglycan. It is thought that the entire extracellular region of betaglycan may be shed into the medium, and hence may be acting as an antagonist; impairing the ability of TGFβ to bind to membrane-bound receptors (Massague 1998). Betaglycan is able to bind all three isoforms of TGFβ with high affinity and facilitates TGFβ binding to the TGFβRII, hence forming a TGFβ/betaglycan/TGFβRII complex. This activity is most evident in TGFβ2 signalling. Although TGFβ2, signals through TGFβRI and TGFβRII (like TGFβ1 & 3), an additional characteristic of TGFβ2 is that is has low intrinsic affinity for TGFβRII and is less potent than TGFβ1 in haemopoietic progenitor cells, myoblasts and endothelial cells that lack betaglycan. Experiments have shown that transfection of betaglycan into these cells augments TGFβ2 binding and activity in these cells (Lopez-Casillas *et al.,* 1993).

The related protein endoglin is expressed at high levels in endothelial cells and at lower levels in other cell types. Endoglin binds TGFβ1 and TGFβ2 but not TGFβ3. Similar to betaglycan, complexes between endoglin and TGFβ receptors have been observed (Lastres *et al.,* 1996).

1.5.3 Signalling pathways via SMAD proteins

SMAD proteins are responsible for relaying signals from cell surface receptors to the nucleus. They are molecules of relative molecular masses between 42kDa – 60kDa, with two regions of homology at the amino and carboxyl terminals, termed the Mad-homolgy domains MH1 and MH2. It has been shown that when in their inactive configurations, the MH1 and MH2 domains of SMAD proteins make contact with each

other (Massague 1998). After activation by receptors, the molecules alter conformation and form hetero-oligomeric complexes and translocate to the nucleus where the transcription of target genes is effected (Massague 1998).

Different SMAD proteins have different roles in signalling and the proteins, which are phosphorylated depending on the ligands that they bind to. There are three main Smad subfamilies: pathway restricted Smads (Smad1, Smad2, Smad 3 and Smad 5) common mediator Smad (Smad 4) a and inhibitory Smads, (Smad 6 and Smad 7) (Massague 1998).

Smads 1, 2, 3 and 5 are thought to interact with and become phosphorylated by specific type I serine/threonine kinase receptors. In response to TGFβ stimulation, Smad 2 and Smad 3 are phosphorylated and translocated to the nucleus. There is also some evidence to suggest that TGFβ induces the phosphorylation of Smad 1. Type I receptors are thought to activate the pathway restricted Smads by phosphorylation of the two most C terminal serine residues on the characteristic ser-ser-X-ser (SSXS) motif (see figure 1.10). The pathway-restricted Smads bind directly to type I receptors. This was demonstrated in an experiment in which Smad 2 or Smad 3 were co-immunoprecipitated with the type I / type II receptor complex. It is thought that Smad proteins are released from the receptors after phosphorylation (Massague 1998).

In mammalian cells, Smad 4 forms complexes with Smad 2 and Smad 3 after activation of TGFβ. This is due to the inability of Smad 4 to directly bind or be phosphorylated by TGFβ, since it lacks the C-terminal SSXS motif (Massague 1998).

Recent studies have shown that accessory proteins also interact with type I and type II receptors and Smad proteins. A classic example is Smad anchor for receptor activation (SARA), a cytoplasmic protein that specifically forms a bridge between the receptor complex and the non-phosphorylated Smad 2. Linkage in this way allows phosphorylation of Smad 2 by type I receptors. The interaction of SARA and non-phosphorylated Smad 2 also inhibits the nuclear import of non-activated Smad2 (figure 1.11). (reviewed in Attisano and Wrana 2002).

1.5.4 Inhibitory Smads

Smad 6 and Smad 7 are structurally different from members of other Smad subfamilies. Smad 6 forms stable associations with type I receptors, and interferes with the phosphorylation of Smad 2 and the subsequent heterodimerization with Smad 4. Smad 7

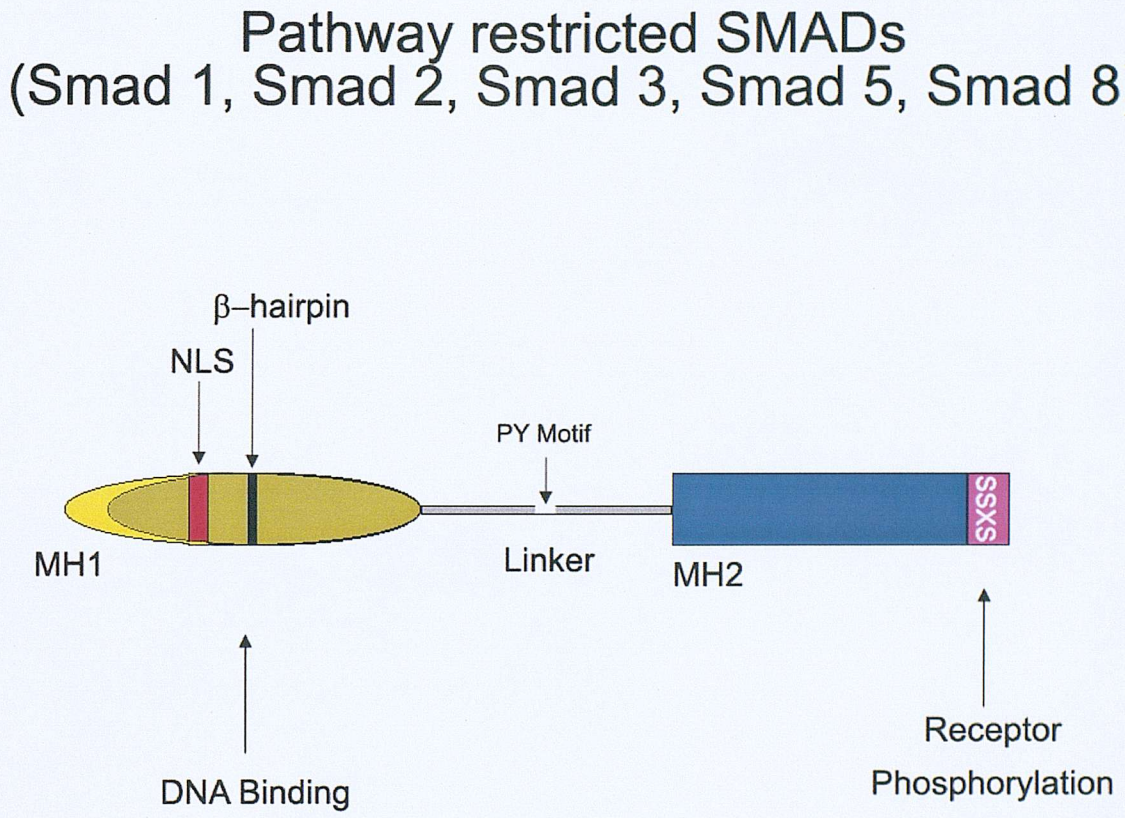


Figure 1.10 The general structure of pathway restricted Smads showing the different domains and regions of interest.

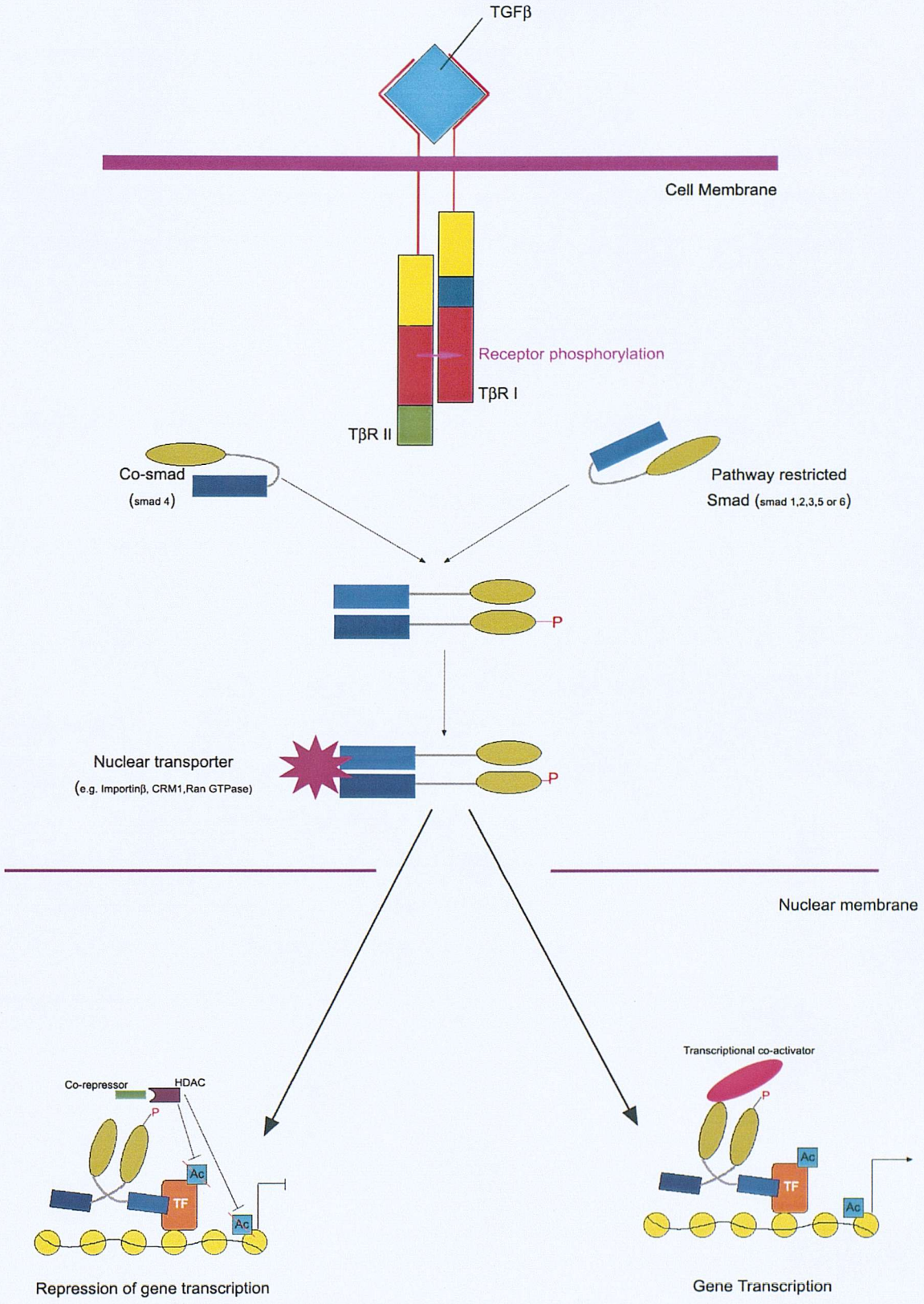


Figure 1.11 The Smad signalling pathway. TGFβ ligands brings the TβRI and TβRII receptors together. Phosphorylation of TβRI results in activation of pathway restricted Smads. The pathway restricted smads then interact with smad4, which becomes phosphorylated. Further interactions, firstly with nuclear transporter proteins and then either transcriptional co-activators or co-repressors results in regulation of gene expression.

has been shown to block responses mediated by TGFβ. Smad 7 is thought to associate with the TGFβ receptor complex (Dijke *et al.*, 2002).

In order for inhibitory Smads to bind to type I receptors, it is necessary for the receptor to be activated by the type II receptor kinase. Inhibitory Smads form more stable interactions with type I receptors than do the pathway restricted Smads and hence probably work by preventing pathway-restricted Smads from binding to the type I receptor in a competitive manner. Inhibitory Smad gene expression is induced by TGFβ stimulation and therefore may act as autoregulatory negative-feedback signals.

1.5.5 Other cytoplasmic signalling pathways – Non Smad dependent

It has been observed that other parallel pathways exist, which transduce TGFβ2 ligand/receptor interactions (Massague 1998). Examples of alternative pathways include members of the Ras or Rac families of small guanosine triphosphate (GTP)-binding proteins, and (TGFβ activated kinase) Tak-1, a serine/threonine kinase of the MAP kinase kinase kinase family. In certain cell types MAP kinases such as extracellular signal-regulated kinses (ERK) 1 and 2 and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK) have also been reportedly activated (Massague 1998).

The Ras GTPase functions as a transducer of cell signals from the membrane bound receptors to intracellular pathways, which control cell growth, survival and differentiation. The Ras signalling pathways are referred to as the mitogen-activated protein kinase (MAPK) cascade. The Ras/MAPK signalling pathways have the ability to cross talk and activate the Smad pathway (Massague 1998).

1.6 The effects of other growth factors on (myo)fibroblasts

1.6.1 Endothelin-1

Endothelin-1 (ET-1) is a bioactive peptide, first characterised as a potent vasoconstrictor (Yanagisawa *et al.,* 1988). However, since this early observation, it is now known to have growth-promoting properties in different cell types (reviewed in Kon and Fogo 1993). It has been shown that in cardiac tissue following myocardial infarction, ET-1 is synthesised and secreted from both cardiomyocytes and fibroblasts. In addition, there are raised plasma levels of circulating ET-1. These have potential deleterious effects on the myocardium, since ET-1, a potent vasoconstrictor, would restrict coronary flow, and also promote hypertrophy of cardiomyocytes and possibly stimulate proliferation of cardiac fibroblasts (Bogoyevitch *et al.,* 1993). Treatment with ET-1 receptor antagonists have been shown to reduce infarct size after experimental myocardial ischaemia, and to have the ability to beneficially effect ventricular remodelling (Wantanabe *et al.,* 1991).

Piacentini and co-workers (2000) studied the pathways by which ET-1 stimulates cardiac fibroblast proliferation. The aim of their study was to determine whether PKC activation was a necessary step for ET-1 induced DNA synthesis. They found that specific inhibition of protein kinase C (PKC) abolished the stimulatory effect of ET-1, and this was confirmed by the observation that down-regulation of cellular levels of PKC by prolonged incubation of the fibroblasts with the PKC activator phorbol 12-myristate 13-acetate (PMA) resulted in a mitogenic effect on both baseline DNA synthesis and cell number. Down-regulation of PKC completely abolished ET-1 induced proliferation.

The receptors for ET-1 are G-protein coupled, both ET_A and ET_B can couple G_q and G_i proteins. The α_q subunit of G_q proteins mediate a PKC-dependent, Ras-independent activation of MAPK. The observed dependence of ET-1 stimulated synthesis of DNA via PKC suggests that G_q is an upstream mediator of the response . Hansen and colleagues (1995) have shown that cardiac fibroblasts express abundant levels of G_q protein. Ju and co-workers (1998) have observed that, in a rat model of myocardial infarction, G_q is significantly up-regulated in border and scar tissue, areas which have large numbers of fibroblasts (Tamirisa *et al.,* 1999).

In fibroblasts, Shahar and colleagues (1999) investigated the proliferative effects of ET-1 on alveolar fibroblasts obtained from the bronchial explants of normal patients, sarcoidosis patients and patients with interstitial pulmonary fibrosis (IPF). They found that

the alveolar macrophages from the patients with IPF secreted significantly greater levels of ET-1 than the macrophages from the control or sarcoidosis patient groups. They also showed that *in vitro* exposure of alveolar fibroblasts to ET-1 promoted DNA synthesis and proliferation. The use of neutralising ET-1 antibodies decreased [^{3}H] thymidine incorporation in the alveolar fibroblasts cultured with the alveolar macrophage supernatants.

Dube and co-workers (2000) investigated the role of ET-1 on bronchial fibroblasts obtained from five normal and seven asthmatic subjects. They showed that the combination of ET-1, PDGF and TGFβ1 resulted in mitogenesis of both the asthmatic and normal fibroblasts. This study was conducted on the basis of a previous study in which they observed a decreased baseline DNA synthesis in the asthmatics when compared to normals (Dube *et al.,* 2000). They postulated that a combination of mediators released following inflammatory insult could activate the asthmatic fibroblasts to proliferate. Their reported observations of decreased DNA synthesis in asthma (a fibrotic disease) is contradictory to observations made by others in fibrotic diseases (Jordana *et al.,* 1988).

Yang and colleagues (1998) have shown that ET-1 is a mitogenic agent for human smooth muscle cells and works by potentiating the mitogenic effect of PDGF, resulting in up to 70-fold increase in proliferation of human aortic smooth muscle cells in response to PDGF. By use of selective receptor antagonists, Yang and colleagues determined that the potentiation of cell growth by ET-1 occurred mainly through the ET_A receptor (Yang *et al.,* 1999).

To date ET-1 has been shown to be an important growth factor with its ability to potentiate the mitogenic properties of other growth factors such as PDGF and epidermal growth factor (EGF).

1.6.2 Proliferation via EGF receptor activation

1.6.2.1 The EGF receptor

The epidermal growth factor receptor (EGFR) subfamily consists of four closely related receptors, the EGFR (also known as ErbB1), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4) (reviewed in Zwick *et al.,* 1999).

On stimulation, the EGFR family members can form homo- or heterodimer complexes, which lead to phosphorylation of a specific set of tyrosine residues on the SH2 domain of the EGFR. Adaptor proteins (e.g. SHC or Grb2), which have a proline rich SH3 domain, bind to the SH2 domain of the EGFR, linking the receptor tyrosine kinases to

downstream signalling events (Hackel *et al.,* 1999). There are a number of different growth factors that have been characterised, and are separated into groups according to their ability to bind and activate distinct hetero or homodimers of this family (Riese *et al.,* 1998). It is believed that ErbB2 plays a central role in receptor dimerisation, since it has been observed to be the preferred heterodimerisation partner for the other three receptors (ErbB1,3,4) (Graus-Porta *et al.,* 1997).

The large number of EGF-like ligands, each with their characteristic signalling cascade, and the variety of heterodimers formed (on activation) between these and EGFR family members are all thought to contribute to the complexity and diversity of EGFR stimulated signalling (Zwick *et al.,* 1999).

It has become evident in recent years that EGFR, rather than being a receptor signalling in response only to EGF-like ligands, has a more central role in the cellular network. The EGFR has been shown to be capable of integrating diverse signals, such as stimuli from G-protein coupled receptor (GPCR) ligands, cytokines, cell adhesion and cellular stress (Zwick *et al.,* 1999; Hackel *et al.,* 1999).

1.6.2.2 EGFR transactivation induction by G-protein coupled receptors

G-protein coupled receptors are the largest group of cell-surface receptors and consist of seven transmembrane domains with an amino extracellular terminus and an intracellular carboxyl terminus (Zwick *et al.,* 1999; Gutkind 1998). A variety of stimuli are able to induce the conformational changes in the transmembrane helices that result in activation and signalling via the G protein.

It has been shown that stimulation of various GPCRs rapidly induces tyrosine phosphorylation of Shc proteins, resulting in the formation of a Shc/Grb2 complex and subsequent activation of the ERK/MAP kinases (Bonfini *et al.,* 1996). This initial observation led Bonfini and colleagues (1996) to suggest the involvement of one or more tyrosine kinases in GPCR-mitogenic signalling. This suggestion was later supported when Daub and co-workers (1997) showed that EGFR was an essential signal transduction element of GPCR-mediated mitogenic signalling. They showed that specific inhibition of EGFR with AG1478, and use of a dominant negative receptor mutant diminished the GPCR activated mitogenic signal. EGFR and ErbB2 became rapidly tyrosine phosphorylated upon stimulation of Rat-1 cells with the GPCR agonists endothelin-1, lysophosphatidic acid (LPA) and thrombin. These observations were also noted in cell types other than Rat-1 cells, namely keratinocytes, primary astrocytes and PCI2 cells

(Zwick *et al.,* 1997; Daub *et al.,* 1997). It has been shown that Lysophosphatidic acid (LPA) is able to induce PDGFR-β tyrosine phosphorylation in cells which lack the EGFR (Ullrich and Schlessinger 1990), suggesting that transactivation of distinct receptor tyrosine kinases can contribute to GPCR mediated mitogenic signalling (Zwick *et al.,* 1999).

1.6.2.3 EGF receptor activation by G-protein coupled receptors

Prenzel and colleagues (1999) have shown that the mechanism by which the EGF receptor is transactivated is via the activation of an EGF ligand. This involves cleavage of pro heparin binding (HB)-EGF by a metalloproteinase that is rapidly activated upon GPCR-ligand interaction (see figure 1.12)

HB-EGF is a member of the EGF family and has the ability to bind heparin sulphate proteoglycans . This prevents the immediate release of the growth factor and increases the local growth factor concentration in the cellular microenvironment. (CRM197 is able to specifically inhibit the mitogenic effect of HB-EGF (Mitamura *et al.,* 1995). Prenzel and colleagues (1999) found that pre-treatment with CRM197 completely inhibited tyrosine phosphorylation of the EGFR induced by LPA and other GPCR ligands. They also found that the pre-treatment reduced SHC tyrosine phosphorylation in cos-7 cells, suggesting that the GPCR activation was coupled to Ras-dependent signalling pathways . However, SHC tyrosine phosphorylation, following stimulation by EGF, was unaffected by pre-incubation with CRM197.

1.6.2.4 Transactivation of the EGFR in fibrosis

The first and, to date, only *in vivo* indication of EGFR transactivation by GPCR ligands has been demonstrated in a model of cardiac hypertrophy. Asakura and co-workers (2002) have shown that when cardiac myocytes were stimulated by GPCR ligands i.e. ET-1, HB-EGF resulting from metalloproteinase activation, resulted in EGFR activation leading to myocyte proliferation and cardiac hypertrophy. They used a specific inhibitor of HB-EGF shedding, KB-R7785, which blocked the increased proliferation. They cloned **a** **d**isintegrin **a**nd **m**etalloproteinase 12 (ADAM 12) as a specific enzyme responsible for shedding HB-EGF. Using a dominant negative expression construct of ADAM12, they found that on stimulation of the GPCR with ET-1, the mitogenic signal was abolished, suggesting that the inhibition of ADAM12 blocked HB-EGF shedding (See figure 1.12).

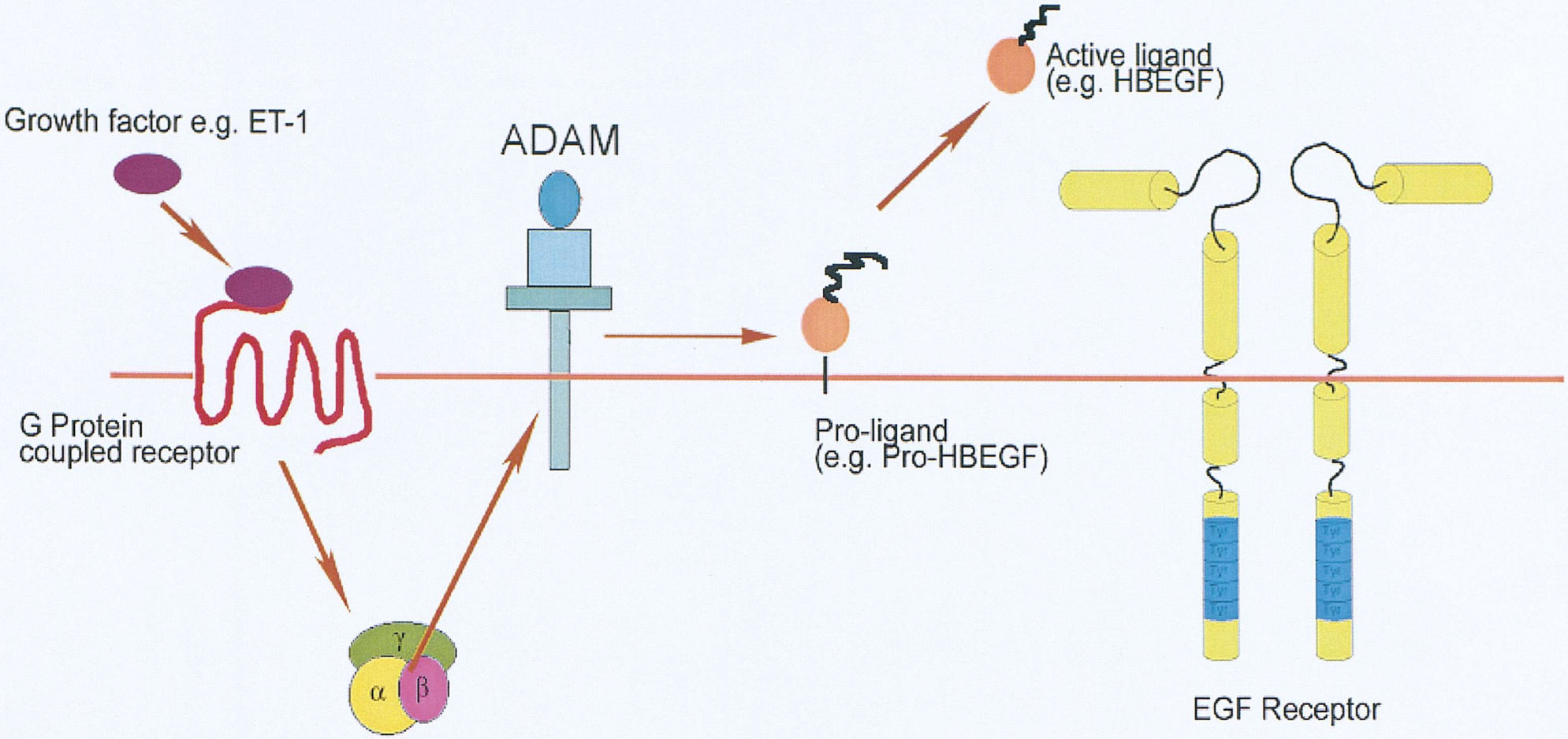


Figure 1.12 The transactivatioin of the EGFR by G-protein coupled receptors. A growth factor (e.g. endothelin-1, angiotensin), binds to the membrane bound G-protein coupled receptor. The activated G-proteins are thought to activate a zinc dependent metalloproteinase (ADAM or MMP). The MMP then cleaves a membrane-bound pro-ligand (e.g. pro-HBEGF) to form an active ligand. The activated EGFR ligand is then able to activate the EGF receptor and initiate EGFR signalling.

1.6.3 Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) is a cysteine-rich, heparin binding, 38kDa protein, originally identified in conditioned media from human umbilical vein endothelial cells (reviewed in Grotendorst 1997).

It is thought that the growth stimulatory action of TGFβ appears to be mediated via an indirect mechanism involving CTGF. Igarashi and colleagues (1993) were the first to demonstrate that CTGF was expressed in fibroblastic cells, in response to TGFβ. The CTGF protein was found to be related to a number of serum-induced immediate early gene products including cyr61 (Grotendorst 1997), fisp12 (Ryseck *et al.,* 1991) and nov (avian oncoprotein) (Joliot *et al.,* 1992). These peptides are characterised by 38 conserved cysteine residues which constitute more than 10% of their total amino acid content. This family of peptides is referred to as the CCN family (**C**TGF/fisp12, **c**ef/10/cyr61 and **n**ov) (Bork 1993).

The domain structure of CTGF provides clues as to what the whole function of this protein may be. CTGF (and the related peptides) contains four distinct modules, all of which are encoded by separate exons (reviewed in Bork 1993). Exons 3 and 4 are separated by a 'linker'domain which is cysteine-free. The two domains either side of the linker region, encoded by exons 3 and 4, are cysteine rich (22 in the amino terminal domain, exon 3 and 16 in the carboxyl domain, exon 4) and seem to be independent of each other with regards to intramolecular disulphide bridges. Experiments conducted by Grotendorst and colleagues (1996), have shown that limited digestion with proteases cleaves in the cysteine-free linker region and allows isolation of pure individual amino and carboxyl terminal domains without denaturing of the protein or reducing of disulphide bridges. Use of extensive proteolytic digestion with proteases fails to cleave the domains to smaller fragments, suggesting that the domains themselves are highly cross linked by disulphide bridges, which are known to be resistant to proteolytic cleavage (Grotendorst *et al.,* 1996; Grotendorst 1997).

It is thought that the two domains have very different functions. The amino domain has two motifs, which are thought to be involved in binding to other growth factor peptides. Adjacent to the signal peptide in the amino terminal is a sequence that is almost identical to a motif found in the low molecular weight insulin-like growth factor binding proteins (IGFBPs) (Bork 1993). This motif, GCGCCxxC, which is present in all members of the

CCN family, is conserved in most IGFBPs and in involved in IGF binding (Kiefer *et al.,* 1991). This IGF binding motif is encoded in exon 2 of the CCN gene found in the vertebrate members of this family. The adjacent domain is related to a von Willebrand factor type C repeat (VWC) (reviewed in Bork 1993), which contains a series of cysteine-rich repeat motifs, that are also found in procollagen and thrombospondin. It is thought that this motif may bind other growth factors (Piccolo *et al.,* 1996).

The carboxyl terminal domain is thought to be the signalling domain. This domain contains other motifs which are involved in binding to heparin, and also share a disulphide linkage pattern with PDGF, TGFβ and nerve growth factor (NGF). The first domain of the carboxyl terminal, after the linker region, is related to a domain identified originally in thrombospondin-1, known as the thrombospondin type I repeat sequence. This sequence has been found to be present in several extracellular matrix proteins (Bork 1993). One activity that has been ascribed to this domain is interaction with sulphated glycoconjugates (Holt *et al.,* 1990). This is similar to other heparin-binding growth factors such as acidic and basic fibroblast growth factor (FGF), and heparin-binding EGF (HB-EGF). The carboxyl terminal is responsible for this binding, as it can be separated from the amino-end of the peptide and still bind heparin (Grotendorst 1997).

The final motif of the carboxyl terminal, contains a spacing and six cysteine residues found in growth factors such as TGFβ, PDGF and NGF and is termed 'the cysteine knot motif' (Murray-Rust *et al.,* 1993). It is thought that this motif is responsible, in part, for a planar conformation of these growth factor subunits, allowing the proteins to form dimers (Murray-Rust *et al.,* 1993).

The dimeric molecules of PDGF and TGFβ are stabilised by the interaction of disulphide bridges, however no such disulphide bridges are found in CTGF. The similarities with other growth factors suggest that the motif in this domain could be involved in receptor binding or signalling (reviewed in Grotendorst 1997).

1.6.3.1 Connective Tissue Growth Factor gene expression

The CTGF gene is strongly induced by TGFβ1 but has not been shown to be induced to any significant level by other growth factors, such as EGF, FGF, PDGF and IGF (Grotendorst *et al.,* 1996). This difference is thought to be due to the different signal transduction pathways activated by TGFβ (see section 1.4.1) compared to the tyrosine kinase receptors for EGF, FGF, PDGF and IGF (Ullrich and Sclessinger 1990).

The kinetics of CTGF gene expression are very different from the kinetics of other growth factor inducible genes e.g. c-fos, c-jun and c-myc, which are expressed over a period from 30 minutes to 6 hours after growth factor stimulation of cells (Grotendorst 1997). However, in the case of TGFβ, a prolonged activation occurs, which lasts for 24-36hours after addition of TGFβ to the cells, and CTGF is unique in the CCN family in exhibiting this pattern of expression after TGFβ stimulation (Grotendorst 1997).

Analysis of the nucleotide sequence of the human CTGF promoter reveals multiple elements that are characteristic of other growth factor or serum-inducible genes, including a CArG box, AP-1 and SP-1 binding sites. Transforming growth factor β can activate the expression of c-jun and junB, and its own production through AP-1 sites. However, these sites do not appear necessary for the TGFβ-induced transcription of CTGF. The CTGF promoter has nucleotide sequences similar, but not identical, to two other reported TGFβ control elements, namely Nuclear factor (NF)1 and TGFβ inhibitory elements (TIEs). The NF1 element appears to act as part of the regulatory sequences for the control of α2 collagen I gene by TGFβ (Oikarinen *et al.,* 1987) and the TIEs are present in a number of genes, such as the stromolysin gene promoter, where this element acts to repress transcription in response to TGFβ (Kerr *et al.,* 1990).

The sequence commonly recognised as the TGFβ-regulatory element (TRE) in the human CTGF promoter is not present in any other CCN family member. The fact that no other TGFβ regulated gene (e.g. collagen, PAI-1 and fibronectin) contain this or any similar sequence in the promoter region suggests that the regulation of CTGF gene expression by TGFβ may be via a mechanism distinct from other TGFβ regulated genes. Proteins that bind to this sequence are constitutively expressed in the nucleoplasm of fibroblasts (Grotendorst 1997).

Studies by Heusinger-Ribeiro and colleagues (2001) have shown that, in human renal fibroblasts, the expression of CTGF is regulated by RhoA and cyclic AMP (cAMP). They also showed that lysophosphatidic acid (LPA) is another potent inducer of CTGF gene expression that was regulated by RhoA. TGFβ mediated induction of CTGF was shown to be strongly reduced when RhoA signalling was inhibited by a Rho kinase inhibitor. Rho proteins are also involved in the regulation of the actin cytoskeleton through the Rho-associated serine/threonine kinase enzymes (Rho kinases), which regulate the polymerisation of stress fibres via inactivation of myosin light chain phosphotases (Kimura *et al.,* 1996). Inhibition of these kinases with specific inhibitors also diminished CTGF

induction, suggesting a possible link between cytoskeletal integrity and the induction of CTGF (Kimura *et al.,* 1996).

Although, not yet clearly understood, there is much evidence to suggest that TGFβ induction of CTGF is RhoA dependent. Since TGFβ has been shown to have a role in the development and maintenance of matrix synthesis and fibrosis, targeting of RhoA may be therapeutically beneficial. Pharmacologically, hydroxymethyl glutaryl-CoA reductase inhibitors may be useful. Although primarily known for their lipid-lowering properties, these compounds have been shown to interfere with the isoprenylation and activation of Rho proteins (Kimura *et al.,* 1996; Goldstein and Brown 1990), and to inhibit CTGF expression (Eberlein *et al.,* 2001).

1.6.3.2 Studies using recombinant CTGF

Studies in which recombinant CTGF was used to stimulate fibroblasts show that it has properties that are similar to other peptide growth factors. Recombinant CTGF exhibits multiple biological activities on cultured fibroblastic cells at concentrations ranging from 1ng/ml to 40ng/ml (Frazier *et al.,* 1996).

CTGF exhibits effects very similar to those seen by TGFβ, i.e. it is able to stimulate cell proliferation in monolayer cultures of NRK fibroblasts, with its activity being enhanced by submitogenic levels of EGF (Frazier *et al.,* 1996). The activity of CTGF can be enhanced by the addition of heparin to the culture, producing effects similar to the ones reported for the mitogenic activity of FGF and HB-EGF (Damon *et al.,* 1989; Gospodarowicz *et al.,* 1990; Besner *et al.,*1992; Robinson *et al.,* 1993; Roghani *et al.* 1994).

In addition to its action as a mitogen, CTGF also stimulates type I collagen, fibronectin and α5 integrin expression in NRK fibroblasts (Frazier *et al.,* 1996). The matrix-stimulating activity of CTGF is another biological effect that CTGF has in common with TGFβ but not with other growth factors such as EGF, FGF and PDGF which do not stimulate significant ECM production (Frasier *et al.,* 1996).

Studies by Frasier and colleagues (1996) investigated the *in vivo* effects of injected PDGF, EGF, TGFβ and CTGF on tissue formation in neonatal mouse skin . They injected TGFβ into the subcuticular area of the skin in neonatal mice and observed a rapid increase in the formation of granulation tissue. The other growth factors were injected in a similar way. Histological investigation of the treated tissue showed that TGFβ and CTGF induced similar alterations of the tissue, characterised primarily by large increases in the number of

connective tissue cells and ECM material. An interesting observation was that although TGFβ treatment resulted in a significantly reduced number of hair follicles at the injection site, CTGF did not cause this phenomenon. This suggested that CTGF did not have a growth inhibitory effect on epidermal cell types. Injection of basic PDGF produced only very mild tissue granulation, whereas administration of EGF produced no detectable alteration in the connective tissue of the dermis.

This study showing differences of action on epidermal cell types between TGFβ and CTGF was one of the first studies to show that CTGF and TGFβ do not share all the same biological effects. Further *in vitro* studies have shown that CTGF is unable to substitute TGFβ to stimulate anchorage-independent growth for fibroblastic cells in agar (Frazier *et al.,* 1996). These led to hypotheses that CTGF may be a downstream mediator of TGFβ for certain biological actions.

A number of observations have led researchers to believe that there are CTGF-dependent and CTGF-independent signalling pathways activated by TGFβ in fibroblasts. It may also be possible that some cellular responses require a synergistic action of TGFβ-dependent and TGFβ independent pathways to produce an effect (Frazier *et al.,* 1996).

Duncan and colleagues (1999) showed that CTGF mediated TGFβ induced collagen synthesis, and that this was downregulated by cAMP. They showed that blockade of CTGF with neutralising antibodies, or inhibition of CTGF synthesis by gene repression, significantly reduced fibroblast collagen synthesis induced by TGFβ by 85-100%. This demonstrated that in the NRK-49F (rat kidney fibroblasts) cell line, collagen induction by TGFβ was exclusively dependent on a CTGF mediated pathway. Furthermore, CTGF protein synthesis must occur in order for TGFβ to up-regulate type I collagen gene expression. It is widely believed that the TGFβ mediated activation of human type VII collagen gene expression is a CTGF-independent and SMAD-dependent process (Roberts and Sporn 1993). Elevation of intracellular cAMP levels have been reported to downregulate the expression of several TGFβ inducible genes such as *c-sis, c-myc,* collagenase and laminin. It has also been proposed that cAMP may downregulate CTGF expression levels. Duncan and colleagues (1999) demonstrated that cAMP inhibits TGFβ induced collagen synthesis, in fibroblasts. This is in line with other published studies that have shown that elevation in intracellular cAMP with phosphodiesterase inhibitors inhibit fibroblast growth and collagen synthesis normally induced by serum and fibroblast activating cytokines (Berman and Duncan 1989; Duncan *et al.,* 1995).

Duncan and colleagues (1995) reported unpublished findings that 8-Br-cAMP inhibited the collagen synthesis of NRK fibroblasts cultured in 5% serum. The increase in cAMP levels may be responsible for the blocking of CTGF synthesis by TGFβ contained in the serum.

Studies by Peterson and co-workers (1993) have shown that pentoxyfilline (which causes an elevation of cAMP) inhibits collagen synthesis in animal models of hepatic fibrosis thought to be dependent on TGFβ for development. This demonstrated that, in addition to 8-Br-cAMP, other agents that elevate intracellular cAMP levels in connective tissue cells can function as effective *in vivo* inhibitors of fibrotic tissue formation.

1.6.3.3 Connective tissue growth factor and remodelling

Published data regarding CTGF suggest that after an initial release of TGFβ, newly synthesised CTGF stimulates a fibroblast chemotactic response and subsequent proliferation of fibroblasts. This expanded population can then be stimulated by paracrine/autocrine CTGF to produce enhanced quantities of collagen and other matrix components (reviewed in Roberts and Sporn 1993).

Using wound chamber experiments, it has been shown that TGFβ was maximally expressed 3 days after dermal wounding in rats, whereas CTGF peak expression occurred between 6 and 9 days (Igarashi *et al.,* 1993). The prolonged expression of CTGF and its long time course of action would potentially allow fibroblasts at the wound site to continue to accumulate, proliferate and synthesise increased quantities of collagen, or other matrix components, for a considerably long time after a brief TGFβ exposure. This may lead to long term scarring and tissue remodelling.

The involvement of CTGF in other conditions such as scleroderma has been widely reported. Scleroderma is a disease characterised by small-vessel vasculopathy and fibrosis of the skin and other organs (Trojanowska 2002). Recently, it has been shown that dermal fibroblasts derived from fibrotic lesions of patients with scleroderma produce high levels of basal CTGF, which is independent of SMAD signalling. (Holmes A *et al.,* 2001). Since CTGF independently promotes fibroblast proliferation and matrix deposition it has been postulated that basal levels of CTGF are responsible for the increased activity of fibroblasts in scleroderma.

1.7 Summary and aims of these studies

Asthma is a disease that manifests as a combination of inflammation and structural changes to the airway wall. Evidence to date has implicated the activation of an epithelial-mesenchymal trophic unit, in which an inflammation-repair cycle is set up. This causes damage to the epithelium resulting in the release of factors which activate mesenchymal cells, causing further changes in the airway wall.

The most prominent structural changes are the increased volume of smooth muscle deeper in the airway wall and a thickened *lamina reticularis* in the subepithelial basement membrane. This thickening has been attributed to the increased deposition of interstitial collagens and extracellular matrix proteins. There have been studies to suggest a correlation between the thickness of the *lamina reticularis* and asthma disease severity (Roche *et al.*, 1989). The mechanisms by which the asthmatic fibroblasts increase in number remains unexplored.

In considering other fibrotic diseases, an increase in ET-1 has been shown to result in the proliferation of cardiac fibroblasts, and CTGF has been shown to cause the proliferation of dermal fibroblasts from scleroderma patients. It is still unclear whether these growth factors have a role in stimulating the proliferation of asthmatic fibroblasts. Activation of the EGF receptor by transactivating ligands (e.g. thrombin, HB-EGF) has been shown to result in the proliferation of some fibroblast lines.

1.7.1 Hypotheses

The increased number of myofibroblasts found in the *lamina reticularis* of asthmatic fibroblasts is a result of hyperproliferation driven by production of autocrine ligands.

Asthmatic fibroblasts exhibit an exaggerated response to TGFβ2 when compared to non-asthmatic fibroblasts.

1.7.2 Aims

The main aims and objectives of these studies are:

(a) To isolate, characterise and compare (myo)fibroblasts from endobronchial biopsy tissue obtained from non-asthmatic and asthmatic subjects.

(b) To compare the immunocytochemical protein expression of these cells, to (myo)fibroblasts derived specifically from the isolated *lamina reticularis* of non-asthmatic airways at baseline and in response to TGFβ2.

(c) To determine if the hyperproliferation of asthmatic fibroblasts is due to the production and subsequent utilisation of autocrine growth factors.

(d) To characterise and compare the production of autocrine growth factors by non-asthmatic and asthmatic bronchial fibroblasts at baseline and in response to TGFβ2. Following characterisation, to determine the role of these growth factors in the pro-fibrotic response.

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Tissue Culture

i. RPMI 1640 medium (1X), liquid, without L-glutamine (Invitrogen, Paisley, UK)
ii. HBSS, Hank's Balanced Salt Solution (1X), liquid, without calcium (Invitrogen, Paisley, UK)
iii. L-Glutamine 200mM (100X) liquid (Invitrogen, Paisley, UK)
iv. Penicillin – streptomycin (100X) liquid (Invitrogen, Paisley, UK)
v. Foetal Bovine Serum (Gamma-Irradiated) Origin: E.C. approved (Invitrogen, Paisley, UK)
vi. Vitrogen 100 collagen I solution (Nutacon BV, Leimuiden, NL)
vii. Trypsin-EDTA (1X) liquid (Invitrogen, Paisley, UK)
viii. Ultraculture tissue culture medium (Biowhittaker, Wokingham, UK)
ix. DMEM tissue culture medium (Biowhittaker, Wokingham, UK)

2.1.2 Immunocytochemistry

i. Acetone : Methanol (1:1) at -20°C
ii. Hydrogen peroxide solution (30%) (Sigma, Dorset, UK)
iii. Methanol (100%) (Sigma, Dorset, UK)
iv. Avidin solution (Vector Laboratories, UK)
v. Biotin solution (Vector Laboratories, UK)
vi. 1% Bovine serum albumin (BSA) solution (Sigma, Dorset, UK)
vii. Tris-buffered saline buffer (see appendix A)
viii. Streptavidin-biotin complex solution (Dako, Bucks, UK)
ix. Sodium Azide solution (1%) (Sigma, Dorset, UK)
x. Harris' Haematoxylin (Sigma, Dorset, UK)
xi. 1% Acid alcohol (Sigma, Dorset, UK)
xii. 70% ethanol (Sigma, Dorset, UK)
xiii. Absolute alcohol (Sigma, Dorset, UK)

xiv. Xylene (Sigma, Dorset, UK)

xv. DPX mounting agent (Sigma, Dorset, UK)

xvi. 3,3'-diaminobenzidine chromogen solution (DAB; Vector laboratories, UK)

2.1.2.1 Antibodies

i. Mouse anti-human vimentin antibody (Sigma, Dorset, UK)

ii. Mouse anti-human desmin antibody (ICN Pharmaceuticals Inc. Ohio, USA)

iii. Mouse anti-human α-smooth muscle actin antibody (Sigma, Dorset, UK)

iv. Mouse anti-human heavy chain myosin (Sigma, Dorset, UK)

v. Rabbit anti-human Muscarinic M_2 receptor (R&D antibodies, Berkley, USA)

vi. Rabbit anti-human SM-22 (generous gift, Dr Julian Solway, Chicago, Illinois, USA)

vii. Biotinylated sheep anti-mouse antibody (Dako, Bucks, UK)

viii. Biotinylated swine anti-rabbit antibody (Dako, Bucks, UK)

ix. FITC conjugated sheep anti-mouse antibody (Dako, Bucks, UK)

x. FITC conjugated swine anti-rabbit antibody (Dako, Bucks, UK)

2.1.3 Proliferation and mitogenesis studies

i. 1% methylene blue in borate buffer solution (see appendix A)

ii. Formal saline solution (see appendix A)

iii. 1:1 0.1M HCl : Absolute alcohol

iv. *methyl* -^{3}H-Thymidine, 37MBq, 1mCi, Aqueous solution sterilised (Amersham Biosciences, Bucks, UK)

v. Microscint-40 Topcount scintillation cocktail (Packard Instrument Company, Meriden, USA)

vi. 0.2M NaOH

vii. 5% TCA

viii. 5mM 5'fluoro-2'deoxyuridine (FudR; Sigma, Dorset.UK)

ix. 40% Ethanol

2.1.4 RNA isolation, quantification and reverse transcription

i. RNase free DEPC-treated water
ii. TRIzol Reagent (Invitrogen, Paisley, UK)
iii. Molecular grade chloroform (Sigma, Dorset, UK)
iv. Molecular grade isopropyl alcohol (Sigma, Dorset, UK)
v. Molecular grade ethanol (80% in DEPC treated water)
vi. High purity analytical grade agarose (Bio-rad, UK)
vii. Ethidium bromide (Sigma, Dorset, UK)
viii. Ribogreen RNA quantification reagent (Molecular Probes, NL)
ix. TRIS-EDTA buffer (Invitrogen, Paisley, UK)
x. Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK)
xi. 18S ribosomal RNA primers and fluorogenic Taqman® probe pre-developed assay reagent (Perkin Elmer, Warrington, UK)
xii. Gene specific sense and anti-sense primers and fluorogenic Taqman® probes (MWG-Biotech, Germany)

2.1.5 ELISA, total protein quantification and Western blotting

i. TGFβ1 E_{max} ImmunoAssay system (Promega, Hants, UK)
ii. Dulbecco's phosphate buffered saline (DPBS; see appendix A)
iii. 1M Hydrochloric acid (Sigma, Dorset, UK)
iv. 1M Sodium hydroxide (Sigma, Dorset, UK)
v. Tris buffered saline with Tween® 20 wash buffer (TBST; see appendix A)
vi. Carbonate coating buffer (sodium bicarbonate and sodium carbonate; see appendix A)
vii. Quantikine Endothelin-1 ELISA system (R&D systems, Abingdon, UK)
viii. Denaturing lysis buffer (see appendix A)
ix. Micro BCA protein detection assay (Pierce, UK)
x. 10% SDS solution (Sigma, Dorset, UK)
xi. Methanol (Sigma, Dorset, UK)
xii. 10% Ammonium persulphate (Sigma, Dorset, UK)
xiii. N,N,N,N'-tetramethylenediamine (TEMED; Sigma, Dorset, UK)

xiv. Water saturated propanol (Sigma, Dorset, UK)

xv. Western blot running buffer (see appendix A)

xvi. Protein molecular weight markers (Amersham Pharmacia, Bucks, UK)

xvii. Western blot transfer buffer (see appendix A)

xviii. Protein loading buffer (Invirogen, Paisley, UK))

xix. Protease cocktail inhibitor tablets (Roche Diagnostics, UK)

xx. Dulbecco's phosphate buffered saline with Tween® (DPBST; see appendix A)

xxi. Mouse anti-human connective tissue growth factor antibody (R&D systems, Abingdon, UK)

xxii. ECL Plus chemiluminescent chromogen (Amersham Pharmacia, Bucks, UK)

2.1.6 Treatment compounds

i. Transforming growth factor β1 human recombinant (Sigma, Dorset, UK)

ii. Transforming growth factor β2 human recombinant (Sigma, Dorset, UK)

iii. Fluvastatin (Calbiochem, UK)

iv. Human recombinant CTGF protein (Western blot positive control; R&D, Abingdon, UK)

v. Endothelin-1 human, Porcine derived (Sigma, Dorset, UK)

vi. N-Acetyl-[D-Trp^{16}]-Endothelin-1 fragment 16-21 (Sigma, Dorset, UK)

vii. BQ788 (Sigma, Dorset, UK)

viii. Epidermal growth factor, human recombinant (Peprotech, UK)

ix. Epidermal growth factor receptor Tyrophostin, AG1478 (Calbiochem, UK)

x. Basic fibroblast growth factor human recombinant (Peprotech, UK)

xi. Broad spectrum matrix metalloproteinase inhibitor, GM6001 (Calbiochem, UK)

xii. Diphtheria toxin analogue, CRM197 (Sigma, Dorset, UK)

xiii. Monoclonal anti-TGFβ1, β2 and β3 antibody (R&D systems, Abingdon, UK)

xiv. Specific anti-TGFβ1 neutralising antibody, (generous gift, Cambridge Antibody Technologies, Cambridge, UK)

xv. Specific anti-TGFβ2 neutralising antibody, (generous gift, Cambridge Antibody Technologies, Cambridge, UK)

xvi. Irrelevant IgG antibody control, (generous gift, Cambridge Antibody Technologies, Cambridge, UK)

2.1.7 Subjects and tissue samples

All patient recruitment, clinical characterisation and biopsy procedures were carried out by trained clinical nurses and physicians at the University of Southampton, Division of Respiratory Cell and Molecular Biology, Southampton General Hospital.

Asthmatic subjects were characterised according to pulmonary function, symptoms and medication. Severity was assessed in accordance with the GINA guidelines on the diagnosis and management of asthma (1995).

Bronchial biopsies were provided for this study by fibreoptic bronchoscopy from nine asthmatic and six non-asthmatic individuals. All subjects were non-smokers and were free from respiratory tract infections for a minimum of four weeks prior to inclusion into the study. Written consent was obtained from the subjects and the work was ethically approved by the Joint Ethics Committee of Southampton University and General Hospital.

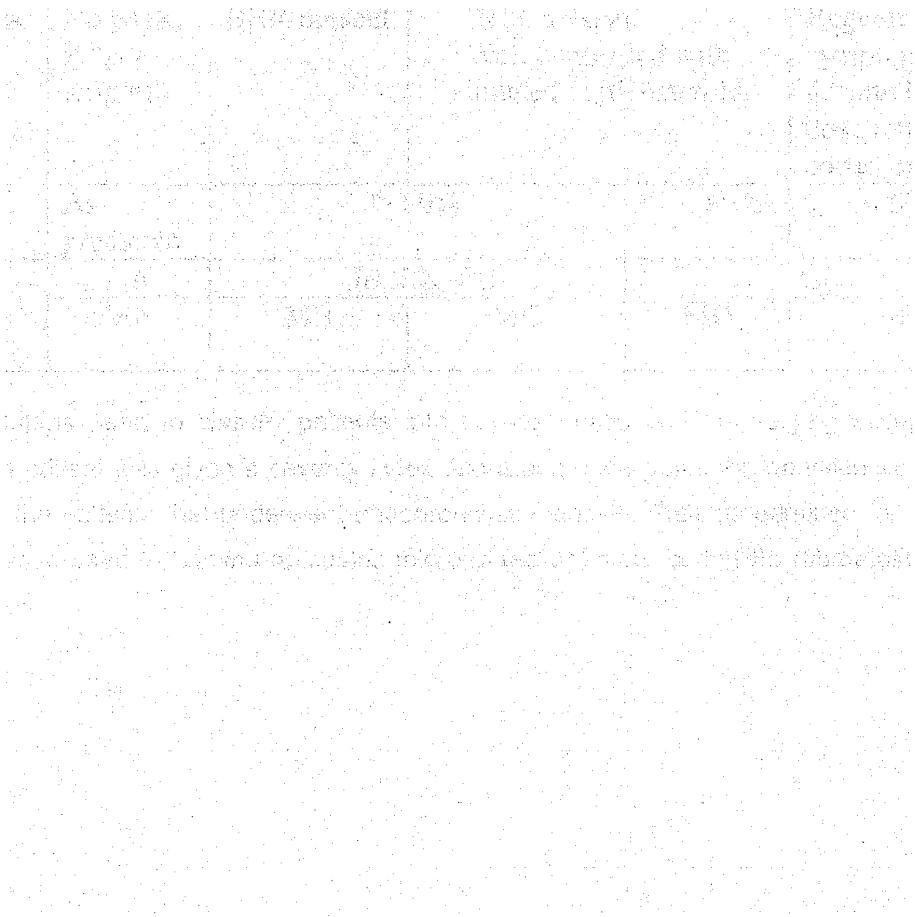
All subjects were tested for atopy by skin-prick tests, using a panel of common aero-allergens including house dust mite extract (HDM), grass pollen, tree pollen, cat dander, dog dander, *candida*, *aspergillus*, positive control (histamine) and negative control (saline). Subjects positive to HDM (determined by a wheal response greater than 3mm in diameter) were selected for this study.

Non-asthmatic subjects were those who were non-atopic and performed at their predicted forced expiratory volume (FEV_1) scores. They were also challenged with histamine and assessed by their PC_{20} (the concentration of histamine required to reduce FEV_1 by 20%). Those with a PC_{20} above 8mg/ml were considered to be non-asthmatic. Table 2.1 shows the phenotype criteria for all patients.

For the microdissection of the *lamina rectiularis* derived fibroblasts, 3 non-asthmatic subjects admitted for surgery, for lobectomy procedures due to lung cancer were recruited. Written consent was obtained to use their tissue for studies into the pathogenesis of respiratory disease. Biopsies were taken from third generation bronchi by Prof W R Roche, University of Southampton, Pathology Department, Southampton General Hospital.

Asthmatic patients were further subdivided into mild, mild to moderate and severe asthmatic. Table 2.2 indicates the clinical characteristics of each subject studied. Bronchial biopsies were obtained using a fibreoptic microscope (Olympus FB-200, Tokyo, Japan)

and removed with alligator forceps, according to standard published protocols (Richter *et al.,* 2001). After an overnight fast, asthmatic subjects received pre-medication with nebulised salbutamol (2.5mg) and intravenous atropine (0.6mg). Light sedation was administered (Midazelam 0.5mg i.v.). Finally, local oropharynx and lower airways anaesthesia was achieved by topical application of 10% Lignocaine spray and 1% Lignocaine solution respectively. The bronchial biopsies were obtained by using alligator forceps.



	Non-asthmatic	ASTHMATIC			
		Intermittent	**Mild Persistent**	**Moderate Persistent**	**Severe Persistent**
Medication	None	Short acting β_2-agonists	Short acting β_2-agonists ***and*** Inhaled corticosteroids	As 'mild persistent' ***or*** Long-acting β_2 agonists	Long acting β_2 agonists ***or*** High dose inhaled corticosteroids (>800mcg/day) ***or*** Oral corticosteroids
Symptoms	No BHR, PC_{20} > 8mg/ml	BHR present	BHR present. Well controlled with inhaled corticosteroids		Regular symptoms despite high dose inhaled corticosteroids
FEV_1	As predicted	> 80%		61 – 80%	< 60%
Severity Index Codes	GA	GB, EX, FG		GC	
	MA	MB	MC	MD	ME

Table 2.1. Criteria used to classify patients into non-asthmatic and the varying severities of asthma. Each patient was given a severity index and a numerical value for identification. These apply only to the patients that underwent endobronchial biopsies. Patients admitted for surgical lobectomy (tissue used for *lamina reticularis* microdissection) were coded Fb (fibroblast) and a numerical value.

Patient Code	Gender	Age	FEV_1 (% of predicted)	PC_{20} (mg/ml)
GA7	M	20	96%	> 32
GA8	M	21	104%	> 32
GA9	M	20	118%	> 32
GA10	M	21	93%	> 32
GA11	F	20	97%	> 32
GA12	F	21	97%	> 32
EX28	F	29	92%	2.3
EX32	F	35	79%	8.5
GB9	M	34	79%	0.7
GB14	F	20	83%	5.7
GB15	F	20	75%	1.5
GC7	M	42	96%	4.5
GC8	M	26	78%	1.17
GC10	F	21	70%	0.50
MD2	M	26	78%	1.17
Fb21	M	60	N/A	N/A
Fb25	M	65	N/A	N/A
Fb26	M	63	N/A	N/A

Table 2.2: shows the clinical characteristics of each subject used in this study.

2.1 Methods - Isolation and culture of primary fibroblasts

2.2.1 Endobronchial biopsy derived fibroblasts

Six submucosal biopsies from each subject were cut into pieces using sterile scalpel blades, then placed in a petri dish with 10% FBS in DMEM:F12 (Invitrogen, Paisley, UK) containing 50 IU/l penicillin, 50μg/ml streptomycin and 2mM L-glutamine. The tissue fragments were incubated in a humidified incubator at 37°C, 5% CO_2, for approximately one week, during which time, fibroblasts migrated out of the tissue and proliferated on the base of the culture dish. The cultures were passaged weekly, up to passage 6.

2.2.2 *Lamina reticularis* derived fibroblasts

Non-asthmatic bronchial biopsies were obtained from the non-involved bronchial wall of pulmonary specimens resected from bronchial carcinoma. Biopsies were washed in RPMI 1640 tissue culture medium (Invitrogen, Paisley, UK) supplemented with penicillin (100 IU/ml) and streptomycin (100μg/ml). The *lamina reticularis* was isolated by microdissection using mounted dissection syringe needles, under a stereomicroscope (Kowa, Tokyo, Japan; figure 2.1).

Any attached blood vessels, glands and loose connective tissue were dissected away from the *lamina reticularis*. The resultant tissue fragments were placed into tissue culture petri dishes and held in position by sterile glass coverslips, cemented by sterile silicon grease. The explants were cultured in RPMI 1640 with 15% foetal bovine serum (FBS), and incubated in 5% CO_2, at 37°C and 98% humidity. On establishment of bronchial (myo)fibroblast growth, the coverslip was removed, and the cells trypsinised and plated directly into a 75cm^2 tissue culture flask and grown to confluence. Cultures were then passaged further for up to six passages.

The explants were processed for Haematoxylin and Eosin staining, in order to assure that the cells isolated were derived from the (myo)fibroblasts of the *lamina reticularis* and that there was no smooth muscle contamination. If any explants were seen to be contaminated with smooth muscle, the cultured cells were discarded.

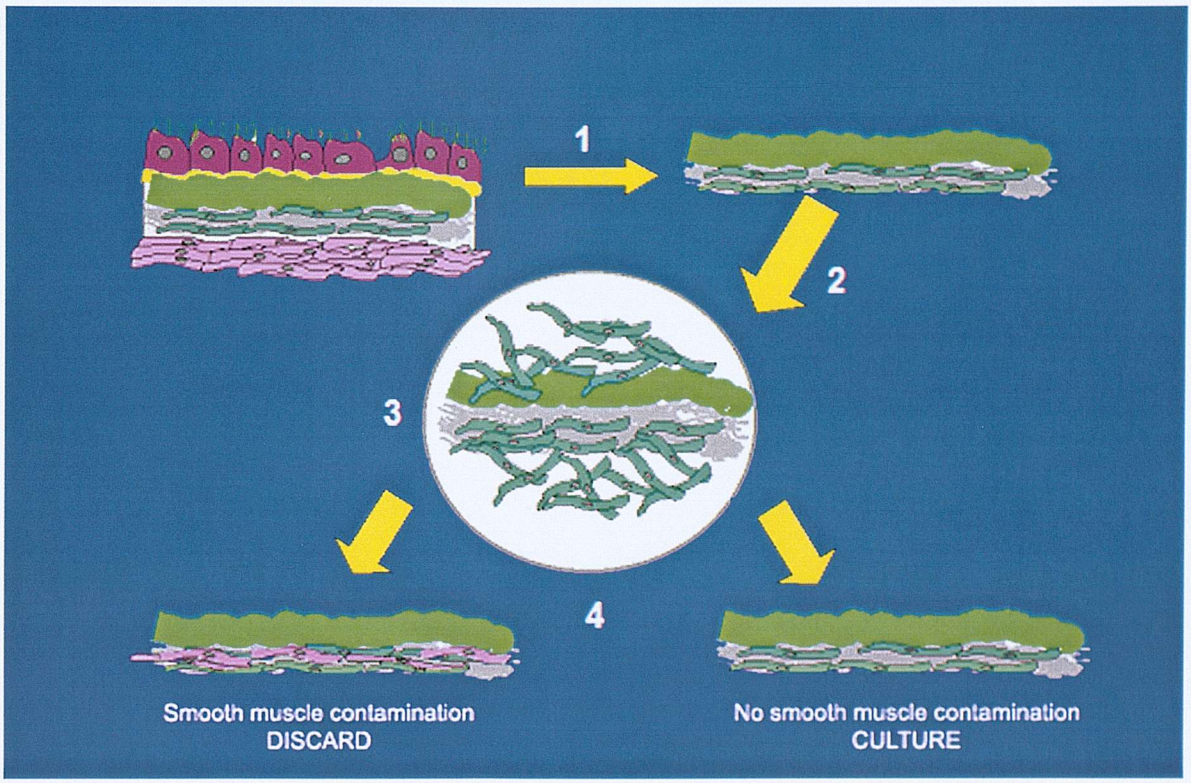


Figure 2.1: Isolation of (myo)fibroblasts specifically derived from the *lamina reticularis* of non-asthmatic airway. A section of bronchail airway was dissected from surgically resected lung. (1) The *lamina reticualris* was micro-dissected out from the airway (2) any blood vessels or nerves were carefully removed. The remaining explant was then cultured *in vitro* (3). Once a confluent monolayer of (myo) fibroblasts was formed, the explant was removed and stained (with haemotoxylin and eosin) and examined for smooth muscle contamination (4). Any cultures that were deemed to have smooth muscle contamination were discarded. Cultures seen to be smooth muscle free were futher cultured and included in the study.

2.2.3 Tissue and cell culture

Once a confluent monolayer of fibroblasts was formed on the surface of the petri dish, the tissue fragment was removed. The cells were then rinsed once with 5ml Hanks' balanced salt solution (HBSS; without calcium and magnesium as they inactivate trypsin) and exposed to 750µl trypsin/EDTA (Sigma, Dorset, UK) for 2-3 minutes, after which time the cells had detached. Ten ml of RPMI 1640 medium (Invitrogen, Paisley, UK) medium containing 10% FBS supplemented with 50IU/l penicillin and 50µg/ml streptomycin (Invitrogen, Paisley, UK) along with 2mM L-glutamine (Invitrogen, Paisley, UK) were added to the cells. This has the effect of inactivating the Trypsin due to the calcium present in the FBS. Five ml of the cell suspension was then transferred to one of two 75cm^2 tissue culture flask (Nunc, UK) containing RPMI 1640 culture medium supplemented as above, and incubated at 37°C, 5% CO_2 overnight, to allow the cells to adhere. The medium was then replaced, and cells incubated for three days, after which the medium was again replaced. Cells were grown till approximately 80-90% confluent, after which they were passaged or cryopreserved.

2.2.3.1 Passaging of cells

When passaging cells, the medium discarded and the cells rinsed with HBSS for 2 minutes. The cells were then exposed to 1.2ml trypsin/EDTA and incubated for 3 minutes at 37°C. Once detached, fresh RPMI 1640 (supplemented as above) was added to the cells to inactivate the trypsin. The cell suspension was then subjected to centrifugation at 1100 rpm for 10 minutes. The supernatant was decanted and the cell pellet resuspended in 3 ml supplemented RPMI 1640. One ml of cell suspension was added to each of three flasks containing 15ml supplemented RPMI 1640. These were then incubated until 80-90% confluent, with medium changes every three days.

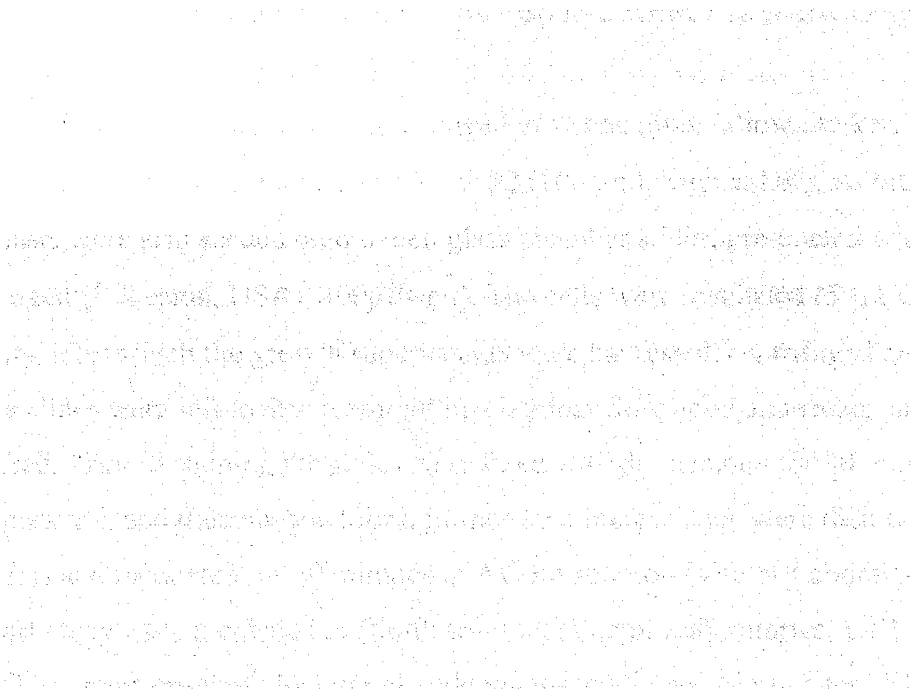
2.2.3.2 Cryopreservation

Once 80-90% confluent, the cells were detached and centrifuged as described above. The cell pellet was resuspended in 3ml RPMI 1640 supplemented with 15% FBS supplemented with 50IU/l penicillin and 50µg/ml streptomycin, 2mM L-glutamine and

10% Dimethyl sulphoxide (DMSO; Sigma, Dorset, UK). One ml of the cell suspension was then aliquoted into each of three labelled cryovials. The crynovials were then insulated with cotton wool, and placed at –20°C overnight, after which they were transferred to –80°C overnight. This allowed a gradual freezing process, making it less likely for ice crystals to form within the cells, and hence damaging them. Finally, the cryovials were placed in liquid nitrogen for long term storage until required.

2.2.3.3 Resuscitation following cryopreservation

The cells were removed from the liquid nitrogen, and were thawed by adding 1ml warm medium. Once thawed, the cells suspension was added to 10 ml supplemented RPMI 1640 and subjected to centrifugation for 10 minutes at 1100rpm, this ensured removal of the DMSO, which can be cytotoxic. The cell pellet was then resuspended in supplemented RPMI 1640, added to a 75cm^2 flask containing 15ml supplemented RPMI 1640, and incubated until 80-90% confluent. As described above, the medium was changed every three days.



2.3 Immunocytochemistry of primary fibroblasts

There are several methods that can be employed to detect antibodies bound to cells, depending upon whether the label used is enzymatic or fluorescent. One of the most common enzymes used is horseradish peroxidase which acts on hydrogen peroxide. The chromogen, Diaminobenzidine (DAB) visualises the reaction and manifests as a brown stain. Fluorescein (FITC) is a common fluorescent label for immunocytochemistry, which appears green when excited by light of wavelength 495nm. Signals often need to be amplified in order to be detected. There are a range of amplification systems, one of the most utilised is the Avidin-Biotin amplification system. Immunocytochemistry and amplification of the signal by the avidin-biotin amplification system is illustrated in figure 2.2.

2.3.1 Immunocytochemistry of primary fibroblasts – Methods

2.3.1.1 Enzyme-mediated detection

The (myo)fibroblasts or fibroblasts were seeded into 8 well glass chamber slides at a density of 1 x 10^5 cells/ml, 300µl/well in 10% FBS in DMEM:F12 (GIBCO, Paisley, UK).

After six hours, the medium was changed to Ultraculture (Biowhittaker, Wokingham, Berks, UK) without or with TGF-β2 (10ng/ml, Sigma, UK). Additionally, the fibroblasts were also seeded onto 8 well glass chamber slides, pre-coated with collagen I (1% Vitrogen (Cohesion, USA), 400µl/well). The cells were incubated (5% CO_2, 37°C) for 72 hours, after which the growth supernatants were harvested, centrifuged and stored (-20°C). The slides were left to dry overnight in a laminar flow hood and frozen at -20°C until required. Prior to staining the slides were fixed with dry acetone for 30 minutes at room temperature, and then air dried for a further 10 minutes. They were then washed in PBS (pH 7.4) and incubated for 20 minutes in Avidin solution (Vector Laboratories, UK). Subsequently they were incubated in Biotin solution (Vector Laboratories, UK) for 20 minutes. These steps ensured blocking of endogenous avidin and biotin sites. The slides were then

incubated for 60 minutes with 1% BSA in 10% FBS/DMEM to block non specific binding. The slides were washed with PBS (pH 7.4) and the primary antibody (diluted in

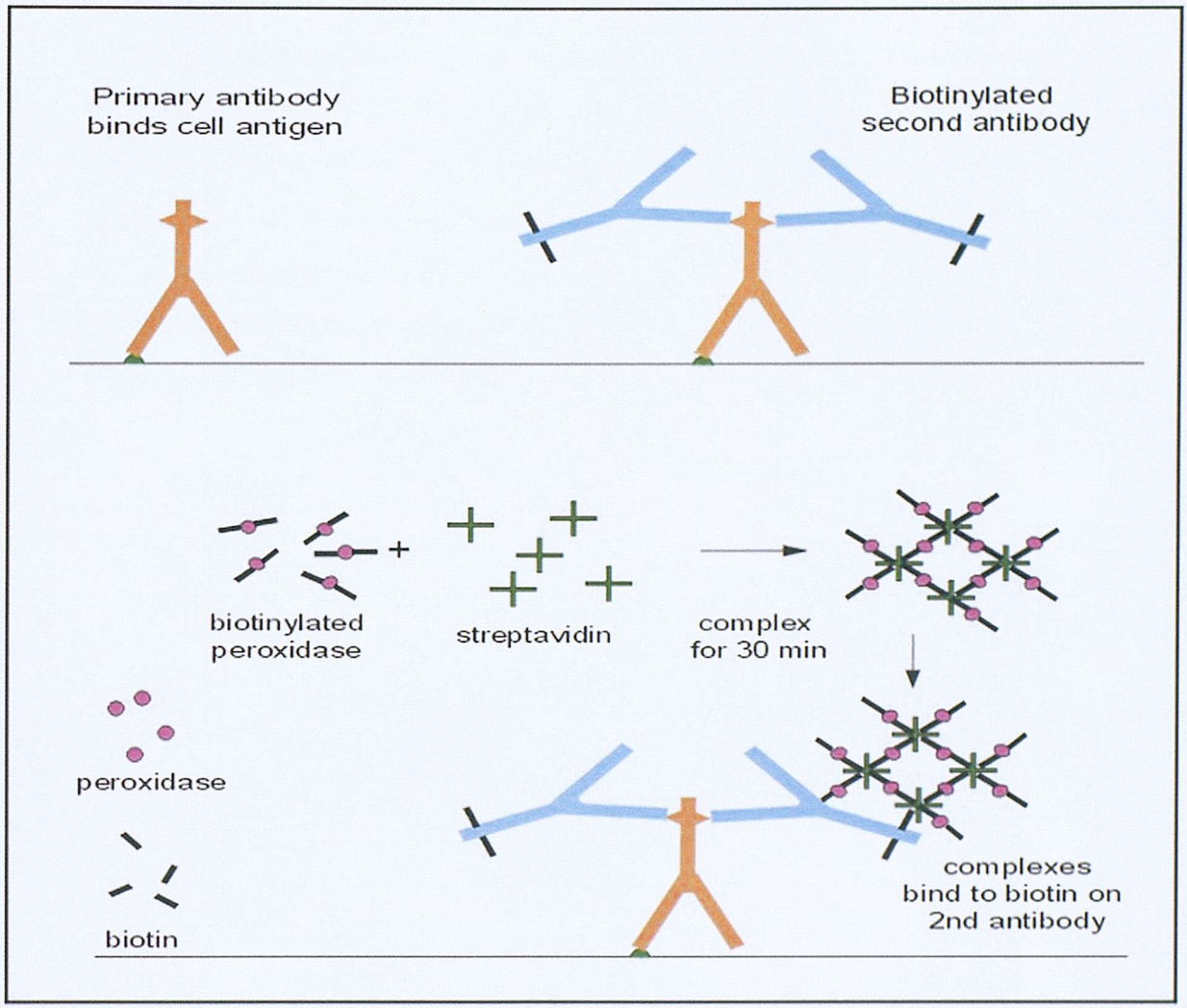


Figure 2.2: The three-layer streptavidin-biotin peroxidase system used to amplify the immunocytochemical signal obtained when using a primary antibody and a biotinylated secondary antibody

PBS with 3% BSA) was added and incubated for 2 hours at room temperature. The slides were then rinsed and secondary antibody was applied (biotinylated sheep anti-mouse (Amersham Life Science, UK) for monoclonals and biotinylated swine anti-rabbit for polyclonals DAKO, UK)) and incubated for 60 minutes, after which endogenous peroxidase was inactivated by incubating for 20 minutes with 1% sodium azide in 3% H_2O_2 solution (diluted in PBS). Then 200µl streptavidin-biotin complex solution (DAKO, UK) was added for 30 minutes and rinsed off. The staining was visualised with a chromogen (3,3'-diaminobenzidine chromogen solution, DAB, Vector Laboratories, UK) and counterstained with Haematoxylin.

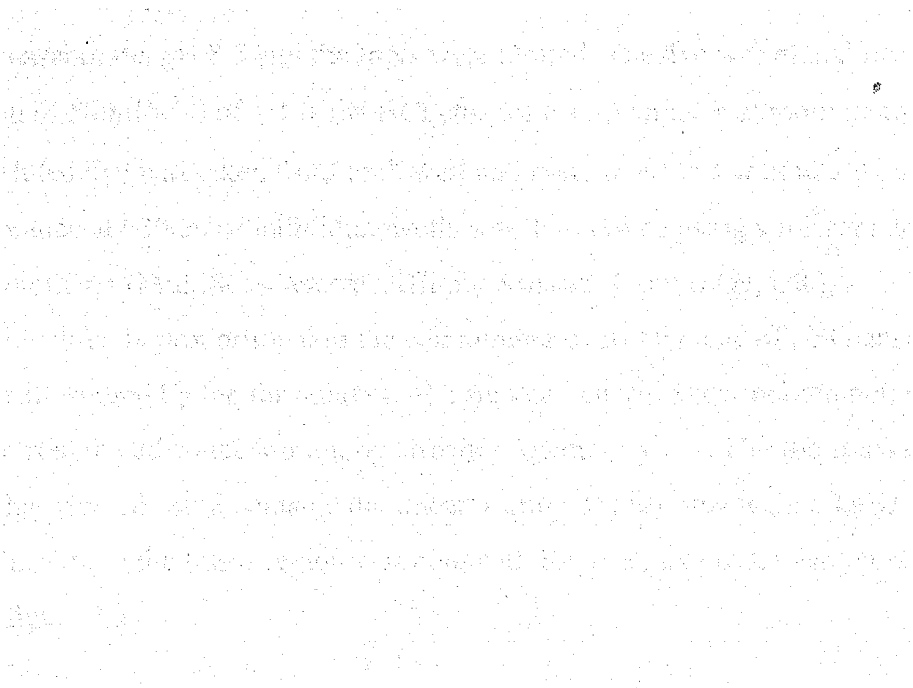
Optimal working concentrations of antibodies were determined by titration and the working dilutions are given in table 2.3.

Antibody	Supplier	Monoclonal / Polyclonal	2° Antibody	Working Concentration
Vimentin	Sigma, USA	Monoclonal	sheep anti-mouse	1 : 200
Desmin	ICN Pharmaceuticals inc.Ohio, USA	Monoclonal	sheep anti-mouse	1 : 40
α-SMA	Sigma, USA	Monoclonal	sheep anti-mouse	1 : 100
Heavy chain myosin (HSM)	Sigma, USA	Monoclonal	sheep anti-mouse	1 : 200
Muscarinic M_2	R&D Abs, Berkley, USA	Polyclonal	swine anti-rabbit	1 : 2000
SM-22	*Gift*; Julian Solway, USA	Polyclonal	swine anti-rabbit	1 : 200

Table 2.3 Shows the primary and secondary antibodies used to investigate the phenotype of the cultured fibroblasts

2.3.1.2 Fluorescence mediated detection

Cells were cultured on 8-well chamber slides and fixed as indicated in section 2.3.1.1. The cells were then hydrated with PBS and then pre-incubated with normal serum (diluted 1:20) in BSA for 30 minutes. This was then removed by tapping the slide after which the cells were incubated with 1° antibody at room temperature for 60 mins. The cells were then washed with PBS for 3 minutes. This was repeated 4 times. The cells were then incubated with 2° FITC conjugated antibody in the dark for 60 mins after which the cells were thoroughly washed with PBS (4 x 3 mins). The slides were then coverslipped, using a mixture of Propidium Iodide (counterstain) and MOWIOL (anti-fade) as mountant. The edge of the coverslip was sealed with varnish. The slides were covered with aluminium foil and stored for up to 3 days at 4°C until viewed under a fluorescent microscope.



2.4 Proliferation Studies

2.4.1 Assessment of fibroblast proliferation by Methylene Blue

The Methylene blue assay is based on uptake of methylene blue dye, according to the method of Oliver et al. (1992).

Cells were seeded into a 24-well tray (Co-star) at $5x10^4$ cells/well, (500µl/well) in growth medium (DMEM:F12, GIBCO, Paisley, UK) supplemented with 10% FBS. The 24-well tray had been pre-coated with collagen I (1% Vitrogen - 500µl per well was applied and then excess collagen was aspirated after 45 minutes). After incubation (6 hours at 37°C, 5% CO2 to allow cell attachment,) the medium was changed to serum free medium (Ultraculture, Biowhittaker, Wokingham, Berks, UK) in the absence or presence of the appropriate stimulus (see table 2.4). At specified time points (0hrs, 6hrs, 12hrs, 24 hrs, 72hrs, 96hrs, 120hrs, 144hrs) the medium was removed and the cells were fixed with 500µl/well of formal saline (4% formaldehyde, 0.15M NaCl) at room temperature for 1 hour. Methylene blue dye was used to assess the growth of fibroblast cell cultures. The fixed cells were stained with 250µl/well with 1% methylene blue in 10mM disodium tetraborate, pH 8.5 for 30 minutes. Excess dye was washed from the trays with 10mM disodium tetraborate, pH 8.5 and the trays were blotted. The dye was eluted from the cells by addition of 500µl/well of 1:1 0.1M HCl/ethanol for 30 minutes at room temperature. 100µl of eluted dye was taken from each well and transferred to a well in a 96 well plate. The absorbance at 630nm of individual wells was determined using a microplate spectrophotometer (MultiScan Ascent, Affinity Sensors, Cambridge, UK). A_{630nm} was shown to be directly proportional to the cell number over the range of cell densities, which was illustrated by the formulation of a standard curve. Spectrophotometer readings were converted to cell concentration by utilising equations yielded by the standard curves. Samples that yielded results outside the linear portion of the curve were diluted serially, until a value within the linear region was obtained. Representative standard curves are shown in figure 2.3.

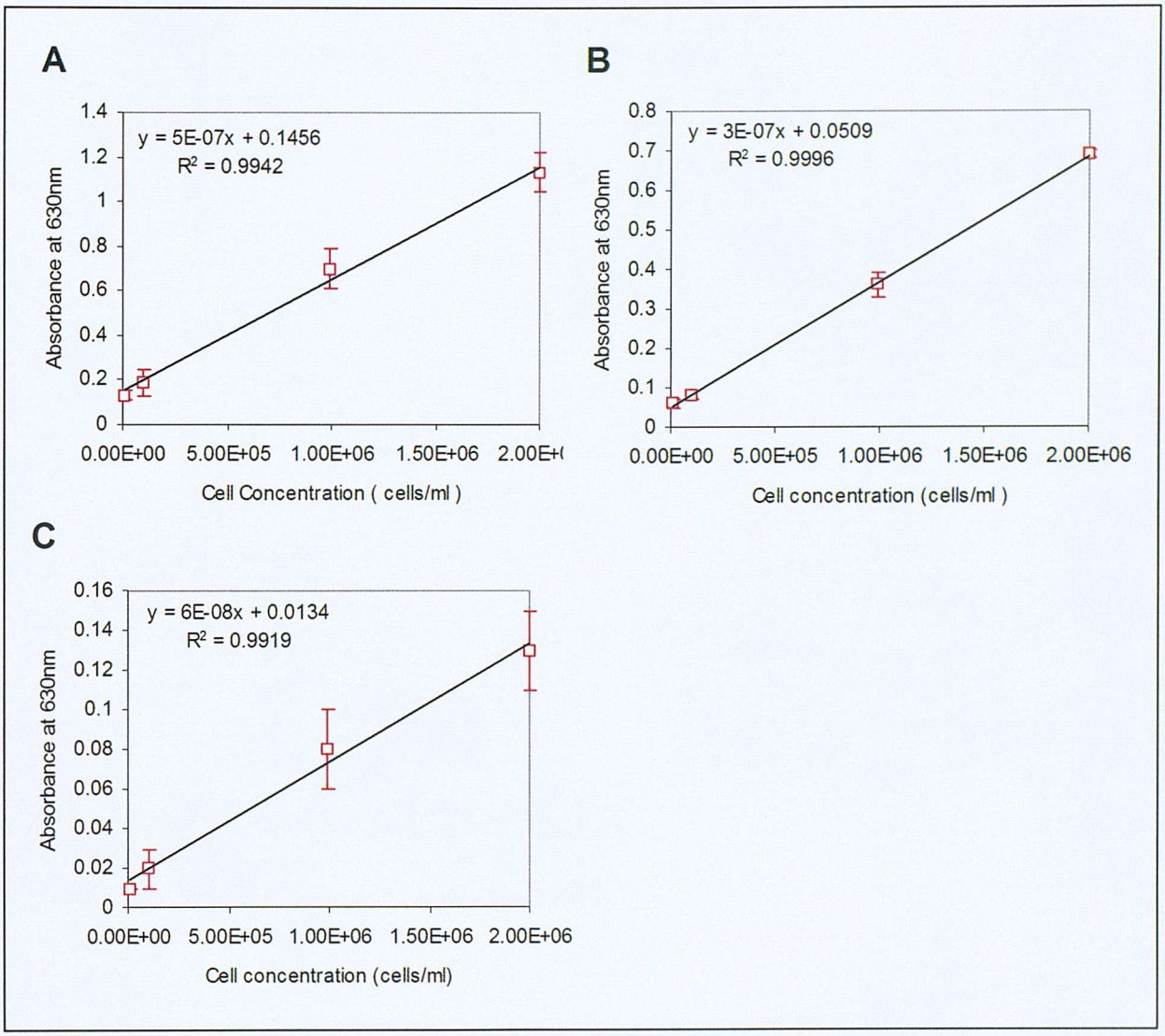


Figure 2.3: Standard curves used to convert absorbance into cell number. The graphs show that the absorbance at 630nM correlates linearly with fibroblast number at (a) Neat (0-fold dilution), (b) 2-fold dilution and (c) 4-fold dilution.

	Stimuli	Working Concentration
1	Transforming Growth Factor β1 (TGFβ1)	0.4nM
2	Transforming Growth Factor β2 (TGFβ2)	0.4nM
3	Endothelin-1 (ET-1)	1 – 10 nM
4	N-Acetyl-[D-Trp^{16}]-Endothelin 1 fragment 16-21	10mM
5	BQ788	10mM
6	Connective Tissue Growth Factor (CTGF)	1 – 10 μM
7	Epidermal Growth Factor (EGF)	5 ng/ml
8	Tyrophostin (AG1478)	1μM
9	Pan TGFβ neutralizing antibody	100 μg/ml
10	Epidermal Growth Factor Receptor Antibody (EGFRAb)	100 μg/ml
11	Basic Fibroblast Growth Factor (bFGF)	1 μg/ml
12	Broad Spectrum MMP inhibitor (GM6001)	?
13	Specific anti-TGFβ1 antibody (CAT-192)	100 μg/ml
14	Specific anti-TGFβ2 antibody (CAT-152)	100 μg/ml
15	Irrelevant IgG antibody control (CAT-001)	100 μg/ml
16	Diphtheria Toxin analogue (CRM197)	10 μg/ml

Table 2.4 shows the different reagents and the concentrations at which they were used to stimulate the cells in the methylene blue cell proliferation assay.

2.4.2 Investigation of mitogenesis in fibroblasts as determined by ^{3}H Thymidine incorporation using the TopCount™ (Microplate scintillation counter)

In order to determine the mitogenic activity in fibroblasts, the ^{3}H thymidine incorporation was determined. Such assays depend on cell cycle synchronisation of all the cells, ensuring that all cells are made quiescent and therefore enter the DNA synthesis stage (S-phase) of their cell cycles at the same time. In order to determine the exact timing of their S-phase, a time-titration experiment was carried out (see appendix D), and was determined to be 28 hours. Following 28 hours of stimulation, the cells were pulsed for 2 hours with ^{3}H Thymidine, and then the DNA was precipitated and a liquid scintillant added (see section 2.4.3).

The TopCount Scintillation counter works by converting the radioactivity emitted by the sample into light by means of a scintillant, which gives off packets of photons or light when stimulated by a beta particle, which is then quantified by a scintillation counter. When ^{3}H thymidine decays it emits a beta particle and a neutrino. The beta particle interacts with the liquid scintillant, which contains solvent molecules along with primary and secondary fluor molecules. When a beta particle interacts with a solvent or a fluor molecule, it causes an excitation. When these excited molecules decay and return to their ground state, they emit energy in the form of photons at wavelengths characteristic for that type of scintillant. The amount of energy emitted is proportional to the amount of energy associated with the beta particle. If the energy is plotted on an energy axis ranging from 0 to E_{max} (a specified end-point as determined by the instrument manufacturer) on the x-axis and the counts per sample on the y-axis the result is an accumulation of counts that forms an energy curve representative for that isotope. This curve, known as a pulse height spectrum (see figure 2.4), can then be used to determine if a comparative sample is being quenched.

Quenching is a phenomenon that leads to a decrease in energy emission from a particular sample. There are three main types of quenching: colour, chemical and physical quenching. Physical quenching involves the physical blockage of energy emission, normally caused by flocculent matter in the sample, thus causing scattering. Chemical quenching is highly dependent on the scintillant being used. Scintillants with aromatic structures or those that contain molecules with multiple carbon-carbon double and triple bonds absorb the energy from the beta particle directly from the solvent or fluor molecule. Chemical quenching is normally concentration dependent and usually occurs before light

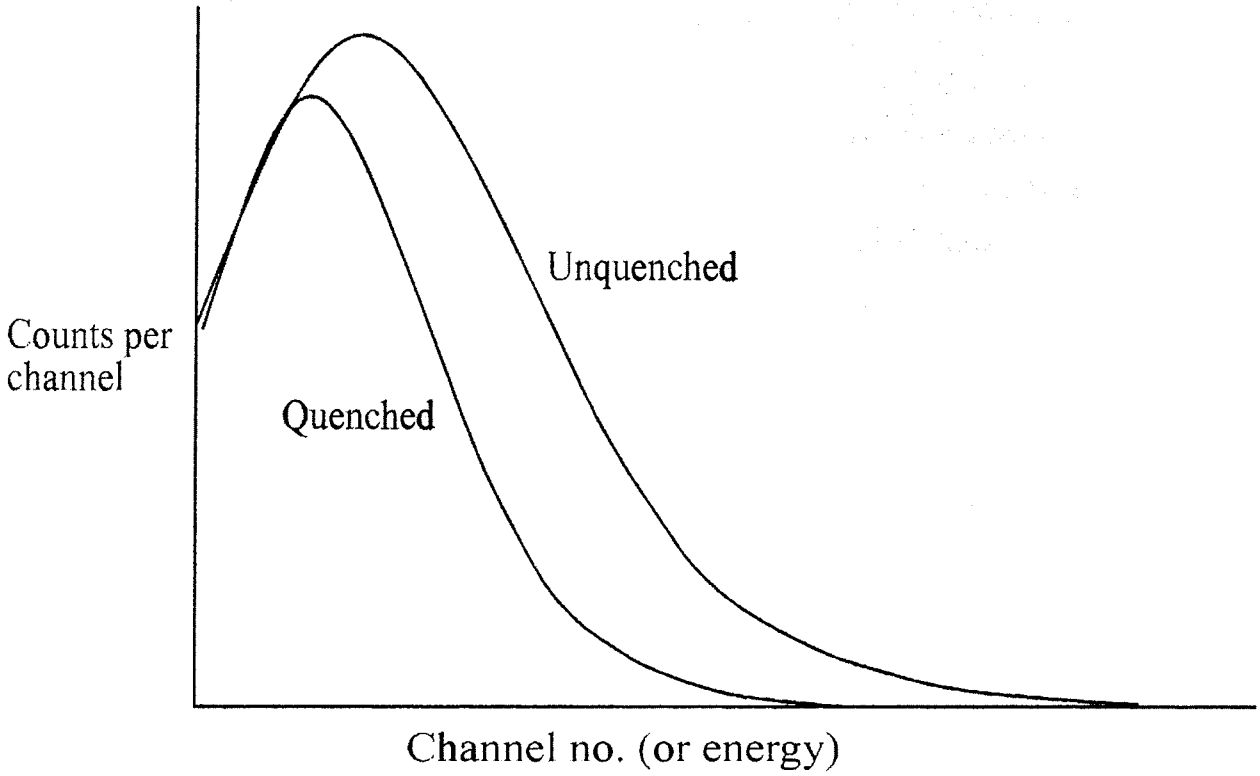


Figure 2.4: Pulse height spectra of quenched and unquenched samples. Quenching reduces the number of photons/keV of β-particle energy resulting in a shift to lower amplitudes of the pulse height spectrum. During isopic decay (β-particle emitters), the energy of the decay is distributed randomly between a β-particle (electron) and a neutrino.
Adapted from: *www.mdyn.com/aptrix/upp00919.nsf/Content/ DrugScr+SPA+TA+Introduction.*

emission from the fluor molecules. Colour quenching is similar to chemical quenching, many large molecules that can absorb light at a particular wavelength have a characteristic colour associated with them. These molecules are referred to as dyes. If a sample is quenched, the pulse height spectrum curve is shifted to the left of normal. This decrease in energy can be quantified by a number known as the quench index parameter (QIP).

2.4.2.1 Quenching and quench correction curves

A quench curve correlates the counting efficiency of quenched standard samples to the amount of quench as measured by the QIP. Quench curves can be constructed by counting a series of quenched standard samples, each containing the same known amount of nuclide and increasing levels of quench. The least quenched sample will generate the most counts and vice versa. The counting efficiency for each sample can be calculated as: **Efficiency = measured CPM / known DPM** (CPM: counts per minute, DPM: disintegrations per minute). If quenching is present the reported CPM would be less than the actual DPM hence decreasing the counting efficiency. Using a quench correction curve, it is possible to convert the CPM into a more accurate DPM whilst taking into account the quenching effect. The quench curve in then plotted as % efficiency vs. QIP. The Packard TopCount™ uses a QIP parameter known as the transformed Spectral Index of the Sample (tSIS), and is evaluated by considering changes in the spectrum of the radionuclide contained in the sample.

The tSIS sample is based on actual sample counts, and the lower the tSIS value, the more the sample is quenched.

It is possible to compare the counts obtained from independent wells without converting the CPM to DPM through a quench correction curve, if the level of quenching in each reaction is equal. Hence, reactions that have similar tSIS values, indicate similar degrees of quenching, and thus can be compared. This method, does not allow appreciation of the actual activity of the isotope, but allows comparison of samples that have been prepared in identical ways, and would be expected to have identical quenching. Samples with very different tSIS values would have to be omitted from the analysis. The distribution in tSIS values obtained in 10 representative plates (600 independent samples) in the present studies, lay between 20 and 22; using published quench correction curves (http://www1.amershambiosciences.com/aptrix/upp00919.nsf) for ^{3}H on the Packard TopCount™, this represents approximately a 2% variation in counting efficiency.

2.4.3 Assessment of proliferation by primary fibroblasts by ^{3}H Thymidine incorporation

The proliferation of primary fibroblasts was measured over 6 days by considering the amount of tritiated (^{3}H) thymidine that was incorporated into acid insoluble cell material at specified time points over the total time period.

Primary fibroblasts obtained from asthmatic and non-asthmatic bronchial biopsies (see section 2.2) were grown until passage 4, after which they were included in this assay.

The primary fibroblasts were seeded into white non-transparent 96-well culture trays (Packard, UK) using cells at a density of $1x10^5$ cells/ml. 100µl of cell suspension in 10% FBS/DMEM was added per well, resulting in a final cell number of $1x10^4$ cells per well. One tray was set up for each time point over the 6 day period. The time points were as those used for the methylene blue assay (see section 2.4.1), 0h, 6h, 24h, 72hr and 144h. This optimal density was determined by conducting a preliminary experiment in which a range of initial starting cell densities were used. For each tray, the outer wells were seeded with medium without cells in order to minimise evaporation of medium from the cells. The trays were incubated for at least 12 hours (37%, 5%CO_2) to allow cell attachment, after which the medium was removed and replaced with 100µl Ultraculture (Biowhittikar, UK) and incubated for a further 24 hours. Starving the cells for this time period was intended to facilitate synchronisation of the cell cycle. After this time, the medium was removed and discarded and replenished with 80µl of Ultraculture. In order to investigate the effect of serum on the growth of the cells, parallel wells were set up containing 1% and 10% FBS.

At the appropriate time point, the plate was processed to determine the ^{3}H thymidine incorporation. The ^{3}H thymidine stock, (37MBq, 1mCi, Amersham, UK) was diluted (30µl ^{3}H-Thymidine, 3µl FUDR and 1470µl Ultraculture) and 25µl was added each well of the culture plate and incubated for 2 hours (37°C, 5% CO_2). The medium was then removed from the cells and disinfected in 1% Virkon prior to disposal of the radioisotope. The cells were then fixed with 200µl per well of 5% tricholoroacetic acid (TCA) at 4°C for 15 minutes. The TCA was then carefully removed and disinfected. The cells were further washed with 200µl 5% TCA and followed by two washes with 200µl per well of methanol. The trays were then left to dry at room temperature for 10 minutes.

The remaining acid-insoluble residue was then dissolved with 40µl per well of 0.2M NaOH. The plate was placed on a plate shaker for 2 minutes to ensure thorough mixing of the acid insoluble residue and sodium hydroxide.

Finally, 150µl of scintillation fluid (Microscint-40, Packard) was added to each well. The plate was shaken thoroughly and then the plate was sealed with a heat sealer (Packard Instrument Company, Meriden, USA).

The incorporated ^{3}H was then measured using the Topcount scintillation counter (Packard Instrument Company, Meriden, USA).

2.4.4 Assessment of Mitogenesis of NR6HER fibroblast cell line

NR6HER cells are a murine derived fibroblast cell line that have been stably transfected with the human epidermal growth factor receptor (a generous gift from Dr. G. Panayatou, Ludwig Institute for Cancer Research, London) and hence exhibit greatest mitogenic responses in response to EGF or related ligands.

The NR6HER cells were grown to confluence in 75cm^2 cell culture flasks in 10% FBS/DMEM, supplemented with penicillin/streptomycin, L-glutamine and 10µM G148 (an antibiotic to which the stably transfected cells are resistant,) incubated at 37°C and 5% CO_2.

The cells were trypsinised and cell number determined by use of a haemocytometer (see appendix B). The cell number was adjusted to yield a final density of 5x10^5 cells/ml using 10% FBS/DMEM. 100µl per well of NR6/HER cell suspension was seeded into a 96 well tray (Packard, Culturplate) excluding the outer wells (see appendix C); 100µl of DMEM was added to the outer wells. The cells were incubated for 48 hours (37°C, 5% CO_2), after which the medium was removed and replaced with 80µl fresh 1% FBS/DMEM and incubated for 24 hours.

Prior to stimulation, the test substances, standards and controls were prepared on a sterile 96 well tray (NUNC) and diluted in Ultraculture. Conditioned medium from cultured primary fibroblasts were also used as stimuli. Each stimulus was serially diluted by a factor of 2 down the tray. Each tray included a recombinant EGF protein standard curve and a baseline and a negative control. An example reagent tray and the appropriate dilutions is shown in appendix C.

To stimulate the cells, 80µl of the stimulus prepared in the reagent tray was added to the culture dish and the cells were incubated for 28 hours (37°C, 5% CO_2). After 28 hours (a timepoint determined by a 'time titration' see appendix D), 25µl of thymidine (see section 2.4.2) was added to each well and the plate incubated for 2 hours, after which the cells were processed to determine ^3H thymidine incorporation as described in section 2.4.2.

2.5 Investigating Gene expression in asthmatic and non-asthmatic fibroblasts

2.5.1 Real time quantitative PCR assays – Taqman™

Real time PCR systems are capable of detecting PCR products as they accumulate during PCR, and thus provide accurate quantitation of a target sequence. The Taqman™ assay uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates.

The Taqman PCR reaction exploits the 5' nuclease activity of Amplitaq Gold DNA polymerase, to cleave a Taqman probe. During the polymerisation of the sense strand, the forward primer displaces a Taqman, probe which has annealed to a specific sequence between the two primers. During displacement, a quencher dye is cleaved from the probe, resulting in increased fluorescence of the reporter dye. Accumulation of PCR products is therefore detected directly by monitoring the increase in fluorescence of the reporter dye. The probe fragments are then displaced from the target and polymerisation of the strand continues (see figure 2.5). The 3'end of the probe is blocked to prevent extension by the DNA polymerase during the PCR, hence non-specific amplification is not detected.

The Taqman reaction mix contains a passive reference dye that does not participate in the assay. This passive dye provides an internal reference to which the reporter dye signal can be normalised.

Normalisation is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference, to obtain a ratio denoted R_n (normalised reporter) for a given reaction.

The magnitude of the signal generated by the given set of PCR conditions is defined as ΔR_n and is defined by the following equation:

$$\Delta R_n = (R_n^+) - (R_n^-)$$

where: R_n^+= $\frac{\text{Emission of intensity of reporter}}{\text{Emission of intensity of passive reference}}$ *With PCR template*

R_n^-= $\frac{\text{Emission of intensity of reporter}}{\text{Emission of intensity of passive reference}}$ *Without PCR template*

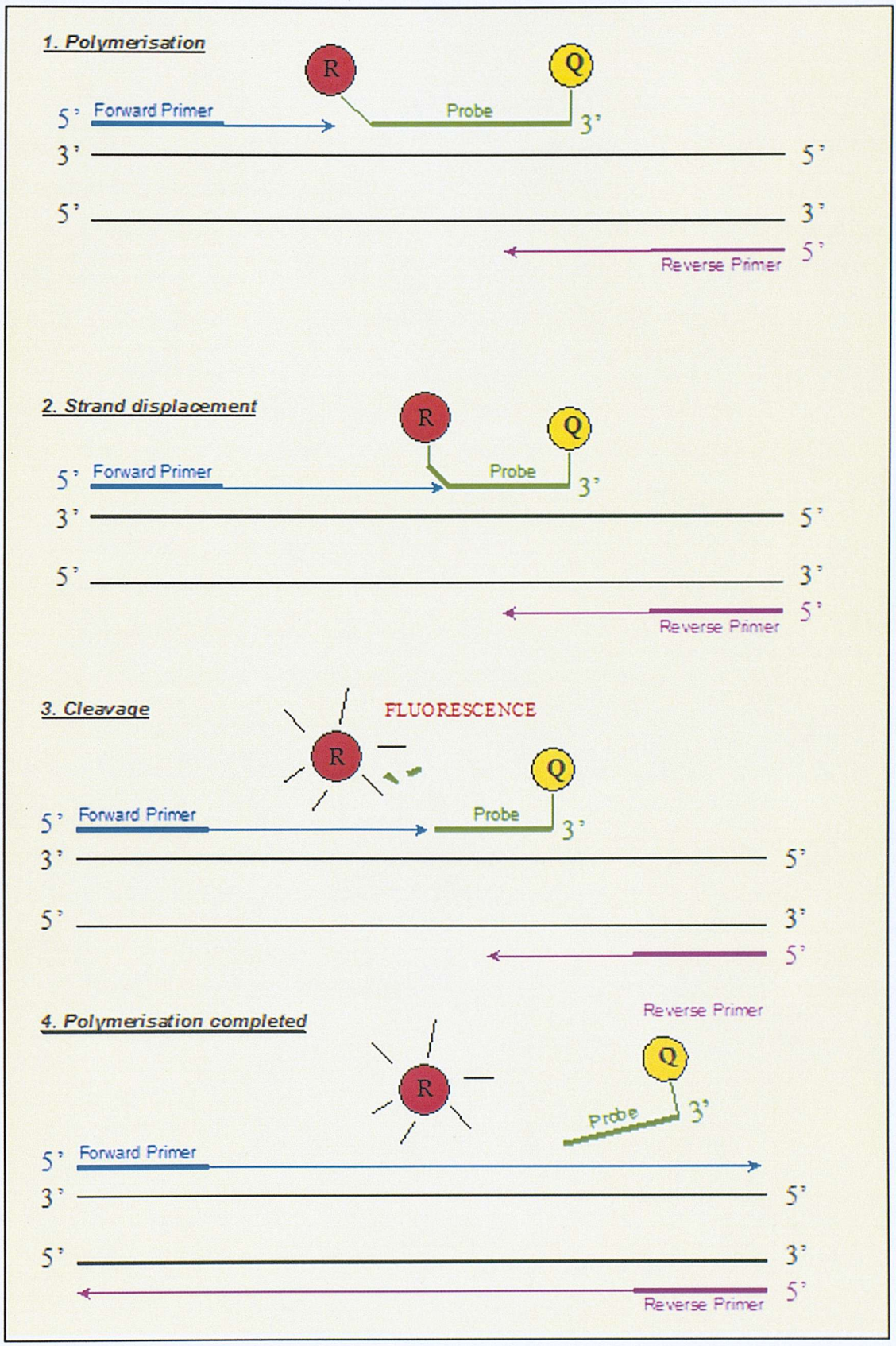


Figure 2.5: The principle of Taqman real time quantitative PCR. During the polymerisation step, both the forward and reverse primers bind to their respective sites flanking the sequence of interest. The probe is placed on a sequence between the two primers. As the Taq DNA polymerase continues to move along the strand, it comes into contact with the probe, which is then displaced. The DNA polymerase causes enzymatic cleavage of the reporter from the quencher resulting in fluorescence. After enzymatic cleavage of the quencher, the polymerase then continues to polymerise along the strand.

2.5.2 Taqman™ Primer and Probe Design

There are general guidelines when designing primers and probes for Taqman™ assays. Many software programmes are available to design Taqman™ probes and primers. An example is Primer Express Software® (Perkin-Elmer, USA).

Primers and probes are normally selected in a region with a G/C content of 20-80%, since regions with a G/C content in excess of this may not denature well during thermal cycling, leading to an inefficient reaction. High G/C content sequences are also susceptible to non-specific interactions. For similar reasons, primer and probe sequences containing more than four adjacent G's are avoided.

Probe and primer sequences rich in A/T bases, require longer primer and probe sequences (due to their reduced melting temperature) in order to obtain the recommended melting temperature. However, sequences 40 base pairs or over exhibit less efficient quenching and produce lower synthesis yields.

The melting temperature of the probe should be 8-10°C higher than that of the primers, this ensures that the probe is fully hybridised during primer extension.

It is also important to ensure that the probe sequence does not end with a G on the 5'end. A G base in this position has a quenching effect, and the probe remains quenched, even after probe cleavage.

Finally, the last five bases on the 3' end of the primers should contain no more than two C and/or G bases. This is another factor that reduces the possibility of non-specific product formation.

Table 2.5 shows the primer and probe sequences for the target genes investigated in these studies.

2.6 Investigating Gene expression in asthmatic and non-asthmatic fibroblasts. Methods

2.6.1 Culturing fibroblasts

Ten lines of passage 5 asthmatic bronchoscopy derived fibroblasts, 6 lines of passage 5 non-asthmatic endobronchial biopsy derived fibroblasts and three lines of passage 5 non-asthmatic *lamina reticularis* derived fibroblasts were cultured in 175cm^2 tissue culture flasks and grown confluent. The cells were then exposed to trypsin and resuspended in fresh RPMI 1640 medium containing 10% FBS and supplemented with penicillin (100 IU/ml) and streptomycin (100μg/ml).

The cells were then seeded into 9cm diameter petri dishes at a density of 1 x 10^5 cells/ dish and incubated overnight, allowing cell adherence. After this time, the medium was removed and replaced with 500μl Ultraculture (Biowhittaker, Wokingham, Berks, UK) in the absence or presence of various stimuli as outlined in table 2.1.

For each of the lines of cells, one dish was seeded for each of the time points (0hrs, 6hrs, 24hrs, 72hrs and 144hrs) At each of the specified time points, the supernatants were harvested and stored at -20°C. The cells were then processed for whole ribonucleic acid (RNA) extraction.

2.6.2 Extraction of whole RNA from cell monolayers

Homogenisation: 750μl of Trizol was added to each petri-dish dish and then using a 5ml syringe, the Trizol was aspirated and ejected back on to the cells several times to ensure that all the cells lysed and homogenised. The lysates were then stored in 2ml eppendorfs at -80°C for up to 2 weeks.

Phase separation: Prior to use, the samples were removed from storage and left to equilibrate at room temperature (10 mins). 300μl chloroform was then added into each tube after which the tubes were vigorously shaken for 15 seconds. The samples were then left to incubate at room temperature (3 mins), after which they were centrifuged for 15 minutes (12 000g, 4°C). After centrifugation, 3 phase layers were visible. The top aqueous layer was carefully removed using a 200μl pipette. 750μl of isopropyl alcohol was then added to the removed aqueous layer, and then stored at -80°C until required.

The samples were removed from storage and left to incubate at room temperature (10 mins), after which they were centrifuged at 12 000g for 10 minutes at 4°C. The RNA formed a transparent gel-like pellet on the bottom of the tube.

RNA washing and re-dissolving: Using a pipette, the supernatant was removed and discarded. 1500µl 75% ethanol was added to the pellet and vigorously mixed using a vortex. These were then centrifuged at 7 500g for 5 minutes, 4°C. The supernatant was removed, and the RNA pellet air-dried for 5 to 10 minutes. The pellet was then redissolved in RNA-ase free water (20µl). The integrity of the RNA was determined by electrophoresis through a 1% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light. A representative gel is shown in figure 2.6. Approximately 1µg is required for adequate real time quantitative PCR. Treating the mRNA with DNase to remove any contaminating genomic DNA typically reduces the mRNA by up to 50%. As the mRNA yields are quite low, the samples under investigation were not treated with DNase. It was hence necessary to check for contaminating genomic DNA. This was achieved by running a negative RT (minus Superscript II™ RT enzyme) reaction. Thus any PCR product from using a negative RT template would indicate genomic DNA contamination (figure 2.7).

2.6.3 Quantifying the RNA concentration

2.6.3.1 Spectophotometery

The amount of RNA in a given sample was determined using the GeneQuant (Amersham, UK) DNA calculator. The absorbance wavelength was set to 260nm. Samples were diluted 1:20 with DEPC treated water and loaded into micro-capillary tubes. The tubes were then placed into the spectrophotometer and the RNA concentration was determined.

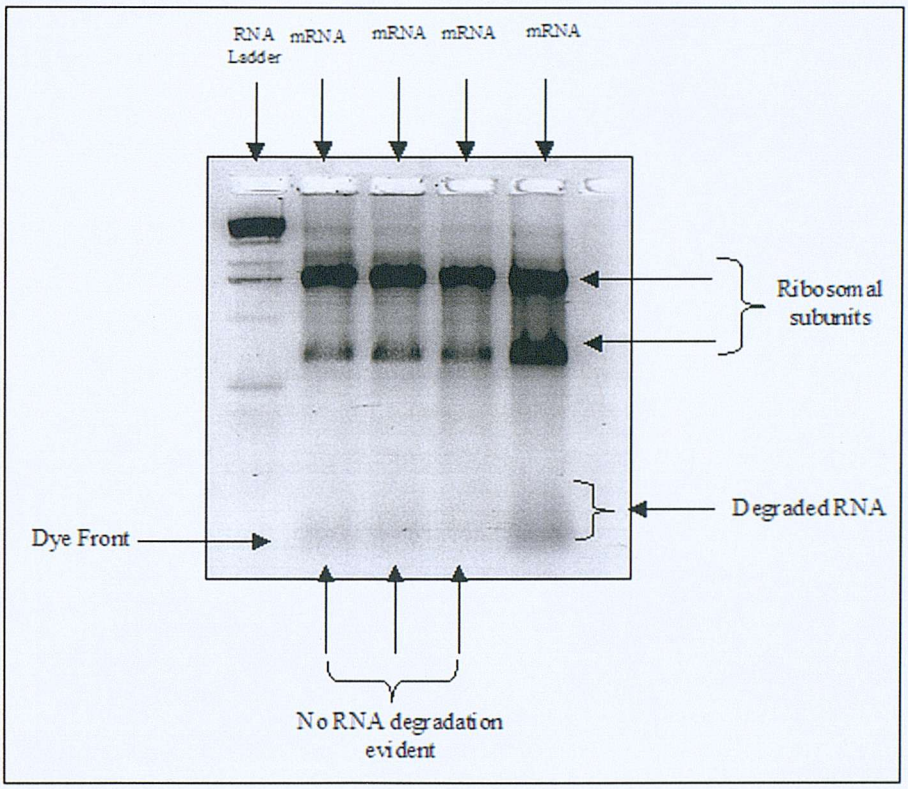


Figure 2.6: RNA integrity gel. 1µl of each mRNA sample was pooled together to produce a mRNA mix. This was then subjected to electrophoresis through a 1% agarose gel for 40 minutes across a 100V potential difference

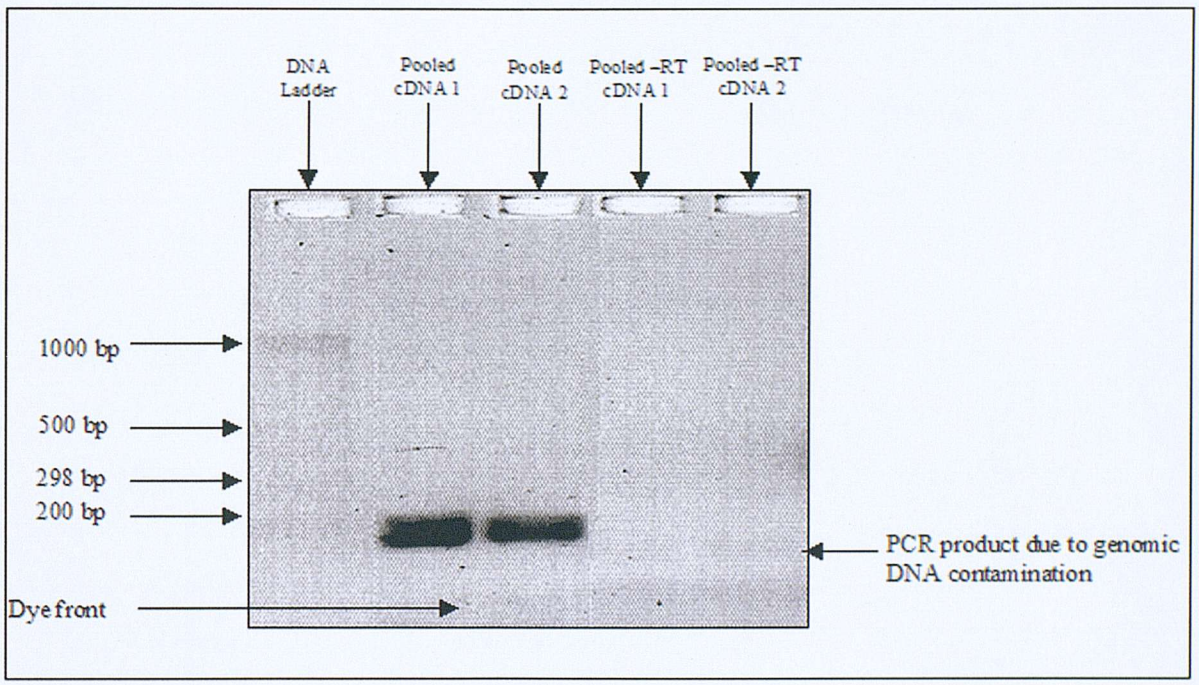


Figure 2.7: α–smooth muscle actin primers. Pooled cDNA mix from (1) asthmatic and (2) non-asthmatic derived fibroblasts. -RT controls were included to detect any genomic DNA contamination.

2.6.3.2 RNA Quantitation using the Ribogreen assay

Ribogreen RNA quantitation reagent is a fluorescent nucleic acid stain for quantitating RNA in solution, and allows detection of as little as 1ng/ml with a fluorescent plate reader using fluorescein excitation and emission wavelengths. Therefore it was found to be suitable for quantitation of preparations of small amounts of mRNA. The linear range for the quantitation is observed over three orders of magnitude in RNA concentration from 1ng/ml to 1ug/ml RNA using two dye concentrations. A high range concentration whereby the Ribogreen reagent is diluted 1:200, allows quantitation of 20ng/ml to 1μg/ml. The low range concentration whereby the reagent is diluted 1:2000 allows quantitation of 20ng/ml to 1μg/ml.

2.6.3.3 RNA Quantitation using the Ribogreen assay – Method

Firstly, a standard curve was prepared. 2μl of 50ng/μl control RNA was diluted 1:500 in 1x Tris-EDTA (TE) to produce a final concentration of 100ng/ml. 100μl of this RNA solution was placed in the well of a 96 well tray. A dilution series was performed across the tray, resulting in an RNA concentration range of 0ng/ml to 100ng/ml. 100μl of 1:2000 (in 1xTE) diluted Ribogreen assay reagent was added to each of the wells, resulting in a final RNA concentration range of 0ng/ml to 50ng/ml. The Ribogreen reagent was added using a pipette with sufficient force to ensure mixing. The mixture was then aspirated and ejected several times to ensure thorough mixing. The tray was then covered with aluminium foil, and left to incubate for 5 minutes. The tray was then shaken for 60 seconds and analysed using a fluorescence plate reader (excitation wavelength 480nm, emission wavelength 520nm, gain 90 ; Cytofluor II Fluorescent reader, PerSeptive Biosystems.) A representative standard curve for the Ribogreen quantification is show in figure 2.8.

The samples were then diluted 1:1000 (1X TE) and pipetted into the 96 well tray. 1:2 dilutions of each sample were then taken 3 times, and 100μl of Ribogreen was added to each sample as before.

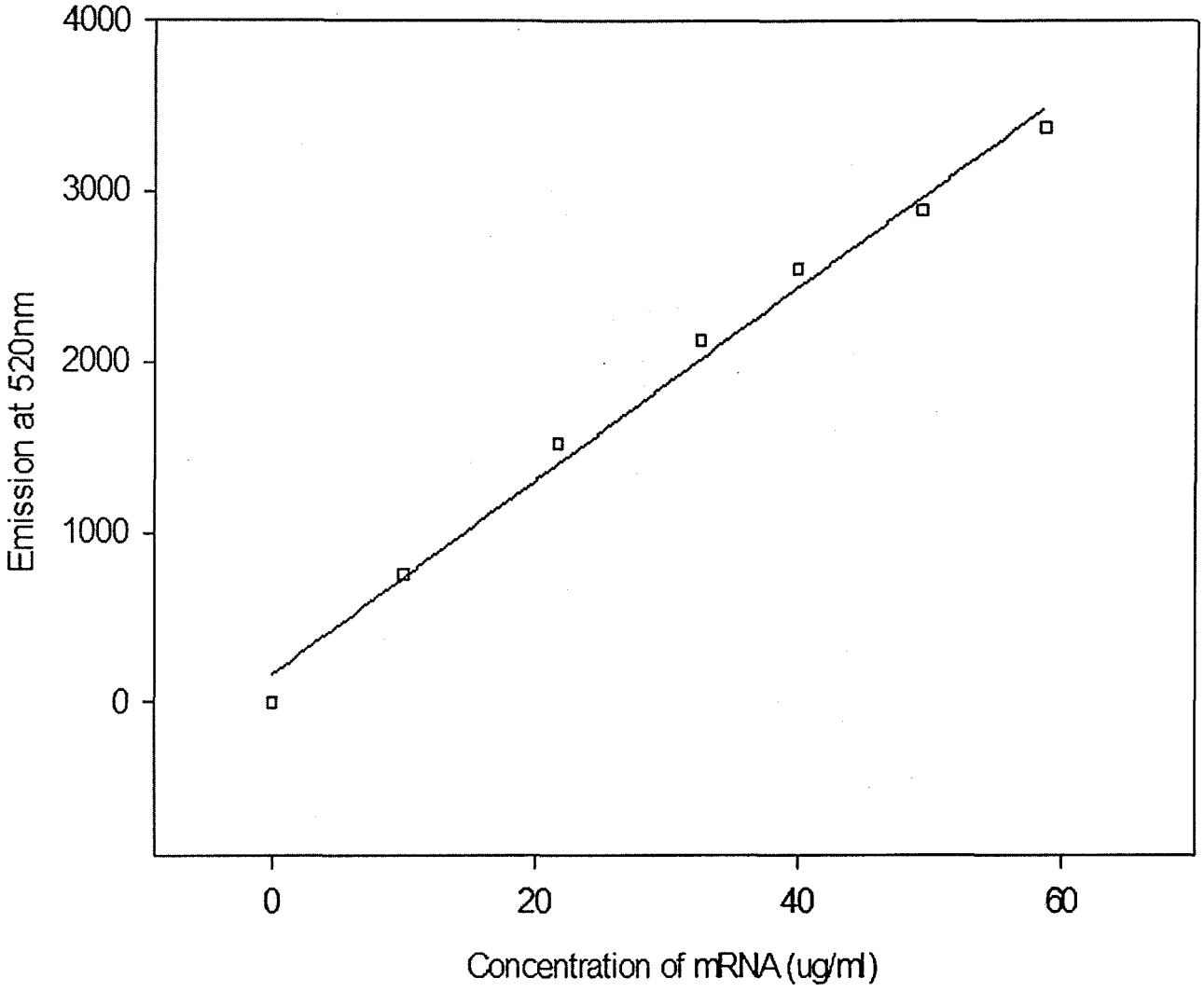


Figure 2.8: Standard curve illustrating the relationship between the concentration of control RNA and the emission at 520nm following excitation at 480nm of Ribogreen fluorescent dye.

2.6.4 cDNA synthesis

2.6.4.1 Reverse transcriptase reaction

The volume containing 1µg of RNA was removed from the stock, and placed in a fresh sterile 96 well plate, and the volume was made up to 10µl with DEPC-treated RNase free water. All reagents were obtained as part of the Superscript™ II first strand synthesis system for RT-PCR (Invitrogen, Paisley, UK). To each sample 1µl of random hexamers (200ng/ml, Invitrogen, Paisley, UK) was added. The plate was then sealed and the samples heated in a PCR heat block (Perkin-Elmer) at 70°C for 10 minutes. At the end of this period, the samples were place immediately on ice.

Next a mastermix was prepared. For each reaction (each well used on the 96 well tray) 2µl of 10X first strand buffer, 4µl of $MgCl_2$, 2µl DTT, 1µl dNTPs and 1µl RNase Out™ was added to a fresh eppendorf These quantities were multiplied by the number of reactions plus an extra 5 reactions.

8µl of mastermix wad added to each sample and then left to incubate at room temperature (25°C, 10 minutes) and then heated to 42°C for 2 minutes. After this, 1µl of Superscript II™ reverse transcriptase enzyme was added to each sample and incubated at 42°C (50 minutes) and then at 70°C (15 minutes). Negative minus RT (-RT) controls were included, in which the Superscript II™ enzyme was replaced with 1µl DEPC-treated RNase free water.

The samples were then transferred to fresh sterile 0.5ml eppendorfs and stored at -80°C until use.

2.6.4.2 Taqman Primer and Probe design

The sequences of the oligonucleotide primers and probes used to amplify the cDNA sequences under investigation, were designed using Primer Express™ . Sequences under investigation were found from the NHLIB genome database (see appendix F). Primer and probe sequences suggested by Primer Express™ were checked to ensure specificity using the basic local alignment search tool (BLAST), a sequence comparison tool accessible on the world wide web. Primers and probes were designed in collaboration with Dr. Mark McHale (AstraZeneca Charnwood, Discovery Bioscience) and Dr. Rob Powell (RCMB division, University of Southampton) and were synthesised by MWG-Biotech AG and Oswell. The sequences are shown in table 2.5.

Target Gene		Sequence
α-Smooth muscle actin	S	5'-GAC AGC TAC GTG GGT GAC GAA-3'
	A	5'-TTT TCC ATG TCG TCC CAG TTG-3'
	P	5'-TGA CCC TGA AGT ACC CGA TAG AAC ATG GC-3'
CTGF	S	5'-GCG GCT TAC CGA CTG GAA-3'
	A	5'-GGA CCA GGC AGT TGG CTC TA-3'
	P	5'-CAC GTT TGG CCC AGA CCC AAC TAT GA-3'
Endothelin-1	S	5'-CGT CCC TGA TGG ATA AAG AGT GT-3'
	A	5'-ACG TGC TCG GGA GTG TTG A-3'
	P	5'-TCT ACT TCT GCC ACC TGG ACA TCA TTT GG-3'
Transforming Growth Factor β1	S	5'-TGG ACA TCA ACG GGT TCA CTA C-3'
	A	5'-AAG CAG GAA AGG CCG GTT-3'
	P	5'-CGA GGT GAC CTG GCC ACC ATT CAT T-3'
HB - Epidermal Growth Factor	S	5'-GAT CTG GAC CTT TTG AGA GTC ACT T-3'
	A	5'-TCC CGT GCT CCT CCT TGT T-3'
	P	5'-AGC CAG AAG CAC TGG CCA CAC CA-3'
ADAM – 12	S	5'-AGC TAT GTC TTA GAA CCA ATG AAA AGT G-3'
	A	5'-CCC CGG ACG CTT TTC AG-3'
	P	5'-ACC AAC AGA TAC AAA CTC TTC CCA GCG AAG AT-3'
ADAM – 33	S	5'-CCT GGA ACT GTA CAT TGT GGC A-3'
	A	5'-GTC CAC GTA GTT GGC GAC TTC-3'
	P	5'-CCA CAC CCT GTT CTT GAC TCG GCA T-3'
18S ribosomal RNA	S	*Obtained as a Pre-developed Assay Reagent from Perkin Elmer*
	A	*Obtained as a Pre-developed Assay Reagent from Perkin Elmer*
	P	*Obtained as a Pre-developed Assay Reagent from Perkin Elmer*

Table 2.5 shows the Taqman primer and probe sequences used to investigate gene expression in these studies. (S) Sense, (A) Anti-sense and (P) Probe

2.6.5 Real-time PCR

The RT-reaction does not include any amplification steps, and hence it is assumed that the amount of cDNA synthesised is equal to the amount of mRNA template.

One µl of each cDNA sample was taken and mixed together to prepare a mixture of all the cDNA's, required to produce a standard curve. 20µl of cDNA (5ng/µl) mix was then added to a fresh eppendorf and serial 10-fold dilutions (with DEPC-treated RNase free water) were conducted to produce a dilution series ranging from 5ng/µl to5pg/µl.

Primers were supplied at 100µM after re-solubilisation. Primers for Taqman reactions are required at 10µM, hence 35µl of sense and antisense primers were diluted 1:10 (in RNase free DEPC-treated water.).

5µl of cDNA (standard or sample) was plated into a well to which 20µl of prepared mastermix (see appendix A) was added, using a pipette and with sufficient force to ensure mixing. Each sample was loaded in duplicate. The plate was then sealed and briefly spun in a centrifuge (1200 rpm 60 sec) to ensure all components were at the bottom of the wells and that no air bubbles were present. After which the plate was placed in the Thermal cycler (ABI Prism™ 7700 sequence detection system) under the cycling conditions indicated in table 2.6. As before 5µl of cDNA was added to each well. As well as the cDNA, 5µl of the corresponding –RT reaction products were included on the plate.

2.6.6 Relative quantitation of gene expression by the standard curve method

In such analysis, the quantity of mRNA target is determined from the standard curve, and divided by the quantity of a housekeeping gene within the same sample and used as the basis for comparative results (calibrator).

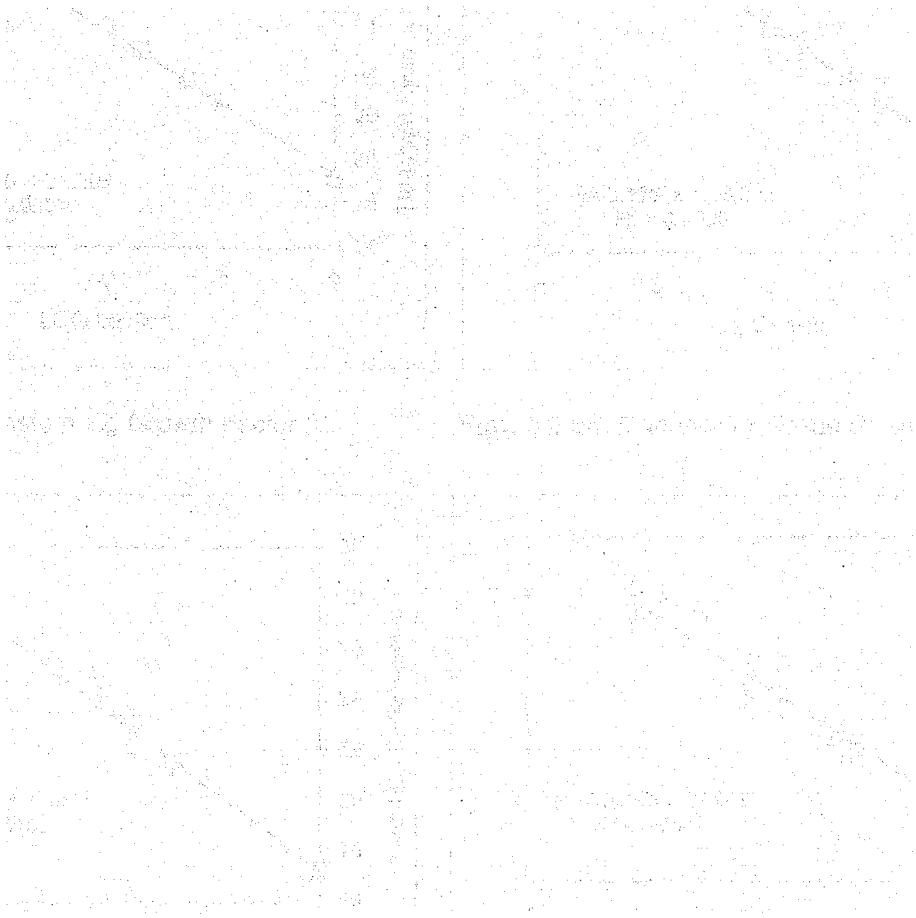
Since the target quantity is divided by the calibrator quantity, the result is dimensionless and thus standards do not require units. All that is required is their relative dilutions.

For quantitation normalised to an endogenous control, standard curves were prepared for both the target and the endogenous reference. For each sample the amount of reference and target were determined from their own standard curve.

In order for the comparisons to be valid, it is important that the efficiency of the PCR reations being compared are approximately equal. The efficiencies can be determined from the gradient of the standard curve as given by the equation:

$E = \{[10^{(-1/m)}]-1\} \times 100$, where E=efficiency and m=gradient of the standard curve. Representative standard curves for each target gene investigated in these studies are shown in figure 2.9 and the calculated efficiences are given in table 2.7

In order to demonstrate the calculations and analysis used to quantitate gene expression by this method, a worked example is given in appendix E.



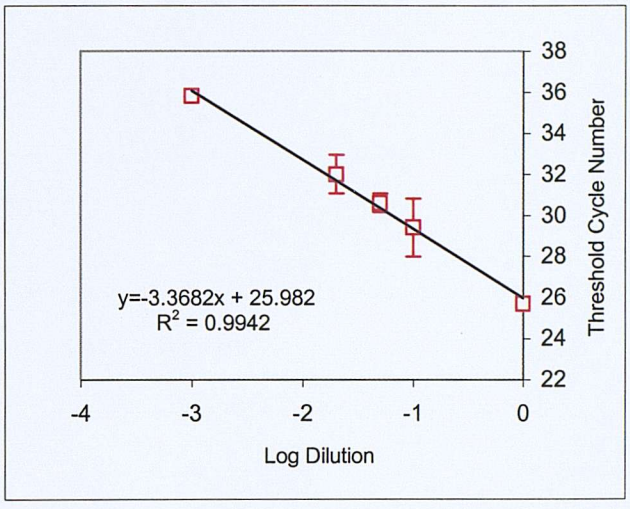


Figure 2.9a: Endothelin-1

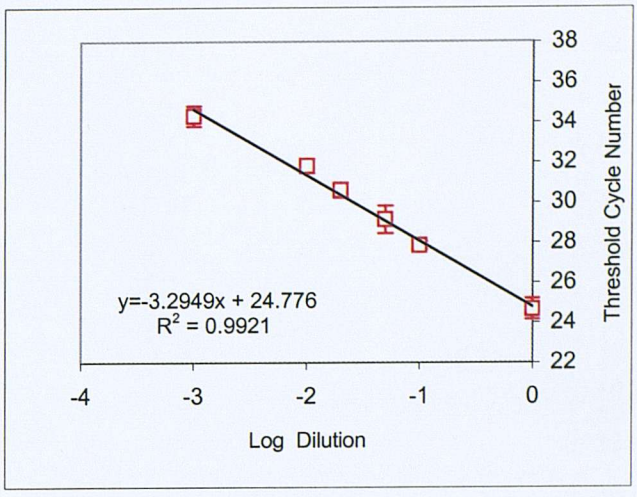


Figure 2.9b: α-Smooth muscle actin

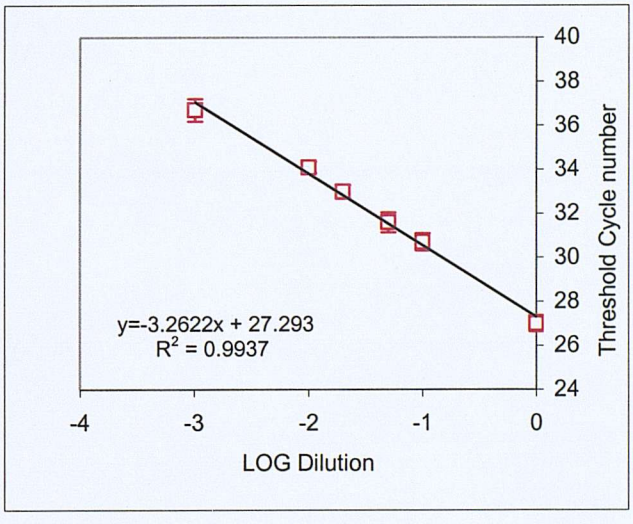


Figure 2.9c: Transforming Growth Factor β1

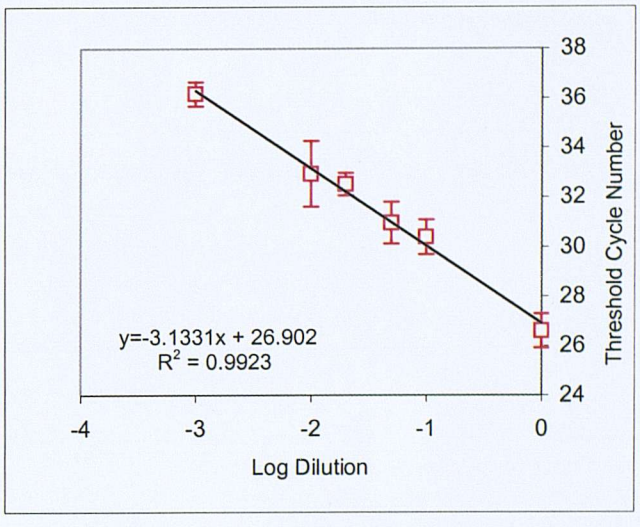


Figure 2.9d: Connective Tissue Growth Factor

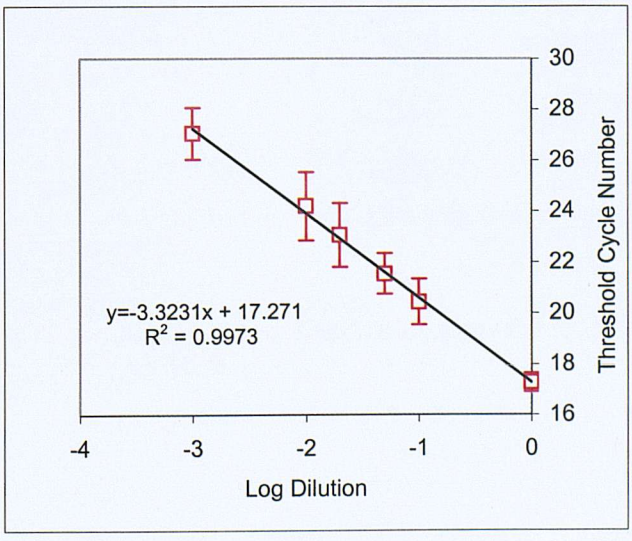


Figure 2.9e: 18S ribosomal RNA

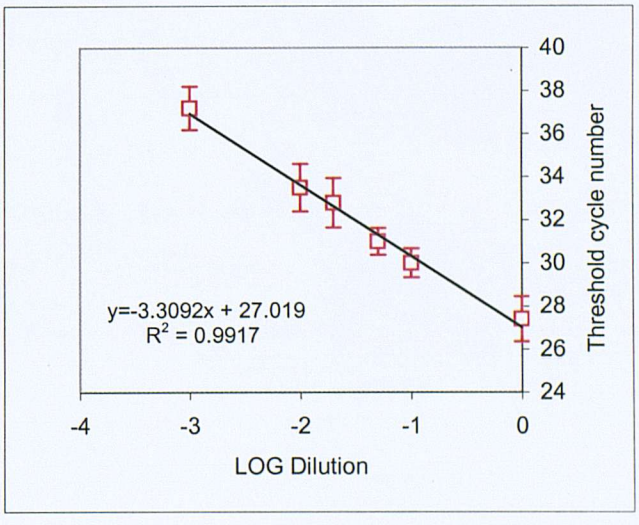


Figure 2.9f: Heparin Binding Epidermal Growth Factor

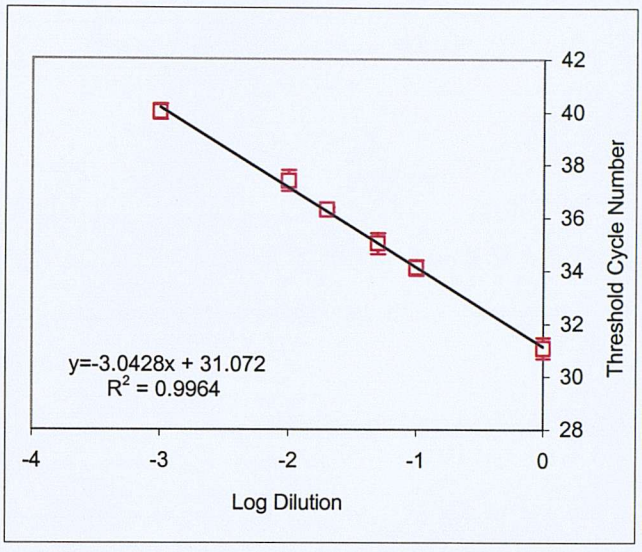


Figure 2.9g: A Disintegrin and Metalloproteinase (ADAM) -12

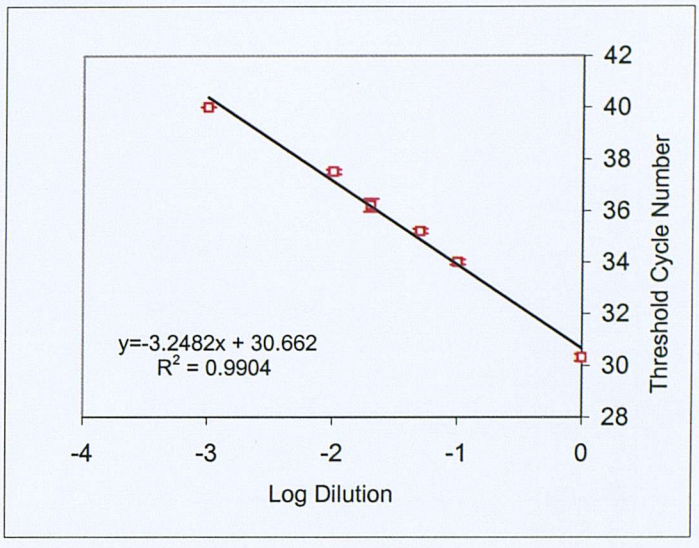


Figure 2.9h: A Disintegrin and Metalloproteinase (ADAM) -33

Figure 2.9: Standard curves indicating the cycle number for a given dilution of cDNA mix

Primer	Gradient	% Efficiency
ET-1	-3.368	98%
α-SMA	-3.295	101%
TGFβ1	-3.262	102%
CTGF	-3.309	100%
18S rRNA	-3.323	100%
HBEGF	-3.133	108%
ADAM-33	-3.248	103%
ADAM-12	-3.143	108%

Table 2.6: Shows the % efficiencies of each primer set

2.7 Investigating Protein expression in asthmatic and non-asthmatic fibroblasts.

2.7.1 Western Blotting

Western blotting is a technique that enables detection of a protein by use of gel electrophoresis and the specific binding ability of relevant antibodies.

2.7.2 Tissue culture and sample preparation

Primary fibroblasts were grown to confluence in 10% FBS/RPMI in 75cm^2 tissue culture flasks (NUNC). When confluent, the cells were enzymatically detached (using Trypsin,) and the cell number determined using a haemocytometer (see appendix B).

The cell density was then adjusted to 0.5x10^5 cells/ml. Four millilitres of this suspension was then seeded into 9cm diameter petri-dishes (pre-coated with collagen I) and incubated for 12 hours to allow cell adherence. After this, the medium was removed discarded and replaced with 5ml of Ultraculture with or without 10mg/ml TGFβ2. After incubation (37°C, 5% CO_2) for 24 hours the medium was removed and stored at -80°C for subsequent protein analysis.

The cells were washed thoroughly with 2ml PBS for five minutes, which was repeated once. Five hundred microlitres (μl) of boiling lysis buffer was added to the cells, and the cells scraped with a cell scraper. The remaining lysates were transferred to a 1.5ml microcentrifuge tube and place on ice. The lysates was then sonicated for 15 seconds (Soniprep, MSE) to reduce the viscosity (by breaking down DNA strands and protein clumps) of the lysates. The lysates were centrifuged and the supernatant was harvested and stored at -80°C until further use.

2.7.3 Determination of protein concentration

The protein concentration was determined using the micro BCA detection kit (Pervio Biosciences, USA) according to the manufacturer's guidelines.

Briefly, the stock concentration of bovine serum albumin (BSA – 2mg/ml) was diluted to 80μg/ml in denaturing lysis buffer and water (for 1ml; 40μl BSA (2mg/ml), 40μl denaturing lysis buffer and 920μl dH_2O). A denaturing lysis buffer based dilutent

was made (1.2ml denaturing lysis buffer and 28.8ml dH_2O), which was used to dilute the samples.

Two hundred microlitres (µl) of BSA standard and diluted samples were added to a row of a 96-well plate, and 4 serial 1:2 dilutions were carried out. The final row contained no BSA standard and just the dilatant as a negative control. The micro BCA reagent was then prepared (5ml solution MA (sodium carbonate, sodium bicarbonate & sodium tartate in 0.2M NaOH), 4.8ml solution MB (4% Bicinhoninic acid in water) and 200µl solution MC (4% cupric sulphate & 0.05% sodium azide) for each plate.) One hundred microlitres (µl) of this reagent was then added to each well. The plate was sealed and incubated at 60°C for 1 hour after which the absorbance at 562nm was determined using a plate reader (Multiskan Ascent, Labsytems, OY). The obtained values were converted to protein concentrations (µg/ml) by use of the BSA standard curve.

Two micrograms (µg) of protein was added to 5µl 5x sample buffer (see appendix A), which was stored at -20°C until separated by gel electrophoresis.

2.7.4 SDS-Polyacrylamide gel electrophoresis

The proteins extracted from the cell lysates were separated according to size using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condtions as first described by Laemlli and co-workers. One millimetre thick 15% polyacrylamide gels were cast using a mini protean II system (Biorad, UK).

Two glass plates were thoroughly cleaned with a 10% SDS solution, followed by methanol. One litre of 1x running buffer (see appendix A) was pre-chilled at 4°C for 1 hour. The glass plates (separated by a 1mm spacer,) were assembled into a mini protean II systems and secured . 50ml of 15% separation gel mix was prepared (4.95µl separation gel stock, 16.5µl 10% (w/v) ammonium persulphate and 2.5µl N,N,N,N',N'-tetramethylenediamine (TEMED) and poured in between the two glass plates. The gel was then overlaid with water saturated propanol to exclude oxygen. The gel was incubated at room temperature for 1 hour to allow polymerisation.

After polymerisation, the gel was rinsed with distilled water, and the excess water was removed with filter paper. Next 5 ml of stacking gel mix (5ml stacking gel stock, 16.7µl 10% (w/v) ammonium persulphate, 3.8µl TEMED) was prepared and poured on

top of the separation gel. A 10 well comb was inserted and the gel left at room temperature for 45 minutes to allow polymerisation, after which the comb was removed.

Each gel cassette was placed into electrode assembly and the resulting chamber was filled with chilled running buffer.

Five microlitres (μl) of protein molecular weight markers (Amersham Pharmacia) were added to the first well. The samples previously prepared (see section 2.7.2) and stored at -20°C were thawed and heated to 95°C for 5 minutes after which 20μl of sample was loaded into the appropriate wells. The electrode assembly was then placed in the running tank filled with chilled running buffer and the gel was subjected to electrophoresis at a constant voltage (160V) and a current of 300mA, until the dye front reached the bottom of the gel (approximately 80 minutes).

2.7.5 Perpendicular electrophoresis (Protein transfer)

The proteins separated on the gel were transferred to a nitrocellulose membrane by electrophoresis.

Firstly the SDS-PAGE gel was placed in chilled transfer buffer (see appendix A) for 15 minutes to equilibrate and pre-shrink the gel. Nitrocellulose membranes of equal size to the gel were soaked for 5 minutes in water and then 15 minutes in transfer buffer. The transfer was conducted in the Transblot apparatus (Biorad, UK). The gel and nitrocellulose membrane were sandwiched in the Transblot cassette pre-soaked in transfer buffer, after which it was transferred to the tank, pre-filled with chilled transfer buffer. The whole tank apparatus was placed on a magnetic stirrer to ensure uniform distribution of the cold transfer buffer. The tank was surrounded by ice block to ensure that the transfer buffer remained chilled. For the transfer, electrophoresis was conducted at a constant 90V for 2 hours with a current of 350mA. The nitrocellulose membrane was then air-dried and stored at 4°C until stained.

2.7.6 Immunostaining and detection

The nitrocellulose membrane was rehydrated for 10 minutes in 10ml of PBS/Tween buffer with 1 protease cocktail inhibitor tablet (Roche) and constantly agitated. Firstly, non-specific binding sites were blocked by incubation for 30 minutes

with 10ml blocking buffer (see appendix A). Residual buffer was then removed by three 10 minute washes in PBS/Tween buffer.

The primary antibody was diluted to yield a final concentration of 1.5μg/ml in PBS/Tween buffer. The membrane was incubated in this primary antibody solution for 2 hours at room temperature, after which the primary antibody was removed and the membrane thoroughly washed with PBS/Tween for 10 minutes. This was repeated twice.

The secondary antibody was then diluted in antibody buffer 1:2000, and the nitrocellulose membrane incubated for 1 hour at room temperature. The membrane was then again washed for 10 minutes a total of 3 times in PBS/Tween.

Specific antibody binding was visualised using chemiluminescence (ECL Plus, Amersham,) according to the manufacturers instructions. Briefly, the blots were placed upwards on blotting paper and then onto Saranwrap. The membrane was then covered with 2 ml of ECL-Plus solution and incubated at room temperature for five minutes. The membrane, handled with forceps was blotted and wrapped in a piece of Saranwrap. The membrane was then viewed under the Gene Gnome (Syngene BioImaging,) and exposed for 30 seconds.

2.8 Enzyme-linked Immunosorbent assays (ELISA)

2.8.1 Transforming Growth Factor (TGF)β1 Enyzme-linked immunosorbent assay

The levels of total and active TGFβ1 in the supernatants of the fibroblasts was determined by ELISA. The TGFβ1 ELISA (Promega, UK) is designed for the specific detection of biologically active TGFβ1 in an antibody sandwich format. The coating antibody is a monoclonal antibody which binds soluble active TGFβ1. The captured TGFβ1 is then bound by a second specific polyclonal antibody. The amount of specifically bound polyclonal antibody is then detected using a species specific antibody conjugated to horse-radish peroxidase as a tertiary reagent. Any unbound conjugate is washed away and is visualised with the colour change of a chromogenic substrate. The kit has a detection range of 32 – 1,000 pg/ml.

2.8.2 Sample preparation

The method was used to detect both active and total (inactive and active) TGFβ1. Normally, *in vivo* TGFβ1 is processed from a latent form to a bioactive form (see section 1.4.6.2). The antibodies provided are able to detect only the active form. Latent TGFβ1 was activated in the cell culture supernatants by acid treatment, according to the manufacturer's instructions. Briefly, 6µl of 1M HCl was added to 300µl of cell culture supernatant, and the pH was tested (using universal indicator paper) to be pH 3.0 or lower, after which it was thoroughly mixed. The acid-treated samples were then incubated at room temperature for 15 minutes. The acid was then neutralised by adding 6µl of 1M NaOH to the sample, and the pH adjusted to pH 7.6 (±0.2). These samples were then stored at -80°C until further use.

2.8.3 Plate coating and blocking

For 1 plate, 10µl of TGFβ coating monoclonal antibody was added to 10ml of carbonate coating buffer (see appendix A) and was thoroughly mixed. 100µl of this prepared antibody solution was then added to each well of a polystyrene ELISA plate

(Corning Co-star 96 well plate) and then sealed with a plate sealer and left to incubate at 4°C overnight.

The plate was then left to warm up to room temperature, after which the contents of the wells were removed by flicking the plate and tapping the plate on blotting paper to remove the excess. 270µl of TGFβ blocking 1x buffer (see appendix A) was then added to each well, ensuring that the pipette tips did not scratch the monoclonal antibody coating on the surface of the plate. The plate was left to incubate at 37°C for 35 minutes, and then washed using tris-buffered saline/Triton-100 (TBST) wash buffer (see appendix A).

The plates were washed by entirely filling each of the wells with TBST wash buffer and then flicking out the contents, finally blot drying the plate by tapping the plate on blotting paper.

Next the TGFβ1 standard provided with the kit at a concentration of 1µg/ml was diluted in TGFβ1 sample 1x buffer (see appendix A) to yield a final concentration of 1000pg/ml. Two hundred microlitres of diluted TGFβ1 standard was then added to the first two wells of the 96 well ELISA plate, and serially diluted (1:2) with TGFβ1 sample 1 x buffer down to 15.6pg/ml. The final 2 wells were used for sample buffer alone (negative control). For the active TGFβ1 measurements, the samples were not diluted and added to the plate in duplicate. For total TGFβ1 measurements, the samples were diluted 1 in 5 and added to the plate in duplicate.

The plate was then incubated for 90 minutes at room temperature and agitated at approximately 500rpm. The plate was then washed with TGFβ1 wash 1X buffer (as described above). This was repeated 4 times. Next the TGFβ HRP conjugate was prepared (100µl HRP conjugate stock, 9900µl TGFβ1 1X sample buffer for 1 plate,) and thoroughly mixed. One hundred microlitres of conjugate was added to each well, taking care not to touch the bound material on the surface of the plate.

The plate was incubated for 2 hours at room temperature and agitated as before. The plate was then washed 5 times using TBST.

Next 100µl of TMB One solution (Promega, UK) was added to each well and left to incubate for 15 minutes at room temperature without agitation. The reaction was halted, by adding 100µl of 1M hydrochloric acid. The absorbance was then read at 450nm on a plate reader within 30 minutes of the reaction being stopped.

The absorbances were then converted to TGFβ1 concentration by use of the standard curve. A representative standard curve is shown in figure 2.10.

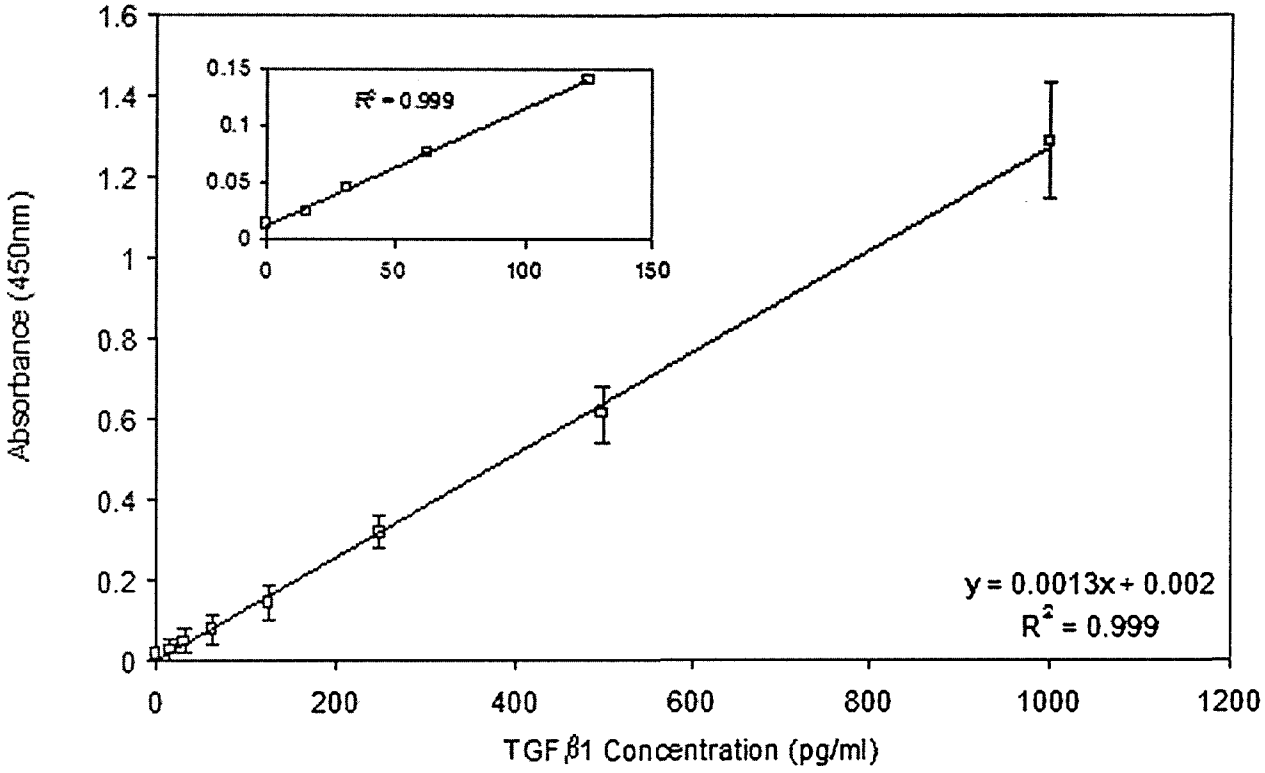


Figure 2.10: TGFβ1 ELISA Standard curve. The magnified portion shows that the curve exhibits linearity at very low concentrations of TGFβ1 as well as the higher concentrations.

2.8.4 Endothelin-1 ELISA

The Endothelin-1 ELISA system (R&D Systems) is a solid phase ELISA designed to measure ET-1 in extracted EDTA plasma and in culture supernatants. It has antibodies raised against synthetic human ET-1, and is able to quantitate both synthetic and human ET-1.

The ELISA was carried out as per the manufacturer's protocol. Briefly, all cultured medium samples were initially used neat. 250µl of the conjugate concentrate was diluted into 11ml of the conjugate dilutent mix. 100µl of diluted conjugate was then added to each well of the pre-coated 96 well plate. 100µl of sample was then added to each well with sufficient force to ensure mixing., The first two columns of the plate were loaded with ET-1 standards supplied with the kit and used to construct a standard curve (see figure 2.11 for a representative standard curve). The plate was then covered with a plastic plate sealer and incubated at room temperature for 60 minutes. The contents from the plate were then aspirated and washed by addition of 300µl per well was wash buffer (see appendix A). This was repeated five times. After the final wash, the contents were aspirated and the excess wash buffer was removed by tapping the plate face down on blotting paper. Next, 100µl of substrate (tetramethylbenzidine) were added to each well, which was covered with a plate sealer and incubated at room temperature for 30 minutes, after which 100µl of Stop solution (sulphuric acid solution) was added to the wells, in the same order as the substrate was added.

2.9 Statistical analysis

The data from the experiments were subjected to statistical analysis to determine levels of significance, The data could not be presumed to be normally distributed, therefore non-parametric tests were used. Within group analysis (i.e. stimulated *vs* unstimulated) was conducted using the Wilcoxon rank sum test. Between groups analysis (i.e. asthmatic *vs* non-asthmatic) was conducted using the Mann-Whitney U test. P values of 0.05 or lower were considered significant.

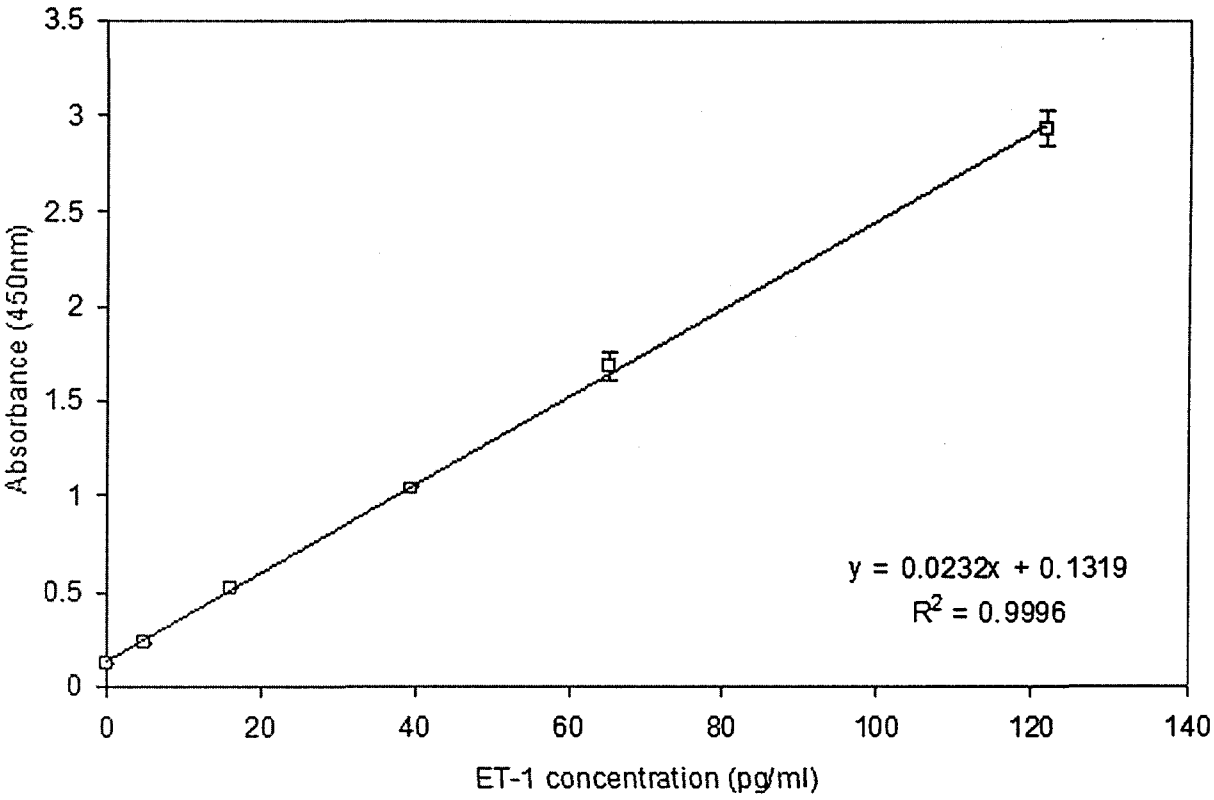


Figure 2.11: Endothelin-1 ELISA standard curve. Standards supplied in the kit were of known concentrations, and were read using a spectophotometer at 450nm. The error bars represent the standard deviation between the standards run on different plates.

CHAPTER THREE

A comparative analysis of the proliferation and the cytoskeletal protein expression in non-asthmatic and asthmatic (myo)fibroblasts.

3.1 Introduction

Subepithelial fibrosis in the *lamina reticularis* of asthmatic subjetcs has been attributed to the increased number of myofibroblasts and production of interstitial collagens. Studies by Roche and co-workers (Roche *et al.,* 1989) have shown that interstitial collagens I,III and V contribute to the fibrosis in asthma and are secreted by myofibroblasts which are normally resident in the *lamina reticularis*. Asthma disease severity has been linked to the number of myofibroblasts resident in the *lamina reticularis.*

Fibroblasts are mesenchymal cells, which have the ability to undergo phenotypic alteration *in vivo* in response to specific factors in the local microenvironment. One of the most potent factors which is able to drive fibroblast to myofibroblast differentiation is TGFβ. Myofibroblasts are typically characterised by the expression of cytocontractile proteins that are also present in smooth muscle cells, the most well characterised of which is α-smooth muscle actin (α-SMA).

The aim of these studies is to compare the proliferation of asthmatic and non-asthmatic myofibroblasts. In order to qualify any results obtained, it was first necessary to show that cells obtained from non-asthmatic and asthmatic bronchial biopsies were of the same origin, since the cells were grown from endobronchial biopsies, there was potential for smooth muscle contamination of the cultures. A number of proteins were analysed in order to distinguish between fibroblastic and smooth muscle cells, in order to be sure that investigations were being carried out on cells fibroblastic in origin and not smooth muscle. In addition cells were grown specifically from tissue microdissected from non-asthmatric *lamina reticularis* (an area known to specifically harbour myofibroblasts; see section 2.2.2,) to enable comparison of their phenotypic properties with cells grown from bronchial biopsies.

As damaged epithelial cells release TGFβ2 (Zhang *et al.,* 1999), this growth factor was added to the cultures, to model the epithelial-mesenchymal trophic unit.

Having established the cytoskeletal and cytocontractile properties of endobronchial-derived cells and those specifically grown from the *lamina reticularis*, the proliferation of the cells was investigated in the absence and presence of 0.4nM TGFβ2.

3.2 Aims

1) To investigate cytoskeletal and cytocontractile protein expression in endobronchial biopsy derived non-asthmatic and asthmatic fibroblasts.

2) To compare cytoskeletal and cytocontractile protein expression between cell from derived from endobronchial biopsies and the *lamina reticularis*.

3) To compare the proliferation of non-asthmatic and asthmatic fibroblasts.

3.3 Hypothesis

1) There is a difference between the cytocontractile and cytoskeletal protein expression in non-asthmatic and asthmatic fibroblasts which may account for the subepithelial fibrobsis observed in asthma.

2) Asthmatic myofibroblasts proliferate more than non-asthmatic myofibroblasts, which may account for increased number in relation to asthma disease severity.

Null Hypothesis

1) There are no differences between the cytocontractile and cytoskeletal protein expression in non-asthmatic and asthmatic fibroblasts.

2) There are no differences in the proliferation of asthmatic and non-asthmatic fibroblasts.

3.4 Comparison of cytoskeletal and cytocontractile proteins in (myo) fibroblasts grown from non-asthmatc and asthmatic endobronchial biopsies and non-asthmatic (myo)fibroblasts grown from the *lamina reticularis*.

Bronchoscopy derived (myo)fibroblasts were grown from asthmatic (BA) and non asthmatic (BN) biopsies which could not be processed for smooth muscle removal, due to their small size. This raised the possibility the cells might be contaminated with smooth muscle cells or be derived from de-differentiated smooth muscle cells. Therefore, to undertake phenotypic analysis of cells derived specifically from the *lamina reticularis*, tissue was obtained from non-involved bronchus from lung resections to provide sufficient material for micro-dissection (Zhang *et al.*, 1999). In this case, after the fibroblasts (LR) had migrated from the explant and proliferated to form a confluent layer, the explant was removed and stained to check for smooth muscle contamination using haematoxylin. Any cultures derived from samples with such contamination were discarded. This increased confidence that the cells obtained via this method were not contaminated with smooth muscle cells or derived from de-differentiated smooth muscle cells. This enabled comparison of the phenotypic properties of the endobronchial biopsy derived fibroblasts with those derived from the *lamina reticularis*.

3.4.1 α-Smooth muscle actin gene expression

α-Smooth muscle actin (α-SMA) expression is characteristic of myofibroblasts and is strongly induced in asthmatic fibroblasts upon treatment with TGF-β. Therefore, initial experiments analysed α-SMA expression in BN, BA and LR fibroblasts. A TGFβ2 concentration of 0.4nM was used throughout these studies, this dose was determined to be the concentration that induced the highest amount of α-SMA gene expressionin both non-asthmatic and asthmatic fibroblasts (see figure 3.1a for dose-reponse curves).

Although LR fibroblasts were derived from the *lamina reticularis*, a region known to contain myofibroblasts (Roche 1990), under basal conditions α-SMA mRNA was low; similarly, α-SMA expression was low in the untreated BA and BN fibroblast cultures. However, in all cases there was a marked induction of gene expression on treatment with TGF-β_2 (figure 3.1b); this increase was evident within 24h of treatment and continued to increase over 3 days.

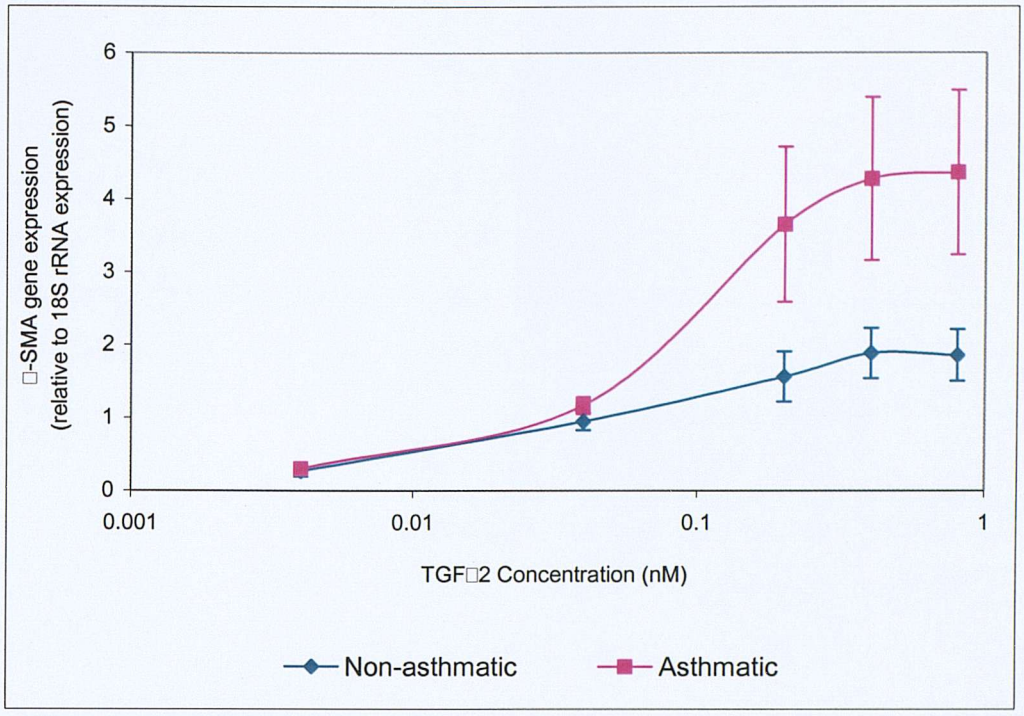


Figure 3.1a: A dose repsonse curve to show the α-smooth muscle actin gene expression in response to increasing doses of exogenous human recombinant TGFβ2 in 5 non-asthmatic fibroblast lines (blue) and 5 asthmatic fibroblast lines (pink). The graphs shows the mean value at each conentration of TGFb2, and the error bars represent the standard error of the mean.

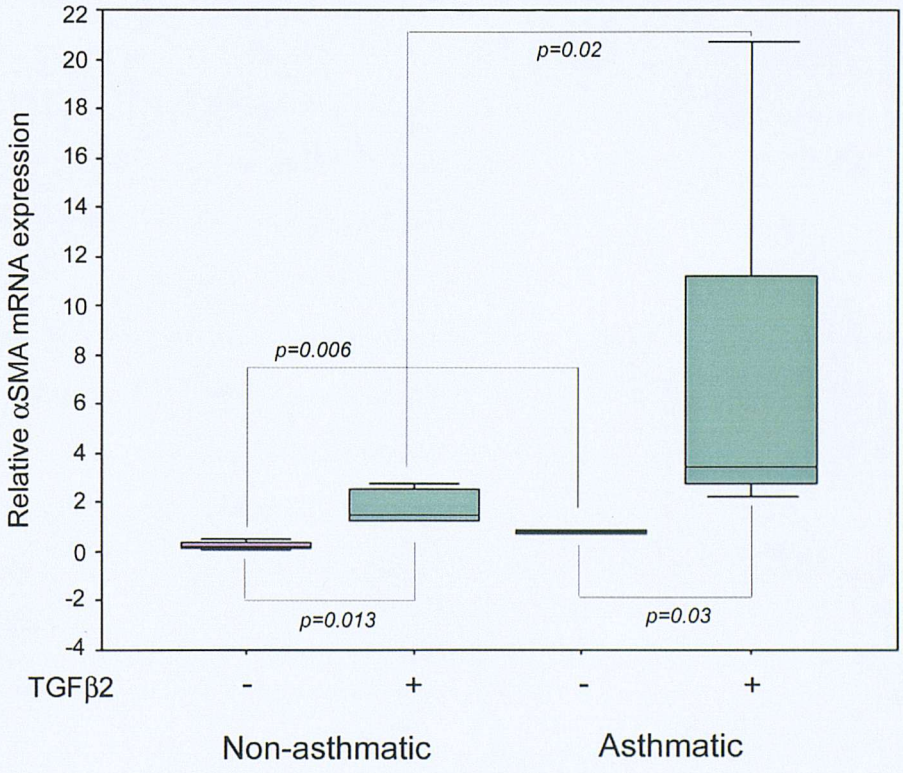


Figure 3.1b: The α-smooth muscle actin gene expression relative to 18S rRNA expression. The total RNA was extracted from endobronchial derived fibroblasts grown from 6 asthmatic and 6 non-asthmatic subjects. The fibroblasts were cultured on a collagen I substratum in serum free medium in the absence (pink) and presence (green) of 0.4nM TGFβ2 for 24 hours. Following reverse transcription, the cDNA was investigated for α-SMA gene expression and expressed relative to the levels of 18S rRNA gene both determined by real-time quantitative PCR. Each assay was conducted in triplicate and the mean value considered. The box plot represents the interquartile range for each of the subject groups when considering the mean value. The central line represents the median; the whisper bars represent the 95% confidence interval. n=18 for each subject group.

3.4.2 Immunocytochemical analysis of cytoskeletal and cytocontractile proteins

Expression of cytoskeletal proteins in LR, BN and BA fibroblast cultures was analysed by immunocytochemistry. As expression of the myofibroblast phenotype required treatment with TGF-β, the study was undertaken on cells cultured in the absence or presence of TGF-β_2 for 3 days. All cells were found to express the intermediate filament, vimentin (figure 3.2) irrespective of treatment; none expressed the smooth muscle marker, desmin (figure 3.3). Consistent with the mRNA analyses for α-SMA expression, little or no α-SMA was detectable in untreated cells (figure 3.4), but this was strongly induced upon treatment with TGF-β_2 (figure 3.5), giving a typical pattern of filamentous staining. The staining intensity was marginally less in the LR myofibroblasts compared with the biopsy- derived cells. Therefore in response to TGF-β_2, LR, BN and BA fibroblasts show characteristic transformation into myofibroblasts.

Even though the cells failed to express the smooth muscle intermediate filament, desmin, three other smooth muscle markers were applied to determine whether the myofibroblasts had any other smooth muscle characteristics, in addition to α-SMA expression. This revealed that SM-22 (transgelin), a 22 kDa protein commonly expressed in smooth muscle cells and fibroblasts (Lawson *et al.,* 1997) gave strong staining irrespective of the presence of TGF-β_2 (figure 3.6). Similarly, muscarinic M_2 receptors gave weak positive staining in all cultures, with no major differences between TGF-β_2 treated and untreated cells (figure 3.7). In contrast, filamentous heavy chain myosin (HCM) protein was present in very few cells in untreated cultures, but became more pronounced in TGF-β_2 treated cells. The HCM staining was similar in appearance to that of α-smooth muscle actin staining, but was not as distinct. It was also noted that untreated BN and LR fibroblasts showed a large number of nuclei staining positively for HCM; this perinuclear staining was not present in the untreated BA fibroblasts. The staining results are summarised in table 3.1.

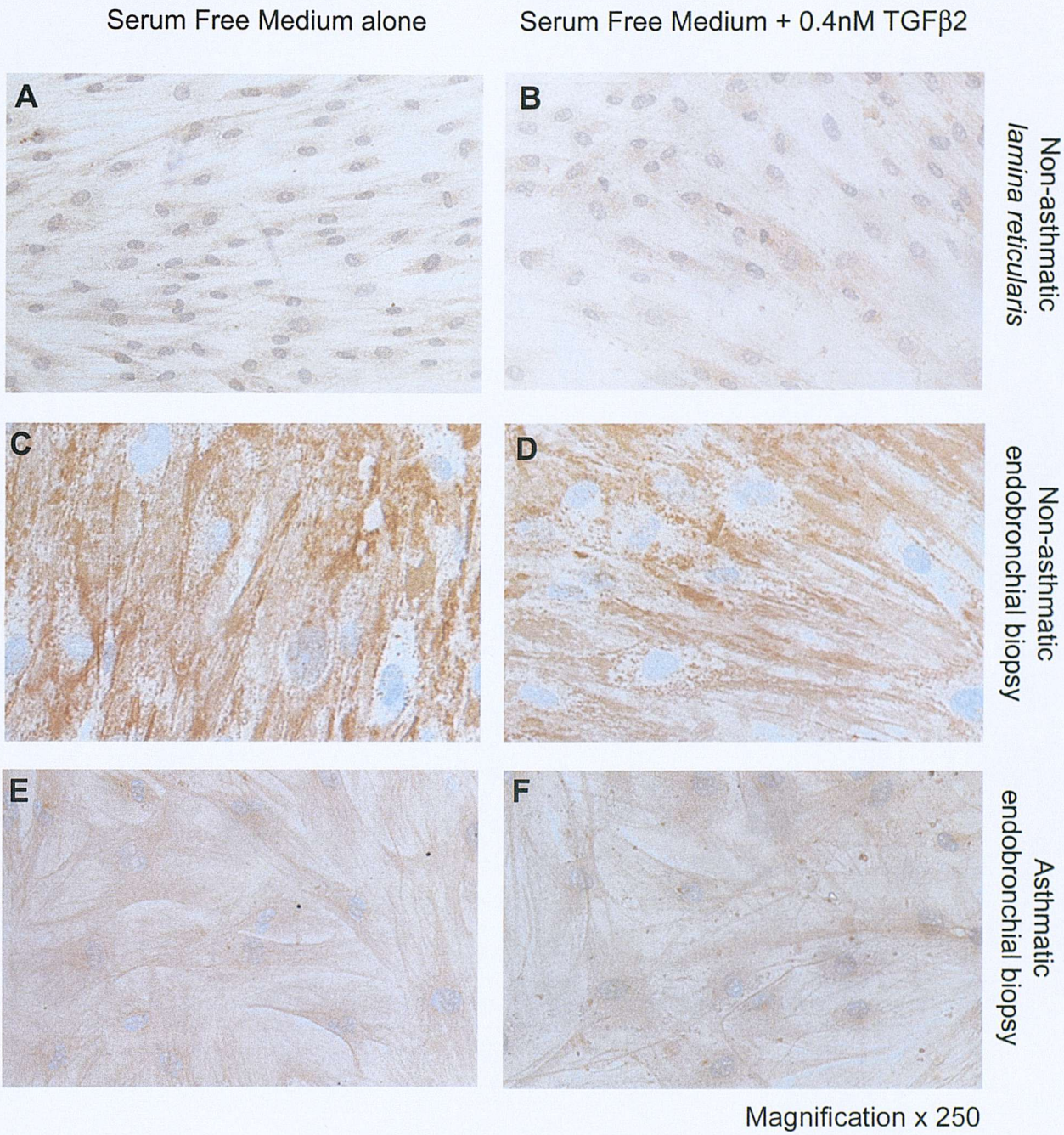


Figure 3.2: Immunostaining for the intermediate filament Vimentin. Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a sheep anti-mouse biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

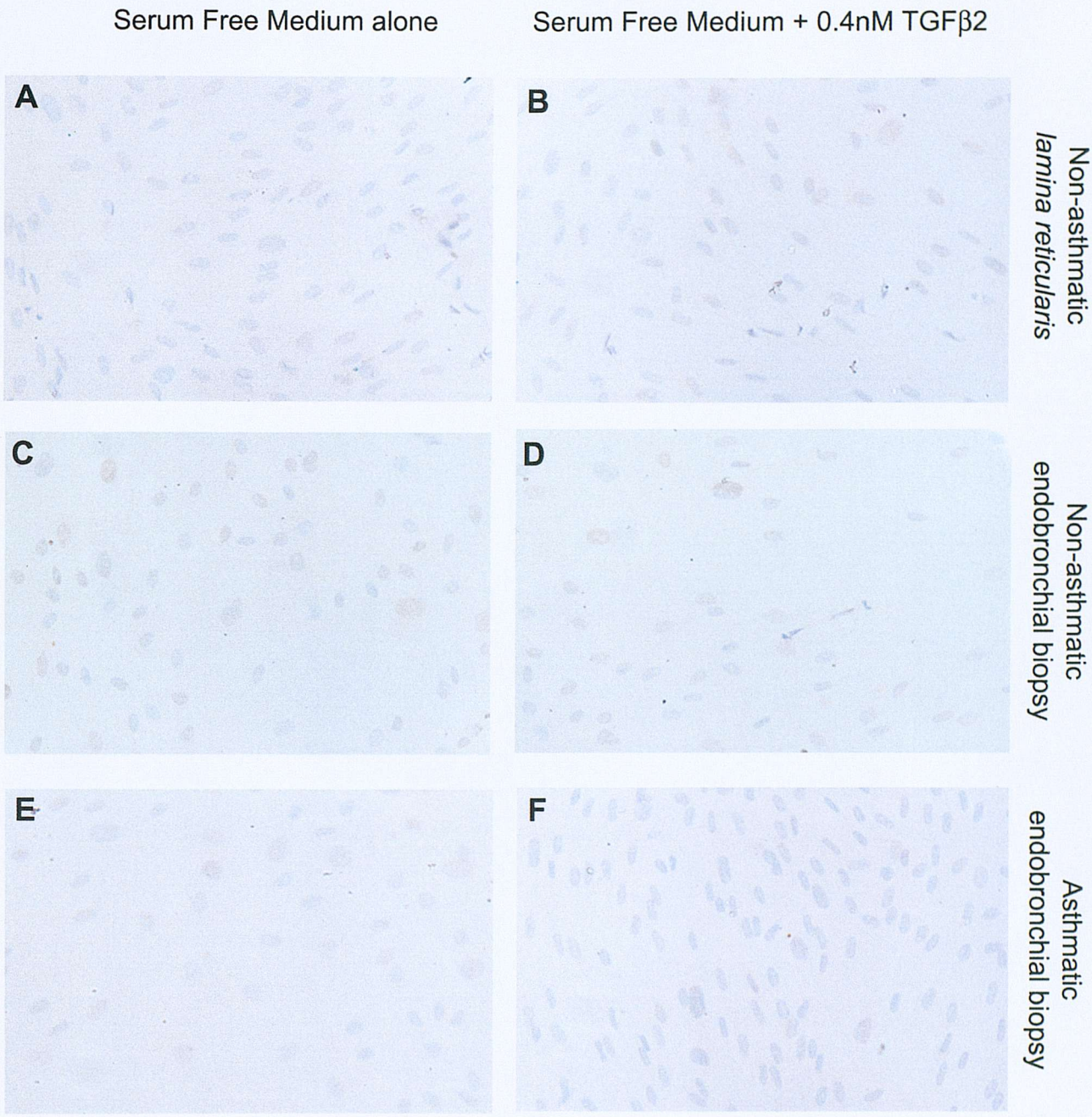


Figure 3.3: Immunostaining for the intermediate filament Desmin. Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a sheep anti-mouse biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

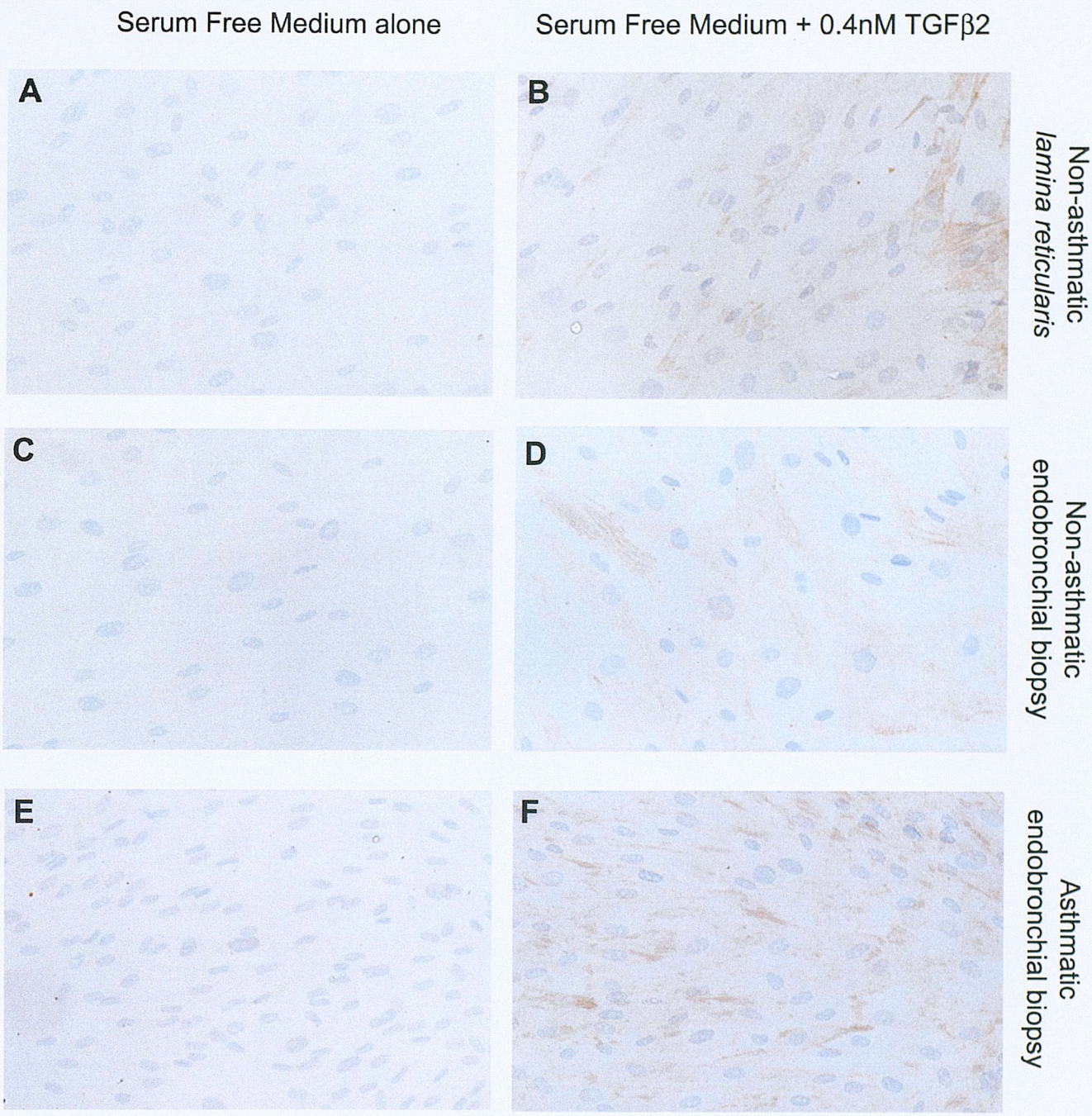


Figure 3.4: Immunostaining for the cytocontractile protein α-SMA . Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a sheep anti-mouse biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

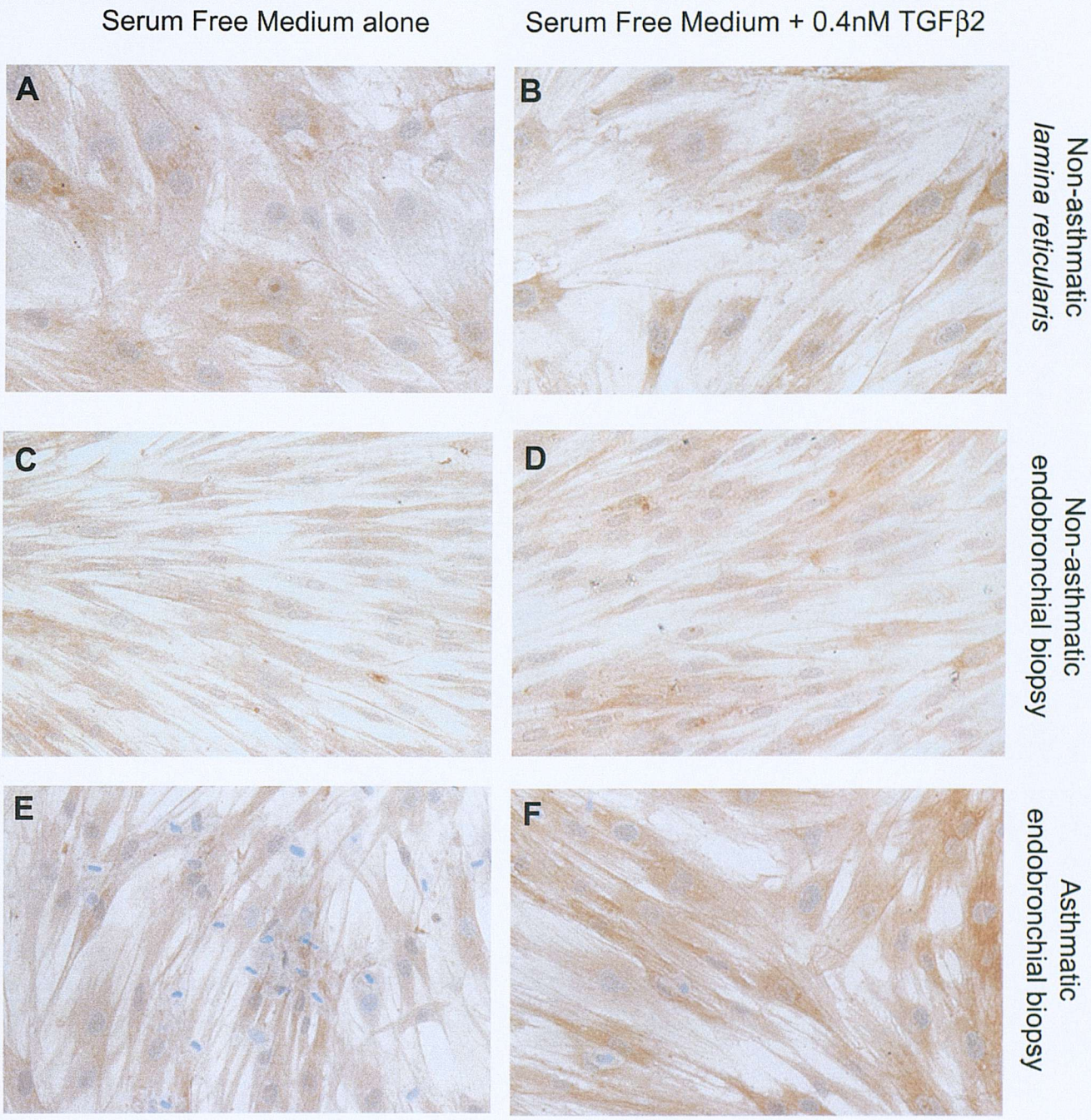


Figure 3.5: Immunostaining for the cytoskeletal protein SM-22. Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a swine anti-rabbit biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

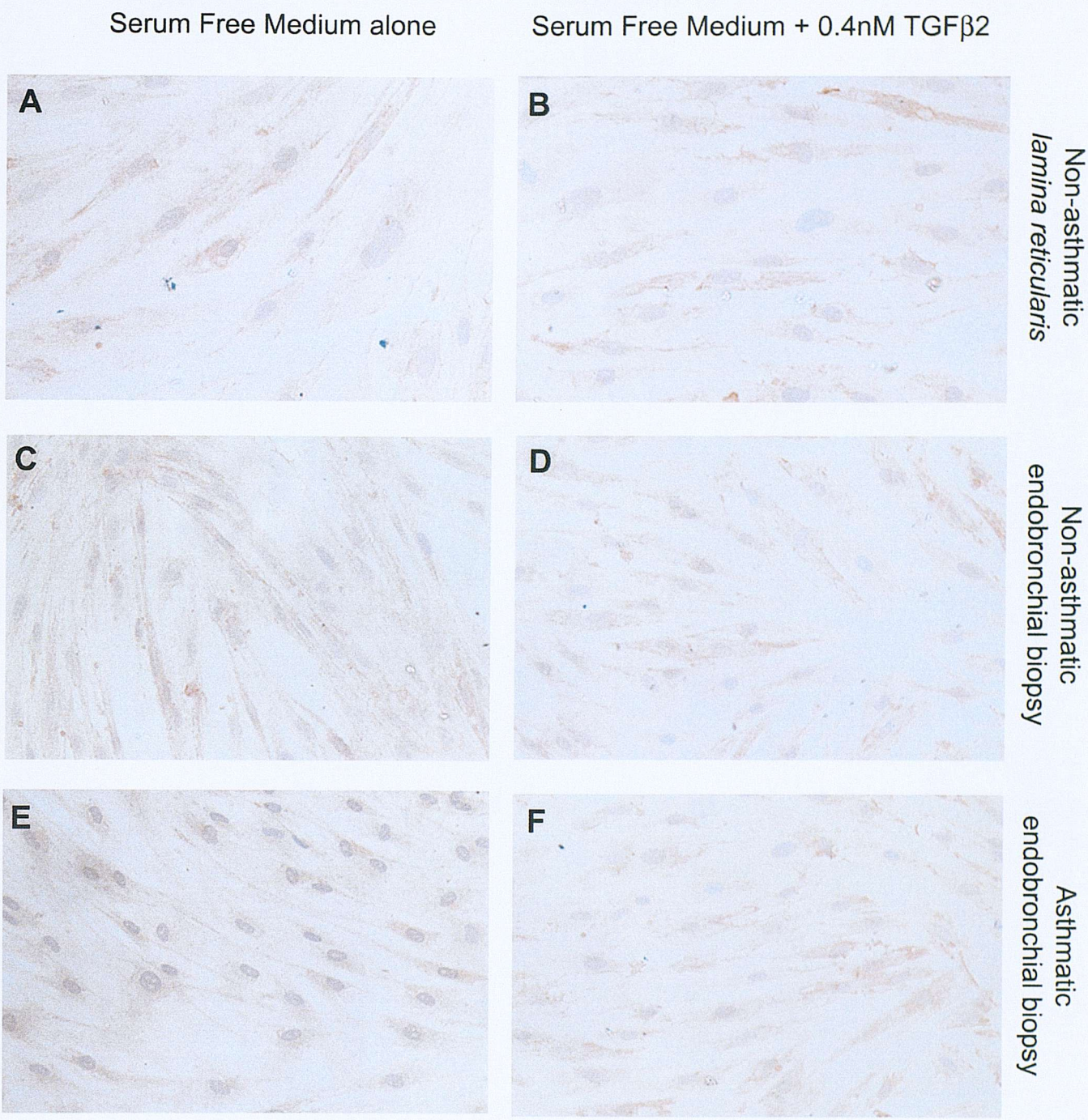


Figure 3.6: Immunostaining for the cell surface Muscarinic, M2 receptor. Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a swine anti-rabbit biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

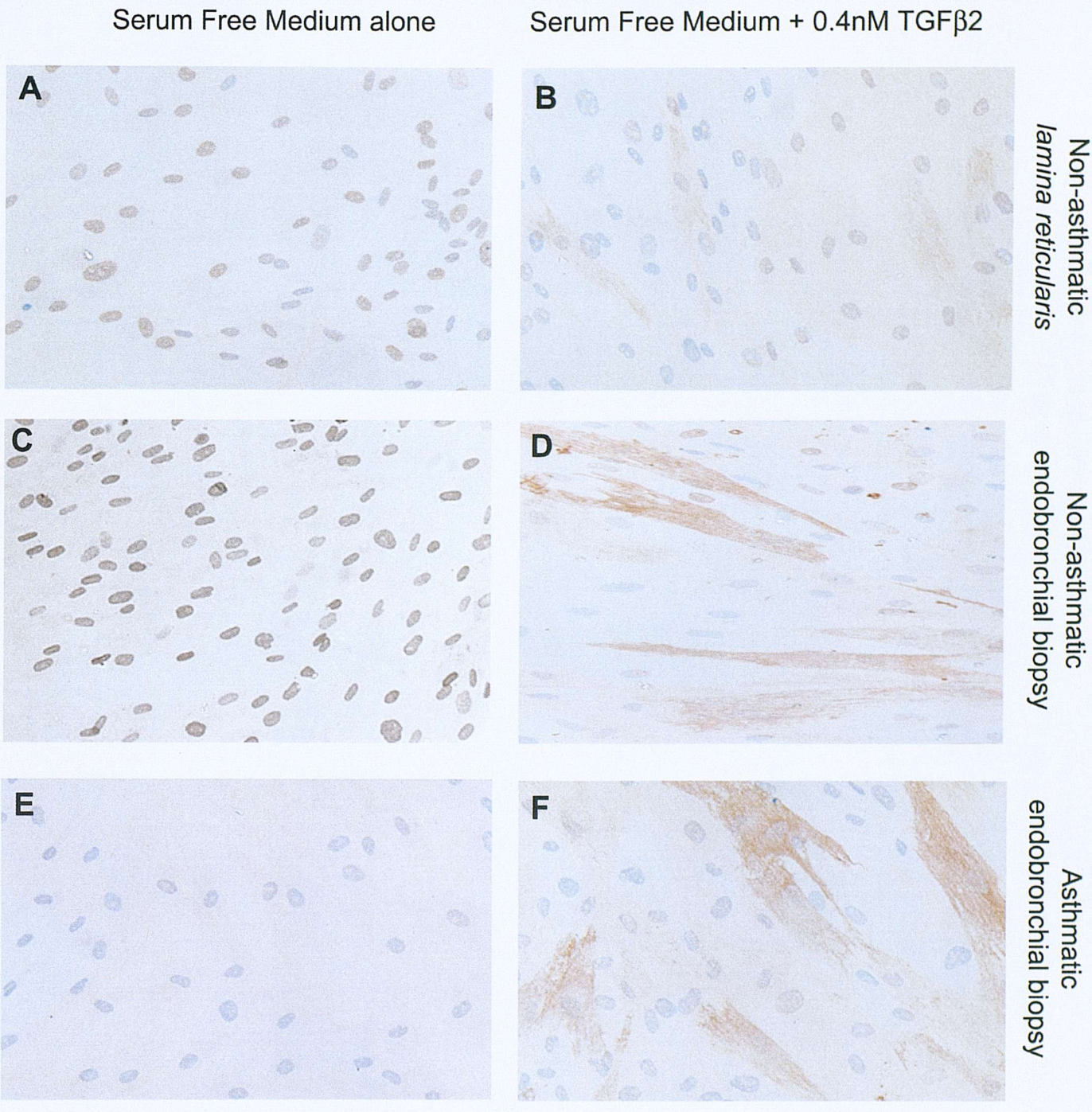


Figure 3.7: Immunostaining for the cytocontractile protein heavy chain myosin (smooth muscle isoform). Five lines of non-asthmatic (myo)fibroblasts derived from micro-dissected *lamina reticularis* and six lines each of asthmatic and non-asthmatic fibroblasts derived from endobronchial biopsies were cultured on 8-well chamberslides pre-coated with collagen I, in serum free medium alone (A,C,E) or in the presence of 0.4nM TGFβ2 (B,D,F) for 72 hours. The medium was then removed and after overnight drying, were frozen at -20°C for at least 48 hours prior prior to investigation. The cells were then fixed in 1:1 Acetone:methanol for 20 minutes and then the antigens were detected using a specific 1° antibody and a swine anti-rabbit biotinylated 2° antibody. The signal was amplified using the three layer Streptavidin-biotin Peroxidase system and visualised using a chromogen (3,3'-diaminobenzidine chromogen solution, DAB) observed as a brown stain. The nuclei were counterstained using Haematoxylin. Magnification x 250.

	ASTHHMATIC		NON-ASTHMATIC	
	+ TGFβ2	- TGFβ2	+ TGFβ2	- TGFβ2
Vimentin	+	+	+	+
Desmin	-	-	-	-
α - smooth muscle actin	+	-	+	-
SM-22	+	+	+	+
Muscarinic (M_2)	+	+	+	+
Heavy Chain Myosin (HCM) (smooth muscle isoforms)	+	-	+	-

Table 3.1: Summary of the staining data, indicating which markers the asthmatic and non-asthmatic fibroblasts stained for.

3.4.3 Comparison of the proliferation of non-asthmatic and asthmatic fibroblasts

Having established that the phenotypic properties of the *lamina reticularis* and biopsy-derived (myo)fibroblasts were comparable, growth experiments were performed. It has previously been shown that asthmatic fibroblasts display the uncharacteristic ability to proliferate in the absence of serum (Warshamana *et al.,* 1998). Therefore, comparisons of growth were made under serum-free conditions using sparsely seeded cultures, which were allowed to grow over 6 days, after allowing initial attachment of the cells in the presence of serum. Under these conditions, the LR fibroblast cultures doubled in number over the first 48h after which they remained quiescent (figure 3.8). Similarly, the number of BN fibroblasts increased slightly over the first 24 h, but showed no further increase over the next 5 days. In both cases, addition of TGF-β_2 did not significantly alter outcome, except in the case of the LR fibroblasts where there was a significant ($p=0.05$), but transient, decrease in cell number 24 h after addition of TGF-β_2. The BA fibroblasts exhibited a similar proliferation profile up to 72 h of culture. However after this time point, there was a progressive increase in cell number up to 144 h, by which time the starting cell number had been increased by more than ten-fold. Similar growth responses were seen when the cells were grown in the presence of TGF-β_2.

Although cell number determined by methylene blue absorbance was checked against cell number obtained from direct cell counts (see appendix B), another method of proliferation was used to confirm the results seen in the methylene blue assays. The proliferation of non-asthmatic (n=3) and asthmatic (n=3) endobronchial derived fibroblasts over 6 days was assessed by thymidine incorporation, in serum free medium, medium containing 1% FBS and medium containing 10% FBS. In the absence of serum, the amount of ^{3}H incorporated by the non-asthmatic fibroblasts was very low and remained so throughout the experiment. Addition of 1% FBS, did not significantly alter the amount of thymidine incorporated, whereas addition of 10% FBS resulted in a significant increase in ^{3}H thymidine incorporated over 6 days (figure 3.9a) In sharp contrast to this, there was no significant dependence on serum for the amount of ^{3}H thymidine incorporated by asthmatic fibroblasts (figure 3.9b) These results agreed with the data obtained from the methylene blue analysis which also indicated that asthmatic (myo)fibroblasts were able to proliferate in the absence of any FBS.

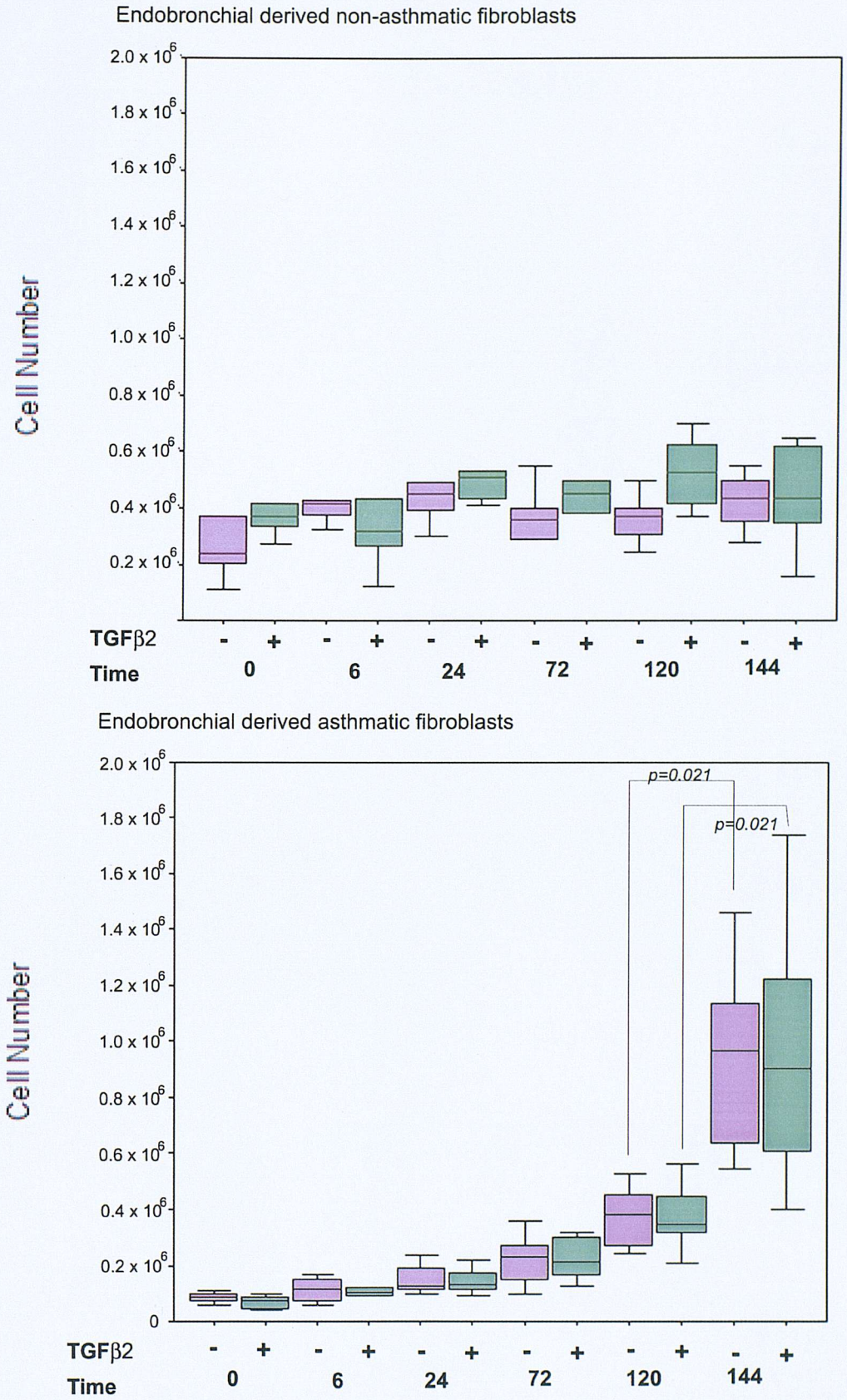
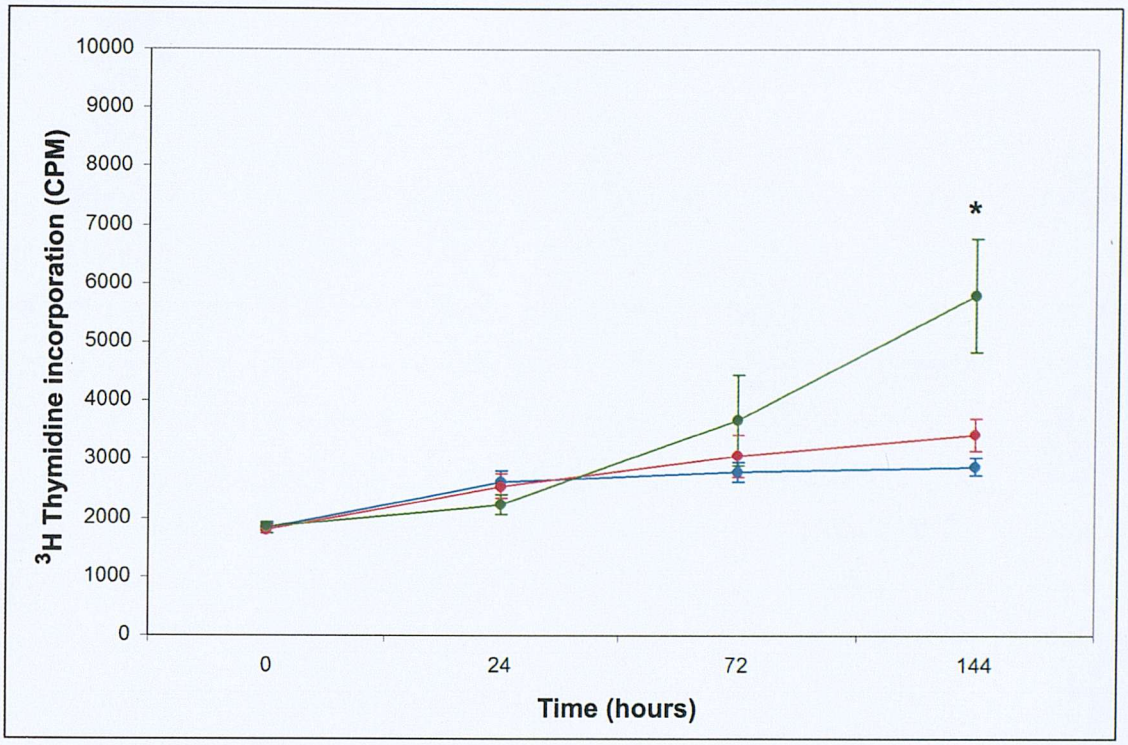


Figure 3.8: The proliferation of endobronchial biopsy derived (a) non-asthmatic and (b) asthmatic fibroblasts. Eight lines of non-asthmatic and ten lines of asthmatic fibroblasts were cultured in serum free medium alone (purple) or in the presence (green) of 0.4nM TGFβ2 for up to 144 hours (6 days) in 24 well dishes pre-coated with collagen I. At different time points (0, 6, 24, 72, 120 and 144 hours) the medium was removed from the designated tray and the cells fixed overnight with Formol saline. Methylene blue dye (500μl) was then added to each well for 30 minutes, after which the excess was removed and the dye eluted from the cells with 500μl HCl/ethanol elution buffer. The absorbance of the eluted solution was then measured in a spectophometer at 620nM. Each assay was conducted in triplicate and the mean value considered. Absorbances were correlated to cell number by means of a generated standard curve (figure 2.3). The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval. n=24 for non-asthmatics and n=30 for asthmatics.



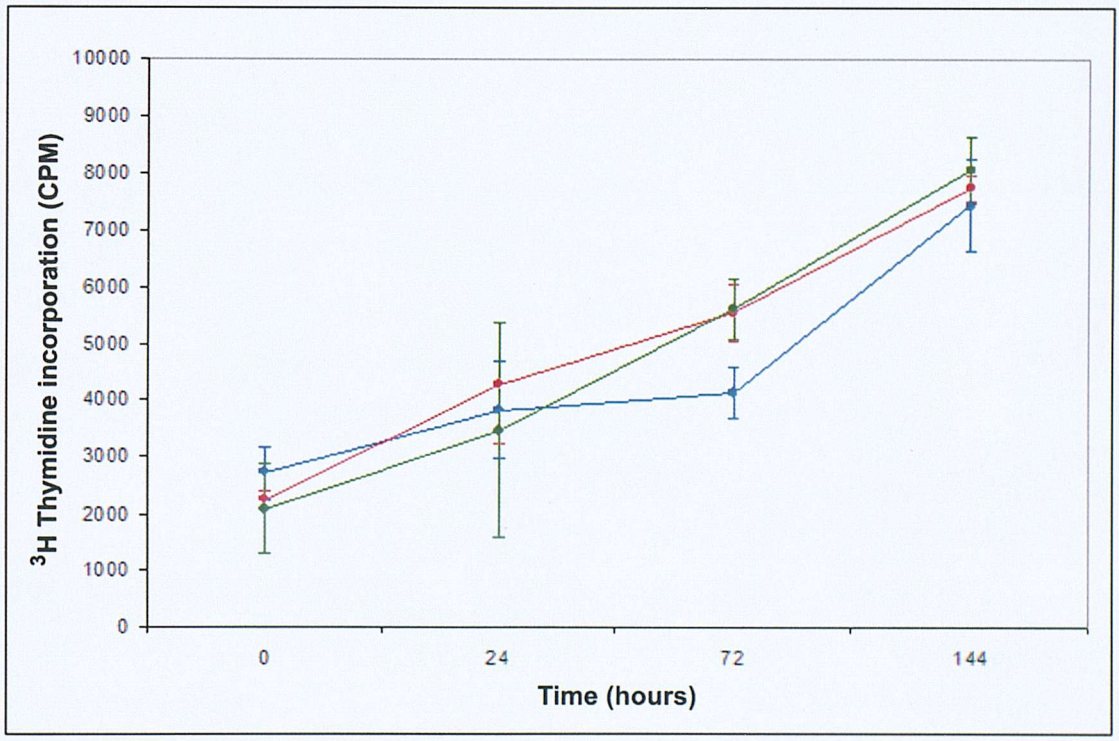


Figure 3.9: 3[H] Thymdine incorporation over 144 hours in (a)3 non-asthmatic and (b) 3 asthmatic endobronchial biopsy derived fibroblast lines. Primary fibroblasts were seeded at a density of 1 x 10^5 cells/well in RPMI medium containing 10% FCS. After 8 hours, following adherence, the medium was changed to serum free medium (red), 1% FBS (blue) or 10% FBS (green). At the appropriate time point the corresponding tray was pulsed with [^{3}H] Thymidine for 2 hours after which it was processed to determine the amount of thymidine incorporated. The data plotted represents the mean of four replicate experiments and the error bars represent the standard deviation between the means of three subjects. (n=12 for each subject group).

3.5 Discussion

Thickening of the *lamina reticularis* is a characteristic feature of the restructured airways in asthma and is evident in young children up to 4 years before asthma diagnosis. This suggests that this region of the airway is intimately involved in disease pathogenesis. As early as 1989, it has been shown that excess collagen deposition in the *lamina reticularis* was due to the increased numbers of myofibroblasts present in this region (Roche *et al.,* 1989). More recently, the potential of myofibroblasts to contribute to the increased smooth muscle mass in asthma has been speculated upon, either through their ability to produce smooth muscle mitogens such as ET-1 in response to TGF-β (Richter *et al.,* 2001) or by potential direct phenotypic transformation. However, this study is the first to compare the phenotypic properties of (myo)fibroblasts specifically derived from the *lamina reticularis* with those of bronchoscopy derived (myo)fibroblasts from asthmatic and non-asthmatic airways.

Using immunocytochemical staining, the profile of expression of cytoskeletal proteins in LR fibroblasts was comparable to that found in fibroblasts derived from endobronchial biopsies. Unlike airway smooth muscle cells, which have been recently characterised from outgrowths of smooth muscle bundles (Halayko *et al.,* 1999) , the cells in the present study failed to express the myofibroblast and smooth muscle marker, α-SMA unless exposed to exogenous TGF-β_2. The low level of α-SMA expression in the LR fibroblasts was not surprising as in this region myofibroblasts do not express α-SMA although certain myofibroblasts markers are expressed (Roche *et al.,* 1989). It is possible that α-SMA was present in these cells in its globular form and was not detected by the monoclonal anti-αSMA antibody used. Nonetheless, following exposure to TGF-β_2, α-SMA mRNA and protein expression was markedly increased. This suggests that the fibroblasts grown from the *lamina reticularis* and those from bronchial biopsies have the potential to be myofibroblast progenitors and that a supply of TGF-β is required to promote or maintain this phenotype. However, it should be noted that induction of α-SMA occurred relatively slowly after TGF-β treatment, being evident at the later 24h and 72h time points, suggesting that induction of α-SMA is unlikely to be the direct effect of TGF-β_2 but is probably the result of a secondary signals induced by TGF-β.

In addition to analysis of α-SMA expression, the expression of other fibroblast and smooth muscle markers was also examined. Fibroblastic cells normally express the

intermediate filament, vimentin, whereas smooth muscle cells express desmin. Since there was strong vimentin staining in all the cultures and a lack of desmin staining, this supports the fibroblastic origin of the cells. As the LR fibroblasts were derived from tissue, which was free from smooth muscle contamination, these cells were unlikely to be de-differentiated smooth muscle cells. Nonetheless, SM-22 expression and muscarinic m_2 receptors were constitutively expressed in these cells and heavy chain myosin (HCM) protein expression was induced upon treatment with TGF-β_2. A similar pattern of expression was also found in the biopsy-derived (myo)fibroblasts. This suggests that TGF-β_2 is an important regulator of the phenotypic properties of these mesenchymal cells causing them to adopt a more smooth muscle-like phenotype. These observations are consistent with a recent study of gene expression in pulmonary fibroblasts which also reported induction of expression of many smooth muscle markers in response to TGF-β treatment (Chambers *et al.,* 2001). The proposal for a direct relationship between myofibroblasts and smooth muscle cells, has been substantiated in studies of the obstructed rabbit urinary bladder, where a gradual transition from fibroblast to myofibroblast and then to smooth muscle was observed and found to be regulated by TGF-β (Buoro *et al.,* 1993). Thus, as TGF-β is increased in the airways in asthma (Redington *et al.,* 1997), it is possible that it contributes the increase in airway smooth muscle mass by modulating myofibroblast function. The findings that airway fibroblasts have the ability to undergo phenotypic differentiation to myofibroblasts and the potential to further differentiate into smooth muscle cells as indicated by expressions of proteins normally associated with smooth muscle cells are in agreement with reports recently published by Chambers and co-workers (2003).

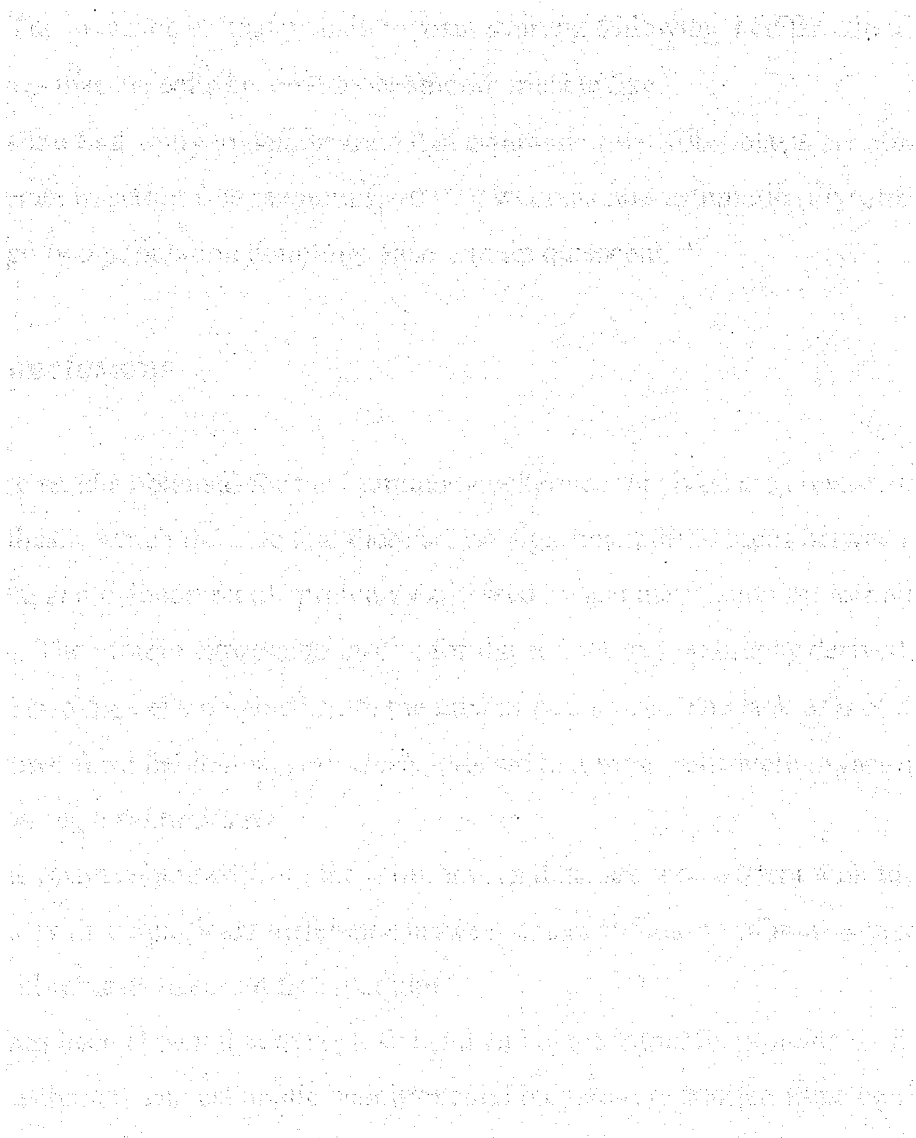
In general, the pattern of cytoskeletal staining was comparable across the three groups of fibroblasts. One significant exception was the expression of HCM in the untreated non-asthmatic and asthmatic fibroblasts. Both the non-asthmatic *lamina reticularis* derived fibroblasts and fibroblasts derived from non-asthmatic bronchoscopies exhibited strong perinuclear HCM staining, whereas this was markedly reduced in the asthmatic fibroblast cultures. Nuclear staining for myosin has been reported in 3T3 fibroblast cell lines (Pestic-Dragovich *et al.,* 2000; Nowak *et al.,* 1997), and the presence of nuclear actin and nucleus specific actin binding proteins have also been reported (Clubb and Locke 1998). The occurrence of actin and myosin in the same nuclear structure may

indicate that actin is involved in energy requiring movements in the nucleus, mediated through myosin molecular motors.

Having characterised the cytoskeletal proteins present in the individual fibroblast cell cultures, their growth in serum-free medium was followed. As shown in figure 3.8, the BA fibroblasts underwent approximately four population doublings over 6 days whereas the LR or BN fibroblasts were relatively static, achieving, at best, one population doubling over the duration of the experiment. Morphological observation of the cells indicated that the non-asthmatic fibroblasts formed a flattened, spread monolayer whereas the asthmatic cells tended to overcome contact inhibition and pile up as they continued to proliferate. In these experiments, the initial growth of the asthmatic fibroblasts was comparable to that of the non-asthmatic fibroblasts, however from about 72h there was a progressive increase in cell number, and this was insensitive to the presence of TGF-β. As the cells were left in the same culture medium for the duration of the experiment, it seems likely that their growth was dependent on the production and accumulation of autocrine factors in the culture medium. The lack of effect of TGF-β is in contrast to a previous reported study (Richter *et al.,* 2001), where a small but significant increase in proliferation was observed on addition of TGF-β_2. However in the former study, the TGF-β_2 was replenished after three days, which may have augmented its growth promoting effects. Consistent with such a mechanism of autocrine growth, it has been shown that asthmatic fibroblasts secrete ET-1 and VEGF, and that this is enhanced by TGF-β (Richter *et al.,* 2001).

It has recently been reported that asthmatic airway smooth muscle cells proliferate at a greater rate than non-asthmatic cells, a finding that is similar to the results with asthmatic fibroblasts in the present study. Based on the observations in the present study that (myo)fibroblasts are able to respond to TGF-β and adopt a more smooth muscle-like phenotype, it may be hypothesised that the asthmatic fibroblasts described in the present study are closely related to the smooth muscle cells studied by Johnson and co-workers (Johnson *et al.,* 2001), perhaps by lying on the same differentiation pathway from a common precursor or progenitor cell. Such a close relationship between myofibroblast and smooth muscle cells might explain why smooth muscle bundles lie closer to the luminal surface in asthma and why there is an increase in migratory, contractile cells in the *lamina reticularis* following allergen challenge (Giyzcki *et al.,* 1997). While TGF-β is involved in the process(es) of differentiation, it is likely that other factors are also

involved. For example, differentiation of vascular smooth muscle and vascular endothelial cells is dependent on the relative amounts of VEGF and PDGF provided to their common progenitor cells (Carmeliet 2000).



3.6 Summary of results and novel findings

- Endobronchial biopsy derived non-asthmatic and asthmatic fibroblasts both exhibit similar cytoskeletal and cytocontractile proteins.
- Fibroblastic cells found in the *lamina reticularis* are not myofibroblasts *per se*, as demonstrated by the lack of α-SMA immunoreactivity.
- All lines of fibroblast demonstrated the ability to transform to myofibroblasts following activation with 0.4nM TGFβ2.
- Bronchial fibroblasts express some proteins normally associated with smooth muscle cells. The presence of heavy chain myosin staining following TGFβ2 stimulation indicates that the cells become more smooth muscle like.
- This is the first study to demonstrate that asthmatic (myo)fibroblasts are able to proliferate in serum free medium ($p=0.02$), whereas non-asthmatic (myo)fibroblasts undergo two population doublings then remain quiescent.

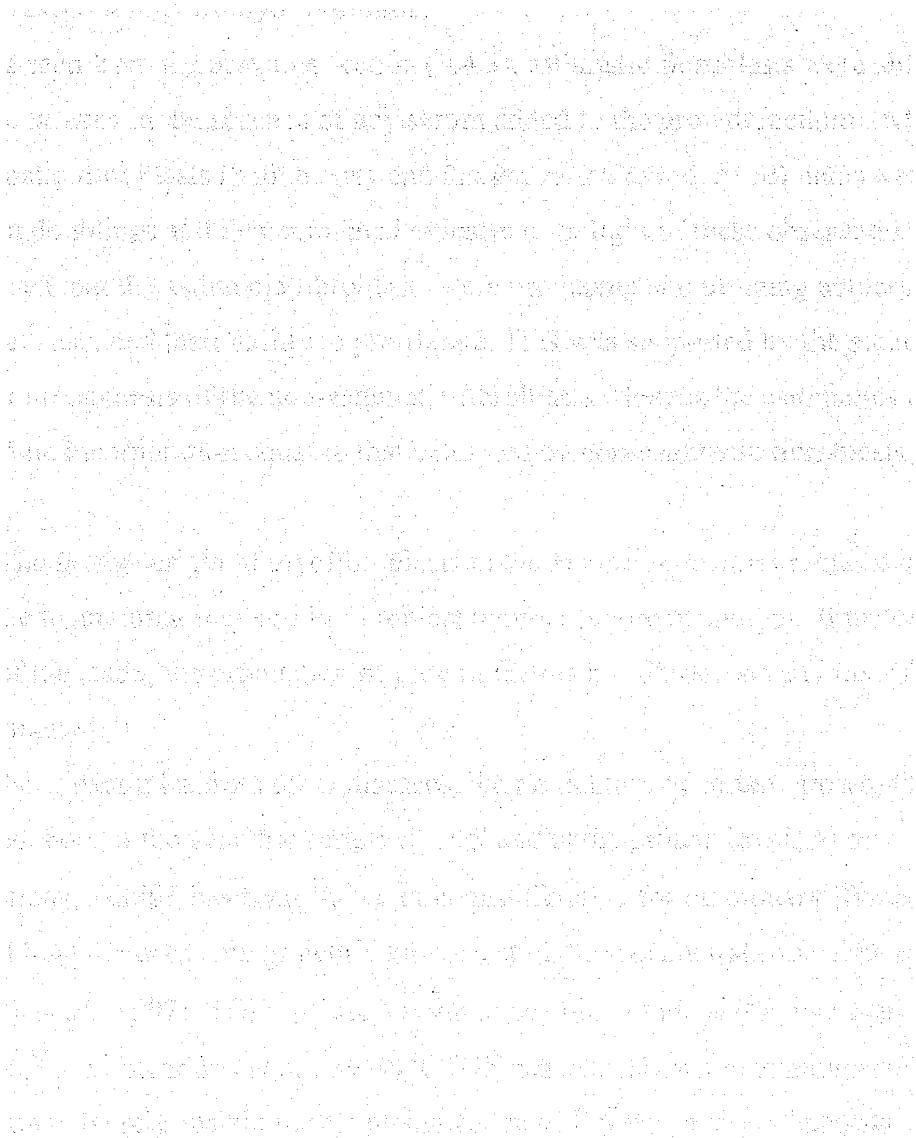
3.7 Conclusions

The results obtained for the immunocytochemical analysis are consistent with the null hypothesis, which indicate that there are no significant differences between the cytoskeletal and cytocontractile proteins expressed by asthmatic and non-asthmatic fibroblasts. The protein expression profile for the endobronchial biopsy derived fibroblasts were similar to the cells obtained from the *lamina reticularis*. The lack of α-SMA staining in cells grown from the *lamina reticularis* indicate that these cells were in fact not myofibroblasts, but fibroblasts.

The results obtained from the proliferation data, are inconsistent with the null hypothesis, with a significant difference between the proliferation of non-asthmatic and asthmatic fibroblasts in serum free medium.

It has been shown that the cytoskeletal and cytocontractile proteins in fibroblasts from non-asthmatic and asthmatic endobronchial biopsies are similar, thus the fibrosis observed in asthma is unlikely to be a result of different cell types. However, the asthmatic fibroblasts differ in their ability to proliferate. In the next chapter, I will investigate whether the intrinsic ability of asthmatic fibroblasts to proliferate is due to the production

of autocrine growth factors. A combination of these factors along with the local microenvironment may result in the proliferation of an activated phenotype *in vivo*.



CHAPTER FOUR

The production of fibroproliferative growth factors

4.1 Introduction

The asthmatic airway is characterised by excess deposition of collagens I, III and V, in the *lamina reticularis* (Roche *et al.,* 1989), which has been attributed to the presence of an increased number of myofibroblasts.

As seen from the previous section (3.4.3), asthmatic fibroblasts were able to proliferate *in vitro* in the absence of any serum added to the growth medium, whereas the non-asthmatic fibroblasts (both biopsy and *lamina reticularis* derived) underwent two population doublings and then remained quiescent. In light of these observations, it was hypothesised that the asthmatic fibroblasts were producing and utilizing autocrine growth factors that sustained their ability to proliferate. This was supported by the serum dependent mitogenesis of the non-asthmatic fibroblasts whereas the asthmatics exhibited ^{3}H thymidine incorporation equal to that exhibited by non-asthmatic fibroblasts in 10% serum.

The *in vivo* origin of myofibroblasts in the *lamina reticularis* remains unclear and may be due to an initial increase in fibroblast number before phenotypic transformation. In this part of the study, the production of growth factors by fibroblasts and myofibroblasts was investigated.

Using examples from other diseases, the production of certain growth factors was determined, both at the gene transcription level and at the protein level. At low concentrations, TGFβ1 has been shown to be proliferative for pulmonary fibroblasts from interstitial lung diseases, and growth inhibitory at higher concentrations in the same cells (McAnulty *et al.,* 1997). TGFβ is also known to be able to induce it's own expression (see section 1.6.3.1; Grotendorst *et al., 1989*). CTGF has been shown in atherosclerosis and in scleroderma to be responsible for the maintenance of fibrosis, and has been shown to induce collagen synthesis in fibroblasts (Duncan *et al,.*1999) (see section 1.6.3.3).

ET-1 is a potent mitogen for smooth muscle cells. In light of the observation in the previous section, indicating that fibroblasts become more smooth-muscle like on

stimulation with TGFβ2, it was postulated that ET-1 may be strongly mitogenic for the (myo)fibroblasts. ET-1 has been shown to stimulate the proliferation of fibroblasts from the left ventricular wall of patients following myocardial infarction. It has also been shown that fibroblasts from the bronchial explants of patients with idiopathic pulmonary fibrosis secreted significantly greater levels of ET-1 than fibroblasts from normal control patients (see section 1.6.1).

The mRNA expression levels for each of the mentioned growth factors were determined by the use of quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Protein levels were measured using enzyme linked immunosorbent assays (ELISAs) or western blotting.

4.2 Aims

To investigate the expression and production of growth factors which may have the potential to drive the proliferation of asthmatic (myo)fibroblasts.

4.3 Hypothesis

1) There is an increased production of autocrine growth factors by (myo)fibroblasts derived from asthmatic bronchial biopsies when compared to (myo)fibroblasts derived from non-asthmatic biopsies.
2) Myofibroblasts (TGFβ differentiated fibroblasts) have a greater ability to produce growth factors when compared to fibroblasts.

Null Hypothesis

1) There is no difference in the production of autocrine growth factors between (myo)fibroblasts derived from asthmatic bronchial biopsies and (myo)fibroblasts derived from non-asthmatic biopsies.

2) There are no increases in the levels of autocrine growth factors which are produced when fibroblasts are differentiated into myofibroblasts.

4.4 The expression of growth factors in non-asthmatic and asthmatic (myo)fibroblasts at baseline and in response to TGFβ2

The induction of gene expression in response to 0.4 nM TGFβ2 in the asthmatic (n=8) and non-asthmatic (n=8; bronchial biopsy derived n=6, *lamina reticularis* drived n=2) fibroblasts, was tested for statistical significance by application of the Wilcoxon statistical test. Comparisons between non-asthmatic phenotypes were tested for significance by application of the Mann-Whitney U test.

4.4.1 Tranforsming Growth Factor β1

TGFβ1 is a profibrogenic growth factor whose autocrine expression is typically regulated by exogenous TGFβ treatment (Bascom *et al.*, 1989 ; see section 1.4.1). Exposure of fibroblasts to 0.4 nM TGFβ2 caused TGFβ1 gene expression. In the case of the non-asthmatic fibroblasts, at 6 and 24 hours there was a statistically significant increase in gene expression on stimulation with exogenous TGFβ2 treatment ($p=0.05$ and $p=0.04$ respectively) but this then decreased by 72 and 144 hours.

TGFβ1 protein release was then determined by using a TGFβ1 specific ELISA (i.e. it did not detect exogenously added TGFβ2) using conditioned medium from the cells used for the mRNA extractions. Unlike the gene expression there were significant increases in response to TGFβ2 at all time points in both the non-asthmatic and asthmatic fibroblasts (figure 4.2). At 6 hours, the protein levels (median (range)) were 50 (22-96) pg/10^5 cells unstimulated and 99 (0-257) pg/10^5 cells ($p=0.017$) ; at 72 hours, the protein levels (median (range)) were 50 (0-191) pg/10^5 cells unstimulated and 114 (0-614) pg/10^5 cells ($p=0.011$).

On comparing the TGFβ1 gene expression between non-asthmatics and asthmatics there was no significant difference in the levels of expression at all time points in the absence of TGFβ2 stimulation. On stimulation with TGFβ2, there was no significant difference in expression levels between asthmatics and non-asthmatics at all time points .The total protein expression was also similar in both the asthmatic and non-asthmatic conditioned medium with no significant differences at all time points, with the exception of 6 hours, at which the levels of total TGFβ1 present was significantly ***higher*** in the ***non-asthmatic cells*** than the asthmatic cells (109 (49-906)pg/10^5 cells *versus*(59 (22-97) pg/10^5 cells; ($p=0.03$)) in the unstimulated cultures. This was also observed in the TGFβ2 simulated cultures cells 261 (129-1467)pg/10^5 *versus* 99 (0-709) pg/10^5 cells; ($p=0.05$) (figure 4.3).

Before TGFβ can elicit its effects, it is required to be activated by cleavage from its latent binding protein (see section 1.4.6.2). The results described above quantified the total TGFβ2 (latent and active TGFβ1) in the conditioned medium of non-asthmatic and asthmatic fibroblasts. As latent TGFβ1 doesn't imply the presence of active TGFβ1, the levels of active TGFβ1 were also investigated.

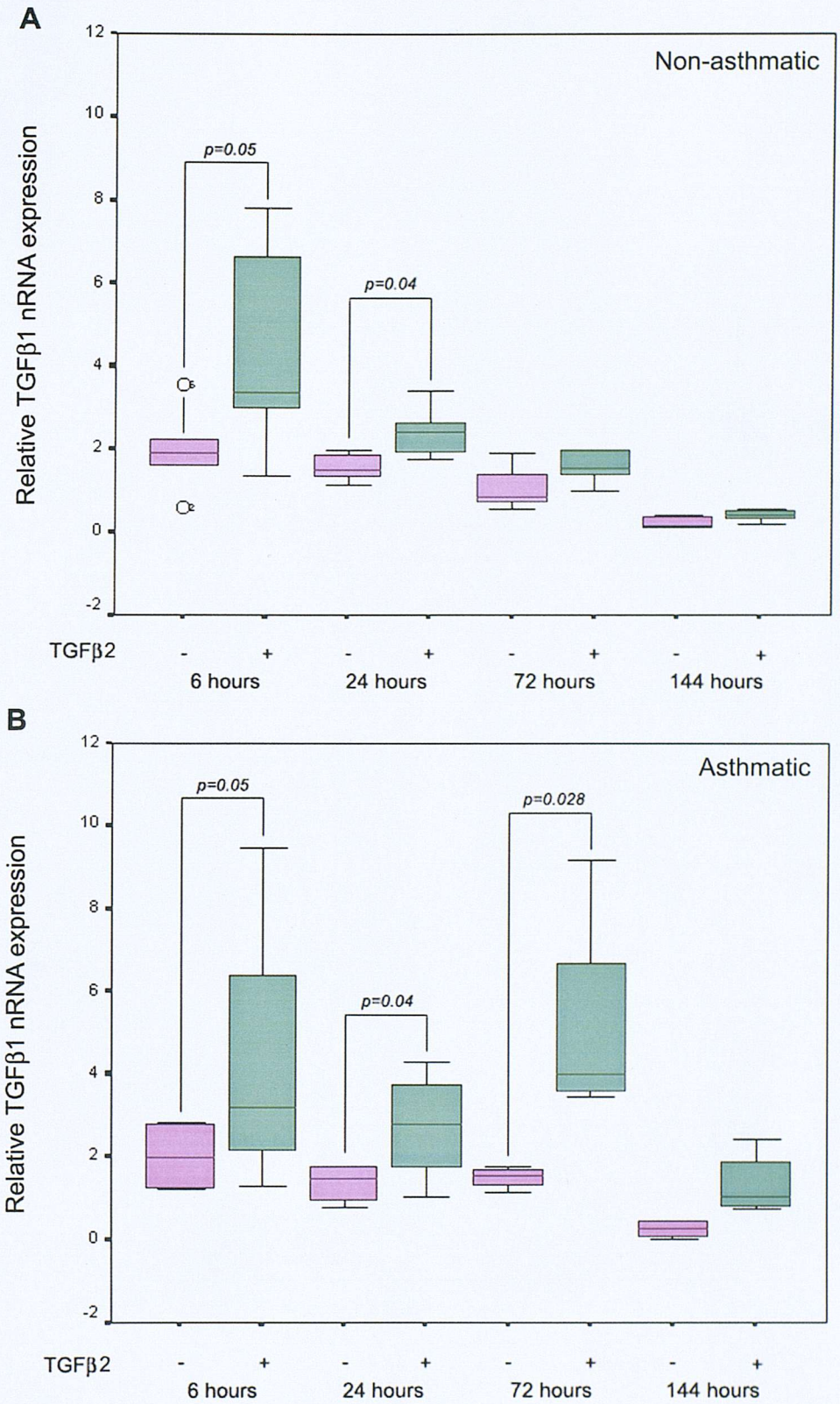


Figure 4.1: Time course for the regulation of TGFβ1 gene expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for TGFβ1 gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

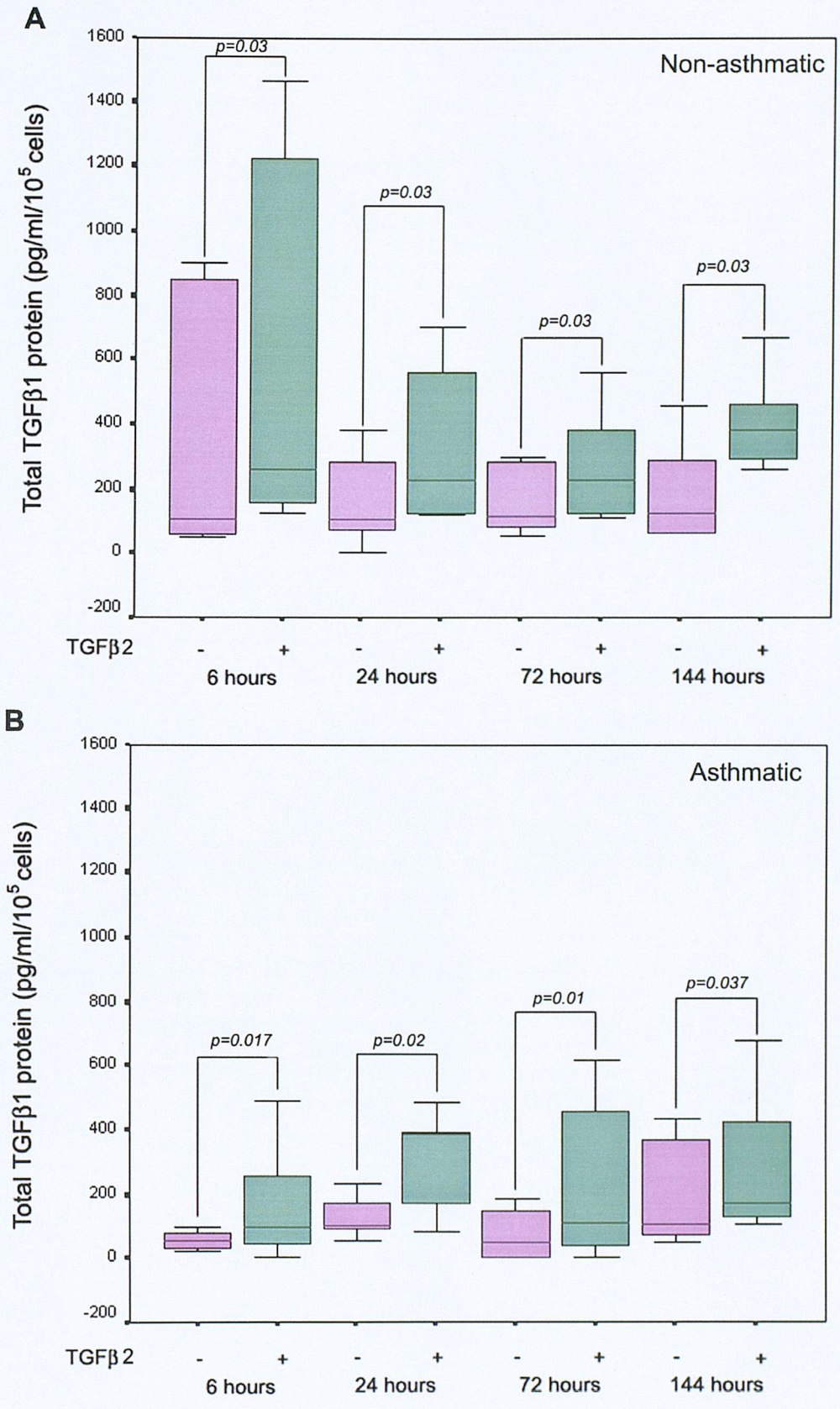


Figure 4.2: Time course for the regulation of total TGFβ1 protein expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). At the specified time points, the supernatant was removed and stored at –80°C for subsequent analysis. The total TGFβ1 protein was assessed after acid activation of latent TGFβ1 in the cultured medium. The TGFβ1 levels were measured by use of an enzyme-linked immunoassay system obtained from Promega (UK). Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

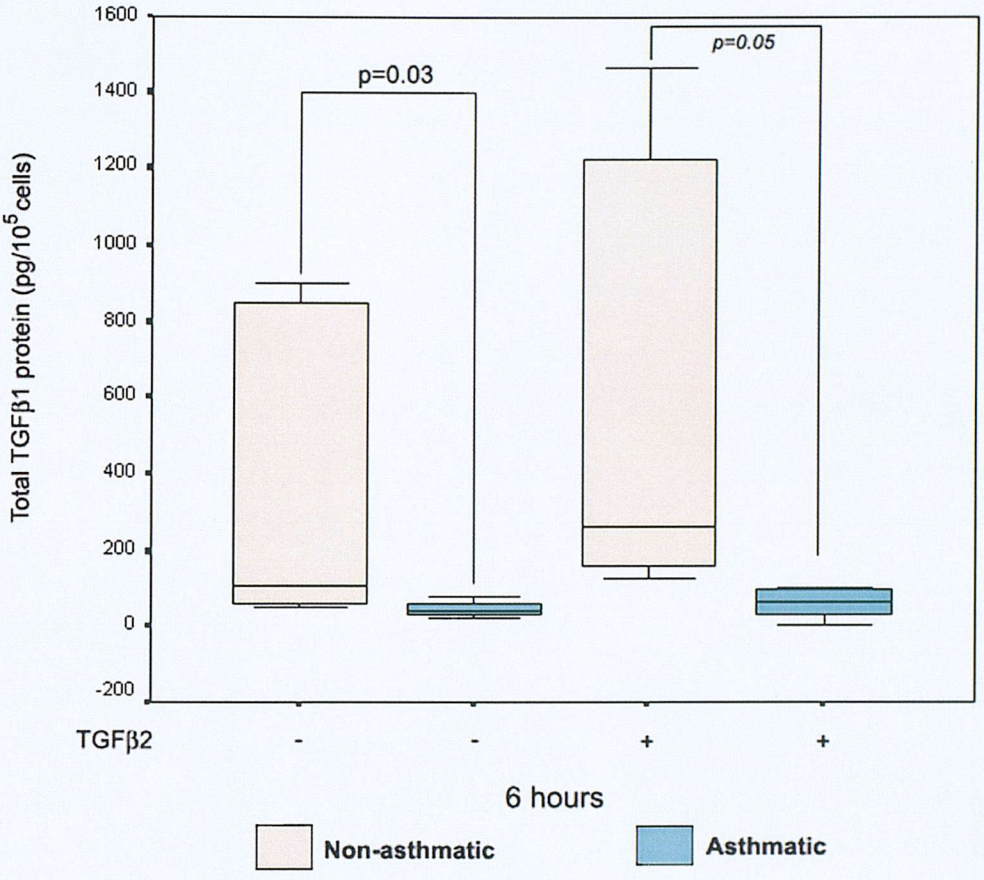


Figure 4.3: The comparison of total TGFβ1 protein levels following culture for 6 hours. The conditioned medium from 8 non-asthmatic (cream) and 9 asthmatic fibroblasts (blue) were analysed following acid treatment in order to determine total TGFβ protein levels. Each assay was conducted in duplicate, therefore for non-asthmatics n=24 and for asthmatics n=27. Statistical significance between subject groups was determined by application of the Mann-Whitney U test. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

As can be seen from figure 4.4, the levels of active TGFβ1 were approximately equal, before and after stimulation. The conditioned culture medium from the asthmatic fibroblasts was however very different. At all time points, the general trend was an increase in active TGFβ1 in response to TGFβ2 stimulation with statistically significant increases at 6 hours (p=0.005), 72 hours (p=0.0018) and 144 hours (p=0.005). Although at 24 hours the active TGFβ1 levels tended to increase in response to 0.4nM TGFβ2 (49 (0-156)pg/10^5 cells in the absence versus (60 (0-104) pg/10^5 cells) in the presence , the increase did not reach statistical significance.

Figure 4.5 illustrates the total TGFβ1 production at baseline and after TGFβ2 stimulation at all the time points and the contribution of active TGFβ1 to the total expressed. The graphs represent the median values for each set of conditions. In general the percentage of active TGFβ1 contributing to the total detected, is higher in the asthmatic than in the non-asthmatic fibroblasts. On comparing just the medians, the asthmatics have a greater percentage of their total TGFβ1 comprised of active TGFβ1 (68 (18.5-99.5) % for the asthmatics *versus* 23 (0-50)% for the non-asthmatics (p=0.003)).

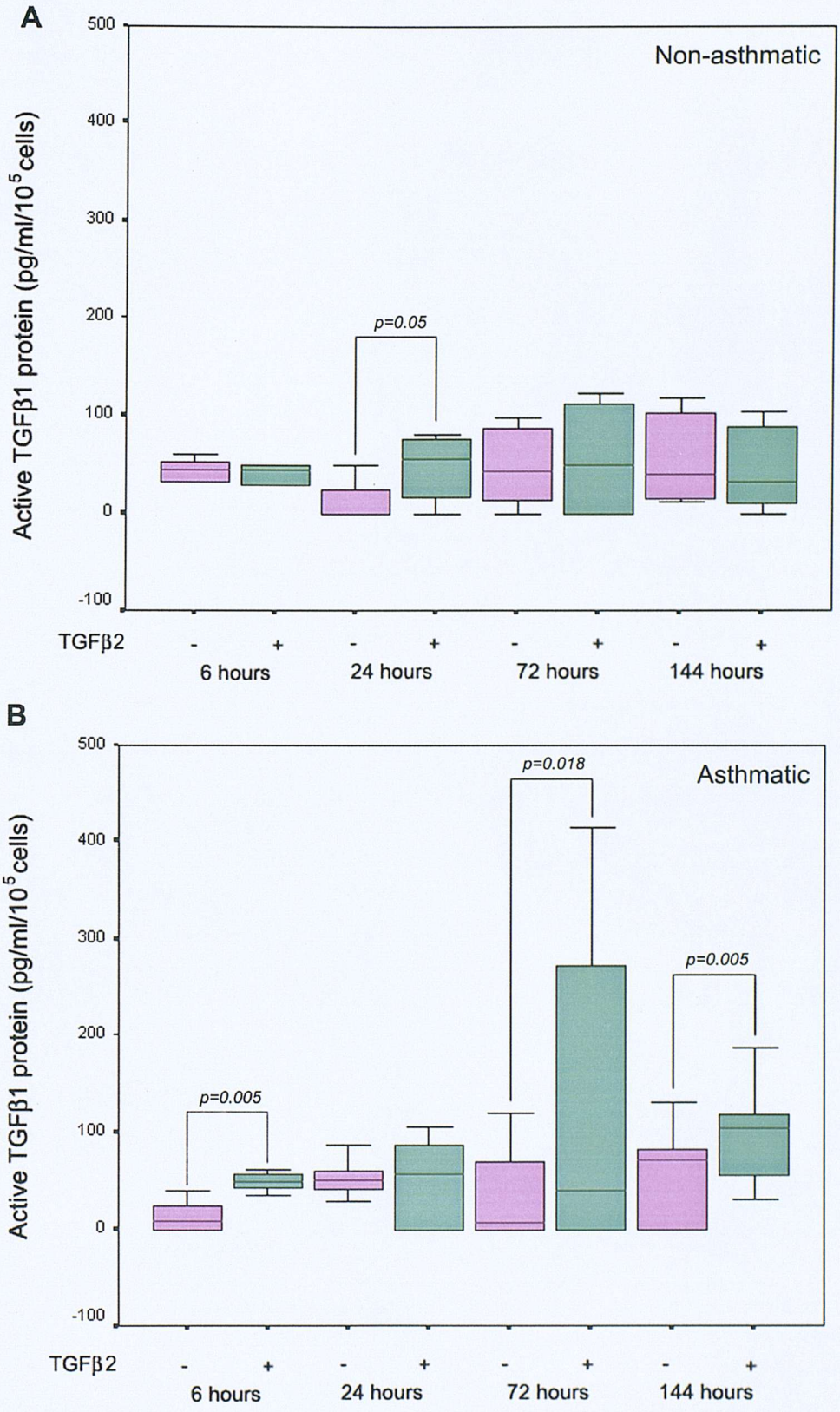


Figure 4.4: Time course for the regulation of activeTGFβ1 protein expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). At the specified time points, the supernatant was removed and stored at –80°C for subsequent analysis. The TGFβ1 levels were measured by use of an enzyme-linked immunoassay system obtained from Promega (UK) *without acid treatment*. Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

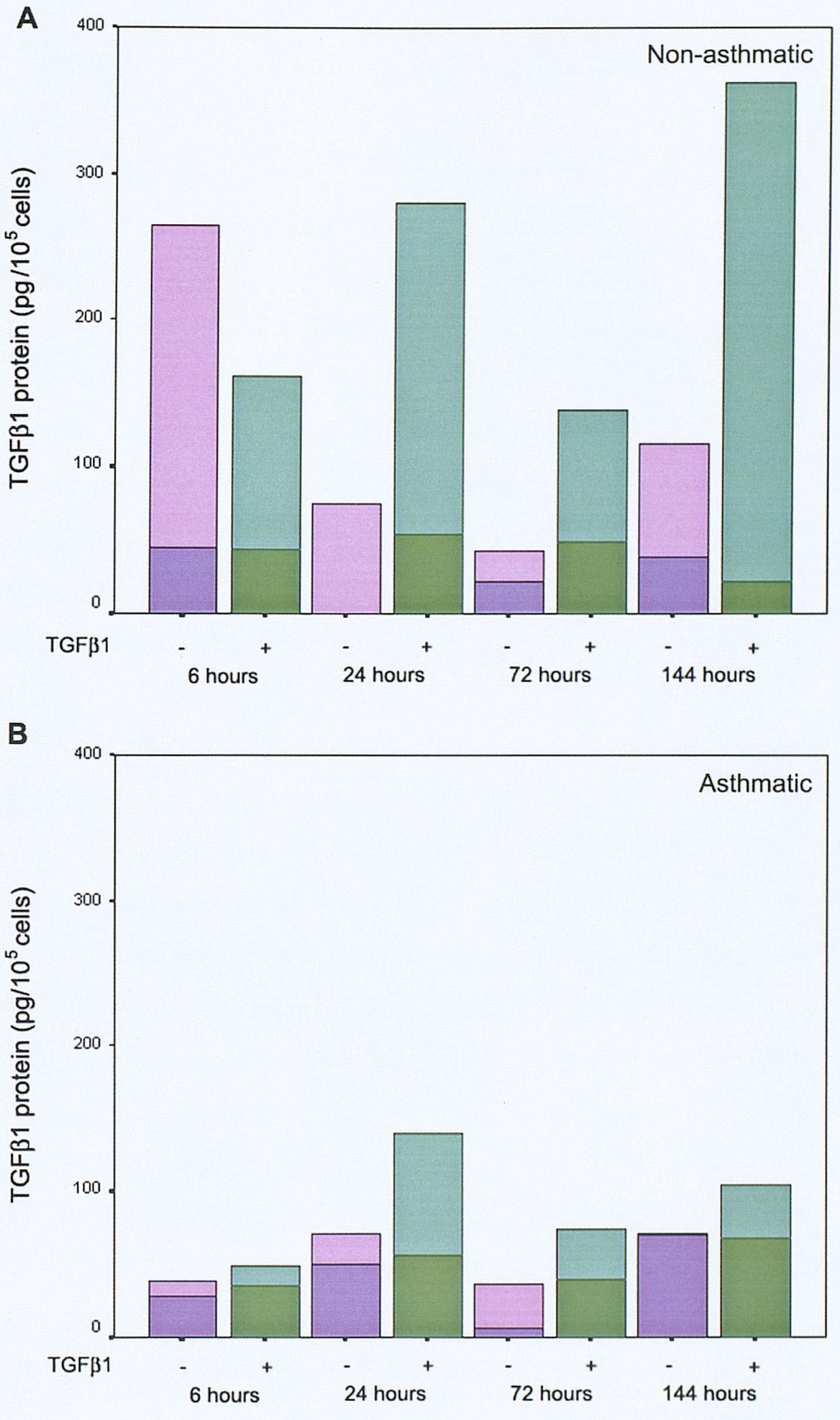


Figure 4.5: The contribution of active TGFβ1 (dark green and dark purple) to the total TGFβ1 protein detected in the cultured medium of (a) 8 non-asthmatic and (b) 9 asthmatic fibroblast lines after culture in serum free medium alone (purple) or in combination with 0.4nM TGFβ2 (green) for up to 144 hours. The total area of each bar represents the total TGFβ1 in the conditioned medium (i.e. after acid treatment) and the inactive component was determined by subtraction of the active TGFβ1 (i.e. without acid treatment) from the total. Each assay was conducted in triplicate, therefore n=24 for non-asthmatics and n=27 for asthmatics

4.4.2. Endothelin-1

As for TGFβ1, endothelin-1 gene expression was investigated by use of quantitative RT-PCR and protein levels by use of ELISA. Cells were treated in the absence or presence of 0.4nM TGFβ2 at 6, 24 72 and 144 hours.

The basal level of ET-1 gene expression at 24 hours was significantly higher in asthmatics when compared to the non-asthmatic fibroblasts (p=0.05; figure 4.7) In both non-asthmatic and asthmatic fibroblasts, endothelin-1 mRNA expression was significantly increased following treatment with 0.4 nM TGFβ2 (figure 4.6). However, the ET-1 gene expression was significantly greater in the asthmatic fibroblasts when compared to non-asthmatics and was sustained from 24 – 144 hours.

The greatest increases in response to TGFβ2 were observed in the asthmatic fibroblasts at the 6 and 24 hours time points (p=0.008).

ET-1 protein expression was similar to the gene expression (figure 4.8) where there was a highly significant increase in the levels of ET-1 protein expressed at all time points in the case of the asthmatic fibroblasts treated with TGFβ2. Modest increases in ET-1 protein expression were observed on stimulation of the non-asthmatic fibroblasts at 24 and 72 hours (p=0.04 and p=0.01 respectively), with no significant increases in the levels of ET-1 expressed in response to 0.4 nM TGFβ2 at 6 and 144 hours.

Under basal conditions, the levels of ET-1 protein detected in asthmatic conditioned medium at 6 hours was significantly higher in the asthmatic fibroblasts than the non-asthmatics (1.3 (1.0-1.5) pg/10^5cells *versus* 2.1 (1.2-6.5)pg/10^5 cells) (figure 4.9). The levels were also higher in the asthmatics at 24 hours (3.3 (2.2-4.6)pg/10^5 cells *versus* 1.6 (0.19-5.7)pg/10^5 cells). On stimulation with 0.4nM TGFβ2, asthmatic fibroblasts also expressed higher levels of protein when compared to the non-asthmatics at both 6 and 24 hours (p=0.03 and p=0.001 respectively; figure 4.9).

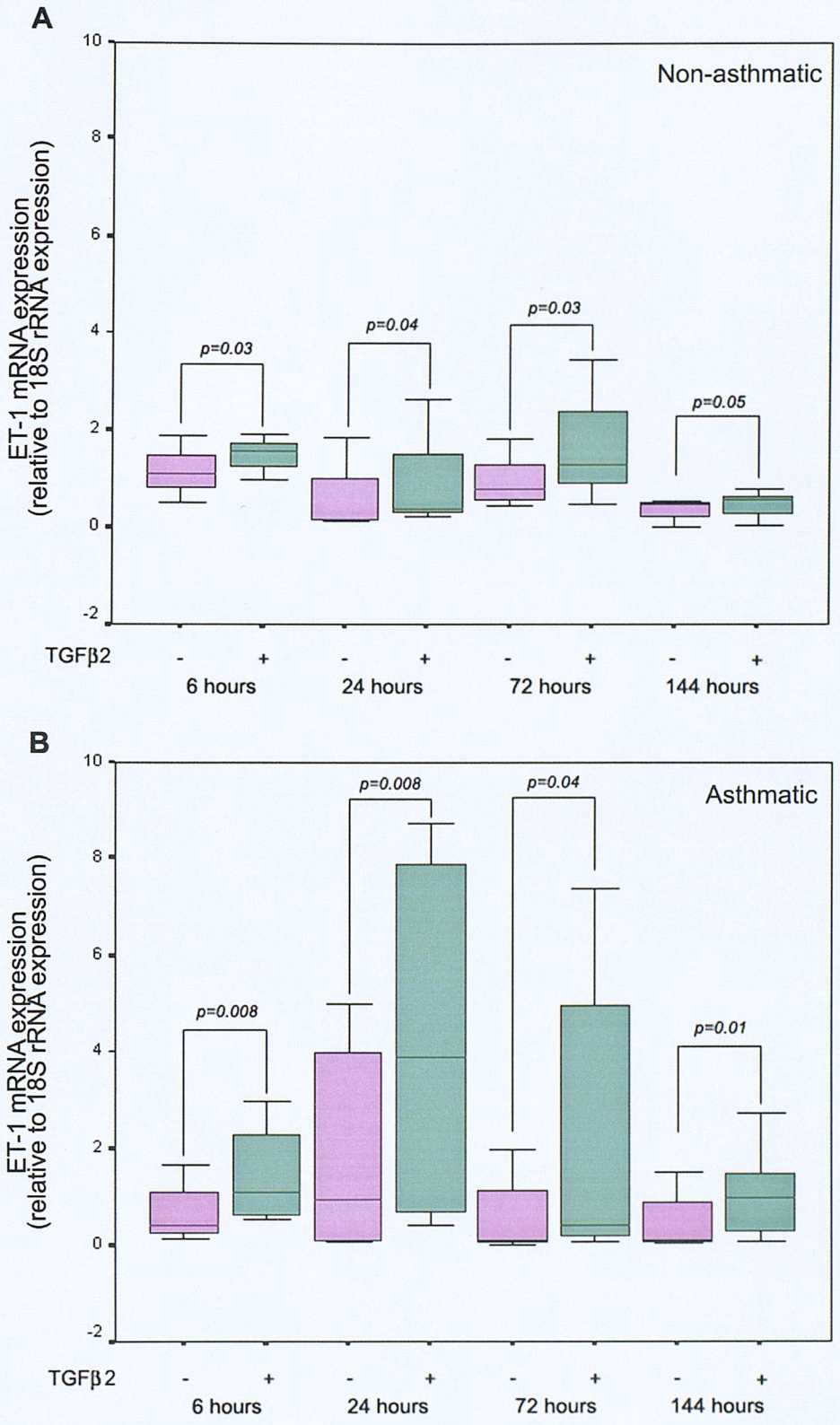


Figure 4.6: Time course for the regulation of ET-1 gene expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for ET-1 gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

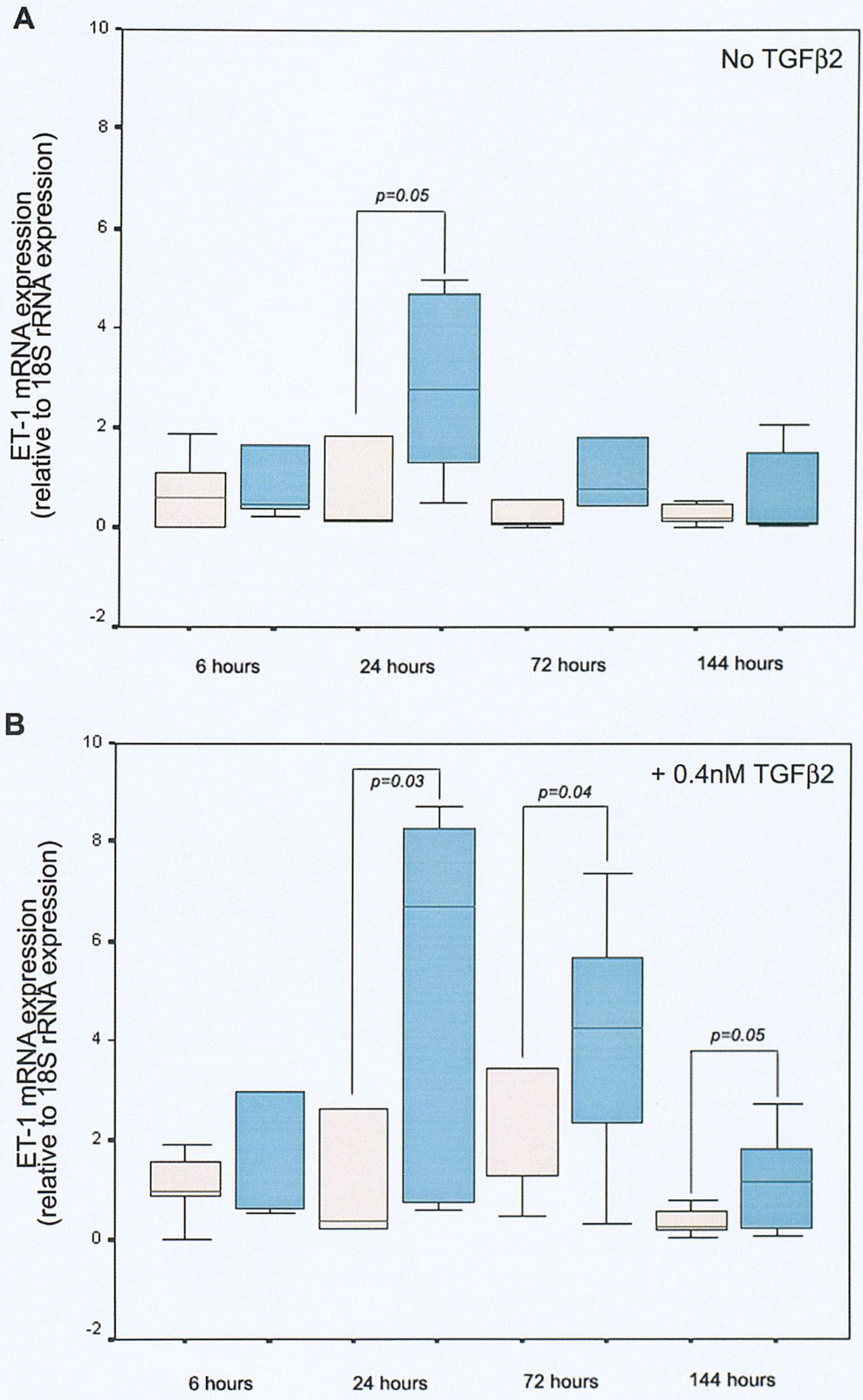


Figure 4.7: Time course for the regulation of ET-1 gene expression in non-asthmatic (cream) and asthmatic (blue) fibroblasts when cultured in (a) serum free medium alone or in (b)combination with 0.4nM TGFβ2. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone or in the presence of 0.4nM TGFβ2 . The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for ET-1 gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Statistical significance between subject groups was determined by application of the Mann Whitney U statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

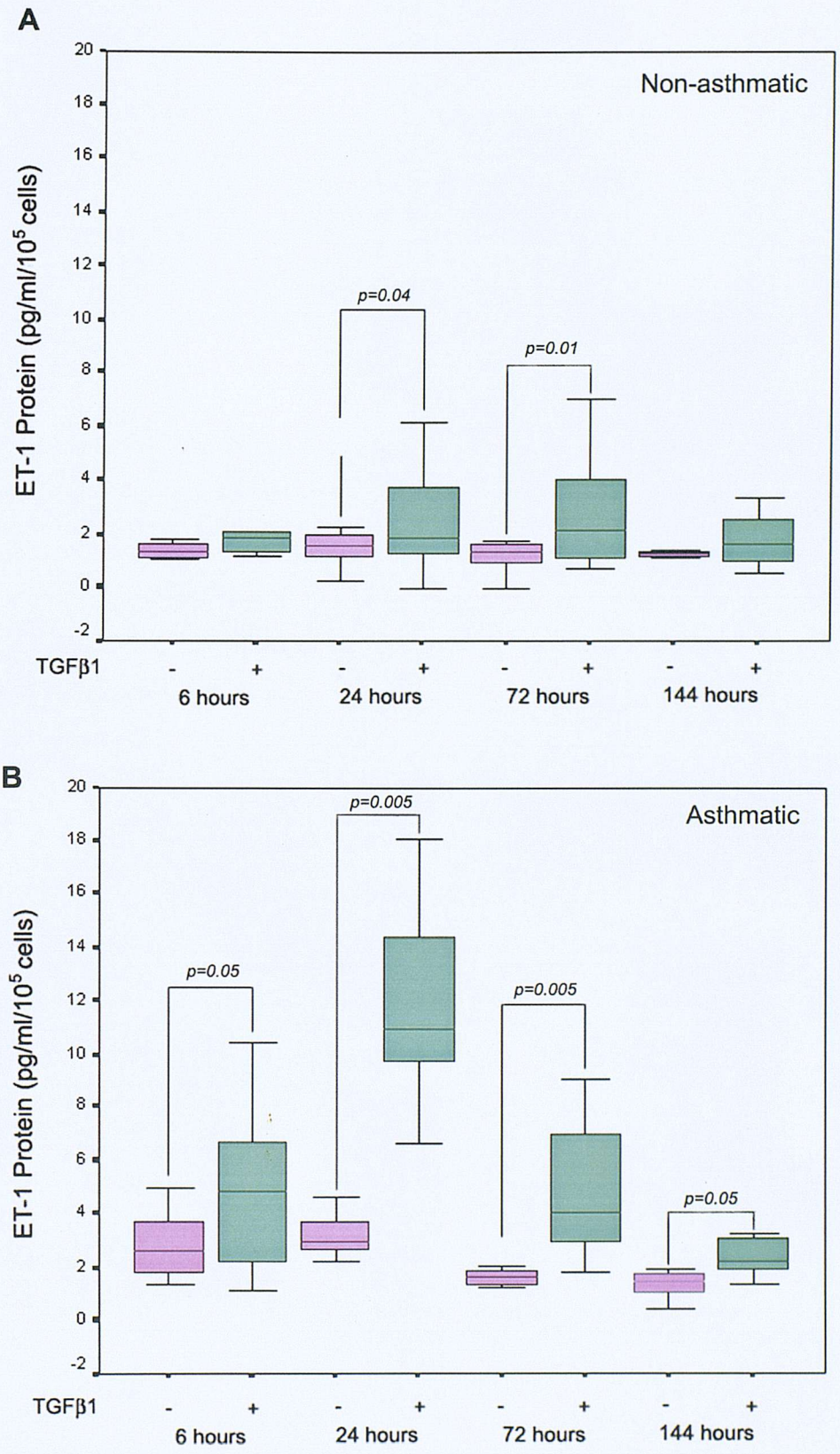


Figure 4.8: Time course for the regulation of ET-1 protein expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). At the specified time points, the supernatant was removed and stored at –80°C for subsequent analysis. The ET-1 levels were measured by use of an enzyme-linked immunoassay system obtained from R&D Systems (UK). Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

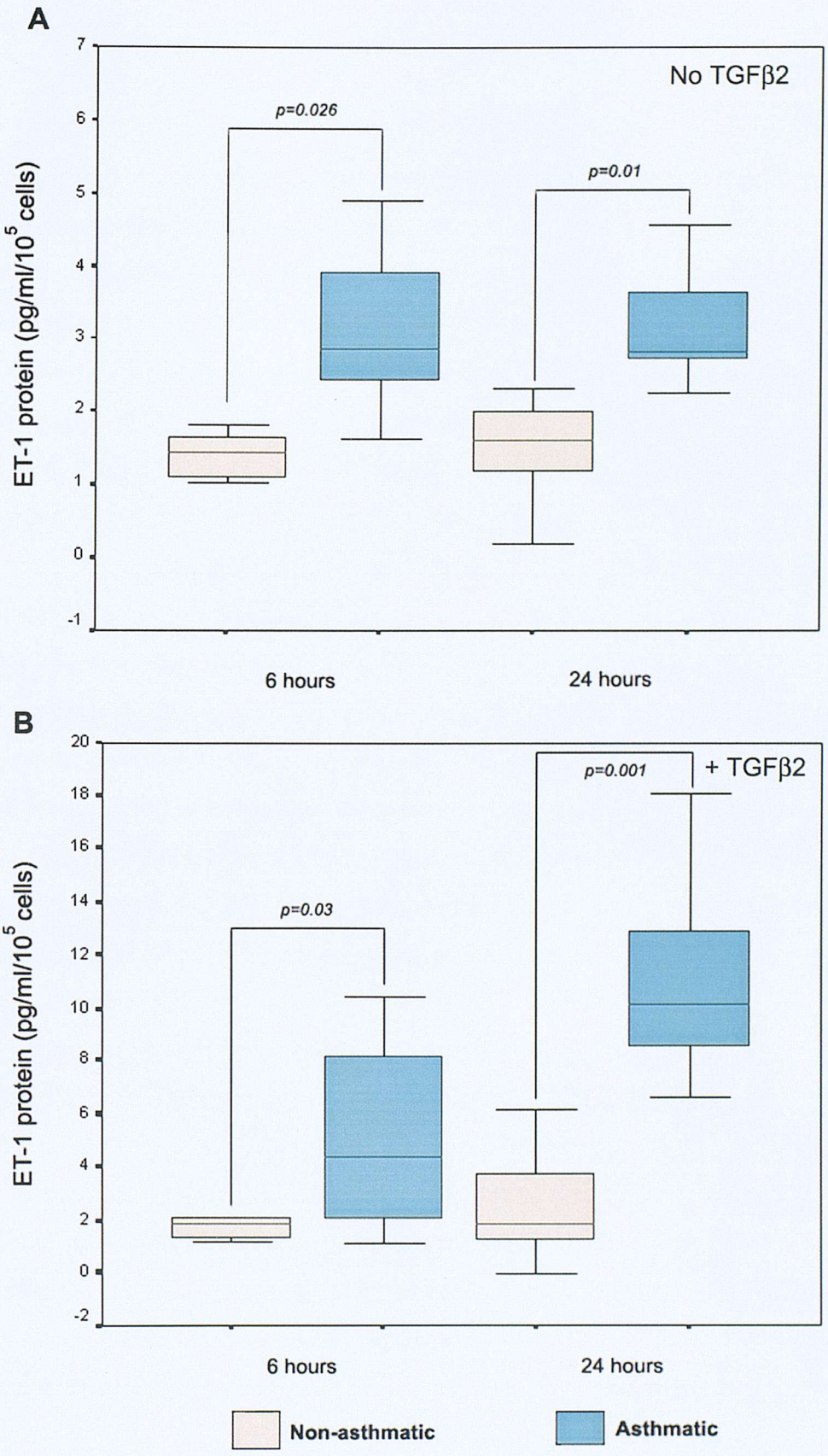


Figure 4.9: The comparison of ET-1 protein levels following culture for 6 and 24 hours. The conditioned medium from 8 non-asthmatic (cream) and 9 asthmatic fibroblasts (blue) were analysed to determine total ET-1 protein levels. Each assay was conducted in triplicate, therefore for non-asthmatics n=24 and for asthmatics n=27. Statistical significance between subject groups was determined by application of the Mann-Whitney U test. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

4.4.3. Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) is a profibrogenic growth factor, known to be expressed in response to TGFβ. It has emerged as an important mediator for classical TGFβ induced effects (Grotendorst 1997).

In this study, CTGF gene expression in asthmatic and non-asthmatic fibroblasts was investigated by quantitative RT-PCR. In both non-asthmatic and non-asthmatic fibroblasts, the levels of CTGF gene expression were increased in response to 0.4 nM TGFβ2 (figure 4.10), although significant in both cases, the increase in the asthmatic fibroblasts was highly significant in particular at 24 hours ($p=0.008$) and at 144 hours ($p=0.008$). These results are consistent the direct regulation of CTGF by TGFβ. The most prominent observation with regards to CTGF gene expression was on comparing the levels of CTGF gene expression between non-asthmatics and asthmatics, both at basal levels and on stimulation with TGFβ2. Under basal conditions at 6 hours, CTGF gene expression was significantly higher in the asthmatic fibroblasts when compared to the non-asthmatic cells ($p=0.02$). At 24 hours, CTGF gene expression was also significantly higher in the asthmatic fibroblasts, when compared to the non-asthmatic cells ($p=0.05$).

On comparison of the TGFβ2 stimulated non-asthmatic and asthmatic fibroblasts, the increased levels of CTGF gene expression in the asthmatic fibroblasts were highly significant higher at all time points, in particular at the 6 and 24 hours time points ($p<0.005$; figure 4.11).

Addition of TGFβ1 antibodies, did not decrease the basal levels of CTGF at 6 hours (figure 4.12), suggesting that the elevated levels were independent of the autocrine TGFβ1 that may have been consumed in an autocrine fashion.

Recombinant CTGF protein was not freely available and there were not adequate antibodies to develop an ELISA. However antibodies for western blotting are commercially available but scarce. A single monoclonal antibody was obtained and used to investigate CTGF protein expression in both the cell lysates and conditioned culture medium of non-asthmatic and asthmatic fibroblasts under basal conditions and in response to 0.4nM TGFβ2.

CTGF protein expression was detectable at very low levels in both the cell lysates of both asthmatic and non-asthmatic fibroblasts, but not in the conditioned medium. The sensitivity of the antibody appeared to be very low. A positive control was obtained from

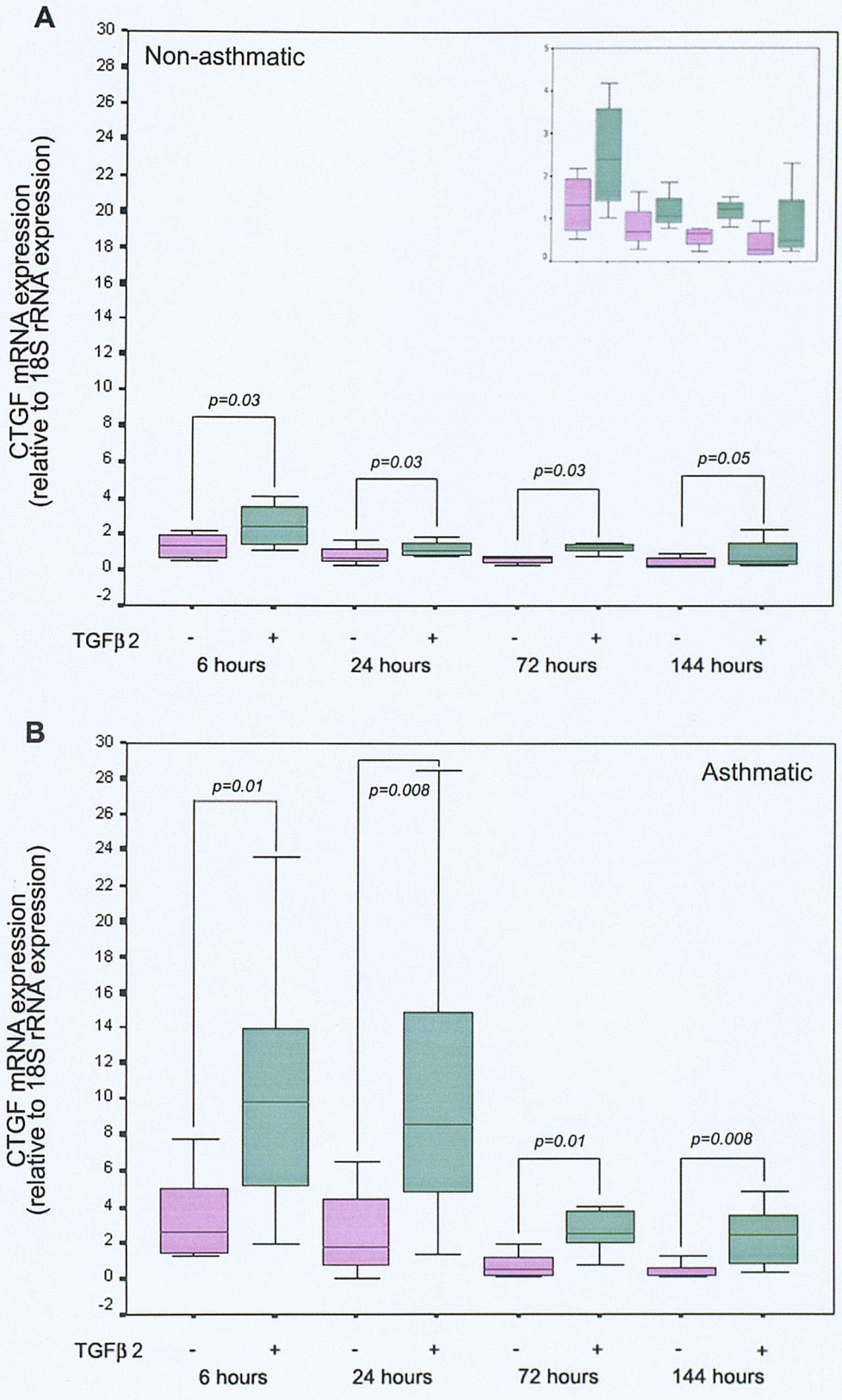


Figure 4.10: Time course for the regulation of CTGF gene expression in (a) non-asthmatic and (b) asthmatic fibroblasts. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone (purple) or in the presence of 0.4nM TGFβ2 (green). The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for CTGF gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Statistical significance following treatment was determined by application of the Wilcoxon statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

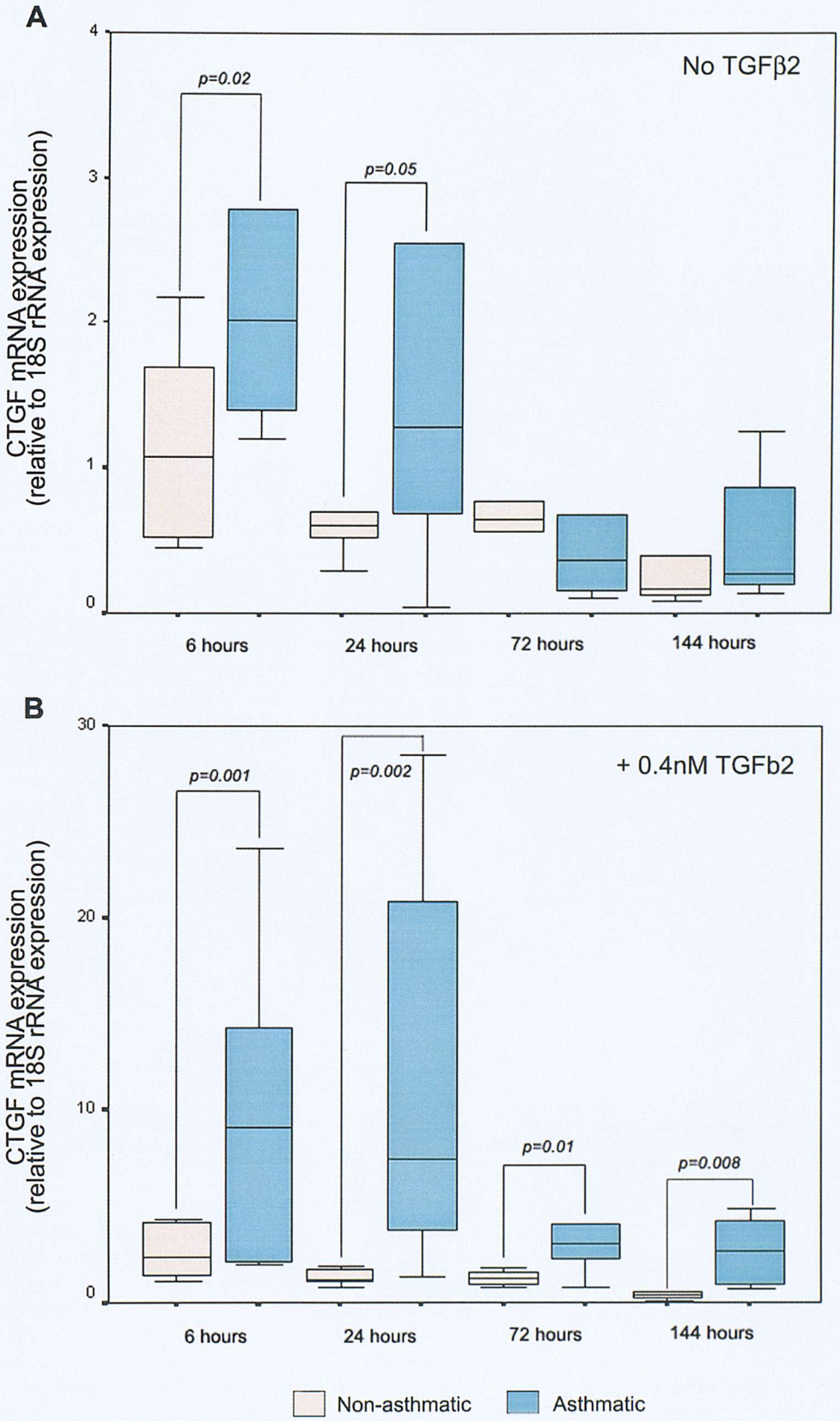


Figure 4.11: Time course for the regulation of CTGF gene expression in non-asthmatic (cream) and asthmatic (blue) fibroblasts when cultured in (a) serum free medium alone or in (b)combination with 0.4nM TGFβ2. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone or in the presence of 0.4nM TGFβ2 . The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for CTGF gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Statistical significance between subject groups was determined by application of the Mann Whitney U statistical test. The results were based on the study of 8 asthmatic and 8 non-asthmatic fibroblast lines and each assay was conducted in triplicate, therefore n=24 for each subject group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval. ***Note the difference in ordinal scales on the two graphs.***

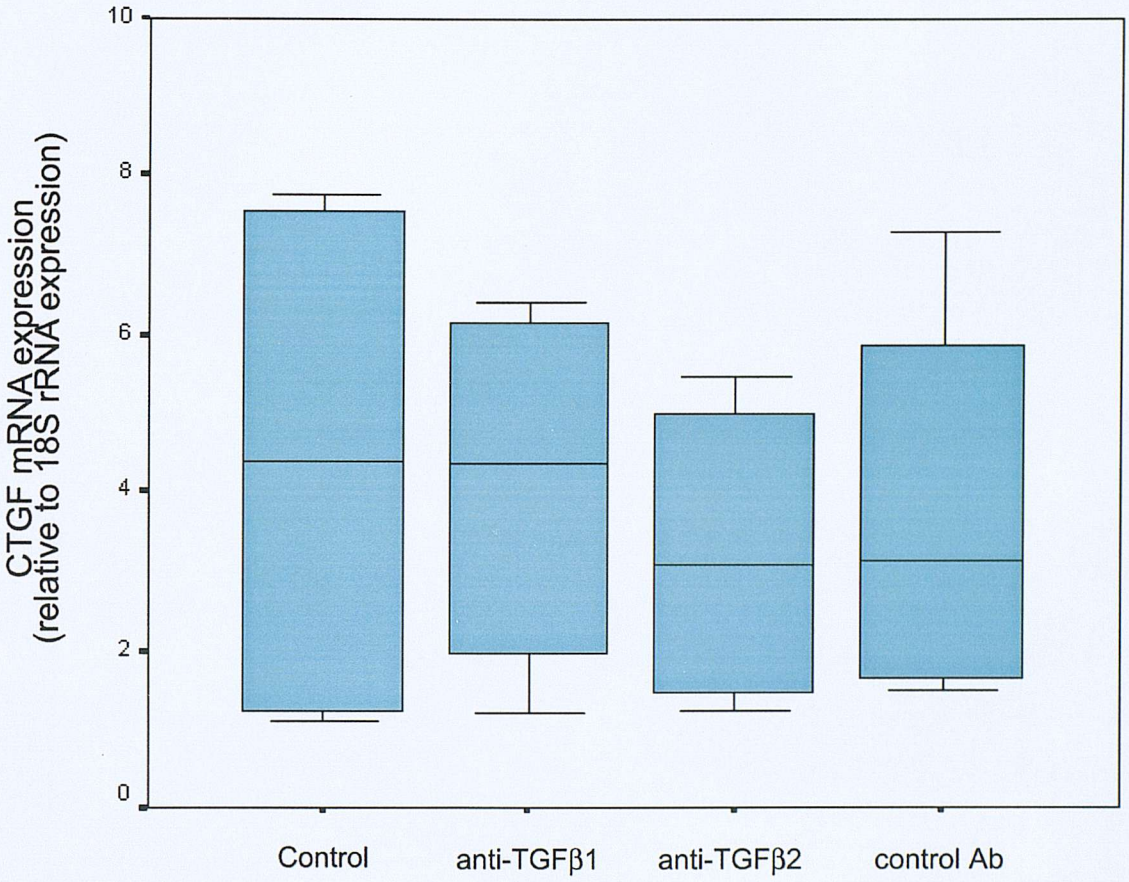
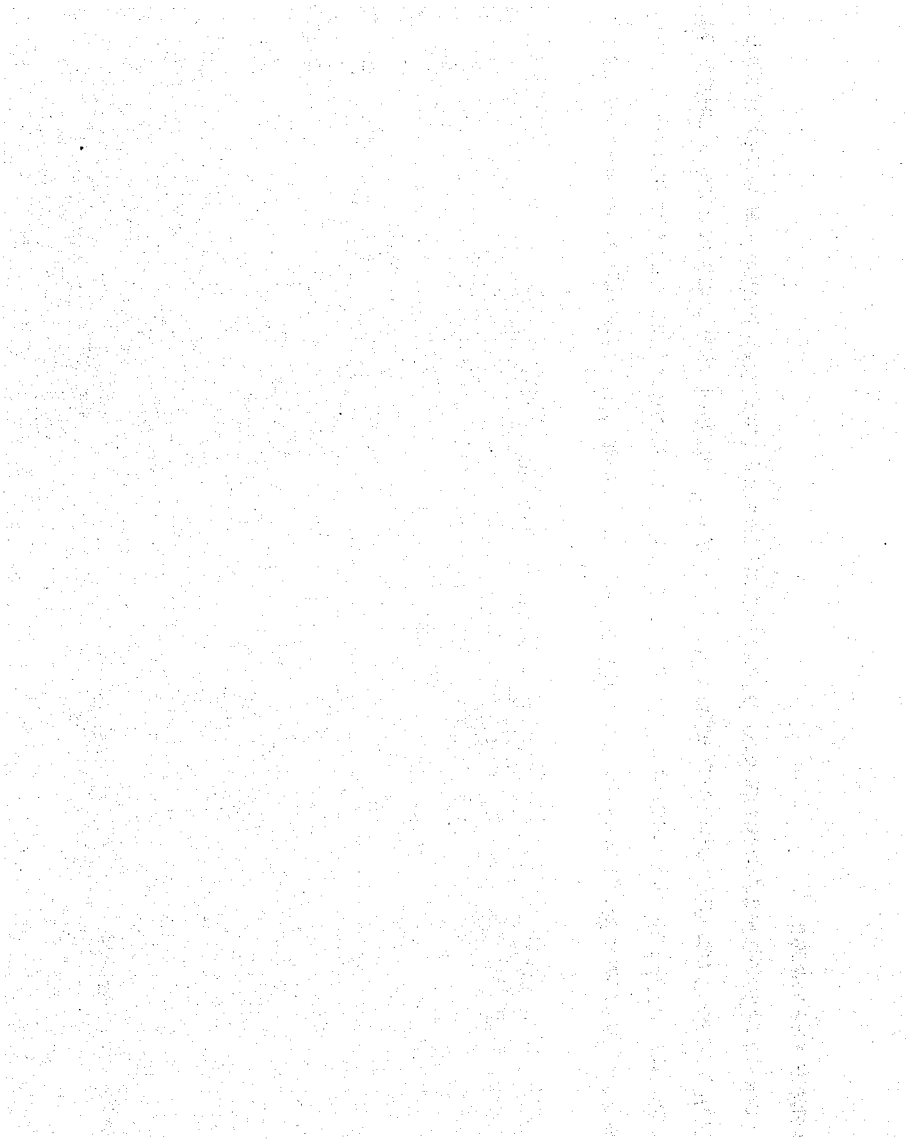


Figure 4.12: The CTGF gene expression in 5 asthmatic fibroblast lines in the presence of TGFβ neutralising antibodies. The fibroblasts were seeded at a density of 5 x 10^4 cells/well in 6-well plates pre-coated with collagen I in serum free medium alone or in the presence of anti-TGFβ1, anti-TGFβ2 or irrelevant control neutralising antibodies. The RNA was extracted from the fibroblasts at the specified time points, and following reverse transcription, the cDNA was analysed for CTGF gene expression using real time quantitative PCR. The values were normalised to 18 rRNA gene expression. Each assay was conducted in triplicate, therefore n=15 for each treatment group. The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval.

the supplier of the antibody, for which a relatively high concentration of antibody was required; 50ng of positive control, required an antibody concentration of 0.5μg/ml. Figure 4.13 shows the western blots obtained from the positive control and those obtained from the cell lysates.



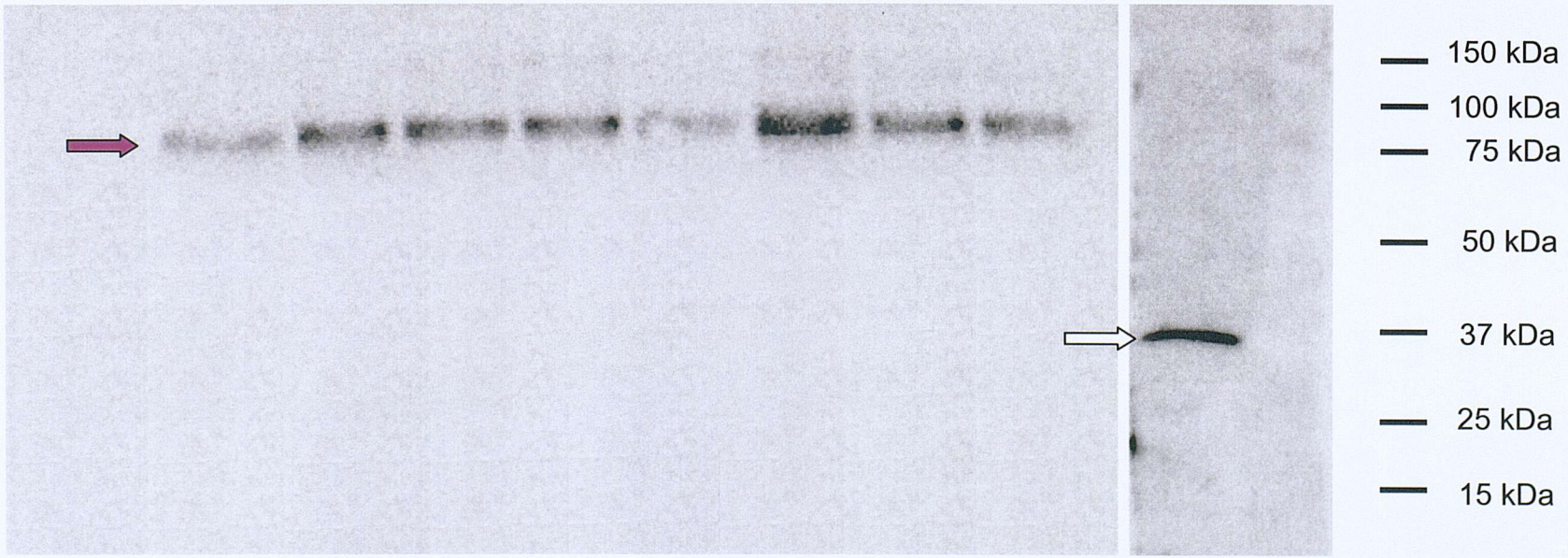


Figure 4.13. Western blots to determine CTGF protein levels The purple arrow shows the presence of a non-specific bovine serum albumin (BSA) at approximately 75kDa in and the white arrow shows the specificity of the antibody to bind to a provided hR CTGF protein (from R&D systems, Abingdon, UK) as is illustrated by a band at approximately 35kDa. This experiment was carried out on cultured supernatants from asthmatic fibroblasts. No CTGF bands were detected when fibroblast culture supernatant was loaded.

4.5 Discussion

Thickening of the *lamina reticularis* is a characteristic feature of asthma. It is evident in children up to 4 years before the diagnosis of asthma, suggesting that events in the *lamina reticularis* are intimately involved in the disease pathogenesis. As early as 1989, Roche and co-workers presented evidence that excess collagen deposition found in the *lamina reticularis* was linked to an increase in the number of activated myofibroblasts present in this region. More recently, Richter and co-workers highlighted the potential of myofibroblasts to contribute to the increased smooth muscle mass through their ability to produce smooth muscle mitogens such as ET-1 and vascular endothelial growth factor (VEGF).

4.5.1 Transforming growth factor β

In several fibrotic diseases, abnormalities in fibroblast function have been identified linked to abnormalities in receptors for members of the TGFβ superfamily or to downstream genes regulated by TGFβ family members. For example, in primary pulmonary hypertension (PPH), a mutation in the bone morphogenetic protein receptor type 2 (BMPR-2) leads to increased fibroblast proliferation due to loss of responsiveness to the growth inhibitory effects of BMP-2 (Lane KB *et al.,* 2000), whereas somatic mutations have been identified in microsatellite repeats of the TGFβ receptor II (Markowitz *et al.,*1995).

Transforming growth factor β is a highly fibrogenic molecule, and it's involvement has been identified in a number of fibrotic diseases. Virtually every cell in the body produces TGFβ and has receptors for it (Massague 1998). TGFβ regulates the proliferation and differentiation of cells, embryonic development, wound healing and angiogenesis.

In the present study, on comparing TGFβ1 gene expression in asthmatic and non-asthmatic fibroblasts, no significant difference between basal or stimulated levels between the two groups was observed (figure 4.1). However kinetic analysis indicated that in the presence of TGFβ2, TGFβ1 mRNA levels remained elevated in the asthmatic myofibroblasts for up to 3 days, after which a significant decrease was observed. The non-asthmatics did not exhibit such elevation in the levels of mRNA gene expression. It is

unclear from the observations in this study, what the reasons for this elevation are. It may represent a shift in sensitivity to TGFβ during the time course of the experiment or a loss of a regulatory feedback loop in the asthmatic cells.

As discussed in section 1.5.3, activation of the TGFβ receptor induces phosphorylation of Smad2 and Smad3 (Moustakas *et al.,* 2001). Nakao and co-workers (1997) were the first to identify a regulatory Smad (Smad7), which was able to block responses mediated by TGFβ in mammalian cells. It was shown that Smad7 associates stably with the TGFβ receptor complex, and is not phosphorylated upon TGFβ stimulation. TGFβ-mediated phosphorylation of Smad2 and Smad3 is inhibited by Smad7, indicating an antagonist effect of Smad7 which regulates TGFβ signalling. Furthermore it has been shown that TGFβ rapidly induces the expression of Smad7 mRNA, which Nakao and colleagues suggested, may participate in a negative feedback loop to control TGFβ responses. In inflammatory bowel disease (IBD), Monteleone and co-workers (2001) have shown that TGFβ1 signalling which normally functions as a negative regulator of T cell immune responses is antagonised by the overexpression of Smad 7. Use of anti-sense Smad7 oligonucleotides allowed TGFβ1 mediated signalling to take place and enabled exogenous TGFβ to modulate T cell immune responses.

In light of these and other observations surrounding the regulation of TGFβ mediated responses by Smad7, it may be possible that, in the case of asthmatic fibroblasts, there is a loss in Smad7 regulatory activity, resulting in a sustained increase in TGFβ1 mRNA expression in response to exogenous TGFβ2.

Although there was no significant difference in the TGFβ gene expression between asthmatic and non-asthmatic fibroblasts, the total TGFβ1 protein expression between the two groups was markedly different at 6 hours under basal conditions. There were significantly higher levels of total TGFβ1 protein in the non-asthmatic fibroblast when compared to the asthmatic cells. In light of there being no significant difference when comparing the gene expression, the reduction in protein expression in the asthmatics at 6 hours, may represent autocrine consumption. At the other time points, the general trend was that levels of total TGFβ1 were higher in the non-asthmatics when compared to the asthmatics, however this did not reach statistical significance at the other time points (figure 4.2). As discussed above, although the levels of total TGFβ1 protein detected in conditioned medium is lower in the asthmatic fibroblasts the percentage of active TGFβ1

which constitutes the total amount is higher. This observation is in line with the above suggestion that the lower levels of protein detected in the asthmatic fibroblast cultured medium is due to activation and subsequent autocrine consumption.

As described in section 1.4.6.2, TGFβ is normally secreted in a latent form, and it is necessary to activate this by proteolytic cleavage of the latent binding protein from the active TGFβ moiety. There are a number of proteinases that have the ability to cause this cleavage including Plasmin, a serine proteinase known to be an important activator of latent TGFβ in the co-culture of endothelial and smooth muscle cells (Lyons *et al.,* 1990), Integrin αvβ6 and thrombospondin. Thrombospondin has been reported to be a major activator for latent TGFβ *in vivo* (Ribeiro *et al.,* 1999). The ability of thrombospondin (TSP)-1 to activate latent TGFβ1 in a epithelial cell-fibroblast co-culture system was investigated by Morishima and co-workers (Morishima *et al.*, 2001). They showed that fibroblasts expressed TSP-1 mRNA, which was upregulated 4 days after epithelial damage. By use of a neutralising antibody, it was possible to block the action of TSP-1 and myofibroblast transformation in response to epithelial –injury was inhibited (see section 1.4.6.2). Neutralising antibodies against TGFβ1 also inhibited TSP-1 up regulation. This suggested that TSP-1 would activate TGFβ1 which would in turn up regulate TSP-1.

As previously suggested, the significantly lower levels of total TGFβ1 protein detected in the conditioned medium of asthmatic fibroblasts may be explained by autocrine consumption. In light of the investigations by Morishima and co-workers, this autocrine consumption, may be driving increased TSP-1 production, which would further activate latent TGFβ1 and result in an increased percentage of active TGFβ1 protein, which was observed in the asthmatic fibroblasts (figure 4.5). The lack of autocrine use in normal fibroblasts would result in lower levels of TSP-1 and hence reduce the amount of active TGFβ1 in the conditioned medium.

4.5.2 Endothelin-1

In contrast with TGFβ1 expression, the asthmatic fibroblasts differed markedly in the basal (at 24 hours) and stimulated expression of ET-1, with significantly higher levels of ET-1 gene expression in the asthmatic cells. TGFβ has previously been reported to induce ET-1 mRNA expression in a number of cell lines. Lee and co-workers (2000)

demonstrated that TGFβ1 induced ET-1 gene expression and ET-1 peptide synthesis in bovine pulmonary artery endothelial cell lines (BPAECs). Markewitz and co-workers (2001) determined that TGFβ was able to significantly induce expression of preproET-1 mRNA and ET-1 production in both rat and human pulmonary arterial smooth muscle cells. Increased levels of endothelin-1 have been observed in dermal fibroblasts obtained from patients with systemic sclerosis. Induction of preproET-1 mRNA has been attributed to a nuclear factor 1 consensus motif found in the promoter region of human preproendothelin-1 gene (Markewitz *et al.,* 2001) which may explain it's induction by TGFβ.

The present study is in agreement with previously published studies by Richter and co-workers in which ET-1 protein expression was shown to be up regulated by asthmatic (myo)fibroblasts (Richter A *et al.,* 2001). The results obtained in the present study are consistent with this, with significantly higher levels of ET-1 found in the conditioned medium of asthmatic fibroblasts at 24 hours. However on stimulation with TGFβ2, it can be seen that although both the non-asthmatic and asthmatic fibroblasts increased their ET-1 gene expression, the induction in the asthmatics was much greater, and the levels remained elevated in the case of the asthmatics. In light of the observations that TGFβ regulates ET-1 gene expression, the loss of TGFβ signalling down regulation may also be used to explain the highly significant differences in ET-1 mRNA expression between asthmatic and non-asthmatic fibroblasts. Whether the ET-1 produced by the asthmatic fibroblasts is used as an autocrine factor driving the increased proliferation will be investigated in section 5.4. The protein levels show a decrease in ET-1 levels between 24 and 72 hours, but this may be due to the breakdown of active ET-1 in the conditioned culture medium.

4.5.3 Connective Tissue Growth Factor

CTGF functions as a downstream mediator of TGFβ action on connective tissue cells, and has been reported to stimulate cell proliferation and extracellular matrix synthesis. CTGF has previously been shown to be strongly induced by TGFβ in fibroblasts (Grotendorst 1997) which can be attributed to a TGFβ response element in the human CTGF gene promoter (Grotendorst 1997). Duncan and co-workers have shown that

the induction of collagen gene expression by TGFβ1 in fibroblasts is mediated via the action of CTGF (Duncan *et al.,* 1999).

In the present study, the observation that 0.4nM TGFβ2 significantly induced CTGF gene expression is line with the evidence presented in the literature. An interesting observation was the significantly elevated CTGF mRNA gene expression in asthmatic fibroblasts in the absence of any exogenous TGFβ2, at 6 and 24 hours.

In scleroderma, Shi-wen and co-workers (Shi-wen *et al.,* 2000) have shown that CTGF levels are higher in scleroderma fibroblasts when compared to normal fibroblasts. They reported substantially elevated CTGF promoter activity in the scleroderma fibroblasts. Holmes and co-workers (2001) later showed that the elevated level of CTGF promoter activity observed in the scleroderma fibroblasts was not dependent on Smad binding site since mutating the Smad binding site in the context of the CTGF promoter did not decrease it's activity. This evidence along with the observations showing that oligonucleotides containing consensus binding sites for the usual factors which normally bind to the TGFβ response element in the CTGF promoter (AP-1, CREB and Sp-1) do not compete for binding to the TGFβRE in electrophoreticmobility shift assays, has been taken to suggest that the maintenance of the scleroderma phenotype is not caused by Smad dependent TGFβ signalling (Holmes *et al.,* 2001). Evidence which further supports this, is the observation that tumour necrosis factor (TNF)α, which suppresses the TGFβ induction of collagen and CTGF in a manner possibly involving the elevation of Smad7 (Inagaki *et al.,* 1995; Bitzer *et al.,* 2000) has no effect on basal CTGF gene expression in scleroderma fibroblasts (Abraham *et al.,* 2000). The Smad responsive *PAI*-promoter is not elevated in scleroderma fibroblasts, further suggesting a Smad-independent mechanism.

The uncoupling of the increased basal levels of CTGF gene expression from TGFβ in scleroderma fibroblasts, may also be the cause for the increased CTGF gene expression in the asthmatic fibroblasts.

It has been shown that CTGF is able to induce proliferation and production of extracellular matrix in dermal fibroblasts (Frazier K *et al.,* 1996). In light of its increased gene expression level in asthmatics, connective tissue growth factor is a likely candidate for an autocrine ligand that may drive the hyper-proliferation of asthmatic fibroblasts. These hypotheses could be readily tested with the use of human recombinant CTGF or neutralising antibodies.

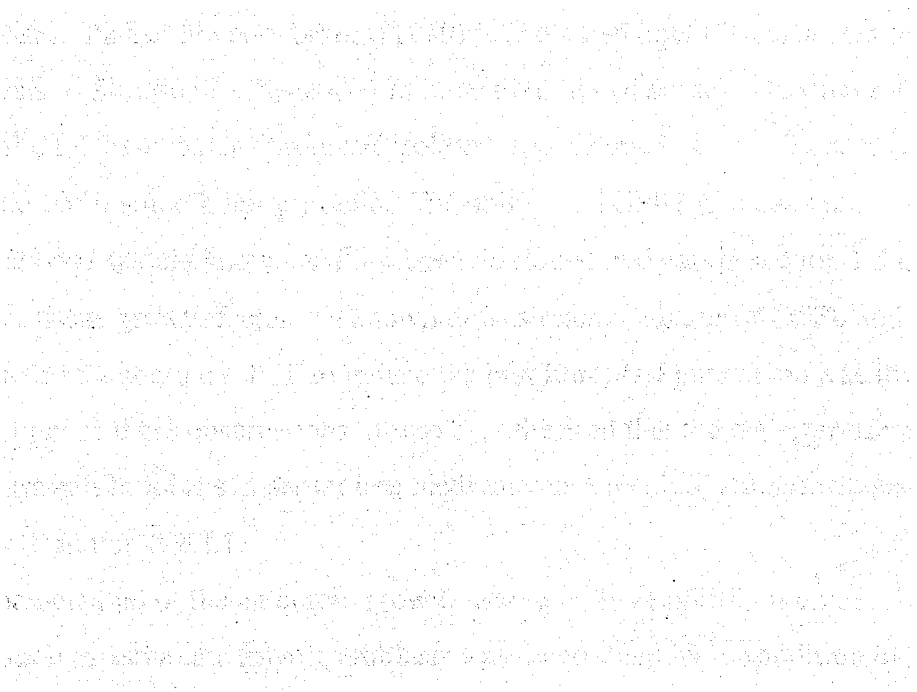
4.6 Summary of results and novel findings

- TGFβ2 induces TGFβ1 gene expression up to 24 hours after stimulation in both non-asthmatic and asthmatic (myo)fibroblasts (p=0.04).
- TGFβ1 gene expression remains elevated in the asthmatic (myo)fibroblasts up to 72 hours after TGFβ2 stimulation.
- Although there is no significant difference in the TGFβ1 gene expression between asthmatic and non-asthmatic (myo)fibroblasts, the levels of TGFβ1 protein detected in the asthmatic fibroblast conditioned medium was lower than in the non-asthmatic (myo)fibroblasts at 6 hours (p=0.03)
- The percentage of active TGFβ1 that contribute to the total TGFβ1 protein levels (latent and active) is greater in the asthmatic fibroblasts than the non-asthmatic fibroblasts.
- TGFβ2 strongly induces ET-1 gene expression in asthmatic and non-asthmatic fibroblasts (p=0.008 and p=0.03 respectively)
- The basal levels of ET-1 gene expression were significantly higher in the asthmatic (myo)fibroblasts than in the non-asthmatic (myo)fibroblasts (p=0.05).
- The levels of ET-1 in the conditioned medium of asthmatic fibroblasts were significantly higher than in the non-asthmatic fibroblasts at 6 and 24 hours, both in the absence (p=0.026) and presence (p=0.03) of TGFβ2.
- In both non-asthmatic and asthmatic (myo)fibroblasts, the levels of CTGF gene expression were significantly elevated in response to TGFβ2.
- Under basal conditions, at 6 hours the levels of CTGF gene expression was significantly higher in the asthmatics than in non-asthmatics (p=0.03).
- Use of an anti-TGFβ neutralizing antibodies did not diminish the elevated basal CTGF gene expression in asthmatic fibroblasts.

4.7 Conclusions

The results obtained are not consistent with the null hypothesis. There are significantly higher levels of ET-1 and CTGF gene expression in asthmatic fibroblasts when compared to non-asthmatic fibroblasts both at baseline and in response to 0.4nM TGFβ2. By analogy with left ventricular hypertrophy following myocardial infarction, ET-1 may have the potential to drive the hyper-proliferation of asthmatic fibroblasts. However, on considering the pathogenesis of scleroderma, the elevated levels of CTGF in the asthmatic (myo)fibroblasts may be responsible for their enhanced proliferation.

In the following section, the contribution of these growth factors to the autocrine proliferation of asthmatic fibroblasts will be investigated.



CHAPTER FIVE

The potential of Endothelin-1, Connective tissue growth factor and Transforming growth factorβ1 to induce autocrine growth in asthmatic (myo)fibroblasts

5.1 Introduction

The asthmatic airway is characterised by a thickened subepithelial basement membrane due to an increased number of myofibroblasts. The presence of these cells may be attributable to a local network of growth factors that may mediate phenotypic transformation from fibroblasts to myofibroblasts that are normally resident in the vicinity.

In chapter 4 endothelin (ET)-1, connective tissue growth factor (CTGF) and transforming growth factor (TGF)β1 were up-regulated in asthmatic myofibroblasts when compared to non-asthmatic cells. There have been some reports observing the potential of exogenous endothelin-1 to induce the myofibroblast transformation in fibroblasts from other diseases. Shahar and co-workers (1999) investigated how ET-1 affected the pathogenesis of fibrotic manifestations in interstitial lung diseases. They have shown that addition of ET-1 to alveolar fibroblasts isolated from interstitial lung diseases results in the induction of α smooth muscle actin. The ability of TGFβ1 to induce phenotypic transformation is widely known and has been discussed in detail in section 1.4.6. Connective tissue growth factor is a known downstream mediator of TGFβ and the potential role of autocrine CTGF to induce the myofibroblast phenotype was investigated.

In light of these observations, it was hypothesised that the hyperproliferation of the asthmatic (myo)fibroblasts in serum free medium was a result of autocrine consumption of ET-1, CTGF and/or TGFβ1.

The potential of the autocrine growth factors to drive proliferation or differentiation of asthmatic (myo)fibroblasts was determined by the addition of recombinant proteins or neutralising antibodies to primary asthmatic fibroblasts cultures and determining cell number by methylene blue. Mitogenesis of the fibroblast cell line NR6/HER in response to recombinant proteins was also investigated. In order to

determine the potential of these growth factors to induce the myofibroblast phenotype, αSMA gene expression levels were determined following treatment with recombinant protein, and protein levels determined by immunofluorescent detection.

5.2 Aims

To investigate the ability of endothelin-1, connective tissue growth factor and transforming growth factorβ1 secreted by asthmatic fibroblasts to function as autocrine growth and differentiation factors.

5.3 Hypothesis

i) The increased levels of endothelin-1, connective tissue growth factor and transforming growth factor β1 secreted by asthmatic fibroblasts are able to drive their proliferation in an autocrine fashion.

ii) The increased levels of endothelin-1, connective tissue growth factor and transforming growth factor β1 secreted by asthmatic fibroblasts are able to drive phenotypic transformation to myofibroblasts in an autocrine fashion.

Null Hypothesis

The increased levels of endothelin-1, connective tissue growth factors and transforming growth factor β1 secreted by asthmatic fibroblasts do not have the ability act in an autocrine fashion.

5.4 The potential of endothelin-1 as an autocrine growth factor

The ability of recombinant endothelin-1 to induce proliferation was determined in primary asthmatic fibroblasts (n=6) by considering growth over 6 days (144 hours) and determining cell number by methylene blue dye uptake at the specified time points. In order to determine whether the endothelin-1 secreted by asthmatic fibroblasts stimulated their autocrine proliferation, antagonists for endothelin-1 receptors (ET_A and ET_B) were used. Mitogenic ability of endothelin-1 was determined by addition of human recombinant protein to the NR6/HER fibroblast cell line.

The induction of α-smooth muscle actin gene expression in response to 1nM ET-1 was determined as a marker of myofibroblasts transformation. Comparisons between unstimulated and stimulated fibroblasts were tested for statistical significance by application of the Wilcoxon statistical test. α-SMA protein expression was determined by immunofluorescent staining for α smooth muscle actin protein (n=4).

5.4.1 Endothelin-1 and autocrine induction of cell proliferation

Endothelin-1 is a potent mitogenic agent for smooth muscle cells and fibroblasts. Exposure of asthmatic fibroblasts to 1nM ET-1 did not result in an increase in proliferation when compared to fibroblasts that were cultured in serum free medium (figure 5.1) The kinetics of cell growth were identical on comparing the ET-1 fibroblasts and the untreated cells. The possibility of endogenous endothelin-1 secreted by the asthmatic fibroblasts already eliciting a maximal proliferative effect was ruled out, since addition of 10nM N-Acetyl-[D-Trp^{16}]-endothelin-1 fragment 16-21 (a ET_A specific receptor antagonist) or addition of 10nM BQ788 (a ET_B specific receptor antagonist) did not result in a reduction of cell number at any time point over 144 hours (figure 5.2a ,b). This suggests that autocrine production of endothelin-1 was not responsible for the hyperproliferation in serum free medium that the asthmatic fibroblasts exhibit.

The mitogenic activity of endothelin-1 was investigated by determining the [^{3}H] Thymidine incorporation following addition of human recombinant ET-1 to NR6/HER fibroblasts. Although the cells responded to basic fibroblast growth factor (bFGF), and exhibited a classical dose-response (figure 5.3a) addition of endothelin-1 did not stimulate mitogenesis (figure 5.3b).

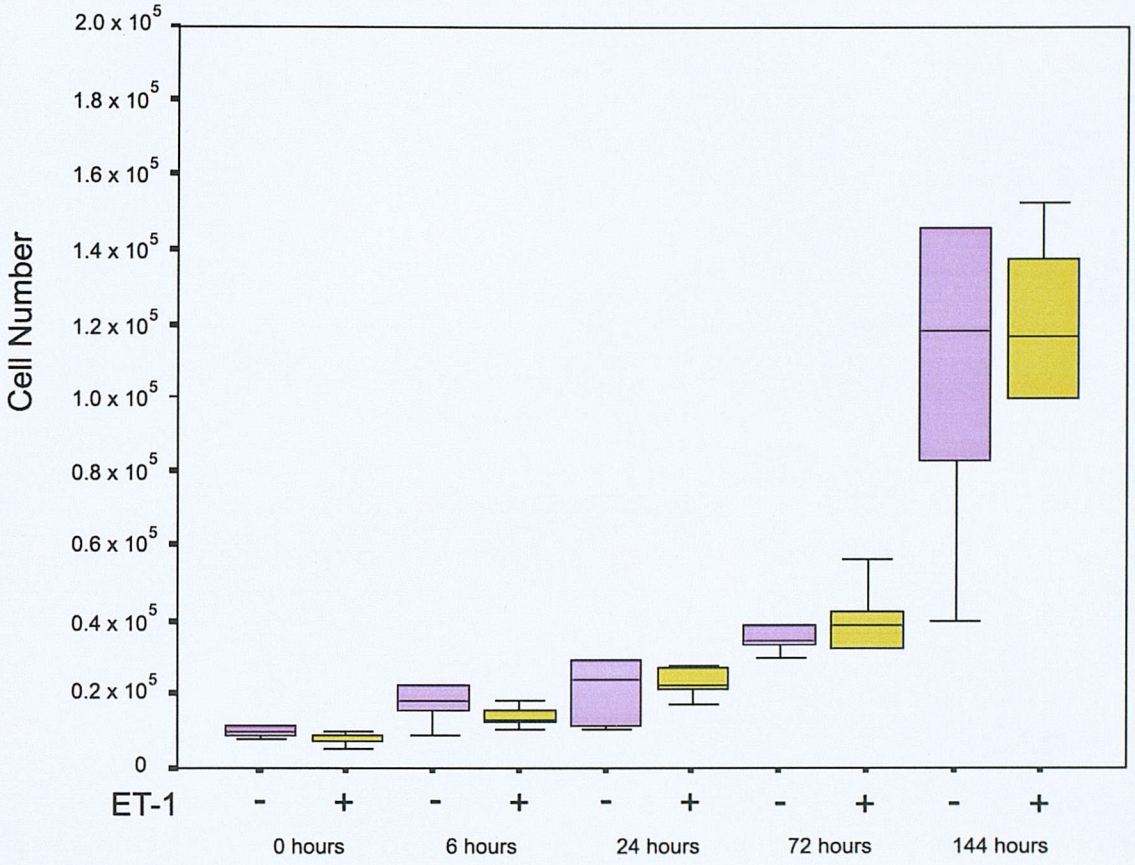


Figure 5.1: Asthmatic fibroblast number over 144 hours when cultured in serum free medium (purple) and in the presence of 10mM ET-1 (green), as determined by methylene blue uptake. Fibroblasts were seeded at a starting density of 5 x 10^4 cells/well in 24 well dishes, and were allocated to specific timepoints. At each timepoint, the corresponding cultures were fixed with formal saline. Methylene blue was added to each culture, the dye was eluted with ethanol/HCl and the absorbance at 620nM was measured. The quantity of methylene blue dye absorbed was translated into cell number by means of a standard curve. Error bars represent the interquartile range (n=6).

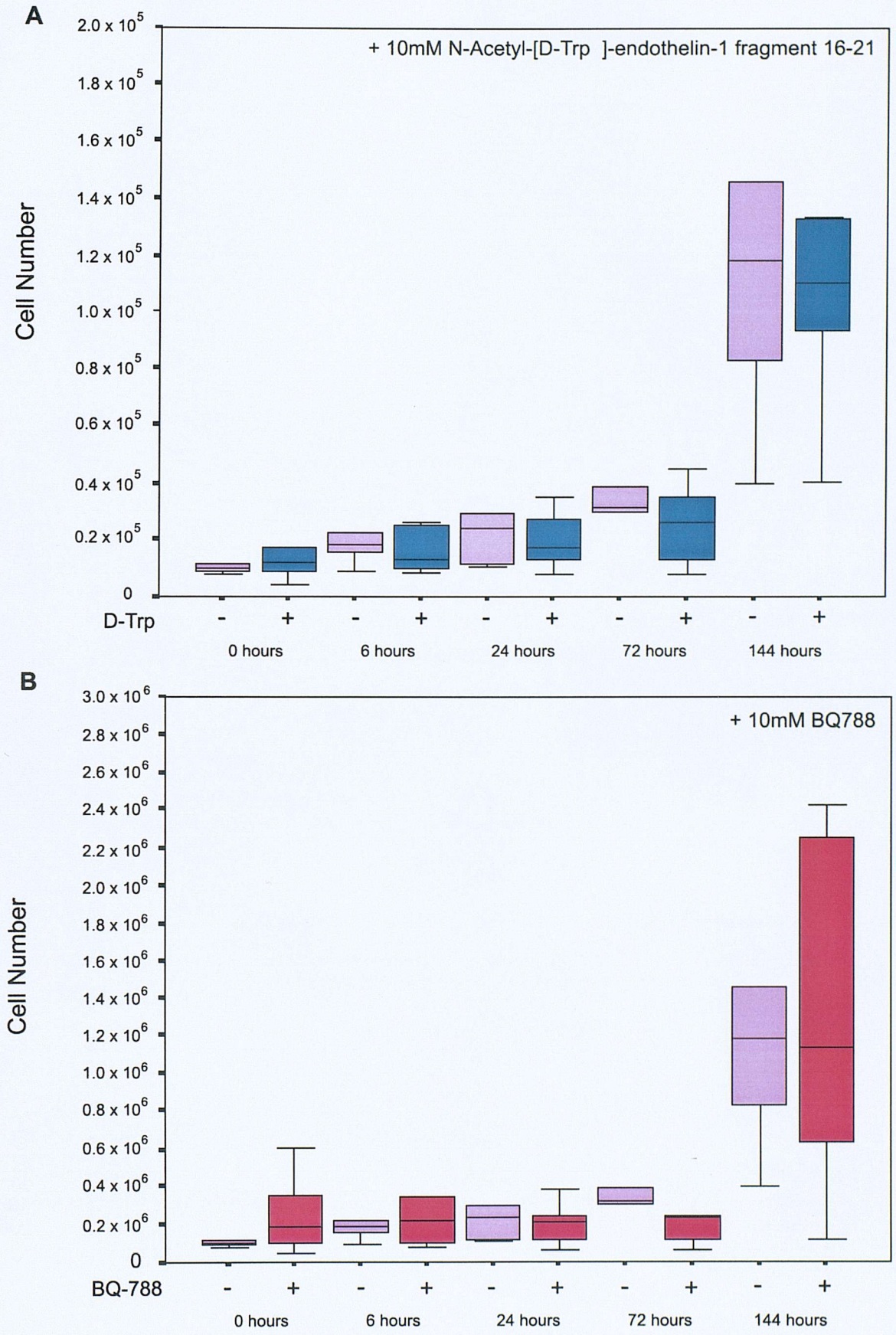
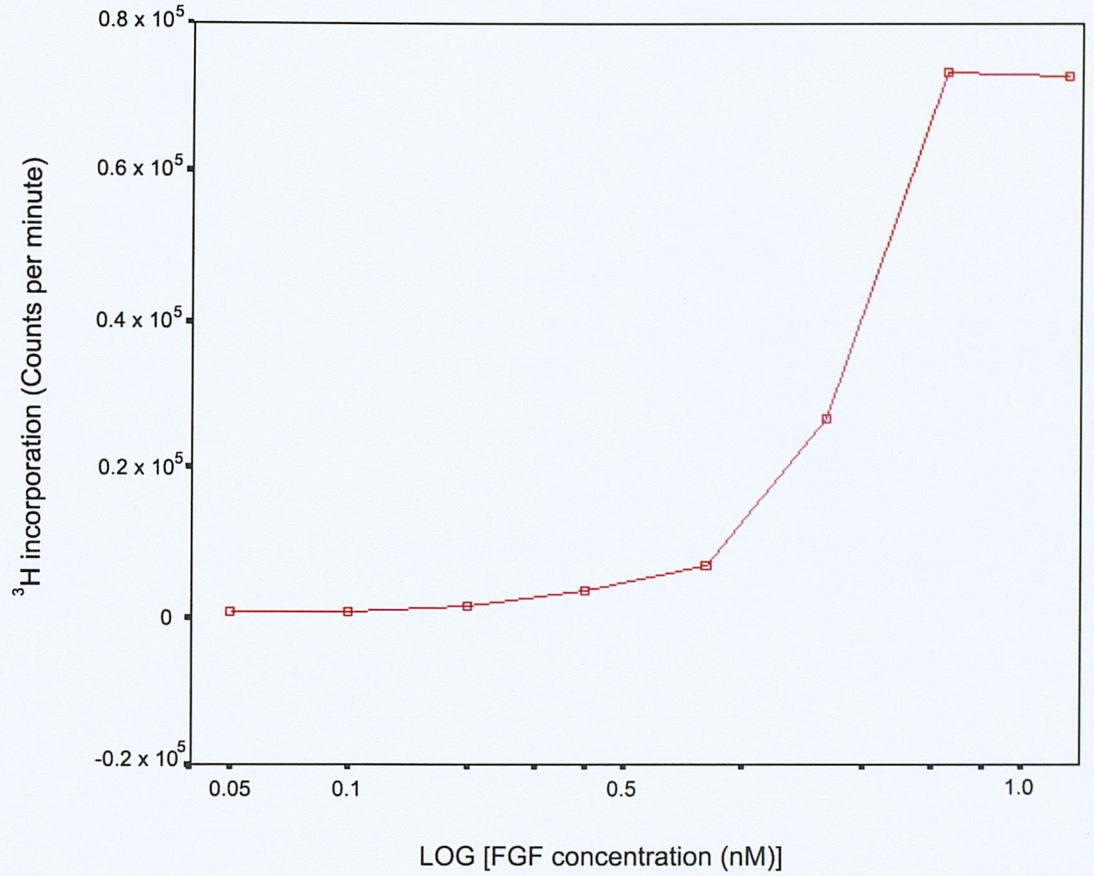


Figure 5.2: Asthmatic fibroblast number over 144 hours when cultured in serum free medium (pink) and in the presence of (a) 10mM N-Acetyl-[D-Trp^{16}]-endothelin-1 fragment 16-21 (an ETA receptor antagonist; blue), and (b) BQ788 (an ETB receptor antagonist; pink) determined by methylene blue uptake. Fibroblasts were seeded at a starting density of 5 x 10^4 cells/well in 24 well dishes, and were allocated to specific timepoints. At each timepoint, the corresponding cultures were fixed with formal saline. Methylene blue was added to each culture, the dye was eluted with ethanol/HCl and the absorbance at 620nM was measured. The quantity of methylene blue dye absorbed was translated into cell number by means of a standard curve. Error bars represent the interquartile range (n=6).



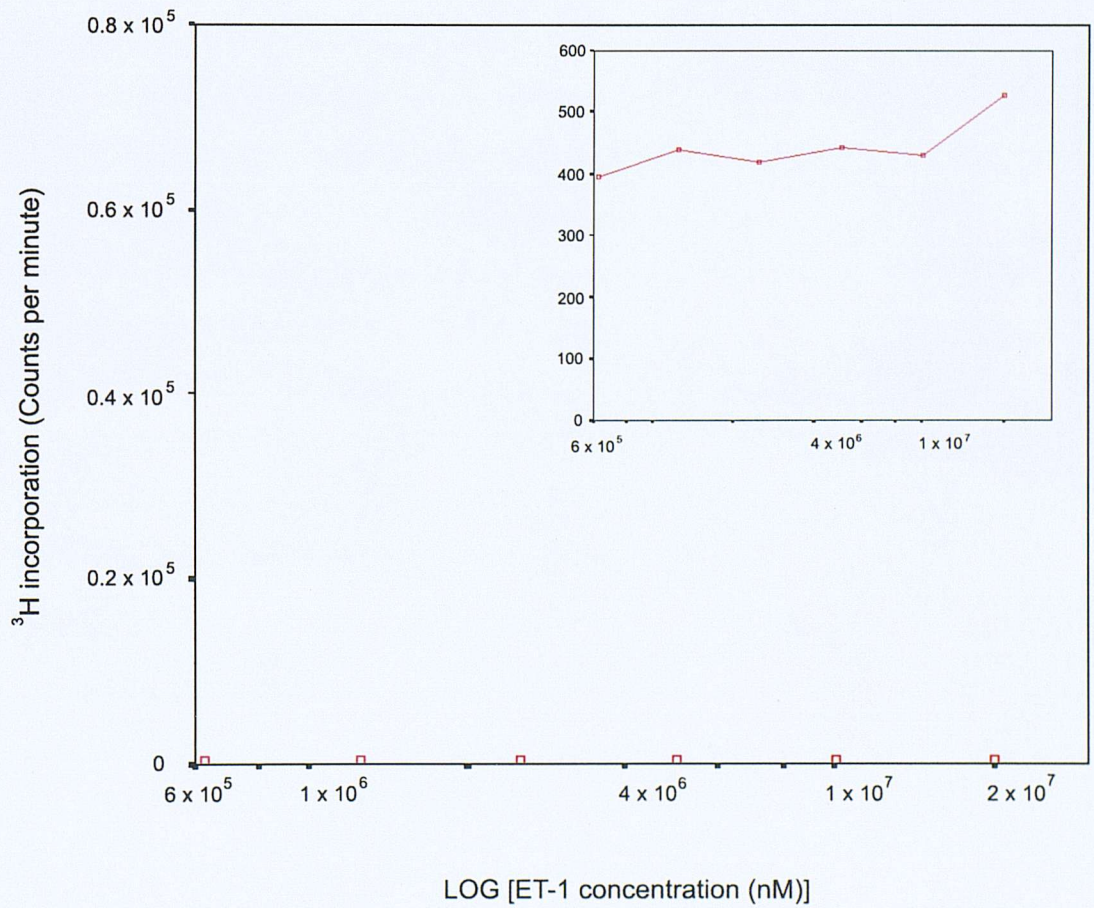


Figure 5.3: Stimulation of NR6/HER mitogenesis by bFGF and human recombinant ET-1. NR6/HER fibroblasts were cultures in 10% FBS and allowed to adhere overnight, after which the medium was changed to DMEM containing 1% FBS and the cells incubated for 48 hours. The stimuli was then added for 28 hours, after which the cells were exposed to ^{3}H thymidine for 2 hours. The DNA was then precipitated and processed for determining the ^{3}H thymidine incorporation.(n=3)

5.4.2 Endothelin-1 and autocrine induction of the myofibroblasts phenotype

Although endothelin-1 did not stimulate proliferation of asthmatic fibroblasts, phase contrast microscopy revealed that treatment with 1nM ET-1 resulted in a altered morphology (figure 5.4). Furthermore, addition of 10nM N-Acetyl-[D-Trp^{16}]-endothelin-1 fragment 16-21 in combination with 10nM ET-1 seemed to inhibit this transformation, the morphology looking similar to the untreated fibroblasts (figure 5.4c). Addition of BQ788 in combination with 1nM ET-1 still exhibited the contracted phenotype (figure 5.4d). This suggested that exogenously added ET-1 was able to induce an altered phenotype in these fibroblasts and that the ET-1 acted via the ET_A receptor.

In light of these observations, the α-SMA gene expression was investigated and determined by quantitative RT-PCR in response to 1nM ET-1 alone and in combination with 10nM N-Acetyl-[D-Trp^{16}]-endothelin-1 fragment 16-21 or 10nM BQ788 (figure 5.5). As observed in section 3.4.1, TGFβ2 induces significant levels of αSMA in asthmatic fibroblasts. The α-SMA induction by ET-1 is less modest, and fails to reach statistical significance, although the trend identifies an increase. Addition of the ET_A receptor antagonist resulted in a complete abolition of α-SMA induction, whereas addition of the ET_B receptor antagonist did not have the reducing effect of ET-1 induced α-SMA.

In order to study this phenomenon further the α-SMA protein expression was determined by immunofluorescent staining. In the absence of any treatment, in serum free medium, there was very little α-SMA immunoreactivity (figure 5.6a). Addition of 1nM ET-1 for 72 hours resulted in characteristic α-SMA filamentous staining (figure 5.6b). The addition of 10nM N-Acetyl-[D-Trp^{16}]-endothelin-1 fragment 16-21 in combination with 1nM ET-1 resulted in a significant decrease in α-SMA immunoreactivity (figure 5.6c) whereas addition of 10nM BQ788 had no effect on reducing the ET-1 induced α-SMA (figure 5.6d).

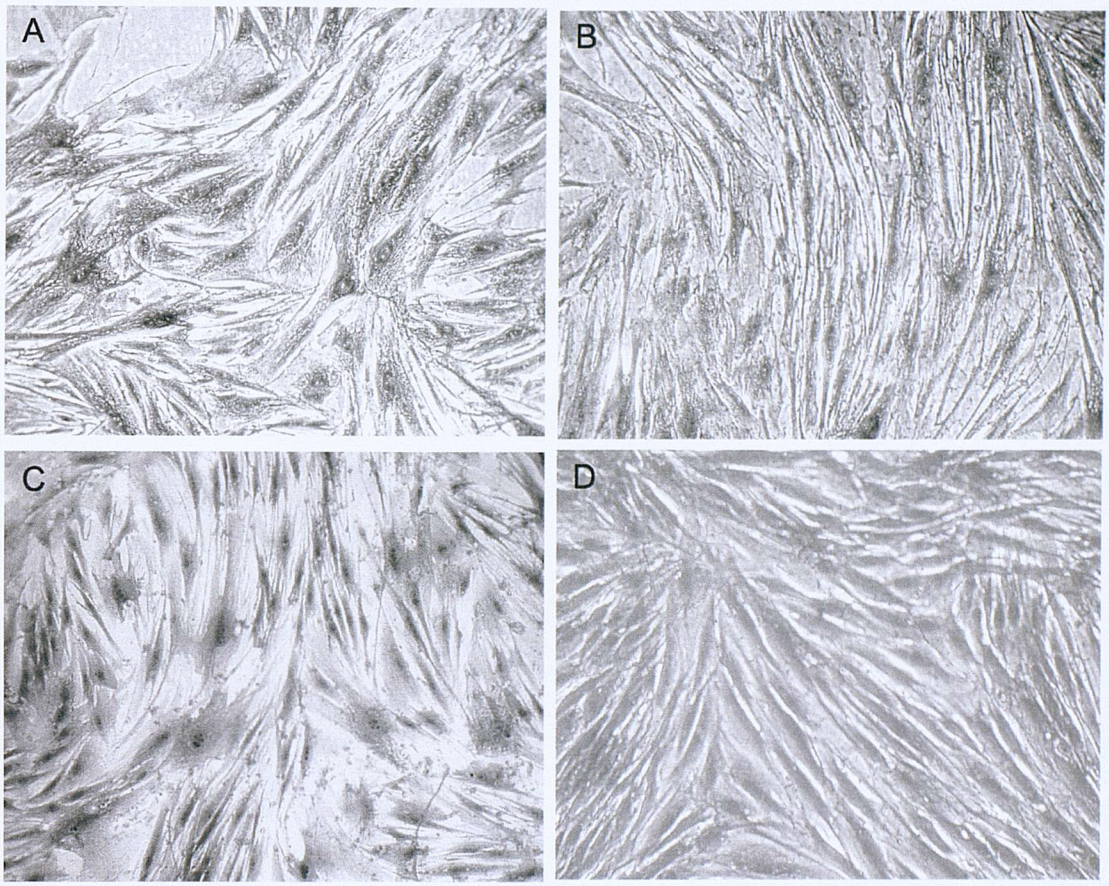


Figure 5.4: Phase contrast micrographs of a typical line of asthmatic fibroblasts after remaining in culture for 72 hours in the presence of a) serum free medium, b) 1nM human recombinant ET-1, c)1nM human recombinant ET-1 + 10nM N-Acetyl-[D-Trp]-endothelin-1 fragment 16-21 and d) 1nM human recombinant ET-1 + BQ788. After culture, the medium was removed and the cells stained with Methylene blue dye to aid visulaisation

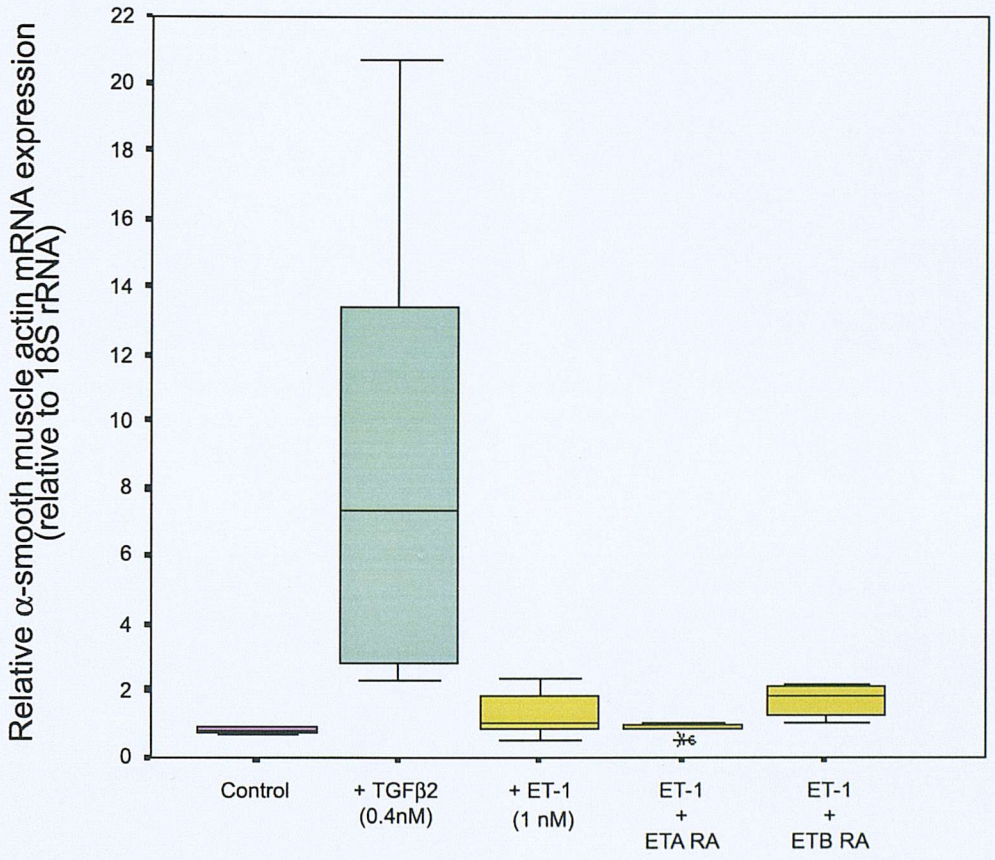
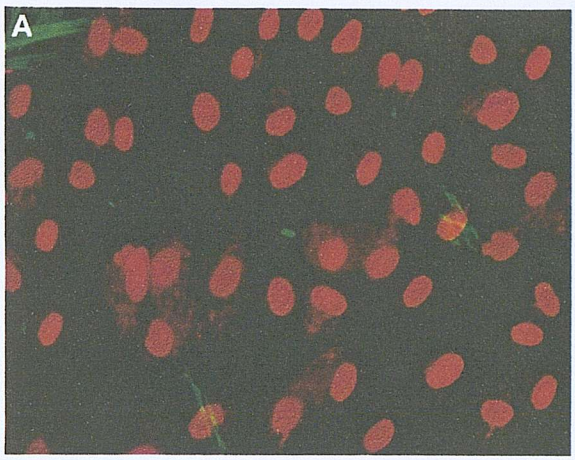
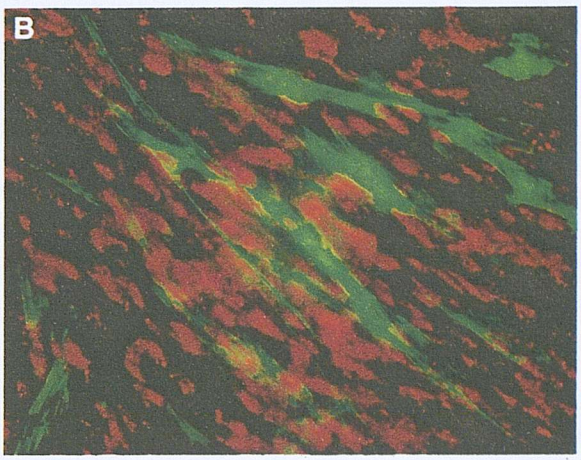


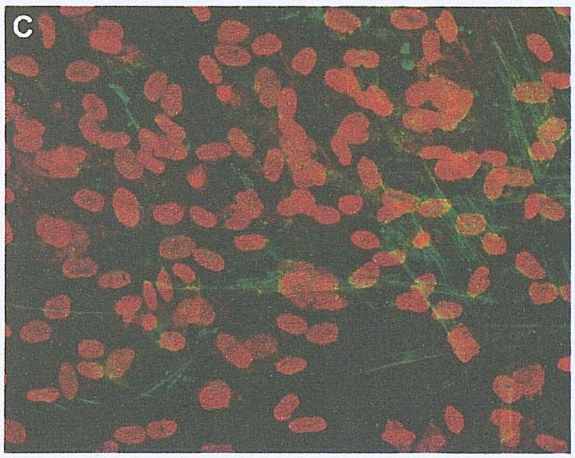
Figure 5.5 : The α–SMA gene expression induced in asthmatic fibroblasts (n=5) in response to 0.4nM TGFβ2, 1nM human recombinant ET-1 alone and in combination with ET-1 receptor A and receptor B antagonists. Gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA.



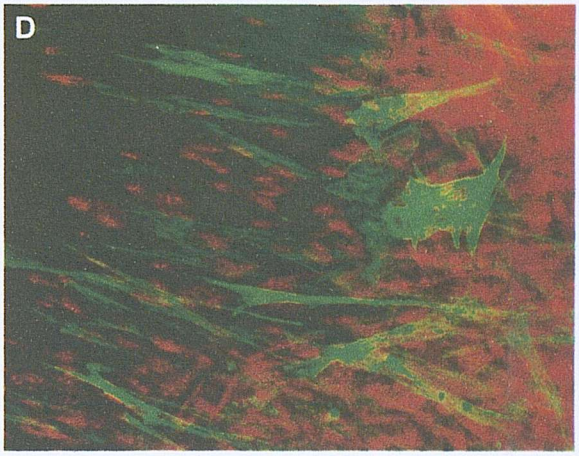
Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 1nM ET-1



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 1nM ET-1 + 10nM D-Trp



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 1nM ET-1 + 10nM BQ788

Figure 5.6: Immunodetection of α-SMA by monoclonal antibody, detected by a secondary fluorescently conjugated rabbit anti-mouse antibody. The asthmatic fibroblasts were cultured on collagen I coated 8 well chamberslides, in serum free medium (a) in the presence of 1nM ET-1, (b) 1nM ET-1 in combination with 10nM D-Trp and (c) 1nM ET-1 in combination with 10mM BQ788.

5.5 The potential of connective tissue growth factor as an autocrine growth factor

The availability of human recombinant CTGF protein as well as neutralising antibodies is extremely limited, and were unavailable for use in these studies. A form of CTGF claimed to be human recombinant protein was obtained from R&D systems, although biological activity was not claimed. In this study, the biological activity of the human recombinant CTGF (hRCTGF) was investigated by addition of the material to primary asthmatic fibroblasts and determining cell number by methylene blue and by use of the compound in a mitogenesis assay using the NR6/HER fibroblast cell line.

5.5.1 Connective tissue growth factor and autocrine induction of fibroblast proliferation and α smooth muscle actin

As can be seen in figure 5.7, addition of the hRCTGF did not result in an increase in cell number at any of the time points over fibroblasts treated with serum free medium.

In view of the absence of neutralising antibodies towards CTGF, the effect of blocking the action of CTGF by the use of CoA HMG reductase inhibitors (statins) was investigated since it has previously been reported that statins are able to block the gene expression of CTGF (Eberlein *et al.*, 2001). Figure 5.8 shows the dose response of fluvastatin on the inhibition of CTGF mRNA expression (as a percentage of maximal stimulation by 0.4nM TGFβ2) in fibroblasts when stimulated with 0.4nM TGFβ2. The CTGF mRNA expression was significantly increased in response to TGFβ2 ($p=0.043$) and this was significantly decreased in the presence of 5μM fluvastatin ($p=0.043$). The CTGF mRNA expression was significantly further decreased in response to 20μM fluvastatin. Addition of 40μM fluvastatin, lead to a further decrease in CTGF mRNA expression, although this decrease failed to reach statistical significance when compared to the reduction exhibited by 20μM fluvastatin.

A concentration of 20μM fluvastatin was used throughout the study to block CTGF gene expression, hence blocking any effects that may be caused by autocrine CTGF protein expression. The ability of 20μM fluvastatin to inhibit TGFβ2 mediated CTGF mRNA expression was investigated in asthmatic fibroblasts ($n=6$) and non asthmatic

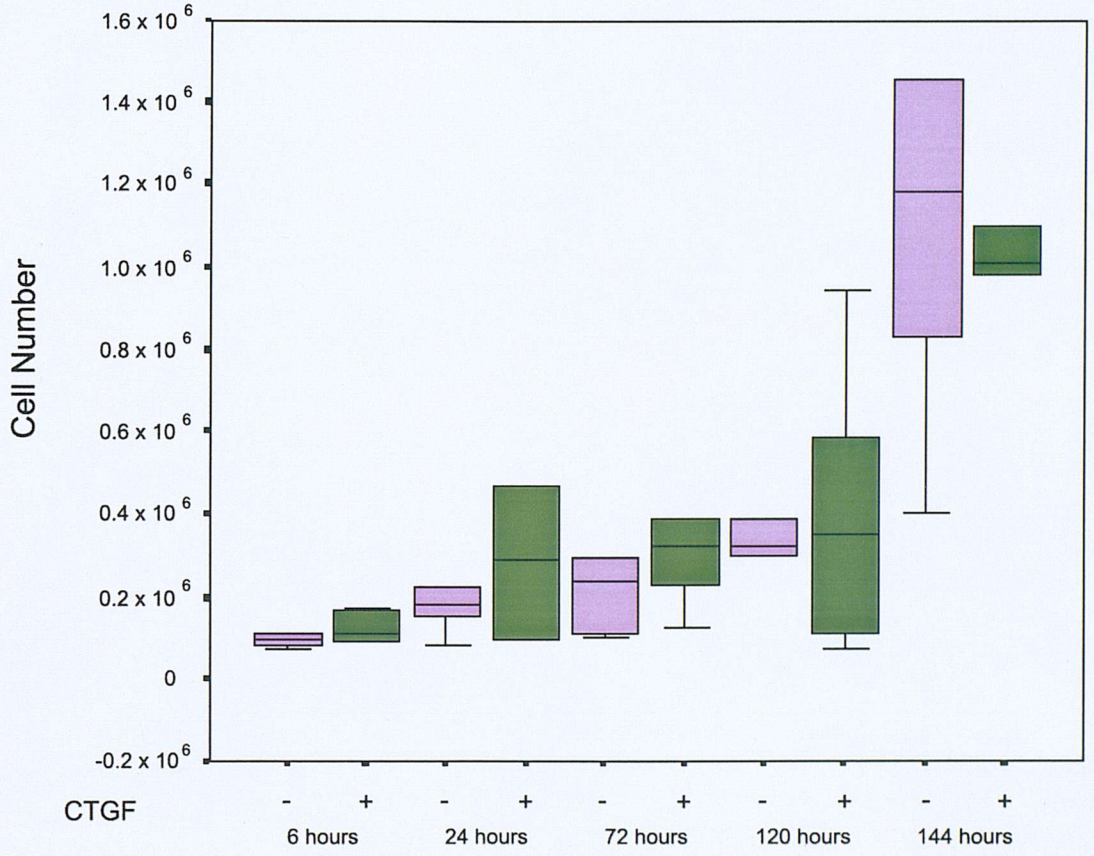


Figure 5.7: Fibroblast cell number as determined by methylene blue uptake. FIbroblasts were seeded at a starting density of $5x10^4$ cells/well in 24 well dishes, and were allocated to specific timepoints. At each timepoint, the correspondng cultures were fixed with formal saline. Methylene blue was added to each culture,the dye was eluted with ethanol/HCL and the absorbance at 620nM was measured. The quantity of methylene blue dye absorbed was translated into cell number by means of a standard curve. Error bars represent the interquartile range. (n=6)

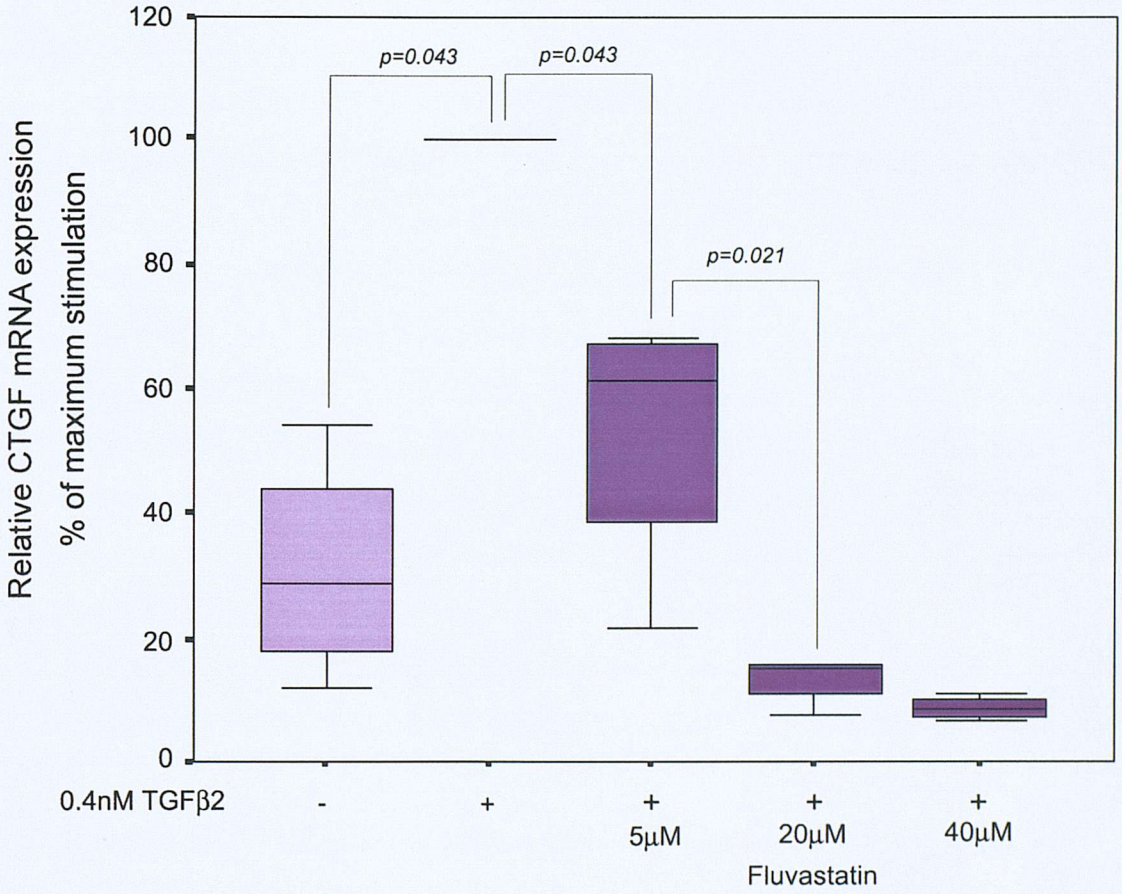


Figure 5.8: The CTGF gene expression induced in asthmatic fibroblasts (n=5) following treatment with 0.4nM TGFβ2 alone and in combination with 5μM, 20μM and 40μM Fluvastatin for 24 hours. The data is expressed as a percentage of the maximumresponse achieved following stimulation with 0.4nM TGFβ2. Significance following treatment was determined by application of the Wilcoxon statistical test.

fibroblasts (n=4). Figure 5.9 shows that in all cases, of both asthmatic and non asthmatic fibroblasts, addition of 20μM fluvastatin in combination with 0.4nM TGFβ2 resulted in a complete abolition of CTGF mRNA expression. In order to test the specificity of 20μM fluvastatin in reducing CTGF mRNA expression, the levels of ET-1 (Figure 5.10a) and TGFβ1 mRNA expression (figure 5.10b) in fluvastatin treated fibroblasts were also determined by quantitative RT-PCR. As can be seen 20μM fluvastatin did not affect the TGFβ2 induced mRNA expression of ET-1 and TGFβ1 in either the asthmatic or non-asthmatic fibroblasts.

Figure 5.11 shows the morphology of asthmatic fibroblasts cultured in response to serum free medium, 0.4nM TGFβ2, 20μM Fluvastatin and HrCTGF for 72 hours. The morphology of the fibroblasts in the absence or presence of CTGF was very similar following treatment in serum free medium after 72 hours, this may be due to the biological inactivity of the rHCTGF. Addition of 20μM fluvastatin caused fibroblasts to exhibit a very different morphology. The fibroblasts were much less stellate in appearance and the culture contained many cells that had lost adherence and appeared to float in the medium, as a result the total cell number following treatment with fluvastatin was decreased. This is unlikely to be a cytotoxic effect of fluvastatin, since it was possible to extract intact RNA and conduct RT-PCR to investigate gene expression on the adherent cells.

The addition of 20μM fluvastatin completed abolished α-SMA mRNA expression in a similar fashion to the abolition of CTGF mRNA expression figure 5.12.

5.6 The potential of transforming growth factor β1 as an autocrine growth factor

Transforming growth factor β has been shown to have both proliferative and anti-proliferative actions on fibroblasts depending on the dose (McAnulty *et al.* 1998). Low concentrations of TGFβ have been shown to be pro-proliferative whereas at higher concentrations (greater than 100pg/ml) TGFβ is seen to be anti-proliferative.

The action of TGFβ2 on both non-asthmatic and asthmatic fibroblasts has been investigated in section 3.4.3 and at the dose used, was shown to have neither proliferative nor anti-proliferative effects on. As discussed in section 4.4.1, the major isotype of TGFβ produced by fibroblasts is TGFβ1. The mRNA expression was up regulated in asthmatic

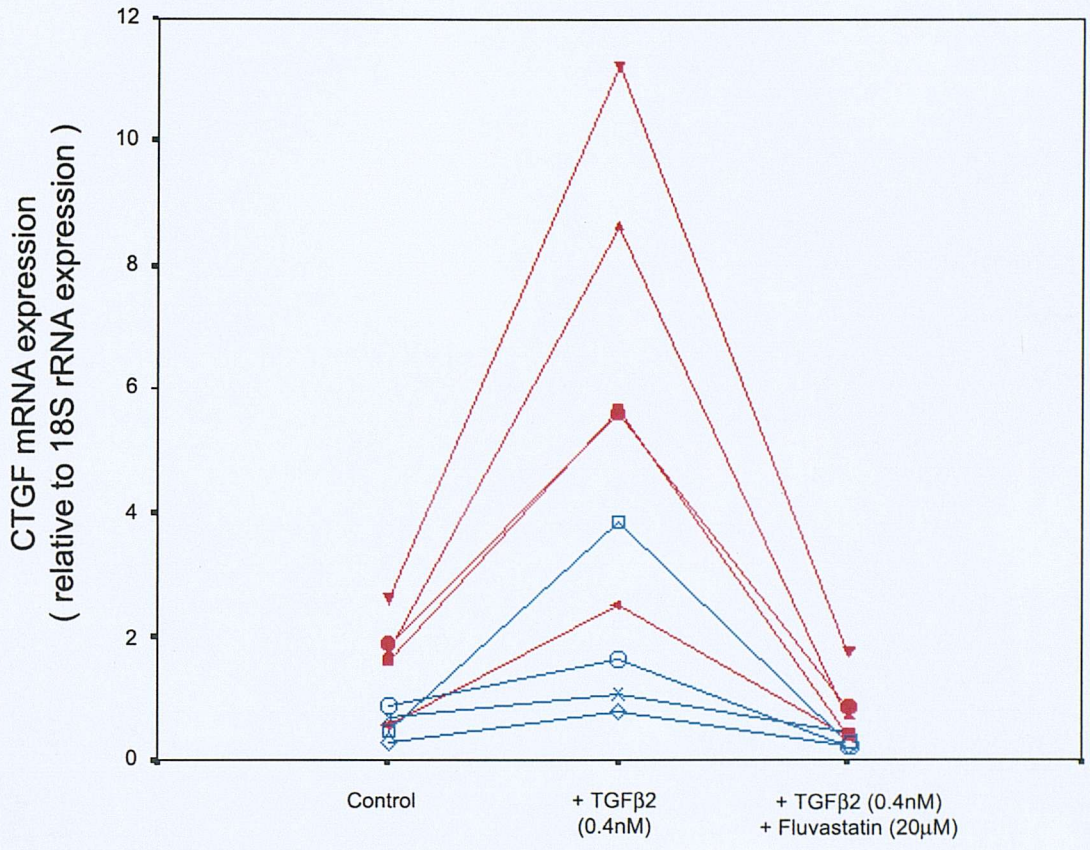


Figure 5.9: The CTGF gene expression following treatment with 0.4nM TGFβ2 alone and in combination with 20μM Fluvastatin for 24 hours. The data represent 4 asthmatic fibroblast lines (red) and 4 non - asthmatic lines (blue). The CTGF gene expression was measured and expressed relative to 18S rRNA expression.

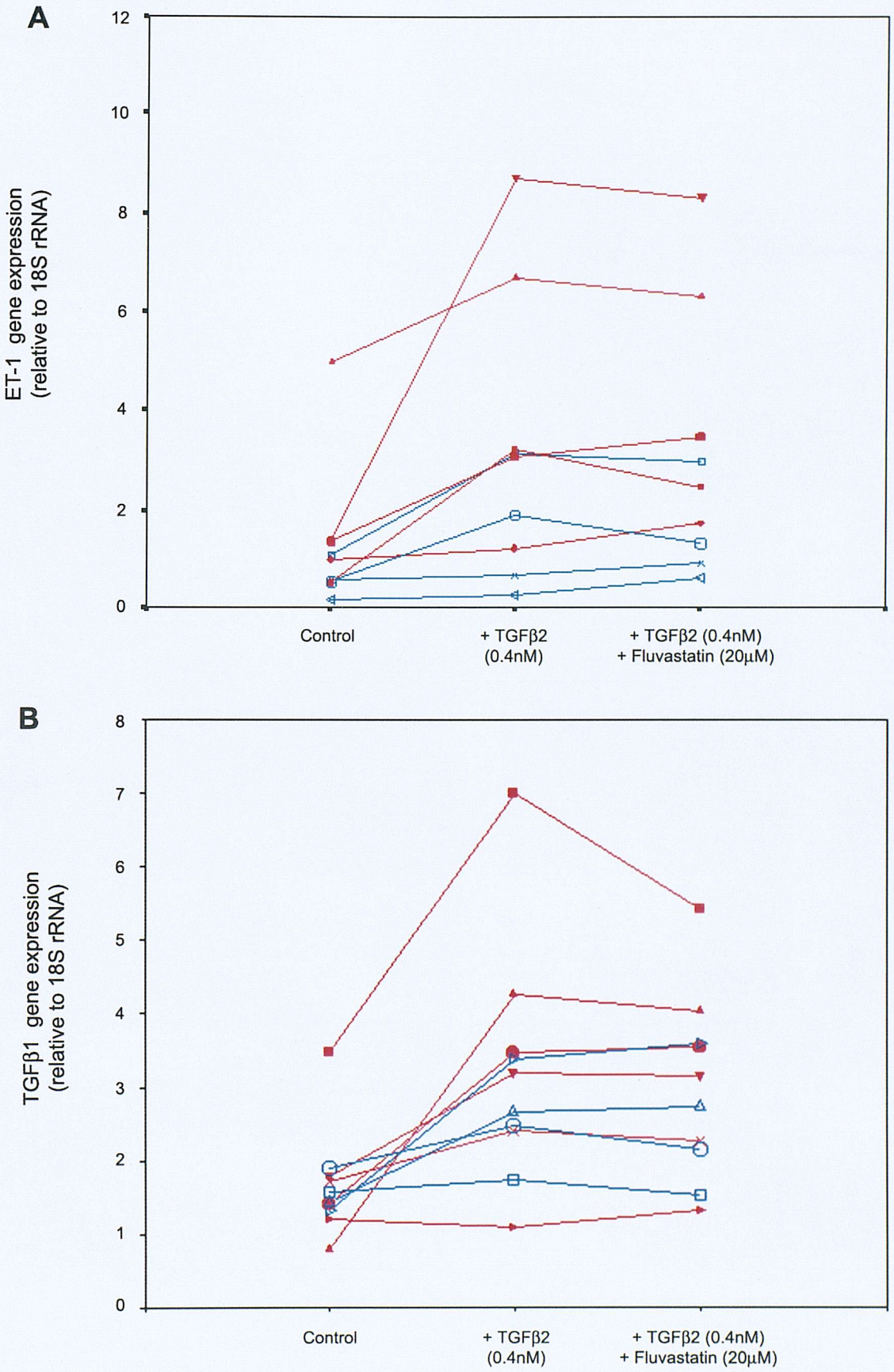


Figure 5.10: The (a) ET-1 and (b) TGFβ1 gene expression in asthmatic (n=6;red) and non-asthmatic (n=4;blue). The fibroblasts were cultured in serum free medium, or with 0.4nM TGFβ2 alone or in combination with 20μM Fluvastatin for 24 hours.

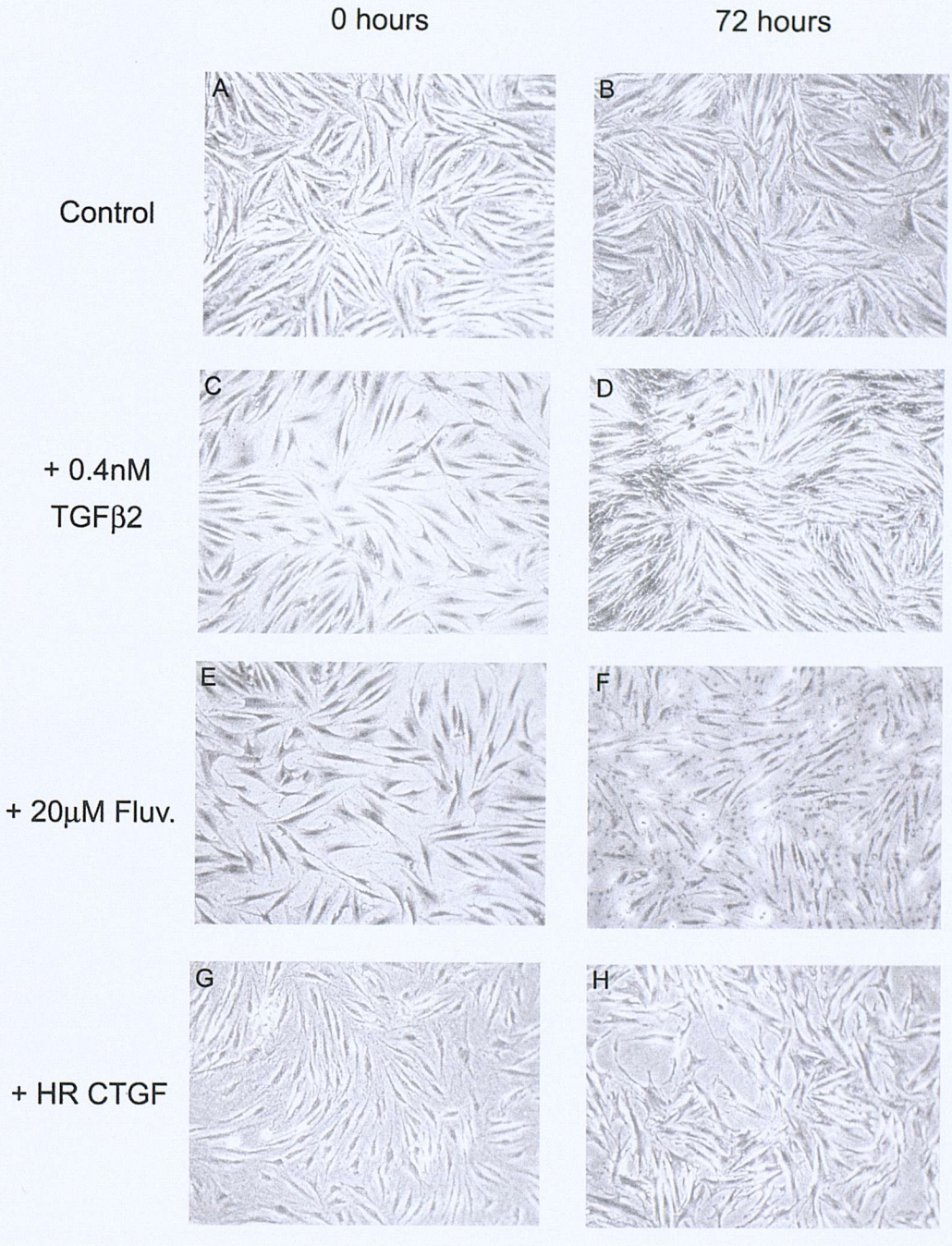


Figure 5.11: Phase contrast micrographs of a typical asthmatic fibroblast line seeded at a cell density of 1 x 10 cells/well at time 0hrs and after 72 hours in culture, following treatment with 0.4nM TGFβ2, 20μM Fluvastatin or human recombinant CTGF protein.

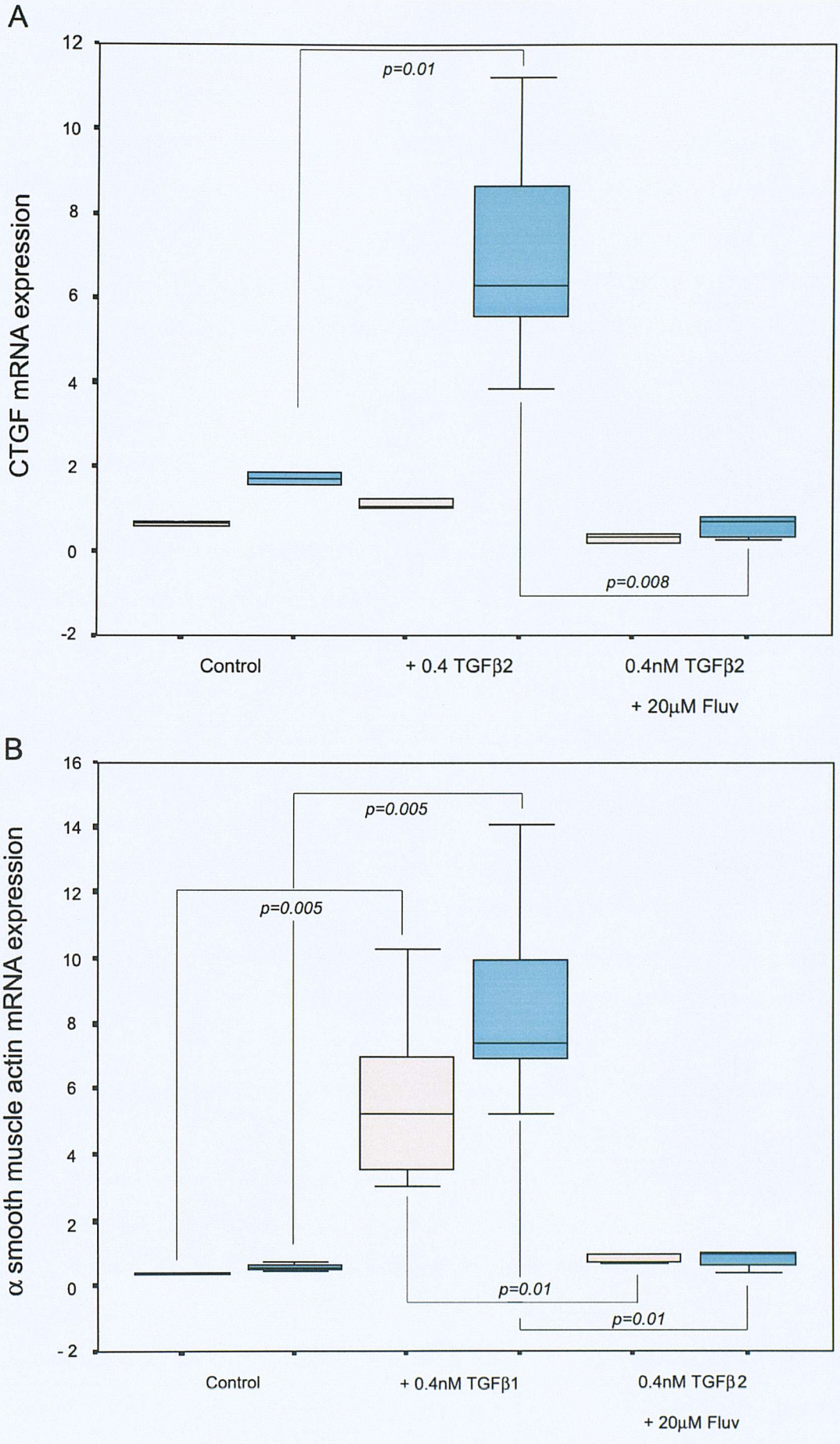


Figure 5.12: The (a) CTGF and (b) α-SMA gene expression in 6 asthmatic (blue) and 6 non-asthmatic (cream) fibroblast lines derived from endobronchial biopsies. The fibroblasts were cultured on a collagen I substratum in serum free medium alone or in the presence of the specified stimulus. Following reverse transcription, the cDNA was investigated for α-SMA and CTGF gene expression, both were expressed relative to the levels of 18S rRNA gene. Gene expression levels were determined by real-time quantitative PCR. Each assay was conducted in triplicate and the mean value considered. The box plot represents the interquartile range for each of the subject groups when considering the mean values. The central line represents the median; the whisper bars represent the 95% confidence interval. n=18 for each subject group.

fibroblasts. In light of those observations, the proliferative effect of TGFβ1 on primary asthmatic fibroblasts was investigated by addition of human recombinant TGFβ1 and use of specific anti-TGFβ1 neutralising antibodies (provided by Cambride antibody technologies) and specific TGFβ2 neutralising antibodies (provided by Cambride antibody technologies) and cell number determined by methylene blue dye uptake at specified time points.

5.6.1 Transforming growth factor β1 and autocrine induction of fibroblast proliferation.

In section 3.4.3, the effects of TGFβ2 on proliferation was investigated. Therefore further experiments were conducted using TGFβ1.

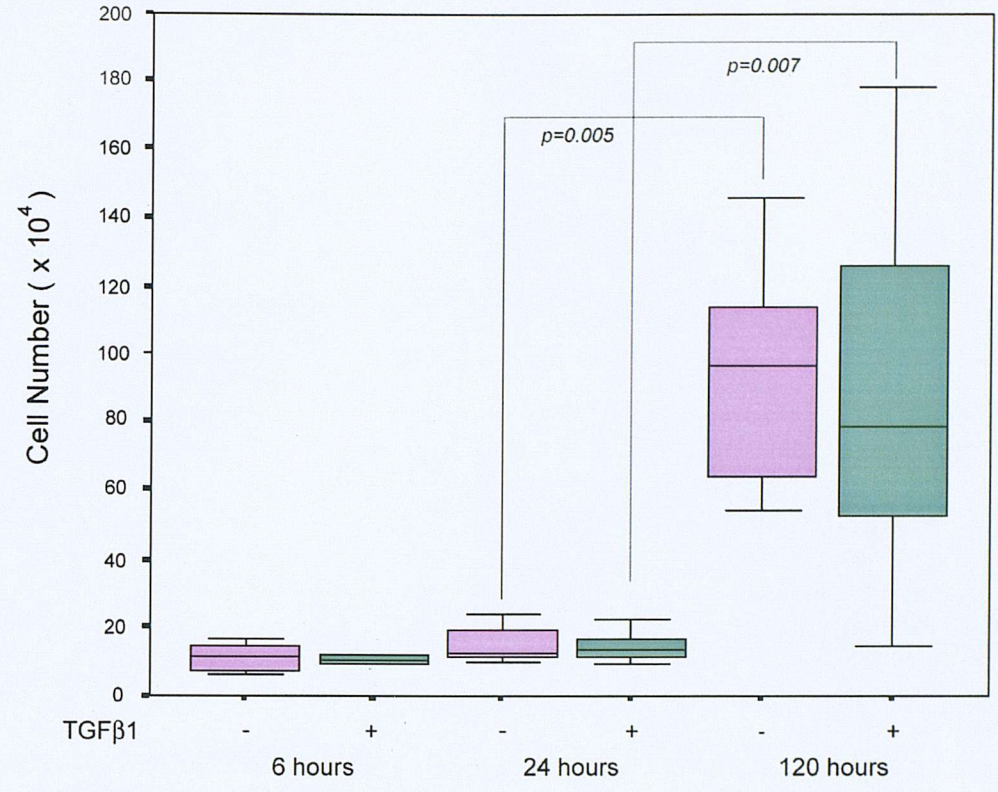
As was seen in previous experiments a significant increase in cell number was seen between 24 and 120 hours when the cells were grown in serum-free medium. As previously seen with TGFβ2, addition of TGFβ1 to the asthmatic fibroblasts did not significantly alter growth observed (figure 5.13).

The lack of an effect may have been due to the possibility of endogenous TGFβ1 secreted by the asthmatic fibroblasts already having elicited a maximal proliferative effect. In order to determine if the levels of TGFβ1 produced by the asthmatic fibroblasts were sufficient to account for the hyperproliferation of asthmatic fibroblasts in serum free medium, neutralising anti-TGFβ1 and neutralising anti-TGFβ2 antibodies were used.

5.6.2 Determining specificity of TGFβ1 and TGFβ2 neutralising antibodies

Initial experiments were performed In order to ensure that the neutralising antibodies were specific for their corresponding isoforms.

Anti- TGFβ1 neutralising antibody was seen to specifically inhibit TGFβ1 stimulated α-SMA mRNA expression in asthmatic fibroblasts ($p=0.028$; figure 5.14a) whereas anti-TGFβ2 neutralising antibody was seen to specifically inhibit TGFβ2 stimulated α-SMA mRNA expression ($p=0.028$; figure 5.14b). In both cases, the control irrelevant antibody (provided by Cambridge antibody technologies) did not effect α-SMA mRNA expression.



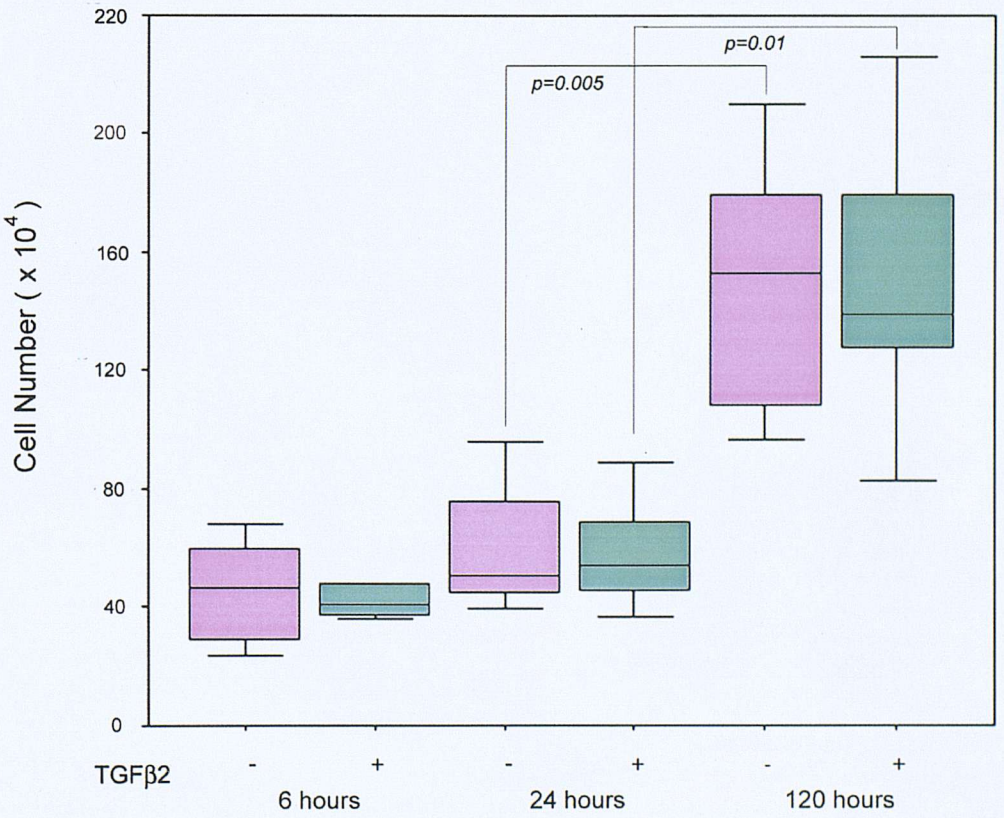
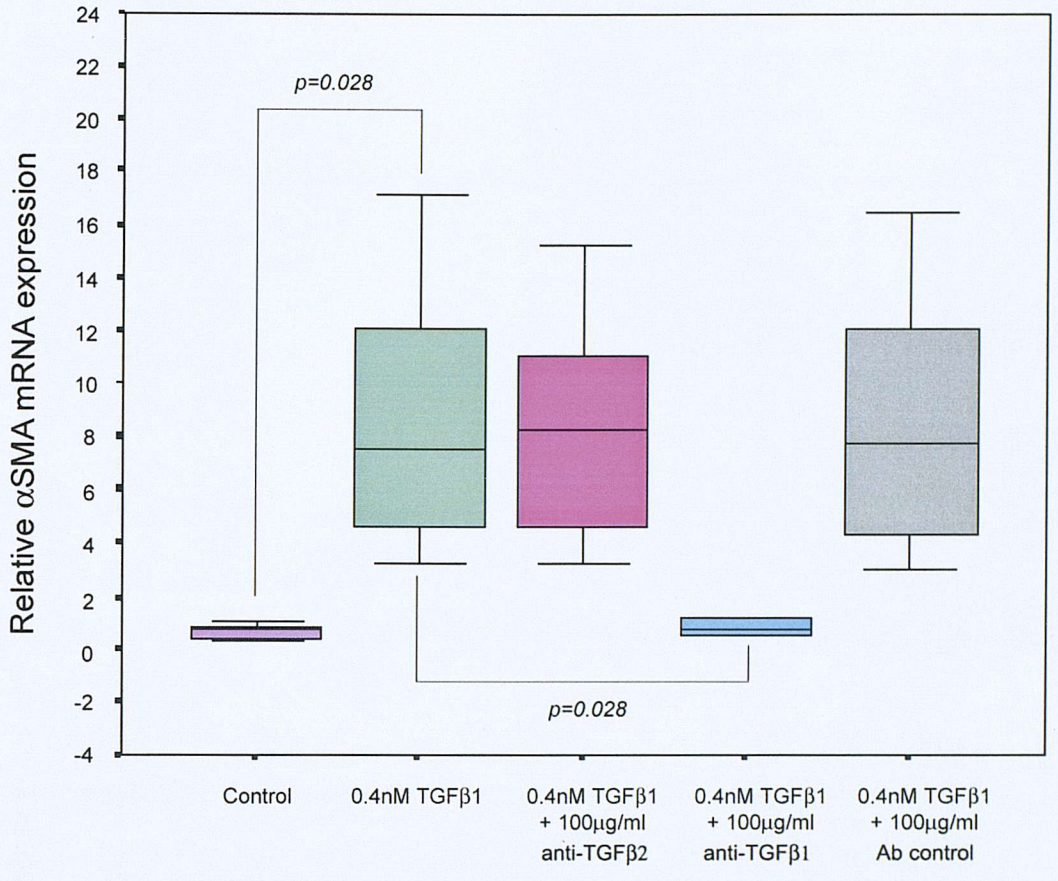


Figure 5.13: The proliferation of endobronchial biopsy derived asthmatic fibroblasts in the presence of TGFβ1 or TGFβ2. Six lines asthmatic fibroblasts were cultured in serum free medium alone (purple) or in the presence (green) of (a) 0.4nM TGFβ1 or (b) 0.4nM TGFβ2 for up to 120 hours (5 days) in 24 well dishes pre-coated with collagen I. At different time points (6, 24, and 120 hours) the medium was removed from the designated tray and the cells fixed overnight with Formol saline. Methylene blue dye (500μl) was then added to each well for 30 minutes, after which the excess was removed and the dye eluted from the cells with 500μl HCl/ethanol elution buffer. The absorbance of the eluted solution was then measured in a spectophometer at 620nM. Each assay was conducted in triplicate and the mean value considered. Absorbances were correlated to cell number by means of a generated standard curve (figure 2.3). The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval. n=18 Statistical significance following treatment was determined by application of the Wilcoxon statistical test.



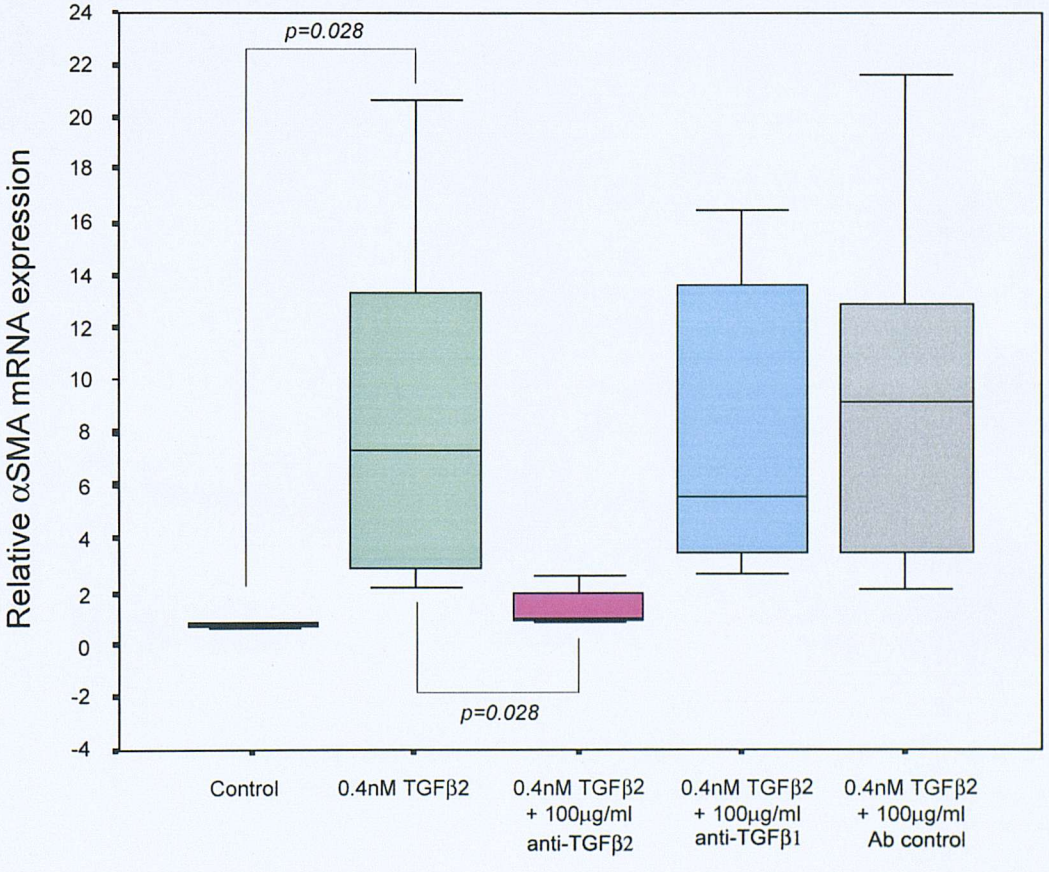


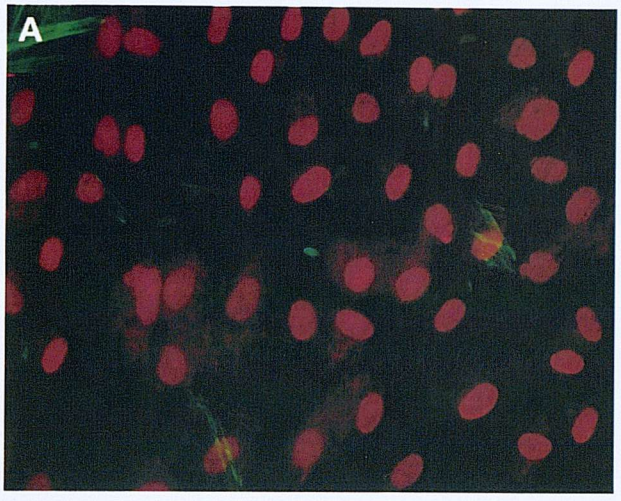
Figure 5.14: The relative α–SMA gene expression in 6 asthmatic fibroblast lines when treated with 0.4nM TGFβ1 (a) or 0.4nMTGFβ2 (b) alone or in combination with 100μg/ml of irrelevant antibody control (Ab control) anti-TGFβ1 neutralising antibody and anti-TGFβ2 neutralising antibody for 24 hours on collagen I substratum.

The specificity of the antibodies observed in the mRNA expression data was mirrored by α-SMA protein expression. Figure 5.15 shows the inhibition of TGFβ1 induced α-SMA by anti- TGFβ1 neutralising antibody, whereas figure 5.16 shows the inhibition of TGFβ2 induced α-SMA by Anti- TGFβ2 neutralising antibody at 72 hours.

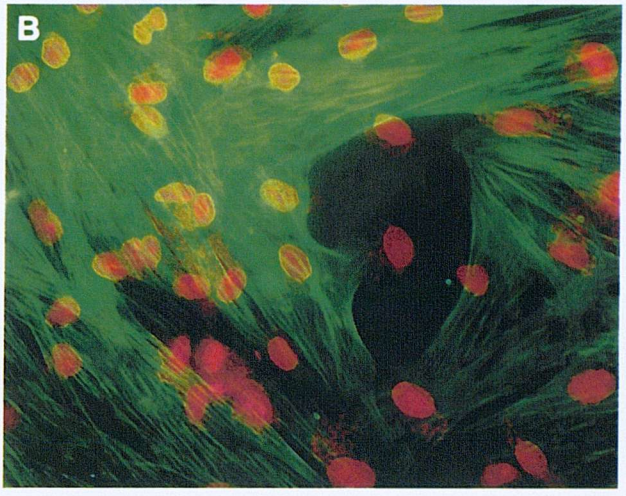
5.6.3 Effect of TGFβ neutralising antibodies on proliferation of asthmatic fibroblasts in serum free medium.

Anti- TGFβ1 neutralising and anti-TGFβ2 neutralising antibodies were added to fibroblasts in order to determine the effect on cell number. Phase contrast photomicrographs illustrate that after addition of anti-TGFβ1 neutralising antibody for 120 hours, there was a clear reduction in cell number of asthmatic fibroblasts (figure 5.17d).

The microscopy analysis was confirmed by the cell number at 120 hours (determined by methylene blue dye uptake),where there was a significant decrease in cell number when treated with anti-TGFβ1 neutralising antibody ($p=0.028$). However no significant decreases in cell number at any of the specified time points were observed following anti-TGFβ2 neutralising antibody treatment (figure 5.18)

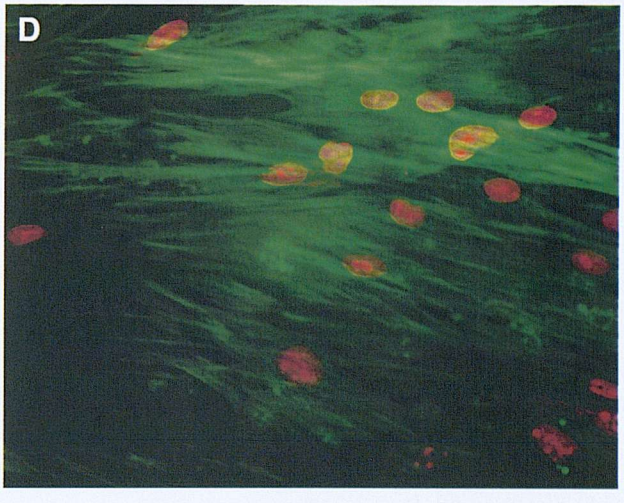


Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium



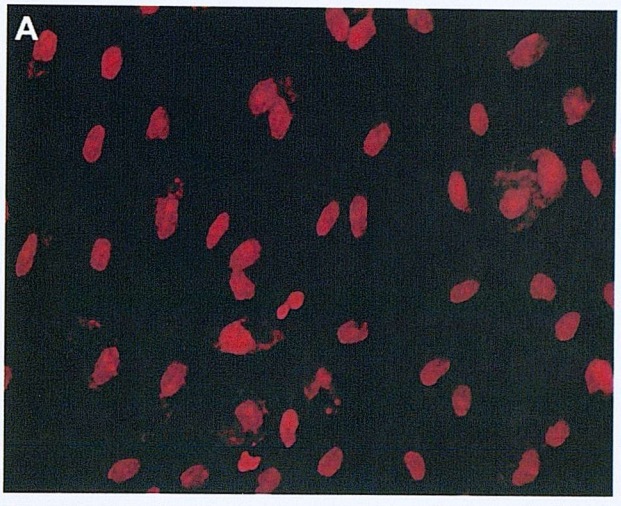
Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ1

Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ1 + 100μg/ml anti-TGFβ1 antibody

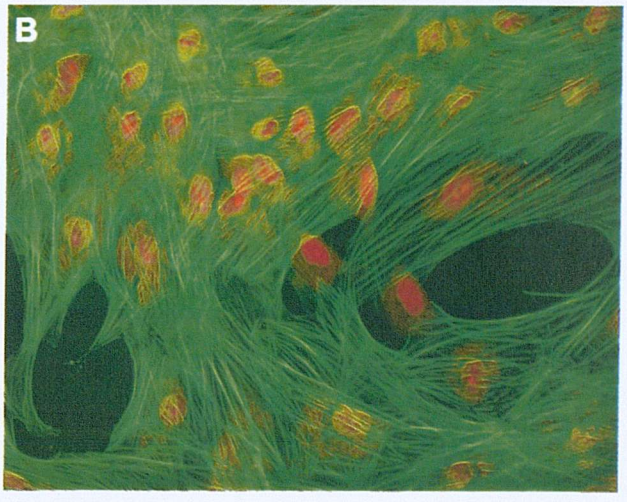


Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ1 + 100μg/ml anti-TGFβ2 antibody

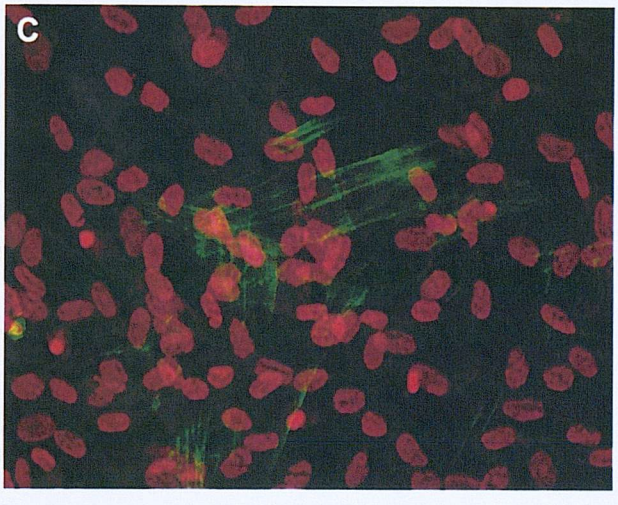
Figure 5.15: Immunodetection of α-SMA by monoclonal antibodiy, detected by a secondary fluorescently conjugated rabbit anti-mouse antibody. The asthmatic fibroblasts were cultured on collgaen I coated 8 well chamberslides for 72 hours in (a) serum free medium (b) 0.4nM TGFβ1 (c) 0.4nM TGFβ1 + 100μg/ml anti-TGFβ1 Ab and (d) 0.4nM TGFβ1 + 100 μg/ml anti-TGFβ2 Ab.



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ2



Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ2 + 100μg/ml anti-TGFβ2 Ab

Immunodetection for α-smooth muscle actin after *in vitro* culture for 72 hours in serum free medium + 0.4nM TGFβ2 + 100μg/ml anti-TGFβ1 Ab

Figure 5.16: Immunodetection of α-SMA by monoclonal antibodiy, detected by a secondary fluorescently conjugated rabbit anti-mouse antibody. The asthmatic fibroblasts were cultured on collgaen I coated 8 well chamberslides for 72 hours in (a) serum free medium (b) 0.4nM TGFβ2 (c) 0.4nM TGFβ2 + 100μg/ml anti-TGFβ2 AB and (d) 0.4nM TGFβ2 + 100 μg/ml anti-TGFβ1 Ab.

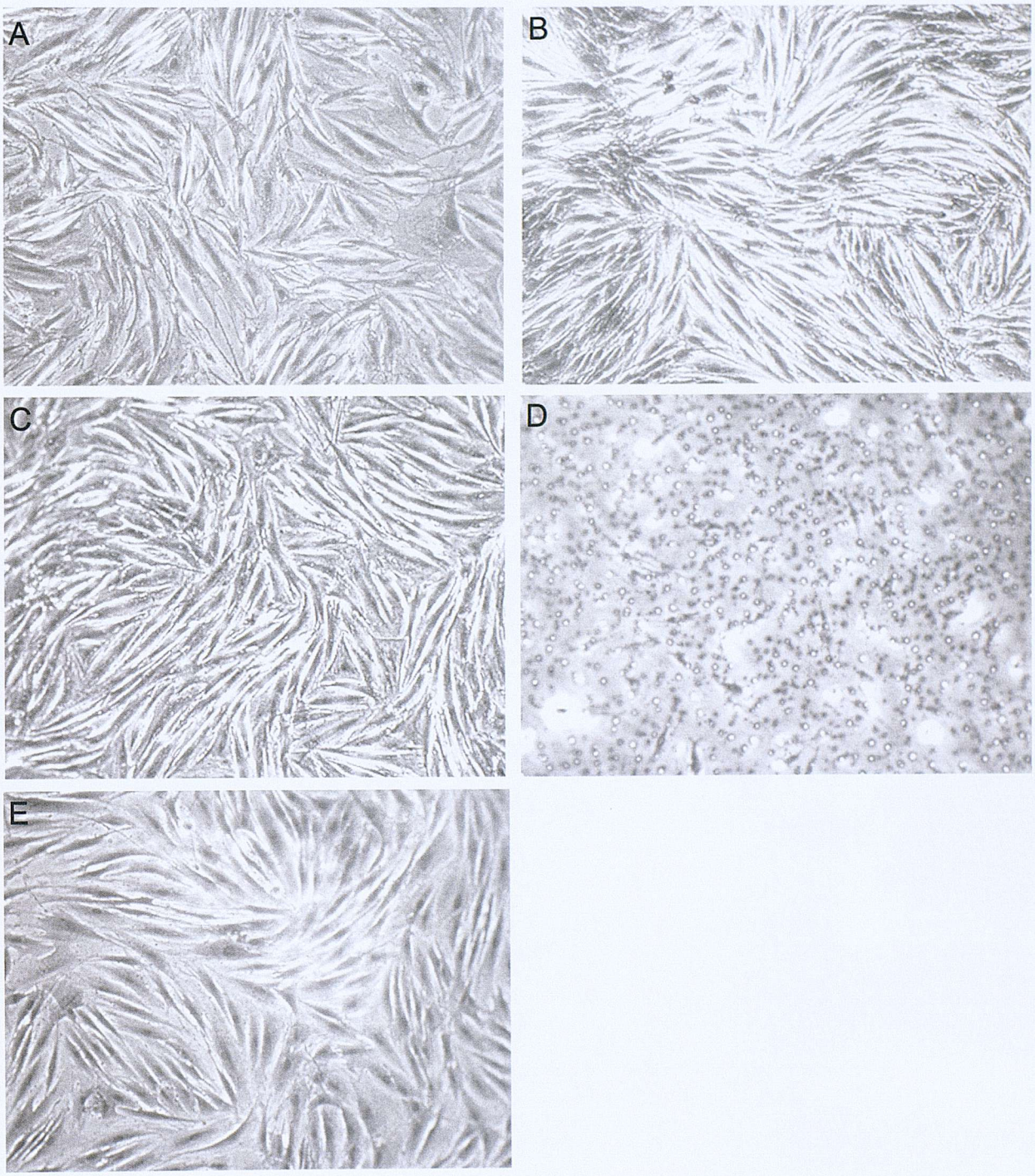


Figure 5.17: Phase contrast micrographs of a typical asthmatic fibroblast line after 120H in culture grown on collagen I substratum. The cells were treated with (a) Ultraculture, (b) 0.4nM TGFβ1 (c) 0.4nM TGFβ2, (d) 100μg/ml anti-TGFβ1 Ab and (e) 100μg/ml anti-TGFβ2 Ab.

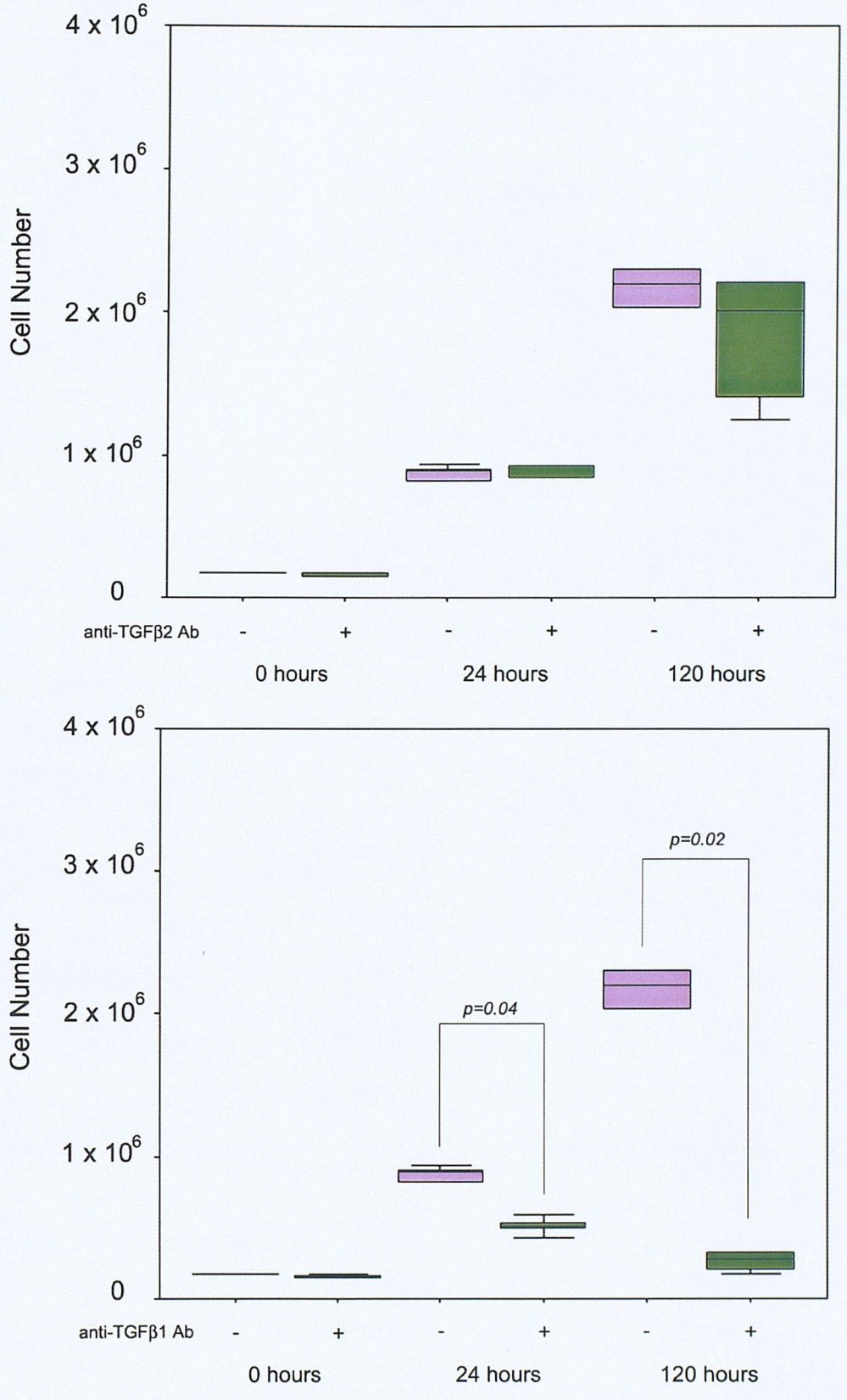


Figure 5.18: The fibroblast cell number of endobronchial biopsy derived asthmatic fibroblasts in the presence of anti-TGFβ1 or TGFβ2 neutralising antibodies. Six lines asthmatic fibroblasts were cultured in serum free medium alone (purple) or in the presence (green) of (a) anti-TGFβ1 neutralising antibodies or (b) anti-TGFβ2 neutralising antibodies for up to 120 hours (5 days) in 24 well dishes pre-coated with collagen I. At different time points (6, 24, and 120 hours) the medium was removed from the designated tray and the cells fixed overnight with Formol saline. Methylene blue dye (500μl) was then added to each well for 30 minutes, after which the excess was removed and the dye eluted from the cells with 500μl HCl/ethanol elution buffer. The absorbance of the eluted solution was then measured in a spectophometer at 620nM. Each assay was conducted in triplicate and the mean value considered. Absorbances were correlated to cell number by means of a generated standard curve (figure 2.3). The bars represent the interquartile range, the central line represents the medium and the whisper bars represent the 95% confidence interval. n=18 Statistical significance following treatment was determined by application of the Wilcoxon statistical test.

5.7 Discussion

As discussed in the previous section (3.4.3) the asthmatic fibroblasts exhibited an increased rate of proliferation in serum free conditions. In the previous section, it was observed that asthmatic fibroblasts produced and secreted increased quantities of growth factors namely ET-1, CTGF and TGFβ1, when compared to non-asthmatic fibroblasts, and in response to 0.4nM TGFβ2. It was postulated that the over production of those growth factors, were able to stimulate their hyperproliferation in an autocrine fashion.

Endothelin-1 (Shahar *et al.,* 1999)), CTGF (Frazier *et al.,* 1996) and TGFβ1 (Blobe *et al.,* 2000) have all been implicated in other disease states as proliferative factors for fibroblasts. There have been no published studies to date, which have implicated these growth factors in autocrine growth with relation to bronchial asthma.

TGFβ1 is well known for it's role in myofibroblast induction in all fibroblast cell lines whereas ET-1 has been shown to induce the myofibroblast phenotype in some fibroblast cell lines (Shahar *et al.,* 1999). Connective tissue growth factor is known to mediate many TGFβ induced effects, although there are no published studies to indicate a role for CTGF in α-SMA induction.

5.7.1 Endothelin-1

Endothelin-1 is a potent constrictor and mitogenic peptide expressed in many pulmonary diseases (Barnes 1994). Previous studies have shown that ET-1 is a mitogen for vascular smooth muscle cells, however it has also been shown to be mitogenic for fibroblasts (Kikuchi *et al.,* 1995).

In the present study, proliferation of asthmatic bronchial derived fibroblasts was not stimulated by ET-1, nor was autocrine proliferation of these cells in serum free medium inhibited by ET_A or ET_B receptor antagonists. These observations are very different from those made by Shahar and co-workers (1999). In this study, alveolar fibroblasts from patients with interstitial lung disease exhibited a dose-dependent increase in mitogenesis in response to ET-1 stimulation, independent of disease status, although the mitogenesis achieved by the fibroblasts derived from patients with interstitial pulmonary disease was greater than the control patients at the higher concentrations of ET-1. This phenomenon was not observed in the asthmatic fibroblasts in the present study (figure 5.1)

nor did endothelin-1 induce mitogenesis in the NR6HER fibroblast cell line (see figure 5.3a). The cells however did exhibit a typical dose dependent increase in mitogenesis in response to basic fibroblast growth factor (bFGF ; figure 5.3b). A possible explanation for the lack of a mitogenic effect by the NR6/HER fibroblasts in response to ET-1 may be that the protein used was a human recombinant protein, whereas the NR6/HER cell line is derived from mice.

The data in the present study is in line with the observations made by Dubé and co-workers (Dube *et al.,* 2000). In their investigation, it was found that ET-1 had no effect on the mitogenic activity of fibroblasts from asthmatic and non-asthmatic fibroblasts. However, in these studies, Dubé and co-workers indicated that ET-1 in combination with TGFβ1 and platelet derived growth factor (PDGF)-BB caused a modest increase of 25% (±20% SEM) in DNA synthesis. In the present study, neither addition of ET-1 to TGFβ1 nor addition of ET-1 to 10% FCS caused an increase the mitogenic activity of the fibroblasts.

In light of the present study and the investigations by Dubé and co-workers, it would seem unlikely that ET-1 is a major pro-proliferative factor for bronchial fibroblasts in asthma.

Although, unable to stimulate proliferation or mitogenesis in primary asthmatic fibroblasts, a distinct morphological phenotype, very similar to TGFβ treated fibroblasts was observed following ET-1 treatment (figure 5.4). Further analyses by means of quantitative real time RT-PCR and immunocytochemistry for α-SMA, clearly showed an ability to transform into myofibroblasts after 72 hours treatment with ET-1.

The relative mRNA expression of α-SMA was not significantly higher in the cells treated with ET-1 than the control (untreated) fibroblasts, although the general trend was towards increased expression (figure 5.5). However, there seemed to be considerably more immunoreactivity in the ET-1 treated fibroblasts (figure 5.6b) although the expression was not as intense as that observed when the fibroblasts were treated with TGFβ2 (see figure 5.17) Shahar and co-workers, as well as investigating the proliferative effect of ET-1 on fibroblasts from patients with IPF, also investigated the ability of ET-1 to induced αSMA expression (1999). They also observed that in response to ET-1, the IPF fibroblasts were able to express α-SMA. However, in the present study, it has been shown that a significant increase in the α-SMA gene expression is not required to result in an ET-1 induction of α-SMA. These observations

suggest that ET-1 may have some role in organising the α-SMA into the characteristic filaments. It may also indicate a 'priming' of asthmatic fibroblasts, whereby only a slight change in the basal levels of α-SMA mRNA results in significant increases in protein expression or assembly.

Sun and co-workers (Sun *et al.*,1997) investigated the effects of conditioned medium from asthmatic bronchial epithelial cells (known to secrete large amounts of ET-1) on α-SMA expression in cultured asthmatic fibroblasts. They found that recombinant human ET-1 closely mimicked the effect of asthmatic epithelial conditioned medium when used to stimulate asthmatic fibroblasts. In the present study, it has been shown that recombinant human ET-1 does have the ability to induce α-SMA in asthmatic fibroblasts, and that the transformation can be down regulated by specific antagonism of the ET_A receptor but not antagonism of the ET_B receptor (figure 5.6c,d). The reduction in α-SMA immunoreactivity was mirrored by the mRNA gene expression data, which showed that addition of ET-1 in combination with ET_A antagonists (but not ET_B antagonists) resulted in a decreases in α-SMA mRNA expression

The ability of ET-1 to induce α-SMA through the ET_A expression in asthmatic endobronchial biopsy derived fibroblasts is a novel finding. ET-1 may have an autocrine role in myofibroblast differentiation and blockade of the ET_A receptor may prevent activation of resident fibroblasts to myofibroblasts and hence aid reduction in airway structural remodelling.

Although there appears to be induction of a myofibroblast phenotype in asthmatic fibroblasts following ET-1 stimulation, the relative potency of TGFβ2 (and TGFβ1) in inducing highly significant increases in α-SMA protein expression suggest that ET-1 is probably playing a minor role in transformation.

Many studies have focussed on the ability of ET-1 to induce mitogenesis and proliferation in airway smooth muscle cells (Panettieri *et al.*, 1996). In light of the observations made in the present investigation, it seems that the increased expression of ET-1 gene and protein observed in asthmatic fibroblasts may be most influential in driving paracrinc proliferation of the underlying smooth muscle cells (SMCs) and not (myo)fibroblasts.

In the future it may be fruitful to investigate the effects of fibroblast derived endothelin-1 on primary bronchial airway SMCs. Such an investigation could be carried out in a number of ways; conditioned medium from asthmatic fibroblasts could be used to

stimulate asthmatic airway SMCs and it would be hypothesised that this would result in increased mitogenic / proliferative activity of the SMCs. Alternatively, a 3D co-culture system, similar to that used by Zhang and co-workers (Zhang *et al.,* 1999), whereby fibroblasts could be immersed in a collagen gel, and smooth muscle cells grown on the surface of the gel. Any growth factors released by the fibroblasts such as ET-1, could then stimulate the SMCs. Endothelin-1 could then be implicated if ET_A or ET_B receptor antagonists resulted in a reduction or abolition of any stimulated mitogenesis / proliferation.

5.7.2 Connective Tissue Growth Factor

Previously, connective tissue growth factor was thought to function only as a downstream mediator of TGFβ signalling, responsible for the stimulation of cell proliferation and extracellular matrix production in fibroblasts (Grotendsort 1997). However, recent evidence has been reported, which suggest that CTGF gene expression can be induced in the absence of TGFβ and SMAD signalling (Holmes *et al.,* 2001 see section 1.6.3.3).

In the previous section, it was observed that the CTGF gene expression was significantly higher in the asthmatic fibroblasts at baseline, when compared to non-asthmatic controls.

As discussed earlier, neither CTGF neutralising antibodies nor receptor antagonists are freely available. Human recombinant protein was made available for use as positive controls in western blots, however biological activity was not assured. Hence it is unclear, if a lack of any proliferative or mitogenic effect observed in the asthmatic fibroblasts was a 'true' observation, or reflected a lack of biological activity of the protein.

Eberlein and co-workers (Eberlein *et al.,* 2001) were the first group to illustrate that HMG CoA reductase inhibitors (statins) had the ability to reduce CTGF mRNA expression in primary renal fibroblasts. Therefore, in the present study statins were used to inhibit CTGF expression.

The results obtained in the present study are in agreement with the observations made by Eberlein and co-workers. Addition of fluvastatin led to a dose-dependent decrease in TGFβ2 stimulated CTGF gene expression. The specificity of reduction in

CTGF gene expression was demonstrated by a lack of an effect in the reduction of TGFβ1 and ET-1 gene expression.

Hahn and co-workers (Hahn *et al.,* 2000) have previously shown that RhoA is critically involved in the regulation of CTGF mRNA expression in rat mesangial cells. The first evidence for RhoA dependent CTGF gene expression in fibroblasts was published by Heusinger-Ribeiro and co-workers (Heusinger-Ribeiro *et al.,* 2001). In this study, inhibition of RhoA proteins by the Rho Kinase family inhibitors Y-27632 and cytochalasin D, resulted in a significant decrease in the TGFβ1 stimulated CTGF gene expression. This provided direct evidence that CTGF gene expression was dependent on RhoA protein. Erberlein and co-workers showed that treatment of the human renal fibroblast cell line TK173 with simvastatin or lovastatin resulted in a reversible alteration in cell morphology, including dissolution of the actin cytoskeleton. This was inhibited by mevalonate , indicating that the changes were caused by inhibition of HMG coA reductase. Products of the mevalonate pathway i.e. the isoprenoid lipids farnesyl- and geranylgeranylpyrophosphate, are involved in the regulation of the activity of the small GTPases of the Ras and Rho family (Zhang and Casey 1996). HMG Co A reductase inhibitors interfere with the isoprenylation, and thus activation of Rho proteins (Goldstein *et al.*, 1990.) From the evidence obtained to date, it follows that HMG CoA reductase inhibitors would result in reduction of CTGF gene expression.

In the present study, it was found that Fluvastatin did specifically reduce CTGF mRNA expression, without affecting ET-1 or TGFβ1 mRNA expression. However, α-SMA gene expression was significantly decreased in line with CTGF mRNA expression. As discussed in section 1.6.3, CTGF is a key regulator of TGFβ signalling. The evidence presented in the present study suggest that induction of αSMA by TGFβ1 in myofibroblast differentiation is controlled by CTGF. The lack of an effect of the statins on ET-1 or TGFβ1 gene expression suggests that their expression is not dependent on CTGF expression.

Heusinger-Ribeiro and co-workers (Heusinger-Ribeiro *et al.,*2001) have also shown that elevated levels of cAMP and the subsequent activation of protein kinase A resulted in a reduction of CTGF mRNA, which in turn led to a disassembly of actin fibre filaments, which could be completely reversed by addition of LPA which is another very potent stimulus for CTGF gene expression. They reported that this disassembly led to changes in cell shaped, characterised by the rounding of the cell bodies and development

of elongated processes. In the present study, addition of Fluvastatin resulted in a rounding of cell bodies. This observation is in line with the observations -reported by Heusinger-Ribeiro and co-workers. However Heusinger-Ribeiro and co-workers indicated that the inhibition of CTGF by elevating cAMP or by use of Rho kinase inhibitors led to a disassembly of the actin fibres, which was easily reversed by addition of LPA (and subsequent induction CTGF). The observation in the present study would dispute this result. In the present study the lack of positive immunoreactivity for α-SMA due to inhibition of CTGF by fluvastatin, has been attributed to the direct inhibition of α-SMA gene expression (figure 5.13). This suggests that CTGF induction by TGFβ is a prerequisite for α-SMA gene and protein expression in myofibroblasts. The activation of fibroblasts to myofibroblasts by TGFβ is a key phenotypic transformation in many fibrotic disorders, such as left ventricular hypertrophy, scleroderma, Crohn's disease and liver fibrosis is well established. In some cases, fibrosis occurs as a consequence of tissue injury whereas in others such as primary pulmonary hypertension, abnormal fibroblast proliferation has a genetic basis. In asthma, the present study provides the first evidence of abnormal fibroblast function linked to abnormal growth factor production. In light of the number of diverse diseases in which myofibroblast mediated fibrosis is an essential component, these observations maybe of interest in fibrosis in general. Furthermore, the observation that statins block myofibroblast differentiation may offer a novel therapeutic approach, which could greatly reduce morbidity and mortality caused by fibrosis.

In section 4.4.3, it was observed that basal levels of CTGF were higher in the asthmatic fibroblasts when compared to non-asthmatics, however, there was no significant induction of α-SMA expression. However, the responsiveness of the fibroblasts to exogenous TGFβ2 stimulation was more rapid, suggesting a 'priming' effect of the basal CTGF gene expression.

As seen in figure 5.11, the number of cells in the fluvastatin treated cultures was markedly reduced due to the rounding up and detachment of the cells. Although from the results obtained, it is not possible to infer whether detachment of the cells is due to cell death or a reduction in adhesion molecules, the ability to obtain intact RNA from these cells would suggest that the cells were not dying.

5.7.3 Transforming growth factor β1

As described in section 4.4.1, both non-asthmatic and asthmatic fibroblasts expressed TGFβ1 mRNA at similar levels, although the protein levels found in the supernatants were higher in the non-asthmatic fibroblasts. This led to the possible suggestion that the reason for the lower levels in the asthmatic fibroblasts was due to autocrine utilization. In this part of the study, the potential role for this autocrine TGFβ1 was investigated using specific anti-TGFβ1 antibodies.

The observed reduction in cell number at 5 days on addition of anti-TGFβ1 neutralising antibody (but not anti-TGFβ2 neutralising antibody), is consistent with the hypothesis that autocrine TGFβ1 production by fibroblasts, is responsible for their autocrine proliferation.

Generally, the effects of TGFβ1 and TGFβ2 are identical, however the specific role of TGFβ1 and not TGFβ2 (as illustrated by the lack of an inhibitory effect by anti-TGFβ2 neutralising antibody in the present study is due to the specific production of TGFβ1 by the fibroblasts (Zhang *et al.,* 1999).

As can be seen from figures 5.16 and 5.17, anti-TGFβ1 neutralising antibody specifically inhibits α-SMA expression induced by 0.4nM TGFβ2 and anti-TGFβ2 neutralising antibody specifically inhibits α-SMA induced by 0.4nM TGFβ1. An incidental observation of this was the fact that 0.4nM TGFβ2 induced significantly more α-SMA protein than did an equivalent concentration of TGFβ1. 0.4nM is a much higher dose of TGFβ that would be physiologically found *in vivo*, results in chapter 4 indicate that a maximum concentration of 350pg/10^5 cells was secreted by the fibroblasts. The fact that asthmatic fibroblasts do not transform to myofibroblasts through the action of autocrine TGFβ1 could be explained by the fact that TGFβ1 is not as potent as TGFβ2 and higher concentrations would be required. A dose-response curve for both TGFβ1 and TGFβ2 on the induction of α-SMA gene expression would be required to support this observation.

McAnulty and co-workers (1997) have previously shown that TGFβ1 exerts a biphasic effect on fibroblast proliferation, with stimulation at low concentrations and growth inhibition at higher concentrations. The mechanism by which TGFβ1 stimulates proliferation of asthmatic fibroblasts has been reported by Soma and colleagues (1989)

and is thought to be mediated via induction of autocrine platelet derived growth factor synthesis. The inhibitory effect of TGFβ1 is thought to occur by inhibiting the progression through the late stages of G1 in the cell cycle., via the inhibition of the expression of G1 cyclins and cyclin-dependent kinases (Howe *et al.,* 1991) which in turn prevents the phosphorylation of the retinoblastoma tumour suppressor gene product Rb (Laiho *et al.,* 1990) and suppression of c-myc gene expression (Pientenpol *et al.,* 1990, Zentella *et al.,* 1991,). TGFβ1 is also known to potently stimulate autocrine production of prostaglandin (PG)E_2 (McAnulty *et al.,* 1995) which has been reported to be a potent inhibitor of fibroblast proliferation (Oliver et al., 1989). McAnulty and co-workers have shown that the prostaglandin synthesis inhibitor Indomethacin, is able to block the growth inhibitory properties of TGFβ1 on fibroblasts and restore the stimulatory effects of the low concentrations of TGFβ1. However, these observations are inconsistent with the data obtained in the present study. In the present study, the TGFβ1 gene expression was similar in both the asthmatic and non-asthmatic fibroblasts, both at baseline and in response to TGFβ2, although there was a difference in protein expression, with the TGFβ1 accumulation in the conditioned medium from non-asthmatic fibroblasts. The lower TGFβ1 has until this point has been attributed to increased autocrine utilisation. A possibility to explain this in terms of the PGE_2 hypothesis, would be that the gene transcription is not translated into protein in the case of the asthmatics. The lower TGFβ1 in the asthmatics may would result in a reduced autocrine production of PGE_2 and hence a lack of control on the proliferation of the asthmatic fibroblasts. Preliminary collaborative studies with McAnulty (UCL, London) have shown that there is no difference in the levels of PGE_2 found in conditioned medium from asthmatic and non-asthmatic fibroblasts.

The decrease in TGFβ1 in the asthmatic fibroblast conditioned medium is unlikely to be a result of a lack of gene to protein translation, since inhibition of autocrine TGFβ1 by anti-TGFβ1 neutralising antibody results in a very significant reduction in cell number. Indeed, if a lack of a PGE_2 effect was the mechanism by which the asthmatic fibroblasts reached a higher cell density, addition of anti-TGFβ1 neutralising antibody would result in an increase in cell proliferation (due to the inhibition of TGFβ1 and subsequent inhibition of PGE_2).

The previously reported observation indicating that low concentrations of TGFβ1 drive proliferation of fibroblasts (McAnulty *et al.* 1998) is consistent with the results obtained in the present study, whereby neutralization of active TGFβ1 in the conditioned

medium (concentration <10pg/ml) results in a suppression of proliferation, whereas, addition of a concentration of exogenous TGFβ2 (10ng/ml) did not effect proliferation.

5.8 Summary of results and novel findings

- Although ET-1 has been found to be pro-proliferative in fibroblasts from many diseases, the evidence presented in study suggests ET-1 has no effect of the proliferation of asthmatic bronchial fibroblasts.
- ET-1 induces the myofibroblast phenotype in asthmatic fibroblasts as illustrated by an increase in α-SMA protein expression in response to human recombinant ET-1.
- ET-1 can induce the myofibroblast phenotype via action of the ET_A endothelin receptor.
- ET-1 is unlikely to act significantly as an autocrine growth factor for asthmatic fibroblast. It's likely role is as a paracrine growth factor for smooth muscle cells deeper in the airway wall.
- Fluvastatin specifically reduces CTGF gene expression in a dose-dependent manner.
- The myofibroblast phenotype induced by TGFβ occurs via the induction of CTGF. Fluvastatin is able to inhibit this transformation and may present a novel therapeutic target for the treatment of asthmatic airway remodeling.
- The increased basal levels of CTGF in asthmatic fibroblasts may represent a 'priming' effect, the result of which is a reduction in the strength of TGFβ stimulus required to activate the fibroblasts to myofibroblasts.
- Anti-TGFβ1 neutralising antibody was specifically shown to inhibit TGFβ1 mediated effects whereby anti-TGFβ2 neutralizing antibody inhibited TGFβ2 mediated effects.
- Neutralization of TGFβ1 by anti-TGFβ1 neutralising antibody resulted in very significant decrease in cell number 5 days after *in vitro* culture ($p=0.028$).
- Autocrine TGFβ1 seems a likely candidate for the survival of asthmatic fibroblasts in culture. It is unclear whether or not autocrine TGFβ1 drives hyperproliferation or more fundamentally required for cell survival.

5.9 Conclusions

The ET-1 produced by asthmatic fibroblasts does not seem to effect proliferation of these cells. The lack of positive immunoreactivity in untreated asthmatic fibroblasts where the levels of ET-1 protein is high (see section 4.4.2) indicates that ET-1 does not act in an autocrine fashion to induce the myofibroblast phenotype either. Both these observation indicate that the role of ET-1 in the asthmatic airways is consistent with the null hypothesis. In light of other published studies, the ET-1 derived from fibroblasts in asthmatic airways is likely to act in a paracrine fashion on smooth muscle cells.

The higher levels of CTGF produced by asthmatic fibroblasts, is also unlikely to contribute to the increased proliferative ability of asthmatic fibroblasts in serum free medium. The results obtained in this chapter strongly suggest a role for CTGF in the TGFβ driven induction of α-SMA in fibroblasts and hence inducing the myofibroblast phenotype. The increased levels of CTGF in asthmatic fibroblasts may represent a 'priming' of the fibroblasts to TGFβ stimulation, and hence reduced stimulus strength would be required to induce phenotypic transformation to myofibroblasts.

Transforming growth factor β1 production was reported to be equal in both asthmatic and non-asthmatic fibroblasts in the previous chapter although there was less TGFβ1 present in the supernatant of asthmatic fibroblasts, suggesting increased utilization. The evidence presented in this chapter is inconsistent with the null hypothesis. The demonstration of the ability of anti-TGFβ1 neutralising antibody to result in a decreased asthmatic fibroblast cell number in serum free medium, is highly suggestive of TGFβ1 acting as an autocrine factor, either in driving proliferation or in promoting cell survival.

This has been the first study to demonstrate roles for CTGF and TGFβ1 as autocrine growth factors for asthmatic fibroblasts. In addition, it has been the first study to demonstrate the ability of ET-1 to induce α-SMA protein expression specifically via activation of the ET_A endothelin receptor.

CHAPTER SIX

Epidermal growth factor receptor ligands as autocrine growth factors for asthmatic fibroblasts

6.1 Introduction

EGF receptor ligands are well known for their ability to induce proliferation in variety of cell types. Epidermal growth factor receptor ligands have been shown to be mitogenic for vascular smooth muscle cells (Dluz *et al.*, 1993). On binding to the receptor, EGFR ligands induce autophosphorylation of the receptor and stimulate various biological activities such as cell proliferation (Thompson *et al.*, 1994). Recently it has been shown that heparin binding growth factor (HB-EGF) is involved in cardiac remodelling following myocardial infarction (Tanaka *et al.*, 2002).

Bulus and Barnard (1999) have shown that in intestinal epithelial cells, HB-EGF is a TGFβ regulated gene. In light of these findings, those found by Tanaka and co-workers and the results from the previous chapter which imply TGFβ1 as having a role in the increased proliferation of asthmatic fibroblasts, it was hypothesized that TGFβ1 may be inducing an EGFR ligand which then drives proliferation.

To detect release of EGFR ligands, a mouse embryonic fibroblast cell line, stably transfected with the human EGF receptor (NR6/HER) was used to determine the presence of any EGF-like ligands in the conditioned culture medium. The use of particular EGF ligand inhibitors then aided identification of the EGF-ligands present in the medium. The ability of the NR6/HER to undergo mitogenesis with EGF-ligands was demonstrated by addition of human recombinant EGF and the construction of a standard curve.

Following the identification of any potential EGF-like ligands, which may stimulate mitogenesis in the NR6/HER fibroblasts, specific inhibitors were added to primary asthmatic fibroblasts in serum-free medium, to investigate their possible contribution to the observed hyperproliferation.

6.2 Aims

To investigate if the *in vitro* hyperproliferation of asthmatic fibroblasts in serum free medium is driven by the production of autocrine epidermal growth factor receptor ligands.

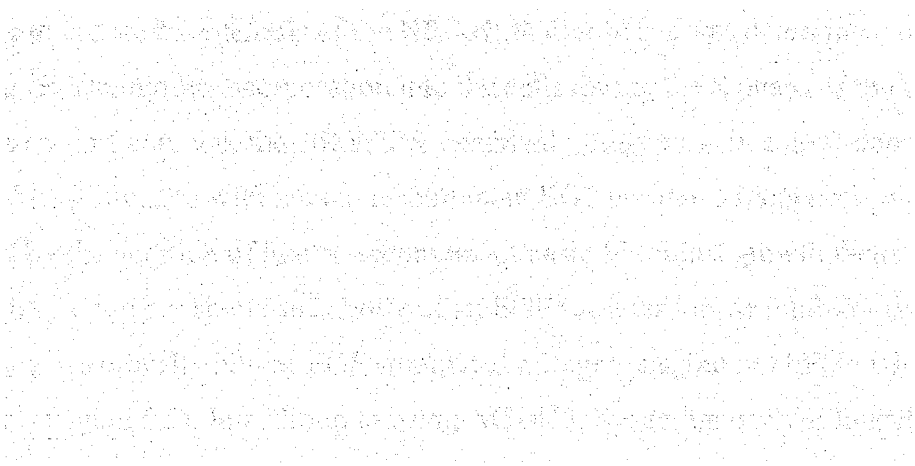
6.3 Hypothesis

i) Asthmatic fibroblasts synthesise and secrete epidermal growth factor receptor ligands in response to TGFβ1 stimulation.

ii) Epidermal growth factor receptor ligands are able to drive the proliferation of asthmatic fibroblasts in an autocrine fashion.

Null Hypothesis

Epidermal growth factor receptor ligands are not secreted by asthmatic fibroblasts, and hence are not responsible for their hyperproliferation in serum free medium.



6.4 Induction of HB-EGF by TGFβ1

Heparin-binding epidermal growth factor gene expression was investigated in 6 asthmatic and 6 non-asthmatic fibroblast lines cultured in serum free medium in the absence and presence of 0.4nM TGFβ2 for 24 hours.

The levels of HB-EGF gene expression were significantly higher in the asthmatic fibroblasts when compared to the non-asthmatic cultures (figure 6.1, $p<0.05$). There were highly significant increases in HB-EGF gene expression following treatment with TGFβ2 in both the non-asthmatic ($p=0.02$) and asthmatic ($p=0.01$) fibroblasts. When considering the fold increases in HB-EGF gene expression (figure 6.2), the trend appeared to indicate that the HB-EGF gene expression increased to a greater degree in the asthmatic fibroblasts, although this did not reach statistical significance. Addition of the TGFβ1 neutralising antibody to asthmatic fibroblasts in serum free medium, resulted in a significant reduction of HB-EGF gene expression ($p=0.05$; figure 6.3), suggesting that the increased HB-EGF gene expression in the asthmatic fibroblasts illustrated in figure 6.1 is due to autocrine produced TGFβ1.

6.5 The mitogenic activity of cultured medium from non-asthmatic and asthmatic fibroblasts

The ability of conditioned medium (CM) from asthmatic and non-asthmatic fibroblasts to induce mitogenesis of the NR6/HER fibroblasts was determined by measuring ^{3}H Thymidine incorporation into the cells during the S phase of the cell cycle.

As see in figure 6.4, the NR6/HER exhibited mitogenesis in a dose-dependent manner when stimulated with human recombinant EGF protein. Mitogenesis was also stimulated by the addition of human recombinant basic fibroblast growth factor (bFGF; figure 6.1b). Figure 6.5 shows the ability of an EGF receptor kinase inhibitor tyrophostin (AG1478) to specifically inhibit EGF stimulated mitogenesis, but not bFGF stimulated mitogenesis (figure 6.5). In addition to using AG1478, the action of EGF ligands can also be inhibited by using the anti-EGFR antibody . As can be seen in figure 6.6a, the antibody specifically inhibited the mitogenesis induced by EGF, but did not significantly affect bFGF induced mitogenesis (figure 6.6b).

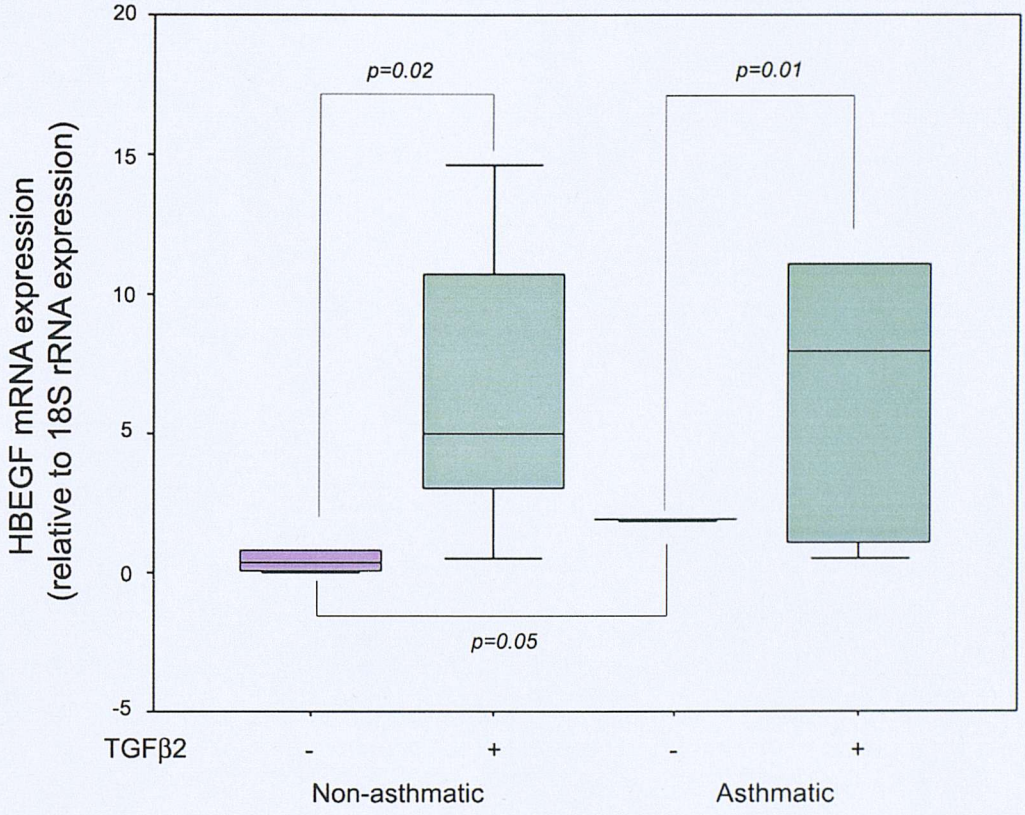


Figure 6.1 : HBEGF gene expression in non-asthmatic and asthmatic fibroblasts after 24 hours, cultured in serum-free medium (purple) and following stimulation with 0.4nM TGFβ2 (green). Gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA.

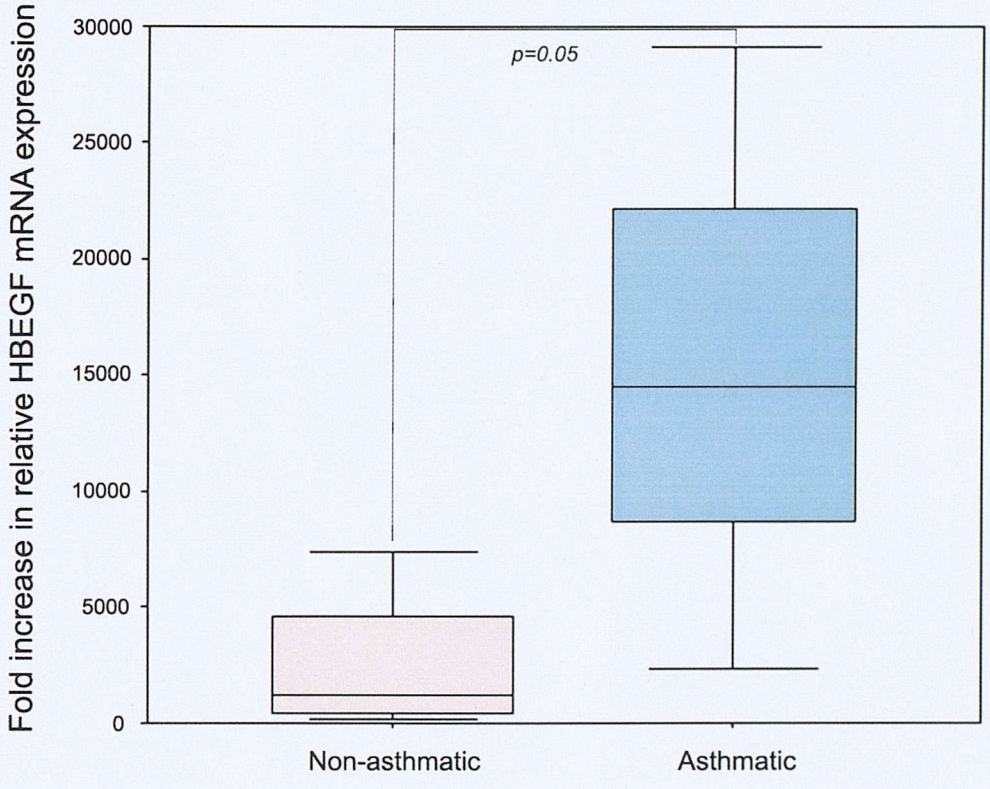


Figure 6.2 : The induction of HBEGF mRNA expression relative to 18S rRNA. The graph represents the fold induction by the non-asthmatic and asthmatic fibroblasts.

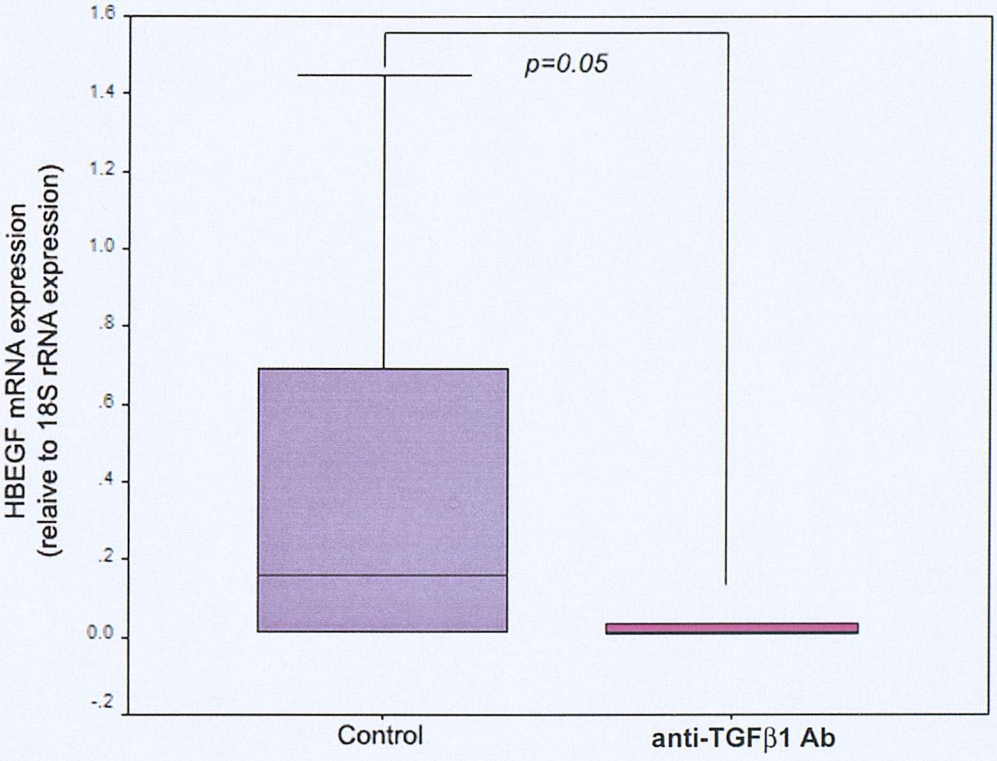


Figure 6.3: HB-EGF gene expression in asthmatic fibroblsts (n=6) folllowing culture for 24 hours in serum free medium alone (purple) or incombination with the TGFβ1 neutralising antibody CAT-192. Gene expression was measured with using real time quantitative RT-PCR and normalised relative to 18S rRNA.

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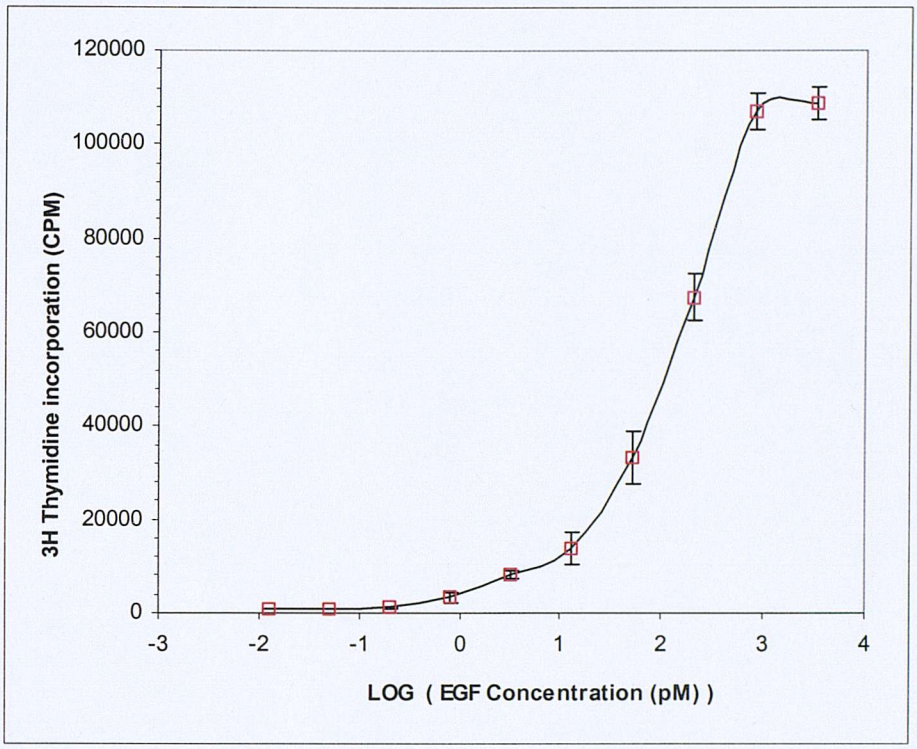

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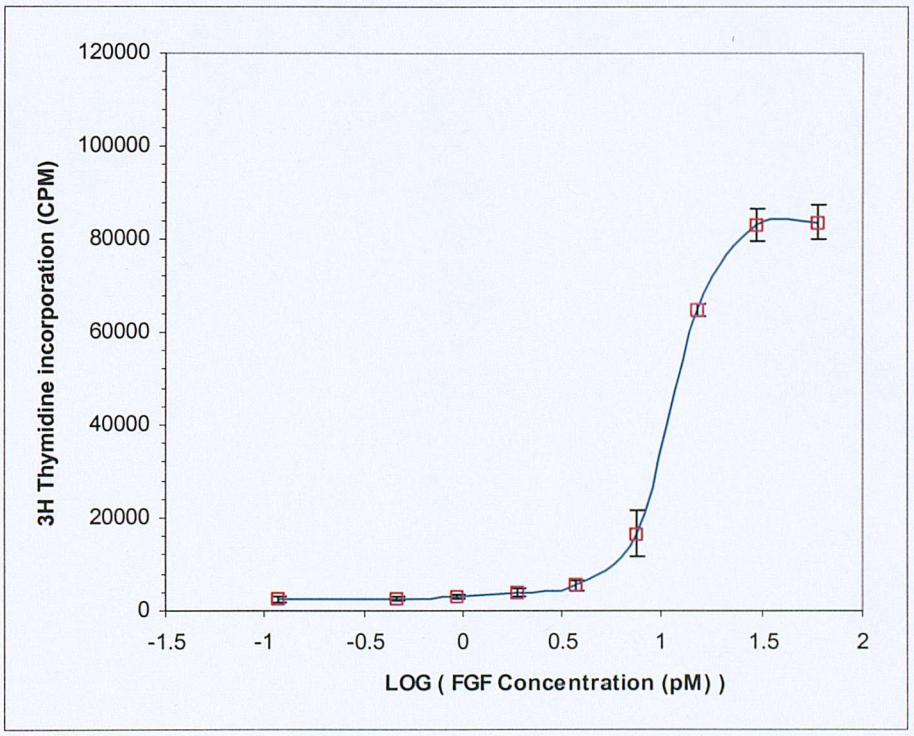
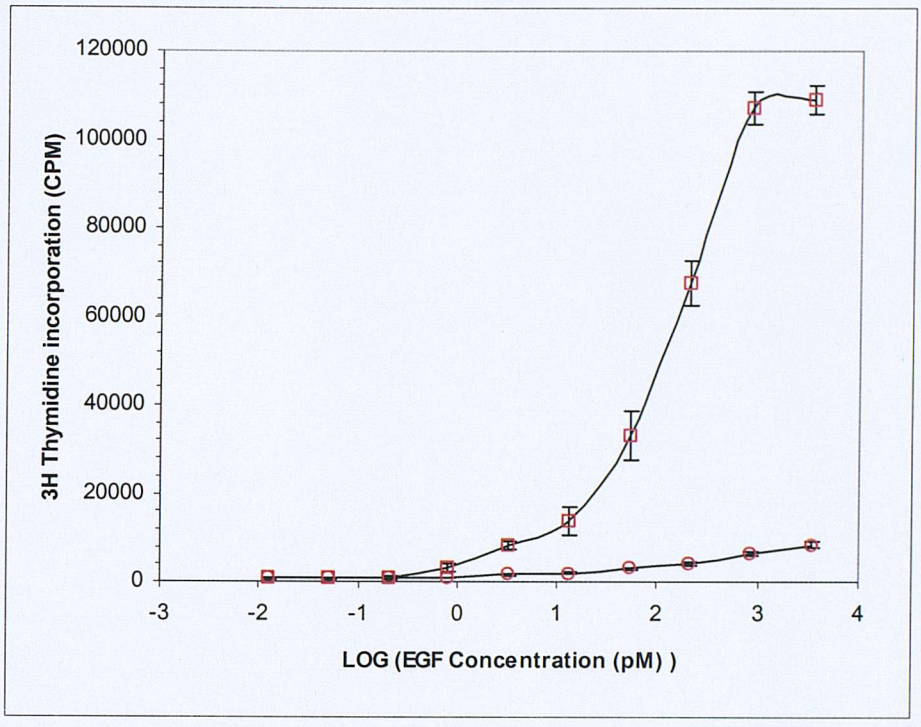


Figure 6.4: Concentration-response curves for the ^{3}H Thymidine incorporation by quiescent NR6/HER fibroblast cells. The cells were pulsed with ^{3}H Thymidine for 2hours between 26 and 28 hours. The fibroblasts were treated for 28 hours with (a) human recombinant EGF and (b) human recombinant bFGF.

A



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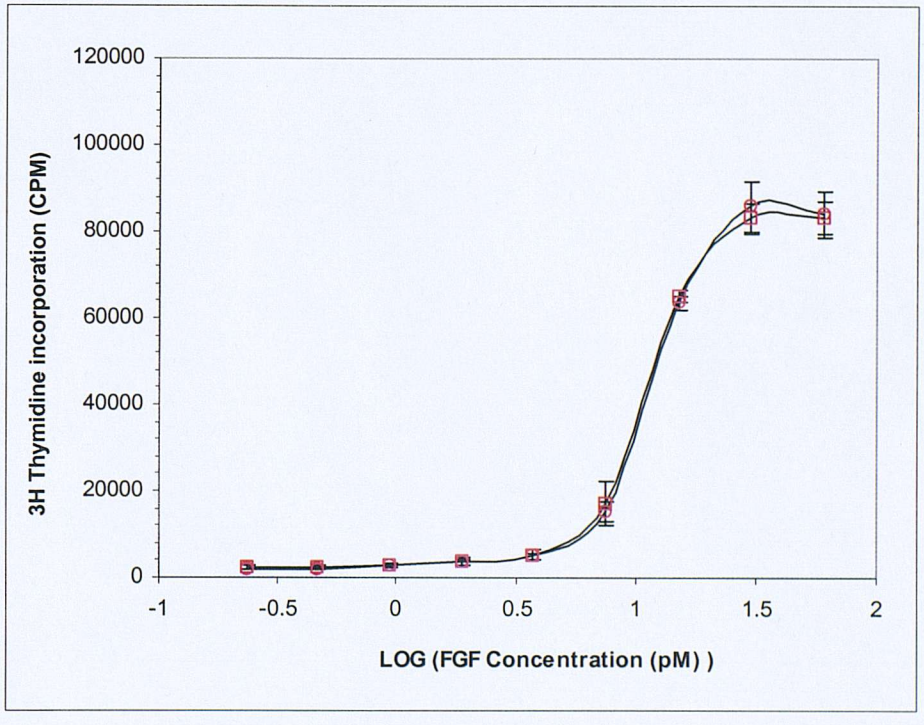
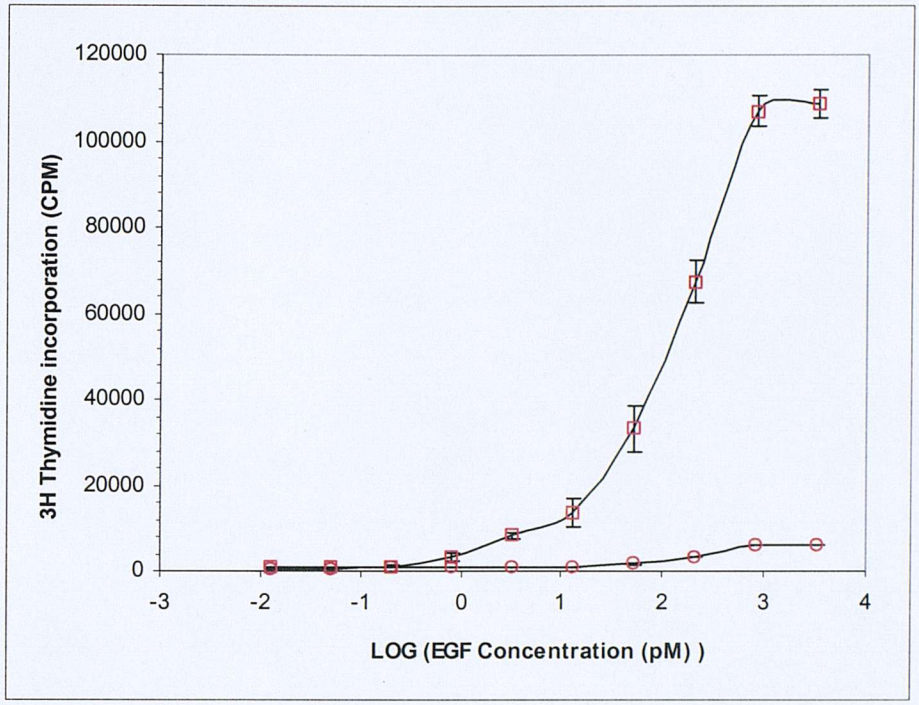


Figure 6.5: Concentration-response curves for the ^{3}[H] Thymidine incorporation by quiescent NR6/HER fibroblast cells. The cells were pulsed with ^{3}[H] Thymidine for 2 hours between 26 and 28 hours of culture. The fibroblasts were treated for 28 hours with (a) human recombinant EGF and (b) human recombinant bFGF alone,(□) or in combination with 1μM AG1478, (○).

A



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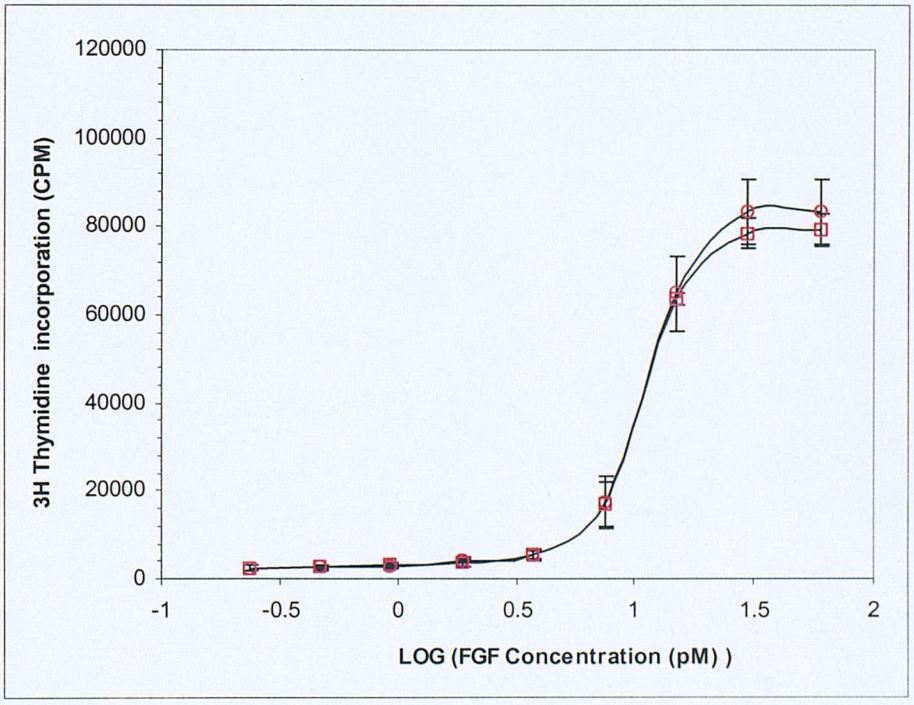
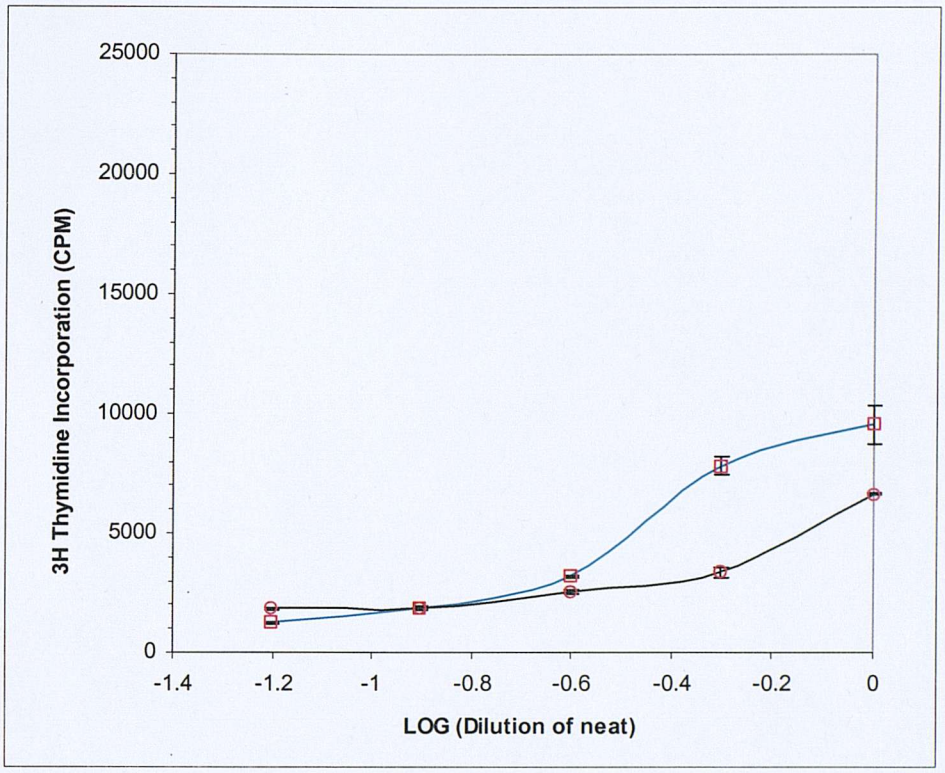


Figure 6.6: Concentration-response curves for the ^{3}[H] Thymidine incorporation by quiescent NR6/HER fibroblast cells. The cells were pulsed with ^{3}[H] Thymidine for 2 hours between 26 and 28 hours of culture. The fibroblasts were treated for 28 hours with (a) human recombinant EGF and (b) human recombinant bFGF alone, (□) or in combination with 100μM anti-EGFR antibody, (○).

Figure 6.7 shows the typical (median) concentration-response curve in response to (a) non-asthmatic and (b) asthmatic fibroblast conditioned medium on the mitogenesis of NR6/HER fibroblasts. The mitogenic responses of the NR6/HER fibroblasts in response to CM from the non-asthmatic and asthmatic fibroblasts were very similar in the untreated cultures, however, when considering the mitogenesis induced when the medium was used neat, there was higher stimulation by the asthmatic CM (figure 6.7). The mitogenic activity of the asthmatic CM was also significantly increased following TGFβ2 treatment of the cultures ($p=0.04$). Following addition of fixed concentration of TGFβ2 (0.4nM) to an EGF concentration-response curve resulted in an early plateau of the dose-response curve, suggesting that the TGFβ2 may itself be exerting a suppressive effect (figure 6.8). Addition of TGFβ2 to the cultures, the mitogenesis stimulated by the asthmatic CM was much greater than the non-asthmatic CM. It was noted that the shapes of the standard curves were very different on comparing the mitogenesis stimulated by untreated cultured medium and that treated with TGFβ2. The unstimulated medium yields the beginning of a sigmoidal curve, however, the stimulated medium, although stimulating mitogenesis to a much higher degree tends to plateau much earlier than that seen with a pure growth factor (e.g. figure 6.4).

Figure 6.9 shows the direct comparison of mitogenesis stimulated by asthmatic and non-asthmatic CM on NR6/HER fibroblasts at a defined dilution (log dilution –0.6). When considering all the fibroblast cultures used, there is no significant difference in the mitogenic activity between the asthmatic and non-asthmatic in unstimulated cultured medium. Addition of Anti-EGFR antibody significantly reduced the mitogenic activity of the asthmatic CM ($p=0.028$) as did the addition of AG1478 ($p=0.028$). The reduction in mitogenic activity of non-asthmatic fibroblast CM on addition of Anti-EGFR antibody or AG1478 failed to reach statistical significance. On stimulation of the primary cultures with TGFβ2, the mitogenic activity achieved by the asthmatic fibroblast CM was much greater than that achieved by the non-asthmatic CM ($p=0.019$). Addition of Anti-EGFR antibody or AG1478 resulted in reductions in mitogenic activity on addition of both the non-asthmatic and asthmatic fibroblast CM ($p=0.05$). Although there were significant reductions in the mitogenic activity of the asthmatic fibroblast CM on addition of Anti-EGFR antibody or AG1478, the activity of the asthmatic fibroblast CM remained significantly higher than that of non-asthmatic fibroblast CM treated with Anti-EGFR antibody ($p=0.033$) or AG1478 ($p=0.038$).

A



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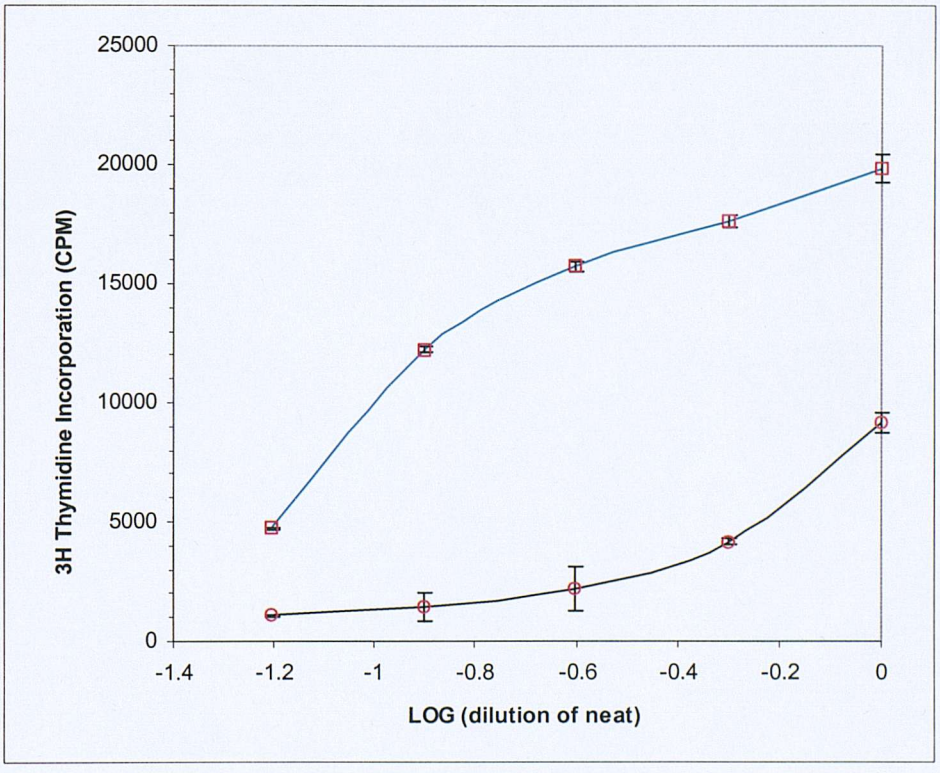


Figure 6.7 : Concentration-response curves for the ^{3}H Thymidine incorporation by quiescent NR6/HER fibroblast cells. The fibroblasts were treated with (a) conditioned medium from untreated non-asthmatic fibroblasts (black) and non-asthmatic fibroblasts treated with 0.4nM TGFβ2 (blue), and (b) conditioned medium from untreated asthmatic fibroblasts (black) and asthmatic fibroblasts treated with 0.4nM TGFβ2 (blue) for 26 hours and then pulsed with ^{3}H Thymidine for 2 hours. Graphs represent the curves for the median non-asthmatic (n=6) and asthmatic (n=6) fibroblast lines. Error bars represent the standard error of the mean for 4 replicates of the same medium.

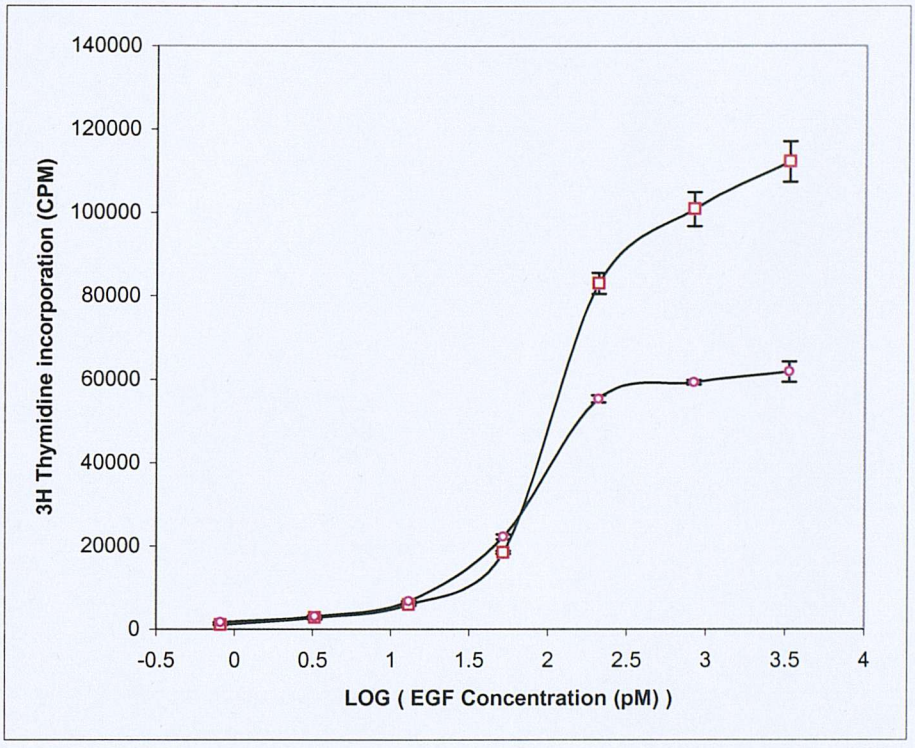


Figure 6.8: Concentration-response curves for the ^{3}H Thymidine incorporation by quiescent NR6/HER fibroblast cells. The cells were pulsed with ^{3}H Thymidine for 2 hours between 26 and 28 hours. The cells were treated for 28 hours with human recombinant EGF alone (□) or in combination with 0.4nM TGFβ2 (○). (Data is the mean of 4 replicate experiments and error bars represent the standard error of the mean)

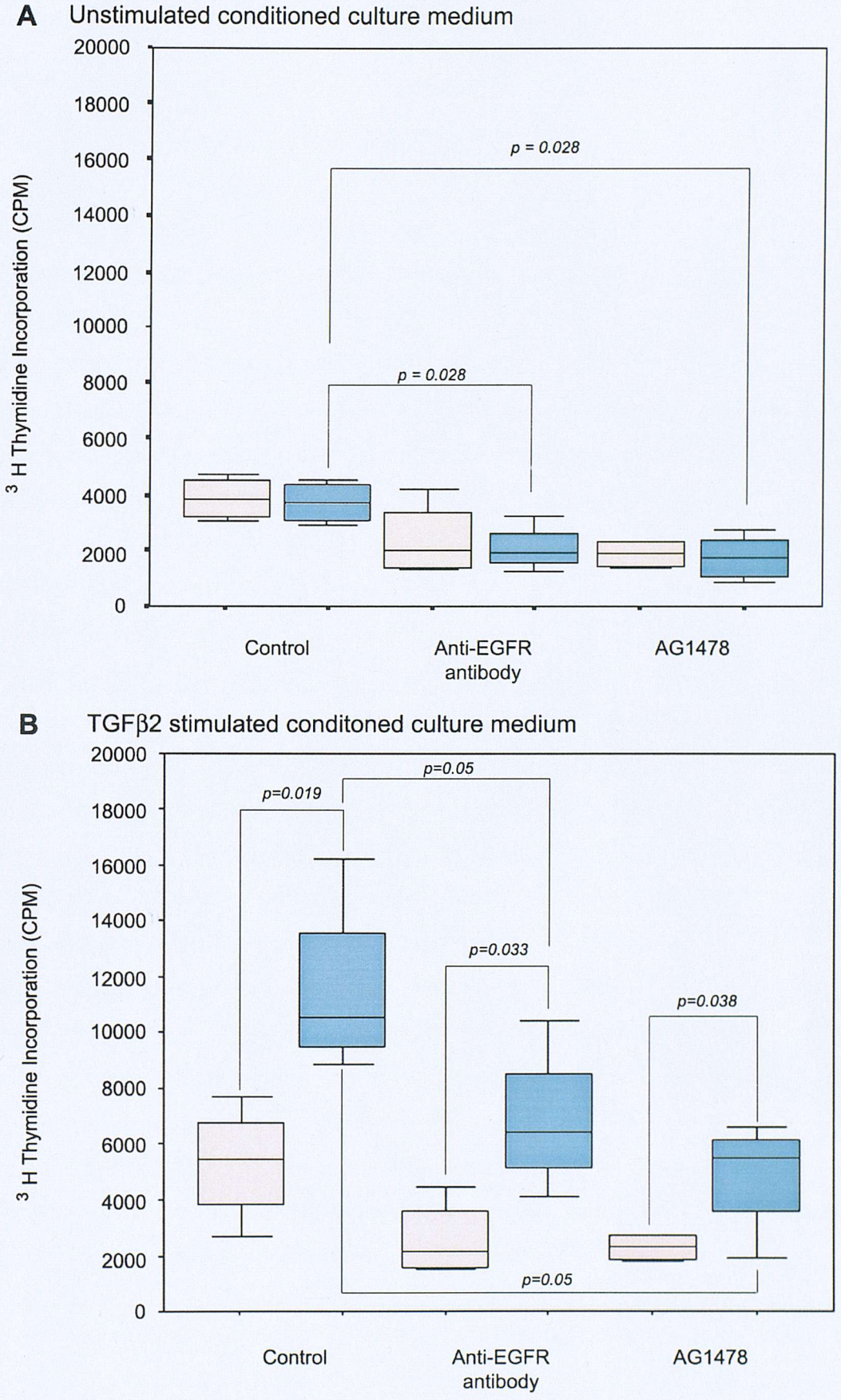


Figure 6.9: ^{3}[H] Thymidine incorporation by NR6/HER fibroblasts. Cells were stimulated for 28 hours with (a) conditioned medium from 6 untreated non-asthmatic (cream) and 6 untreated asthmatic (blue) fibroblasts cultured for 72 hours and (b) conditioned medium from 6 non-asthmatic (cream) and 6 asthmatic (blue) fibroblasts treated with 0.4nM TGFβ2 cultured for 72 hours. Treatments were added alone (control) or in combination with the specified stimulus. Statistical significance between non-asthmatics and asthmatics were determined by application of the Mann-Whitney U test. Each assay was conducted in triplicate therefore n=18 for each subject group.

6.6 Addition of EGF, AG1478, Anti-EGFR antibody and CRM-197 to primary asthmatic fibroblasts

In order to determine if EGFR ligands were responsible for the autocrine proliferation of the asthmatic fibroblasts in serum free medium, Anti-EGFR antibody and AG1478 were added to the cultures and cell number was assessed by methylene blue dye uptake. It became clear that addition of these agents to the primary fibroblasts resulted in distinct morphological change, with cells adapting hypo- and hypertrophic morphology. The assessment of cell number by methylene blue uptake in these cases would yield incorrect results (a cell that has become hypertrophic would absorb more methylene blue dye than a non-hypertrophic cell). In order to overcome this discrepancy, the optical density readings obtained from measuring the amount of methylene blue absorbed were not converted to cell number and the readings were considered as readings of total cell mass. Figure 6.10 shows phase contrast micrographs of the fibroblasts at the time points at which total cell mass was assessed. At the beginning of the experiment, the cell density in each of the cultures is identical (figure 6.10 a,d,g,j). On addition of AG1478 for 24 hours (figure 6.10h), there are a number of unattached cells floating in the medium, indicating apoptosis or necrosis. The number of cells attached is much lower than those in the control and EGF stimulated cultures. The addition of Anti-EGFR antibody to the fibroblasts (figure 6.10k), resulted in a very distinct morphological appearance when compared to the other cultures. The morphology was very 'spindle-like' with some cells beginning to round up and beginning to detach. The morphology of the cells remained similar till 120 hours (figure 6.10l), although the cell density was much lower than in the control (figure 6.10c) and EGF stimulated (figure 6.10f) cultures.

Figure 6.11a shows that the total cell mass significantly increased in both the control cultures and in the cultures treated with human recombinant EGF ($p=0.04$). There was no significant difference between the control and EGF treated fibroblasts at 0 and 24 hours. However at 120 hours, there were a significantly greater total cell mass in the EGF treated cultures ($p=0.04$; figure 6.11b). This confirmed the visual observation as shown in figure 6.10 and indicated that EGF was mitogenic for asthmatic fibroblasts.

On addition of the EGFR antibody, there was no significant change in total cell mass when compared to the control cultures at 24 hours, however at 120 hours, there was

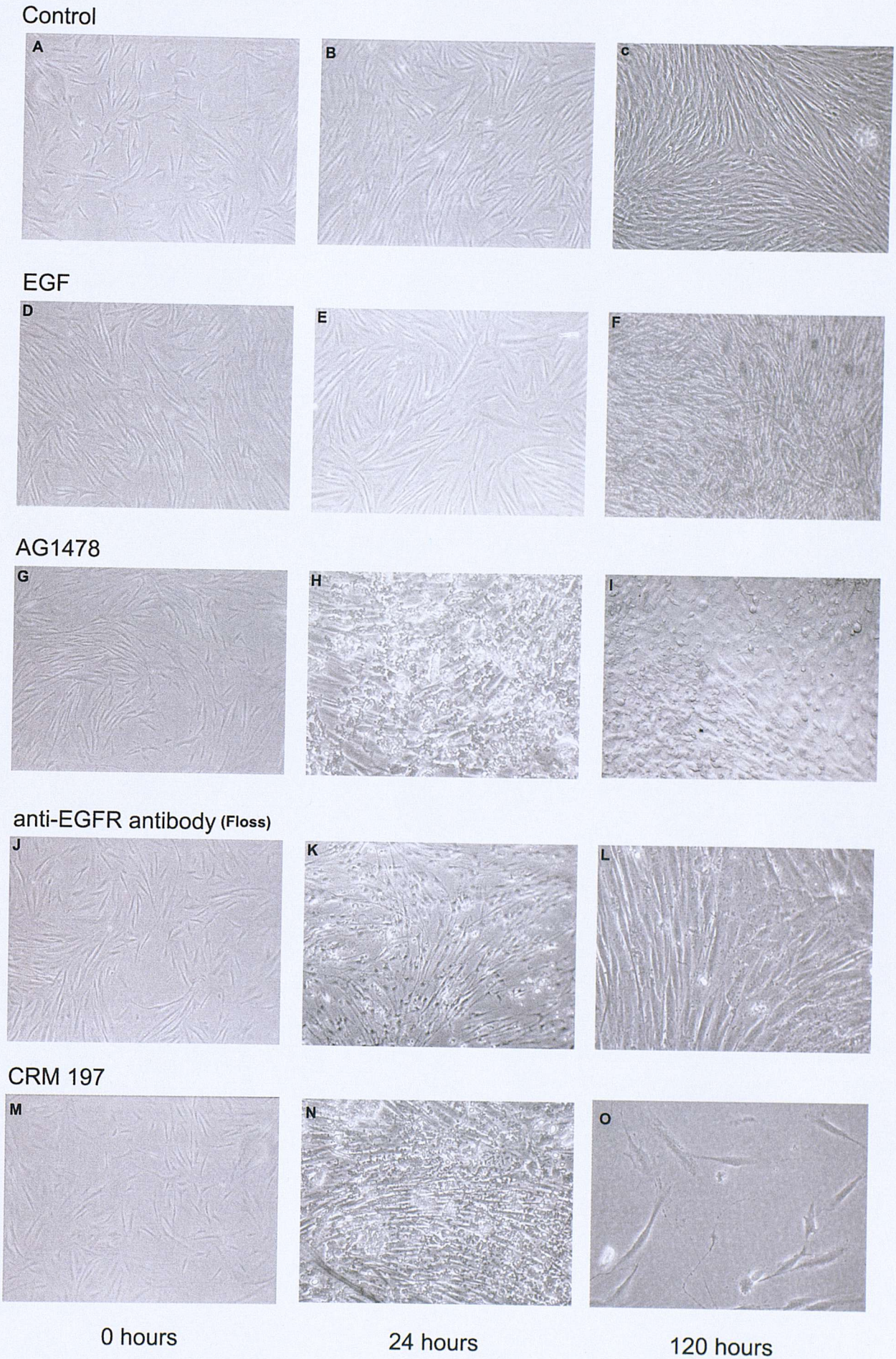
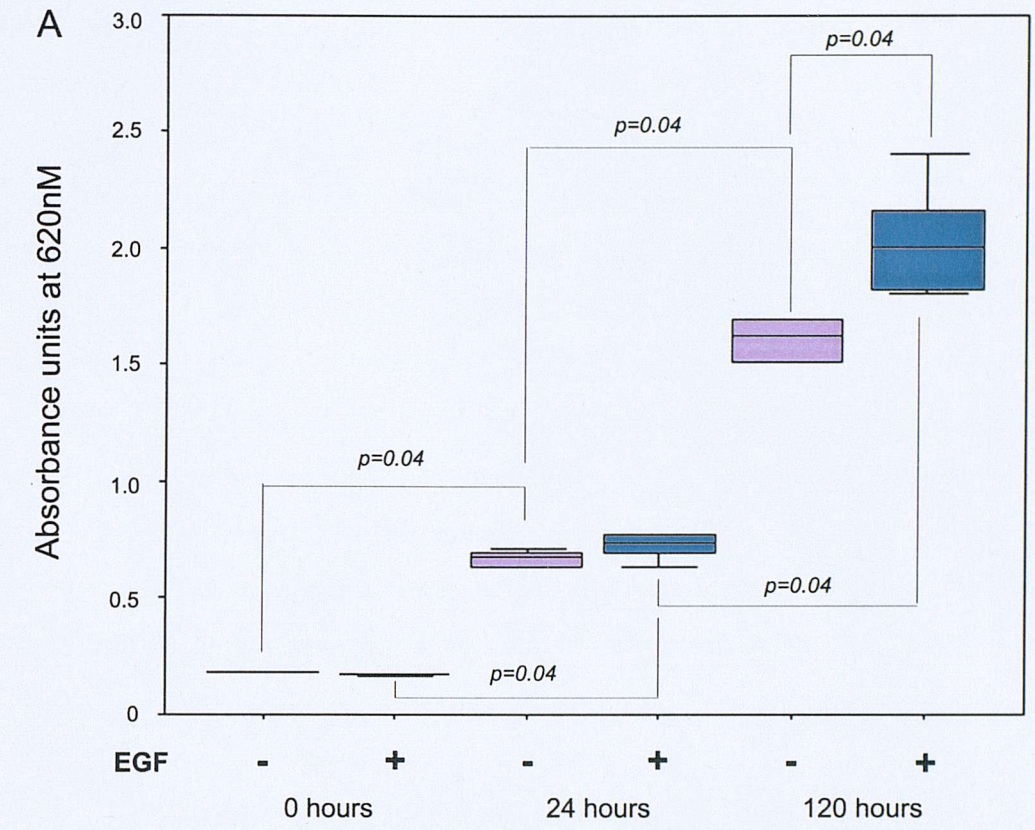


Figure6.10:Phase contrast micrographs of a typical asthmatic fibroblast line in serum free medium (A,B,C) and following treatment with human recombinant EGF (D,E,F), 1μM AG1478 (G,H,I), 100μM anti-EGFR antibody (J,K,L) or 100μM CRM197 (M,N,O) at the start of the treatment (0 hours) and at 24 hours and 120 hours following stimulation. Magnification x120.



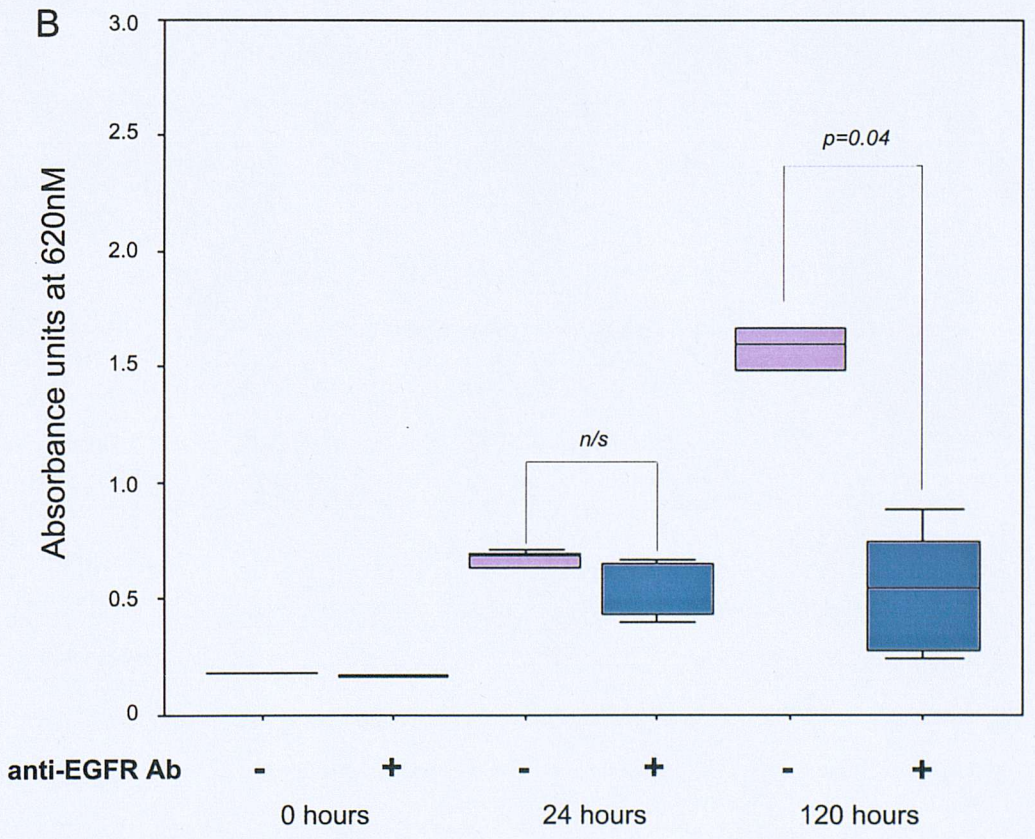


Figure6.11:Total fibroblast cell mass on addition of (a) 2nM EGF and (b) 100μM anti EGFR antibody as determined by methylene blue uptake. Fibroblasts were seeded at a starting density of 5x10 cells/well in 24 well dishes, and were allocated to specific timepoints. At each timepoint, the corresponding cultures were fixed with formal saline. Methylene blue was added to each culture then eluted with ethanol/HCl and the absorbance at 620nM was measured. Error bars represent the interquartile range (n=5)

a significant reduction in cell mass following Anti-EGFR antibody treatment (p=0.04). The addition of AG1478 also resulted in a significant reduction (p=0.05) in total cell mass at 120 hours (figure 6.12a).

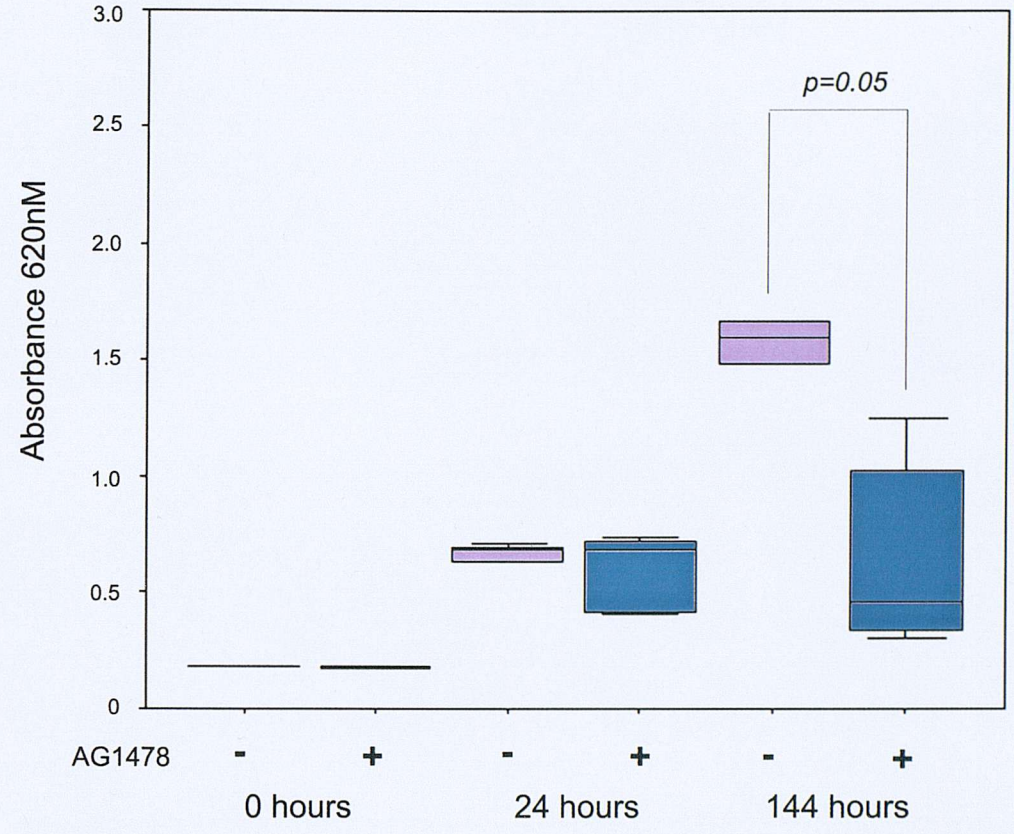
As discussed earlier, HB-EGF has been shown to result in the proliferation of cardiac myofibroblasts and is involved in cardiac hypertrophic remodeling. In order to determine if whether the EGFR ligand allowing an accumulation of asthmatic fibroblasts in culture in serum free medium was HB-EGF, an analogue of naturally occurring diptheria toxin, CRM-197, was used. CRM-197, binds HB-EGF, and hence acts to neutralize it. Addition of CRM-197, resulted in a significant reduction in cell number when compared to control cultures at 24 hours (p=0.04) and at 120 hours (p=0.05; figure 6.12). Since CRM-197 is highly selective, the reduction in total cell mass following treatment, was similar to levels seen when the cells were treated with AG1478 or Anti-EGFR antibody. This suggests that an EGFR ligand that the cells were producing was HB-EGF.

6.7 Expression and possible processing of HB-EGF

HB-EGF, like many other EGFR ligands is produced as inactive pro-form, which is normally bound to the surface of the cell membrane. In order to produce a mature, secreted form of the growth factor, a metalloproteinase is required for cleavage from the pro-form.

In order to accumulate evidence for the generation of an EGFR ligand (which was likely to be HB-EGF) which required metalloproteinase action to activate, the broad spectrum MMP inhibitor GM6001 was added to the primary asthmatic fibroblast cultures to investigate the morphology and cell number at 0, 24 and 120 hours (figure 6.13).

As can be seen from the phase contrast micrographs seen in figure 6.13, the GM6001 treated fibroblasts exhibited similar cell density at 0 and 24h. However, at 120 hours, the treated fibroblasts are morphologically different from the control cultures. The cells appeared to be rounded in areas and the cell density was much lower than in the control cultures. This was confirmed by the methylene blue cell mass data, where there were significantly decreased cell mass in the treated cultures when compared to the untreated cultures (p=0.028; figure 6.13g).



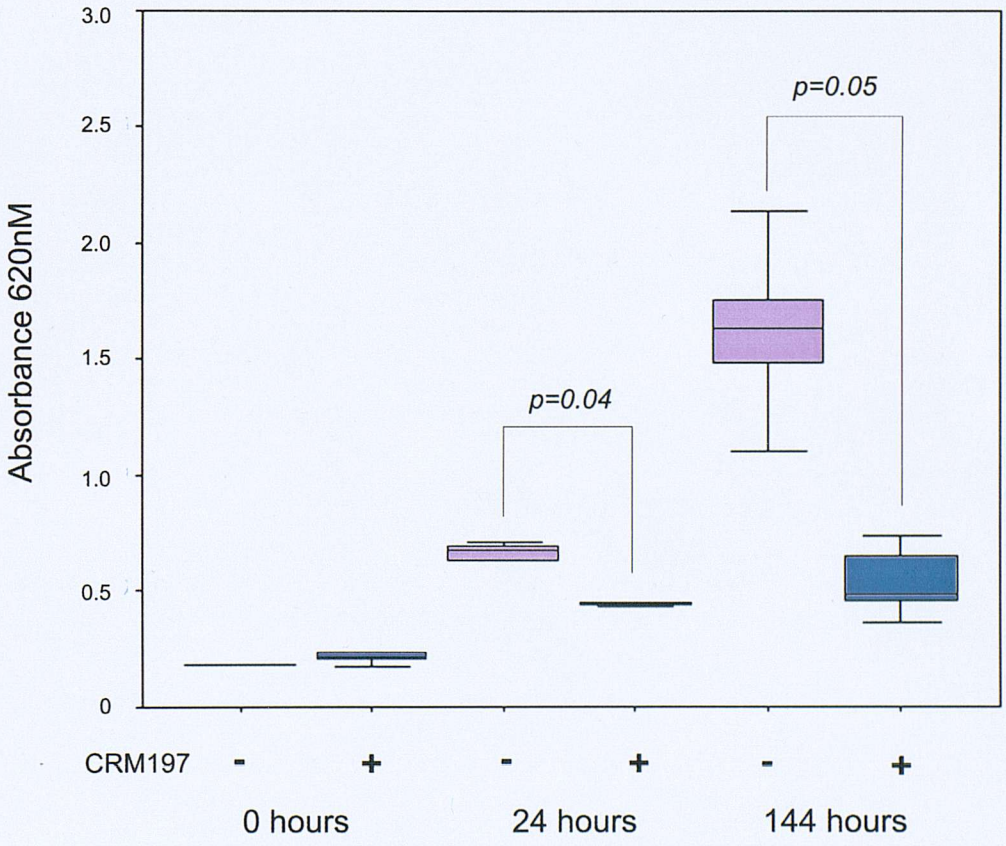


Figure6.12:Total fibroblast cell mass on addition of (a)1μM AG1478 and (b) 100μM CRM197 as determined by methylene blue uptake. Fibroblasts were seeded at a starting density of $5x10^4$ cells/well in 24 well dishes, and were allocated to specific timepoints. At each timepoint, the corresponding cultures were fixed with formal saline. Methylene blue was added to each culture then eluted with ethanol/HCl and the absorbance at 620nM was measured. Error bars represent the interquartile range (n=5)

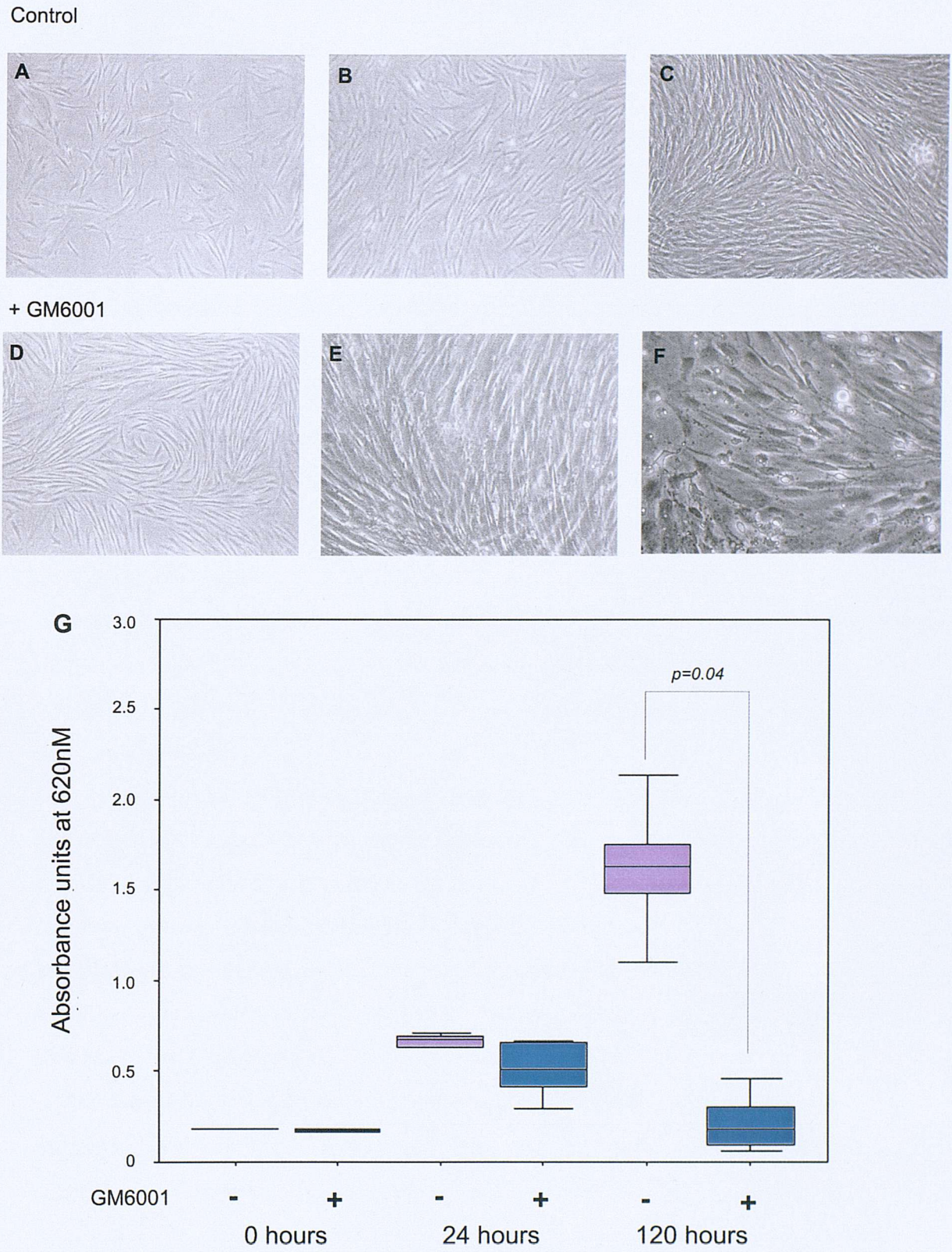


Figure 6.13: Phase contrast micrographs of asthmatic fibroblasts cultured in serum free medium (a,b,c) and in the prescence of 1μM GM6001 (d,e,f) at specified timepoints. The total cell mass at each timepoint was then determined by methylene blue absorption. Fibroblasts were seeded at a starting density of 5 x 10 cells/well in 24 well dishes, and allocated to specific timepoints. Methylene blue was added to the fixed cells and then eluted with ethanol/HCL. The absorbances were determined at 620nM and the results are shown in (g).

As discussed above, the broad spectrum MMP inhibitor GM6001, resulted in a much lower total cell mass than when compared with that measured in the asthmatic fibroblasts. A disintegrin and a metalloproteinase (ADAM)-12 has been shown to be the metalloproteinase responsible for cleavage of pro-HB-EGF to produce active HB-EGF. Recently, it has been reported that polymorphisms in the closely related molecule ADAM-33, have been highly associated with bronchial hyperresponsiveness (BHR) (Van Eerdewegh *et al.,* 2002).

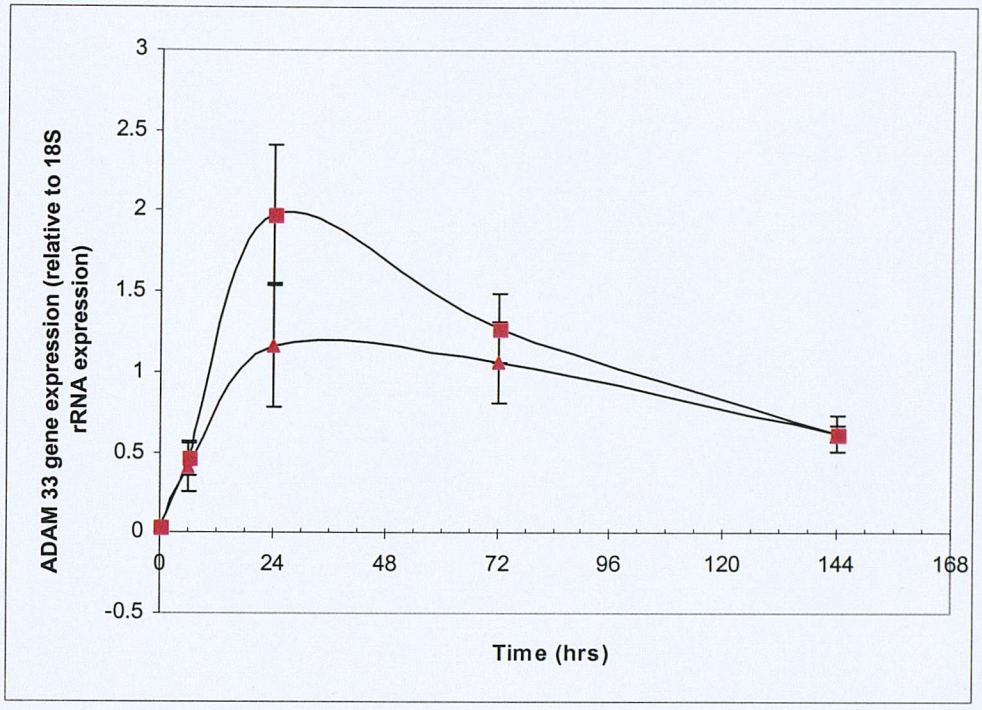
The levels of ADAM-12 and ADAM-33 gene expression in asthmatic and non-asthmatic fibroblast were measured in serum free medium in the absence and presence of 0.4nM TGFβ2.

The levels of ADAM-33 gene expression in both the non-asthmatic (figure 6.14a) and asthmatic (6.14b) fibroblasts peaked at 24 hours and then continued to decline thereafter. The addition of TGFβ2 to these cultures resulted in a reduction of ADAM-33 gene expression. Figure 6.15a shows that ADAM-33 gene expression in non-asthmatic fibroblasts both in the absence and presence of 0.4nM TGFβ2. Similar data for asthmatic fibroblasts is shown in figure 6.15b. The levels of ADAM-33 gene expression was significantly lower in TGFβ stimulated cultures at 6hours ($p=0.05$) 72 hours ($p=0.03$) and 144 hours ($p=0.008$).

The ADAM-12 gene expression over the time is very different to the ADAM-33 gene expression. Figure 6.16 shows the time course for ADAM-12 gene expression. In the case of the non-asthmatic fibroblasts, the levels of ADAM-12 gene expression peaked at 24 hours, however whereas the levels of ADAM-33 gene decreased thereafter, ADAM-12 gene expression remained elevated, and did not significantly decrease. The levels of ADAM-12 gene peaked at 72 hours in the asthmatic fibroblasts and slightly decreased after that point (figure 6.16b).

Figure 6.17a compares the ADAM-12 gene expression in non-asthmatic and asthmatic fibroblasts in the absence of TGFβ2. The levels were significantly higher in the asthmatics at 72hours ($p=0.004$) and at 144 hours ($p=0.002$). Similar results were observed in the TGFβ2 treated cultures, where there was significantly higher ADAM-12 gene expression at 72hours ($p=0.002$) and 144hours ($p=0.002$).

A



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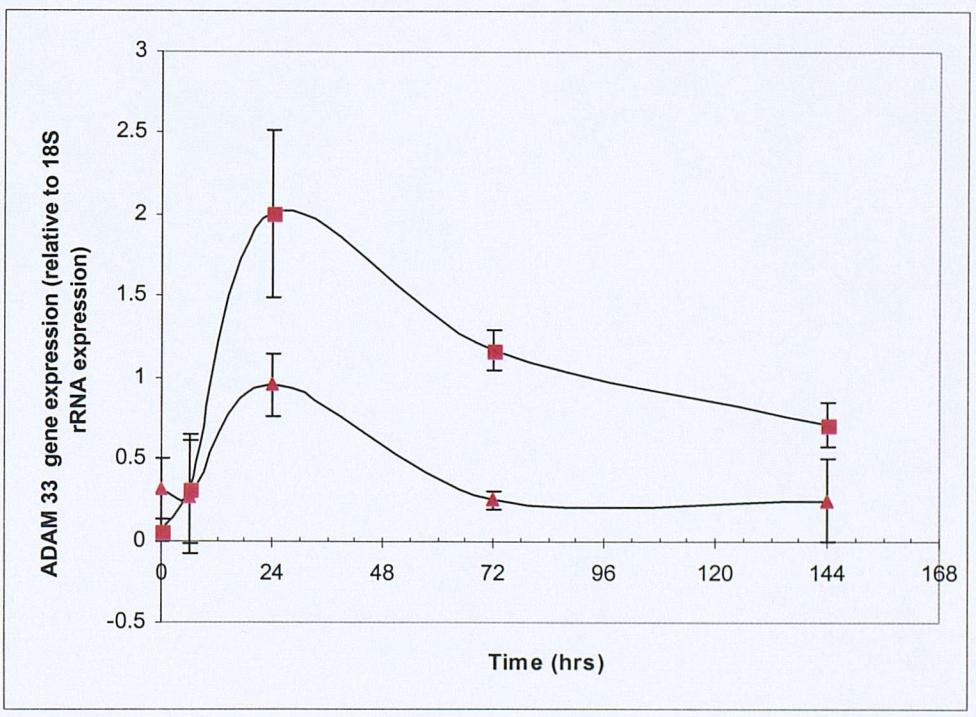

Figure 6.14: Time course for the regulation of ADAM-33 gene expression in (a) a typical non-asthmatic fibroblast line (6 non-asthamtics, each assay in triplicate, n=18) and (b) a typical asthmatic fibroblast line (6 asthamtics, each assay in triplicate, n=18) both at baseline (▲) and in response to 0.4nM TGFβ2 (■). The gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA. Error bars represent the standard error of the mean.

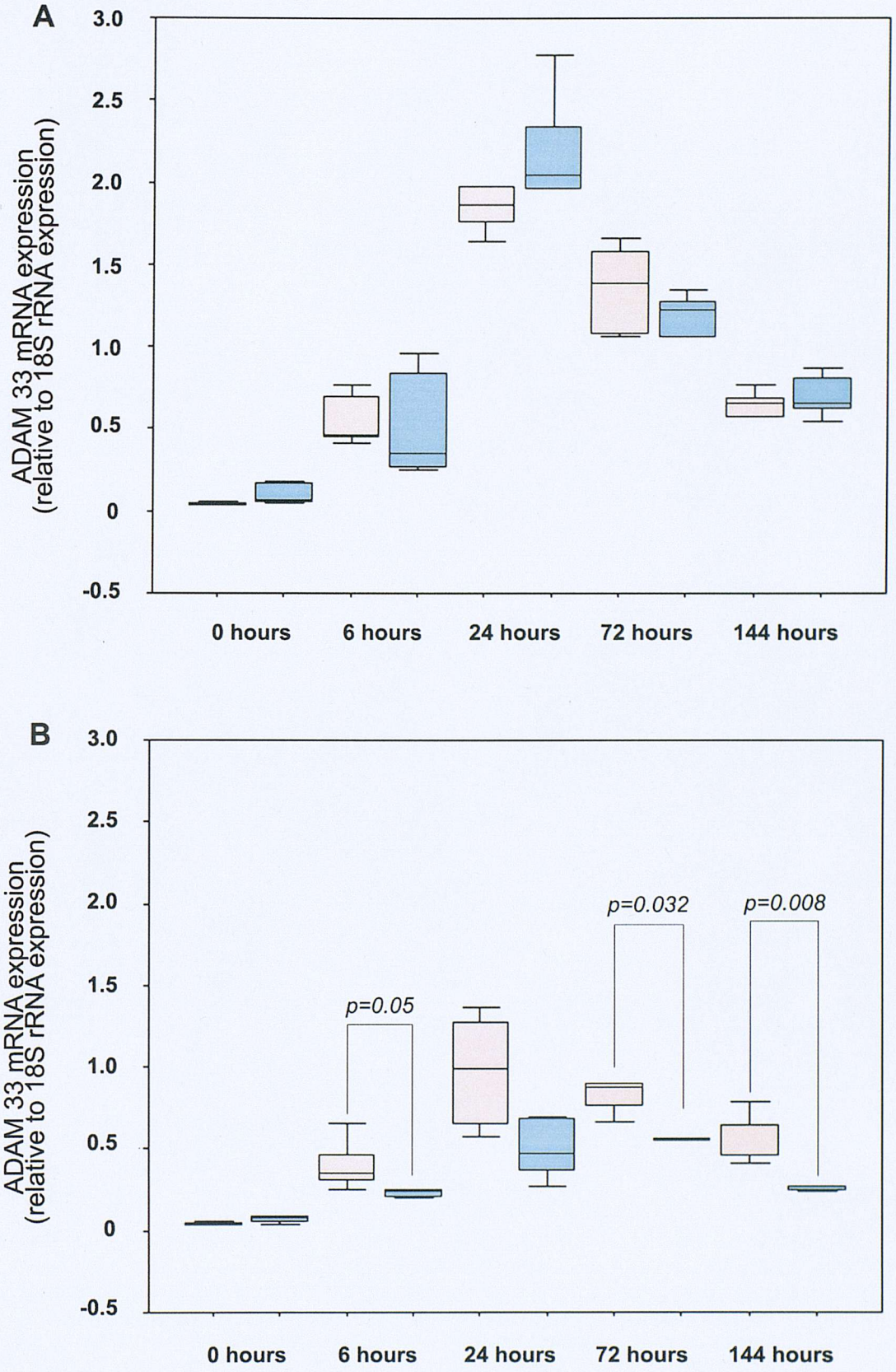

Figure 6.15: The ADAM-33 mRNA expression in 6 non-asthmatic fibroblast lines (cream) and 6 asthmatic fibroblast lines (blue) cultured for up to 144 hours stimulated with (a) serum-free medium and (b) 0.4nM TGFβ2. Gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA.

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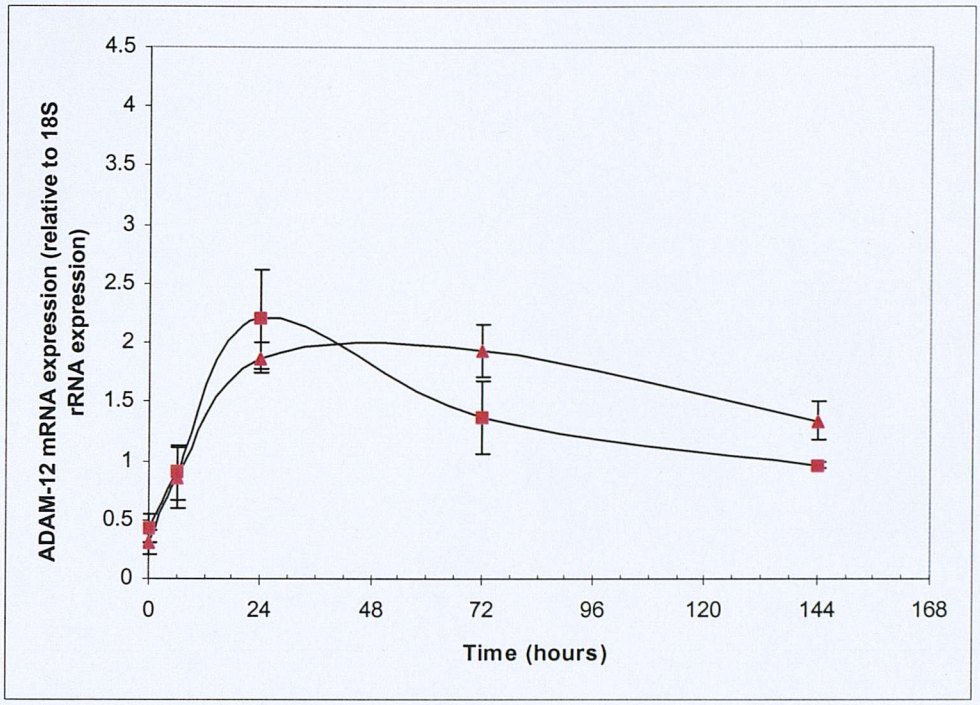

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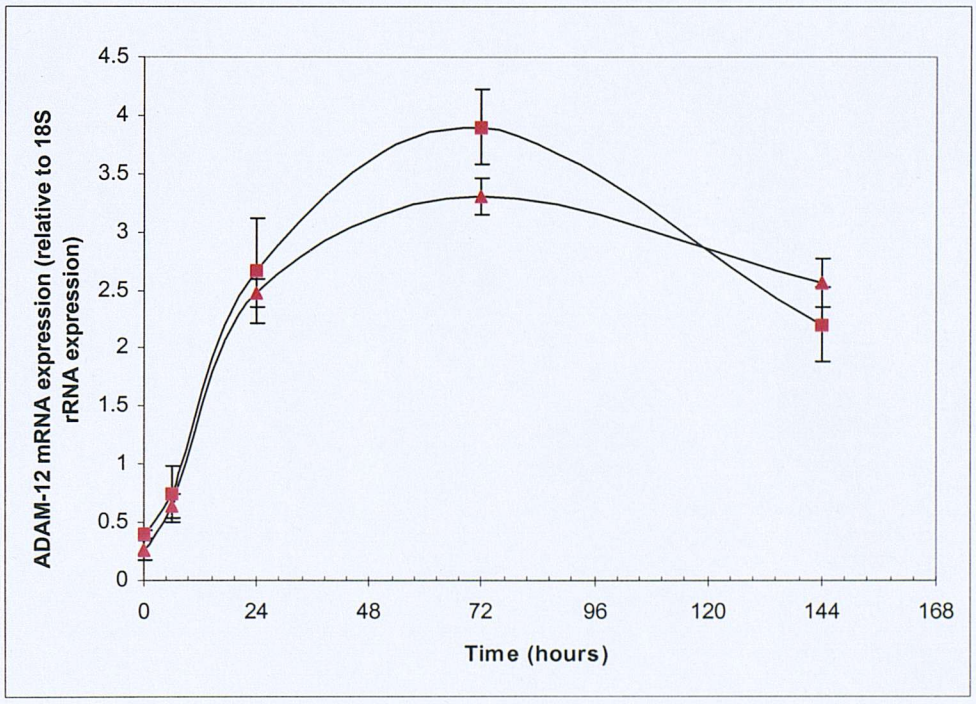


Figure 6.16: Time course for the regulation of ADAM-12 gene expression in (a) a typical non-asthmatic fibroblast line (6 non-asthamtics, each assay in triplicate, n=18) and (b) a typical asthmatic fibroblast line (6 asthamtics, each assay in triplicate, n=18) both at baseline (▲) and in response to 0.4nM TGFβ2 (■). The gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA. Error bars represent the standard error of the mean.

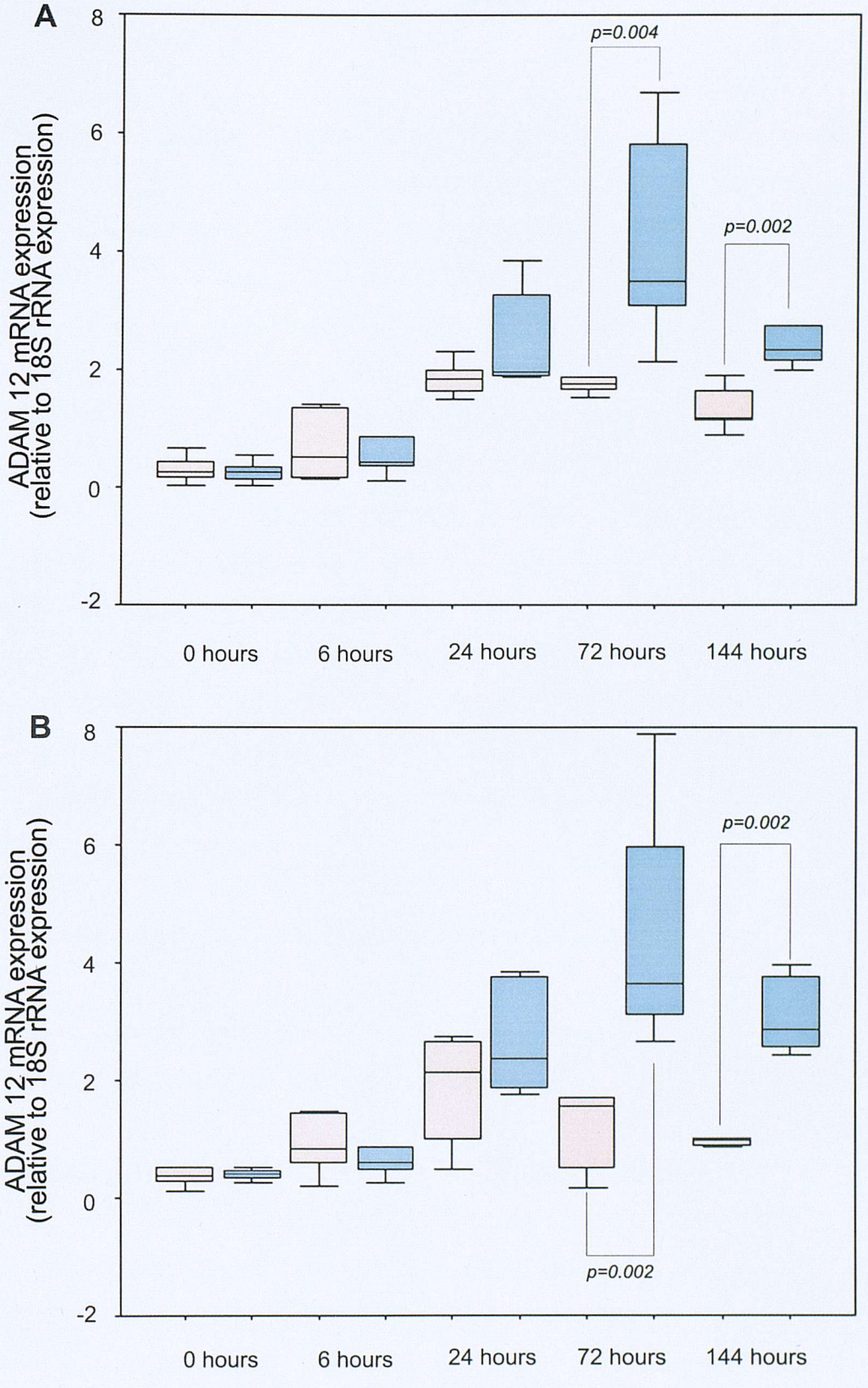


Figure 6.17: The ADAM-12 mRNA expression in 6 non-asthmatic fibroblast lines (cream) and 6 asthmatic fibroblast lines (blue) cultured for up to 144 hours stimulated with (a) serum-free medium and (b) 0.4nM TGFβ2. Gene expression was measured using real time quantitative RT-PCR and normalised relative to 18S rRNA.

6.8 Discussion

The remodelled airways in asthma have a number of pathological features which are thought to be the result of activated fibroblasts. In the previous chapter, the roles of ET-1 and CTGF were considered, ET-1 was not responsible for the hyperproliferation of the asthmatic fibroblasts even though it has been implicated in other diseases. Inhibition of CTGF by fluvastatin resulted in a reduced total cell mass, although from the data is it unclear whether this is due to an inhibition or growth of a reduction in the ability of the cells to survive. Epidermal growth factor ligands have previously been shown to be highly mitogenic for a variety of cell types including fibroblasts. In this part of the study, a role for EGFR activation in the hyperproliferation of fibroblasts was investigated. In order to determine this, the conditioned culture media from non-asthmatic and asthmatic fibroblasts were added to NR6/HER fibroblasts and tested with EGFR inhibitors to determine if EGFR activation mediated mitogenic activity. In other disease models, e.g. ventricular hypertrophic remodelling following myocardial infarction, the EGFR ligand HB-EGF has been shown to result in activation of resident myofibroblasts (Duncan *et al.*, 1997). Recent evidence has shown the involvement of ADAM-12 in activating HB-EGF from its latent pro-form.

6.8.1 Epidermal growth factor ligands are produced by fibroblasts

Epidermal growth factor ligands have the capacity to stimulate a wide variety of effects in a large number of different cell types (Zwick *et al.*, 1999). The tyrphostin AG 1478 was used to inhibit the action of any EGFR ligands. Tyrphostins are compound that inhibit protein tyrosine kinases by binding to the substrate binding site (Gazit *et al.*, 1989; Gazit *et al.*, 1991; Nowak *et al.*, 1997). The Tyrphostin AG1478 is a specific EGF receptor kinase inhibitor (Levitzki and Gazit 1995) and would hence specifically inhibit any ligands present in the CM (as shown in 6.9), which could directly or transactivate the EGF receptor.

In the present study, addition of CM from both the non-asthmatic and asthmatic fibroblasts resulted in a significant increase in mitogenesis of the NR6/HER fibroblasts. Following treatment with TGFβ2, the mitogenic activity of asthmatic fibroblast CM was

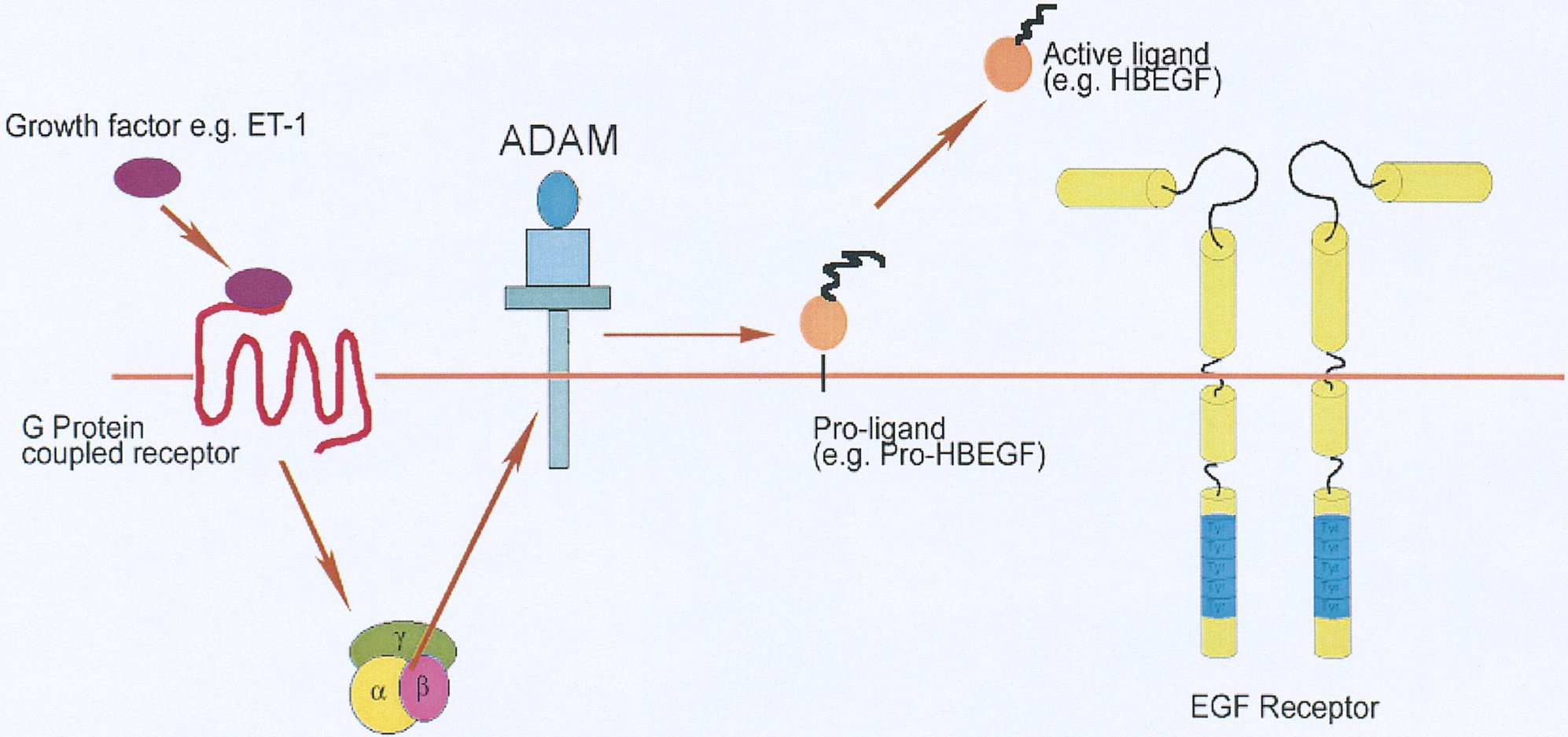


Figure 6.18 The transactivatioin of the EGFR by G-protein coupled receptors. A growth factor (e.g. endothelin-1, angiotensin), binds to the membrane bound G-protein coupled receptor. The activated G-proteins are thought to activate a zinc dependent metalloproteinase (ADAM or MMP). The MMP then cleaves a membrane-bound pro-ligand (e.g. pro-HBEGF) to form an active ligand. The activated EGFR ligand is then able to activate the EGF receptor and initiate EGFR signalling.

significantly increased (p=0.02), whereas the mitogenic activity of non-asthmatic fibroblast CM was not significantly altered. As discussed above, the standard curve obtained from stimulation with TGFβ2 treated CM reached a plateau at a much lower dilution than CM from untreated cultures. A possible explanation for this could be the ability of TGFβ2, not only to induce mitogenic growth factors, but also to produce factors that may suppress mitogenesis. It may be likely that that the TGFβ2 itself is causing the suppression in mitogenesis, since addition of a fixed amount of human recombinant TGFβ2 (0.4nM) to the EGF standard curve did lead to an early plateau in the concentration-response curve. The net proliferation exhibited by asthmatic fibroblasts in serum free medium would be the result of an equilibrium which may exist between proliferative and anti-proliferative factors. The results in these studies suggest that although TGFβ2 may also induce suppressive growth factors in asthmatic fibroblasts, it induces factors, whose influence seems to shift the equilibrium towards proliferation.

An important observation was the ability of AG1478 to significantly reduce the mitogenic activity of the CM from both unstimulated and stimulated asthmatic fibroblasts. The ability of anti-EGFR antibody to also decrease the mitogenic activity of the asthmatic fibroblast CM reinforced this observation. The observation that TGFβ2 had the ability induce expression of EGFR ligands (or ligands which are able to transactivate the EGF receptor), in the asthmatic fibroblasts, but not the non-asthmatic fibroblasts, suggest that autocrine TGFβ may have the ability to induce ligands that directly activate or transactivate the EGFR. These observations are consistent with the observations outlined in the previous chapter (section 5.6.3; figure 5.21). In that section, it was shown that addition of an anti-TGFβ1 neutralising antibody to serum free medium resulted in an inhibition of asthmatic fibroblast hyperproliferation.

The concept of transactivation of the EGF receptor may be an important consideration in explaining the obtained results. It has been shown by Prenzel and colleagues that a mechanism by which the EGF receptor is transactivated is via the activation of an EGF ligand (Prenzel *et al.,* 1999). They showed that EGFR transactivation upon GPCR stimulation involves proHB-EGF and the activity of a metalloproteinase that is rapidly induced upon GPCR-ligand interaction (see figure 6.18) Prenzel and co-workers found that CRM197 pre-treatment completely inhibited tyrosine phosphorylation of the EGFR induced by LPA and other GPRC ligands. Asakura and co-workers have shown that when cardiac myocytes were stimulated by GPCR ligands e.g. ET-1, HB-EGF

resulting from metalloproteinase activation, activated the EGFR leading to myocyte proliferation and cardiac hypertrophy (Asakura *et al.,* 2002). They used a specific inhibitor of HB-EGF shedding, KB-R7785, which blocked the increased proliferation.

Asakura and co-workers cloned a disintegrin and metalloproteinase 12 (ADAM 12) as a specific enzyme that interacted with HB-EGF. Using a dominant negative expression construct of ADAM12, they found that on stimulation of the GPRC with ET-1, the mitogenic signal was abolished, suggesting that the inhibition of ADAM12 blocked HB-EGF shedding. Using a mouse model of cardiac hypertrophy, Asakura and colleagues inhibited HB-EGF shedding with KB-R7785 and attenuated the hypertrophic changes (Asakura *et al.,* 2002).

Based on these observations, there is the possibility that there are factors secreted into the conditioned culture medium which may transactivate the EGF receptor via the mechnanism suggested by Prenzel and co-workers (1999). As in the model proposed by Asakura and co-workers (2002), ET-1 may function as EGFR transactivating ligand. In chapter 4 it was shown that asthmatic fibroblasts produce more ET-1 than non-asthmatic fibroblasts, however, addition of recombinant ET-1 protein had no effect on cell number over 6 days. It may be possible that other GPCR ligands may be produced by asthmatic fibroblasts which have the ability to transactivate the EGF receptor by this mechanism. Chambers and co-workers (2000), have shown that thrombin is a potent inducer of connective tissue growth factor in fibroblasts. Thrombin, which acts on protease activated receptors (PAR)-1 through cleavage of a tethered ligand, may result in transactivation of the EGFR, and in light of the results reported by Chambers and co-workers (2000), may also account for the increased basal levels of CTGF expressed in asthmatic fibroblasts. However, it is unlikely that thrombin is itself produced by fibroblasts, but secretion by nearby cells e.g. epithelial cells may make it available to the fibroblasts.

In light of the results obtained from the NR6/HER mitogenesis assays, AG1478 and Anti-EGFR antibody were added to primary cultures. Addition of these inhibitors reduced the increase in total cell mass exhibited by asthmatic fibroblasts in serum free medium, normally observed at 120 hours (see figure 3.8). The morphological appearance of the cells (figure 6.10) would suggest that not only are the EGFR inhibitors inhibiting growth, but may in fact be resulting in the death of some cells, which could be responsible for keeping total cell mass low. Clearly EGF is mitogenic for asthmatic fibroblasts (see figure 6.11a). The observations as illustrated in figures 6.10h and 6.10i, would suggest that cell death is a contributory factor to the reduction of total cell mass at 120 hours. The

phase contrast micrographs of the asthmatic fibroblasts following treatment with AG1478 or Anti-EGFR antibody show that there is significant decrease in total cell mass after 120 hours in culture. At 24 hours however, there seems to be a large degree of cellular death, either though apoptosis or necrosis. The abolition of EGFR mediated effects by these factors, clearly has a profound effect on cell survival, suggesting that EGFR activation is pivotal in allowing asthmatic fibroblasts to survive and hence reach a higher density.

From figure 6.10, it can be seen that some of the cells following the treatment with Anti-EGFR antibody or AG1478, adapted a different morphology. The cells in figure 6.10k appeared more spindle-like and appeared to be much more hypotrophic than the control. This observation along with the observations from section 6.5 which indicate AG1478 and Anti-EGFR antibody inhibit mitogenesis of NR6/HER fibroblasts do indicate signal mechanisms through EGFR are important in mediating the autocrine increase in cell mass in asthmatic fibroblasts.

6.8.2 Heparin binding growth factor is an EGFR ligand produced by primary bronchial fibroblasts.

As discussed above, HB-EGF has been implicated in cardiac remodelling following myocardial infarction. HB-EGF has also been closely associated with the formation of atherosclerosis in human aorta and coronary artery (Miyagawa *et al.,* 1995; Nakata *et al.,* 1996), conditions which have been attributed to activation of fibroblasts. The HB-EGF molecule exists in 2 forms, each with distinct activity. The transmembrane form of HB-EGF functions as a juxtacrine growth and adhesion factor (Higashiyama *et al.,* 1995; *Raab et al.,*1996) The soluble mature form is proteolytically processed from the larger membrane-bound precursor pro-HB-EGF which is highly mitogenic for bladder smooth muscle cells (Higashiyama *et al.,* 1991; Higashiyama *et al.,* 1993). It has been shown that ADAM-12 is responsible for this proteolytic cleavage (Asakura *et al.,* 2002). A very distinct feature of HB-EGF is its high affinity for binding the potent microbial toxin, Diphtheria toxin (Naglich *et al.,* 1992). Diphtheria toxin elicits its effect by irreversibly stopping all protein production in cells (Pappenheimer *et al.,*1977). Giannini G and co-workers (1984) developed a non-toxic analogue of Diphtheria toxin, cross reacting molecule (CRM)-197. This analogue does not have the enzymatic ability to inhibit protein production by cells, but retains the ability to bind HB-EGF (Kaefer *et al.,* 2000).

Kaefer and colleagues (2000) showed that CRM-197 was able to inhibit the proliferation of bladder smooth muscle cells in a non-toxic manner, by binding and thus inhibiting HB-EGF.

The results obtained in the present study, are in line with the observations made by Kaefer and co-workers (2000). The present study is the first to demonstrate that asthmatic fibroblasts require HB-EGF in order to maintain an increased cell mass, as demonstrated by the ability of CRM-197 to specifically reduce the total cell mass after 120 hours in serum free culture when compared to non-treated cells.

In the previous chapter (figure 5.19) it was shown that the anti-TGFβ1 neutralising antibody, was able to significantly reduce the number of asthmatic fibroblasts in serum free medium, suggesting that TGFβ1 was an autocrine ligand necessary to maintain an increased fibroblast cell mass. In this part of the study it has been shown that TGFβ2

highly significantly induces the expression of HB-EGF in both asthmatic and non-asthmatic fibroblasts, but to a greater degree in asthmatic fibroblasts (figure 6.1). As discussed in section 4.5.1, the TGFβ1 gene expression by asthmatic and non-asthmatic fibroblasts at baseline were not different, although the protein levels in the CM of the asthmatic fibroblasts was less than that of the non-asthmatic fibroblasts. It was suggested that a possibility to explain the decreased levels in the asthmatics could have been autocrine consumption. This would be consistent with the observations made in this part of the study; the basal levels of HB-EGF gene expression are significantly higher in the asthmatics than in the non-asthmatics, suggesting that the autocrine TGFβ1 consumed by the asthmatic fibroblasts may be responsible for this. The data presented in figure 6.3 supports this hypothesis, where treatment of asthmatic fibroblasts with anti-TGFβ1 neutralising antibody, resulted in a reduced HB-EGF gene expression. This is the first study to indicate HB-EGF as a possible autocrine ligand for the hyperproliferation of asthmatic fibroblasts in serum free medium. Furthermore, it confirms the findings of reports by other members of the present group (Chrissy Boxall, personal communication, *ATS 2003 abstract*) which illustrate the ability of TGFβ to induce HB-EGF gene expression in asthmatic fibroblasts. The literature generally indicates antagonising roles for TGFβ receptor and EGFR ligands on cellular processes. The present study in fact illustrates a co-operation of the two ligands to promote cellular growth.

6.8.3 ADAM-12 and ADAM-33 in the processing of HB-EGF

The a distintegrin and a metalloproteinase (ADAM) family of proteins are type-I transmembrane proteins with a unique domain structure composed of a signal sequence and pro, metalloproteinase, disintegrin, cysteine-rich, EGF-like, transmembrane and cytoplasmic domains (Yoshinaka *et al.*, 2002); Black and White 1998; Blobel 1997). ADAM-12 has been shown to be a key metalloproteinase in the activation of HB-EGF from pro-HB-EG in cardiac hypertrophy, a disease in which ventricular remodelling, due to fibroblast and smooth muscle proliferation, is an important feature.

In the present study, addition of GM6001, a broad spectrum MMP inhibitor resulted in a decrease in total cell mass at 120 hours when compared to unstimulated asthmatic fibroblasts in serum free medium (figure 6.13) suggesting MMP cleavage of a growth factor was involved in the observed increased cell mass exhibited by asthmatic fibroblasts.

ADAM-33 is the latest ADAM protein to have been identified. Recently, Van Eerdewegh and co-workers have shown a highly significant association of polymorphisms within the ADAM-33 gene with asthma and bronchial hyperresponsiveness (Van Eerdewegh *et al.,* 2002). ADAM-12 and ADAM-33 have a high degree of homology and indeed are both considered to be part of the same sub-family of ADAM protein, which also consists ADAMs 8,12,13,15 and 19. As discussed above, ADAM-12 is responsible for the proteolytic activation of HB-EGF, so in light of their homology, it was speculated that ADAM-33 may be responsible for the proteolytic cleavage of pro-HB-EGF to active HB-EGF.

There was no significant difference between the levels of ADAM-33 in the asthmatic and non-asthmatic fibroblasts at baseline. An interesting observation was the reduction of ADAM-33 expression in response to TGFβ2 stimulation. Since TGFβ induced HB-EGF, it seemed unlikely that a protein down-regulated by the same stimulus would be responsible for HB-EGF activation. The levels of ADAM-33 gene were significantly reduced in the asthmatic fibroblasts when compared to the non-asthmatic fibroblasts at 6, 72 and 144 hours of being in serum free culture. The levels of ADAM-33 gene expression peaked at 24 hours and then reduced, however, in culture, the hyperproliferation of asthmatic fibroblasts was not observed till 120 hours.

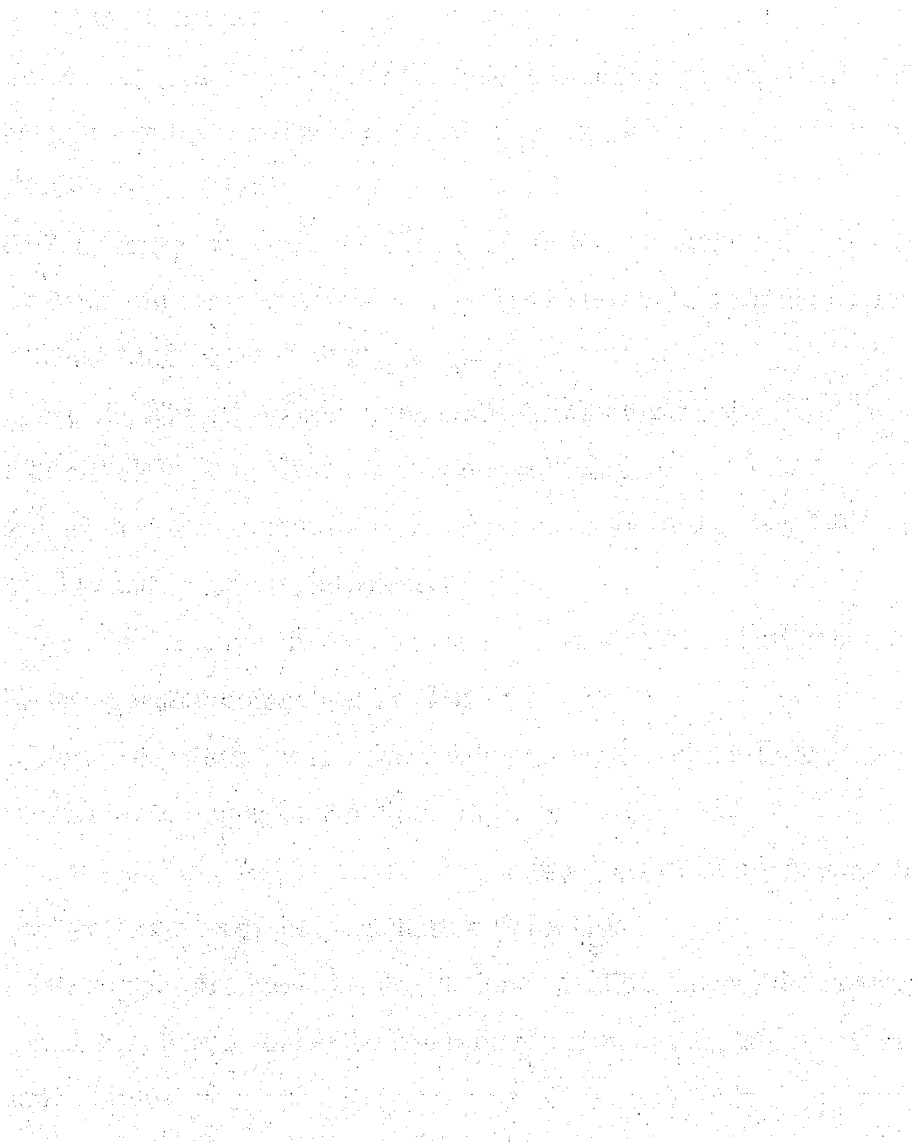
The observation indicating that ADAM-33 gene expression was decreased in asthmatic fibroblasts in response to TGFβ2 may suggest that ADAM-33 may activate a protein which normally inhibits the fibroblasts from proliferating. This would lead to a hypothesis that the hyperproliferation is not caused by a gain of a proliferative stimulus, but a loss of regulatory stimulus.

The levels of ADAM-12 were significantly higher in the asthmatic than in the non-asthmatic fibroblasts at 72 and 144 hours after being in culture at baseline. This is consistent with the hyperproliferation of the asthmatic fibroblasts, which typically occurs between 72 and 120 hours (figure 3.8b). This data may suggest that ADAM-12 is responsible for activation of HB-EGF in asthmatic fibroblasts, and that HB-EGF is an important factor that is involved in the hyperproliferation of asthmatic fibroblasts.

6.8.4 Measurement of total cell mass and cell proliferation

As discussed above, the work presented in this part of the study has considered total cell mass as determined by methylene blue uptake. The distinct differences in morphology make it impossible to use the standard curves constructed in section 2.x to convert to cell number, this would only be possible if methylene blue standard curves are constructed for each set of conditions. Other measures of cell proliferation would be required to determine if HB-EGF does indeed cause proliferation. Such techniques could include MTT assays or 3H thymidine incorporation, however these techniques themselves have disadvantages. MTT assays, which depend on the reduction of MTS to MTT by mitochondrial activity, are dependent on the number of mitochondria present in the cell. Cells, which have undergone hypertrophy, are likely to have a larger number of mitochondria, rendering the MTT assays with the same problems as the methylene blue assay. ^{3}H Thymidine incorporation is a measure of DNA synthesis and not proliferation. It is possible to use ^{3}H Thymidine incorporation as a measure of proliferation by not allowing the cells to become quiescent, as was conducted in section 3.x. This assay would work under the assumption that, if there are more cells, there would be a greater proportion of cells in the S-phase of the cell cycle at any given time, and hence more cells with the ability to incorporate ^{3}H thymidine. The use of thymidine incorporation would provide evidence for the proliferative ability of HB-EGF on primary asthmatic fibroblasts.

It is also possible to conduct direct cell counts, which would give definitive values, however this method is subject to, typically a 15% error (www.bhtafe.edu.au/bhi2/toolbox4/_TEST501/task33.htm).



6.9 Summary of results and novel findings

- The mitogenic activity of conditioned medium from asthmatic fibroblasts is significantly increased following treatment with 0.4nM TGFβ2 (p=0.04).
- Conditioned medium from asthmatic fibroblasts treated with 0.4nM TGFβ2, stimulated mitogenesis of NR6/HER fibroblasts to a significantly higher degree than conditioned medium from non-asthmatic fibroblasts treated with TGFβ2 (p=0.019).
- AG1478 and Anti-EGFR antibody significantly reduced the mitogenic ability of asthmatic fibroblast cultured medium (p=0.05), suggesting the cultured medium contained EGFR ligands.
- Addition of AG1478 and Anti-EGFR antibody to primary asthmatic fibroblasts, resulted in a significant reduction in total cell mass after 120 hours in culture (p=0.05 and p=0.04 respectively).
- CRM197, a compound which specifically binds and therefore inactivates HB-EGF also resulted in lower total fibroblast mass at 120 hours (to a similar degree as AG1478 and Anti-EGFR antibody, p=0.05)
- The ability of CRM-197 to inhibit the proliferation of asthmatic fibroblasts, identified a role for HB-EGF in mediating their hyperproliferation.
- At baseline, the asthmatic fibroblasts express a higher level of HB-EGF mRNA when compared to non-asthmatic fibroblasts (p=0.05).
- Following TGFβ treatment, both non-asthmatic (p=0.028) and asthmatic (p=0.018) fibroblasts expressed higher levels of HB-EGF gene.
- HB-EGF gene expression was induced to a greater degree in asthmatic fibroblasts when compared to non-asthmatic fibroblasts.
- Addition of a specific TGFβ1 neutralising antibody significantly reduced the basal HB-EGF gene expression in the asthmatic fibroblasts.
- These data suggest that autocrine production of TGFβ1 induces the expression of HB-EGF which may have a role in the hyperproliferation or maintaining the survival of asthmatic fibroblasts.

6.10 Conclusions

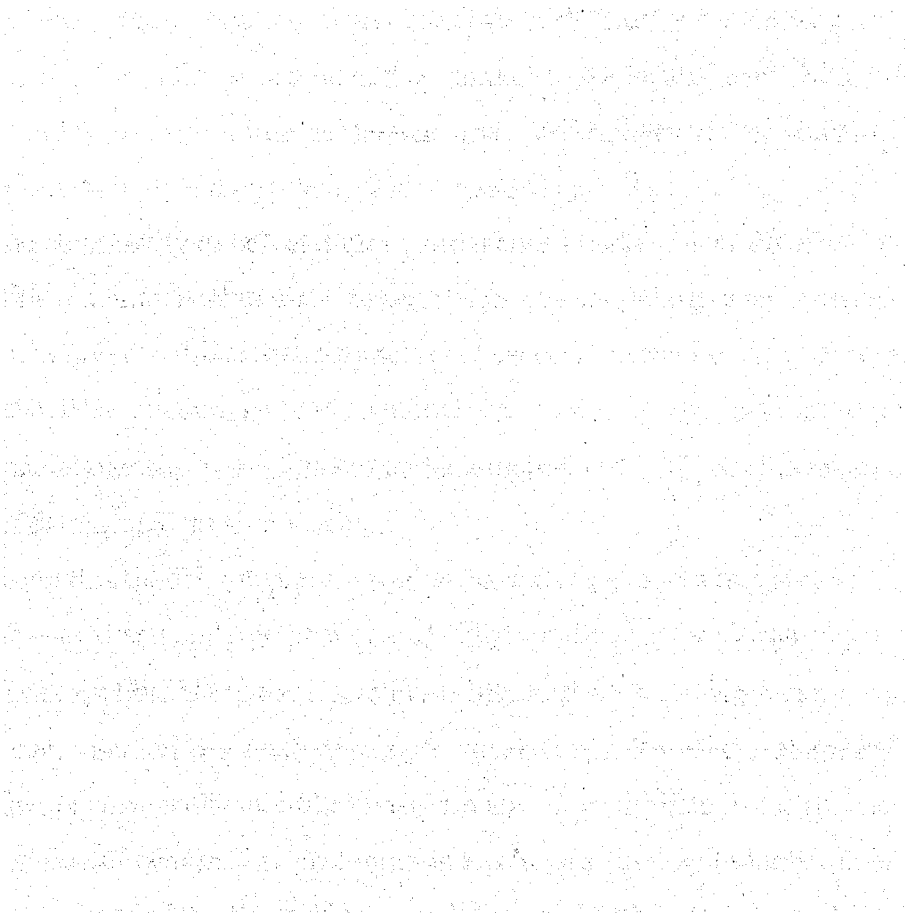
The results presented here are consistent with the hypotheses outlined at the beginning of the chapter. Conditioned medium from both non-asthmatic and asthmatic fibroblasts induced mitogenic responses in the NR6/HER fibroblast line, however, the mitogenesis induced by conditioned culture medium from asthmatic fibroblasts which had been treated with 0.4nM TGFβ2 induced significantly higher levels of mitogenesis. The mitogenesis was significantly reduced by AG1478 and an anti-EGFR antibody, indicating that there were factors capable of activating the EGF receptor present in the medium.

The hyperproliferation of asthmatic fibroblasts in serum free medium observed in chapter 4 may be due to the production of factors which activate the EGFR either directly or through transactivation. The evidence from the last chapter that TGFβ1 is consumed as an autocrine growth factor, along with the data that TGFβ1 is able to induce HB-EGF in bronchial fibroblasts suggest that HB-EGF may be one of the factors important in maintaining increased cell number in asthmatic fibroblasts. It may be possible that TGFβ1 is not directly regulating HB-EGF expression (even though the HB-EGF promoter has been shown to contain a TGFβ1 response element). In light of the studies by both Prenzel and co-workers (1999) and Asakura and colleagues (2002), it is possible that TGFβ1 may be inducing other factors which may be GPCR ligands, which then lead to HB-EGF processing as in cardiac hypertrophy.

The present and previous chapter have concentrated solely on the production of growth factors to explain the increased proliferation of asthmatic fibroblasts, however there may be other potential mechanisms by which the asthmatic (myo)fibroblasts are able to proliferate, it is equally possible that the hyperproliferation of the asthmatic fibroblasts is due to a lack of regulatory control that normally exists. This phenomenon has been explored by McAnulty and co-workers (1995). They have shown that TGFβ1 has the ability to stimulate the autocrine production of prostaglandin (PG)E_2 by normal fibroblasts. PGE_2 has been shown to be a potent inhibitor of fibroblast proliferation (Oliver *et al.*, 1989), which can be completely abolished on addition of Indomethacin (a potent inhibitor of prostaglandin synthesis). As discussed in the previous chapters, TGFβ1 is an autocrine growth factor produced by both asthmatic and non-asthmatics and the gene expression data suggested that there was no significant difference between the two groups

however, the ELISA data, suggested that since there was autocrine utilization of TGFβ1. In light of these observations in combination with the observations made by McAnulty and co-workers (1995) would lead to the hypothesis that asthmatic fibroblasts lack the ability to produce PGE_2 in response to TGFβ1 stimulation. Further exploration of these mechanisms are beyond the scope of this thesis.

The identification of HB-EGF as a possible factor important in mediating the hyperproliferation of asthmatic fibroblasts (either directly or through promoting their survival) may lead to a novel therapeutic target by which survival of newly proliferated fibroblasts may be inhibited and hence may dampen the structural changes that occur in chronic asthma.



CHAPTER SEVEN

Discussion and Future Work

7.1 Summary of findings

There is now accumulating evidence that airway remodeling is a major component of bronchial asthma. The most prominent features of the structural changes that characterize airway wall remodeling include subepithelial fibrosis and increased smooth muscle volume. Myofibroblasts, resident in the *lamina reticularis*, have been shown to be responsible for the interstitial collagen deposition that results in subepithelial fibrosis. The increase in smooth muscle mass is not clearly understood, and it is unclear whether the increase is due to proliferation or hypertrophy or both. Studies by Holgate and co-workers (Holgate *et al.,* 1998) have postulated that damage to the airway epithelium which occurs in asthma can be translated into permanent structural changes via the activation of (myo)fibroblasts in the *lamina reticularis* (figure 7.1).

The complexity of cell and tissue interactions make it very difficult to study the mechanisms that may lead to the various aspects of remodeling in an *in vivo* setting. Therefore, in order to disseminate some of the aspects of remodeling with respect to (myo)fibroblast activation, an *in vitro* model was used, whereby (myo)fibroblasts grown from bronchial biopsies were cultured and stimulated with TGFβ2, the major secretory product of damaged airway epithelium.

Using this model, the present studies have demonstrated that airway fibroblasts do become activated and undergo phenotypic differentiation to myofibroblasts in response to TGFβ2. The myofibroblasts then have the ability to secrete growth factors, which have the ability to cause paracrine growth of smooth muscle cells. However, the results also indicate that *in vitro*, asthmatic fibroblasts are able to proliferate in the absence of any exogenous growth factors. The proliferation has been attributed to the production and subsequent consumption of autocrine growth factors. Furthermore, myofibroblasts may have the potential to differentiate into smooth muscle cells, as demonstrated by the

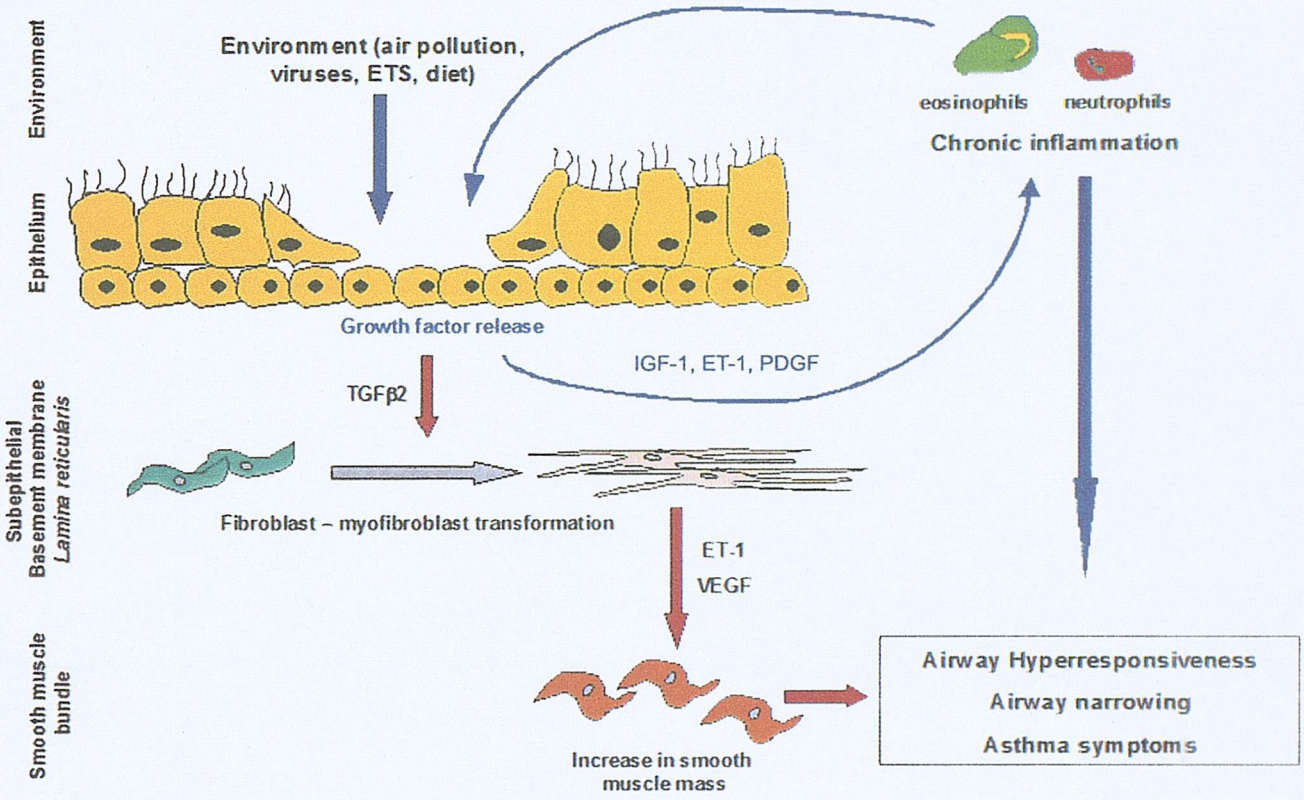


Figure 7.1: The epithelial mesenchymal trophic unit in bronchial asthma. The scheme shows that damage to the epithelium by direct environmental insult or by cationic proteins secreted from inflammatory cells. The damaged epithelium releases a number of growth factors which have the potential to contribute to inflammatory cell infiltration and chronic inflammation. Also secreted is TGFβ2, which results in the phenotypic transformation of fibroblasts to myofibroblasts. This phenotypic switch is central to the hypothesis, since myofibroblasts then have the ability to produce growth factors which may lead to increased smooth muscle volume. The increase in smooth muscle then results in bronchial hyperresponsiveness and airway narrowing.

increased expression of cytoskeletal and cytocontractile proteins normally associated with smooth muscle cells.

Directly implicated in the proliferation of these cells, has been TGFβ1 and HB-EGF. It has been shown that TGFβ1 has the ability to induce HB-EGF gene expression, both in non-asthmatic and asthmatic fibroblasts, but to a greater degree in the latter case. The specific neutralisation of TGFβ1 or HB-EGF both result in a lower total cell mass when compared to asthmatic fibroblasts grown in serum free medium.

In the course of these investigations, autocrine production of CTGF and ET-1 was also observed, however, although having proliferative roles in other diseases (i.e. scleroderma and left ventricular cardiac hypertrophy respectively) they seem to have little role in driving autocrine proliferation of asthmatic fibroblasts. Instead it has been demonstrated that differentiation of fibroblasts to myofibroblasts by TGFβ is dependent on the expression of CTGF. The increased levels of autocrine CTGF in asthmatic fibroblasts has be postulated to be involved in 'priming' a TGFβ induced phenotypic differentiation, resulting in a lower concentration of TGFβ being required to induce the myofibroblast phenotype. Endothelin-1 was also shown to have the ability to induce differentiation through the ET_A receptor, although at concentrations much higher than those detected in the cultured medium.

The treatment of both non-asthmatic and asthmatic fibroblasts with TGFβ2 resulted in phenotypic differentiation to myofibroblasts, characterised by an induction of the cytocontractile protein α-smooth muscle actin. However, the induction of other cytocontractile and cytoskeletal proteins normally associated with smooth muscle cells e.g. heavy chain myosin in TGFβ2 treated fibroblasts, suggests the possibility that the cells have the potential for further differentiation into smooth muscle cells. This phenomenon has previously been demonstrated in the obstructed rabbit urinary bladder, in which repeated stimulation of fibroblasts with TGFβ1 resulted in differentiation, firstly into myofibroblasts and further into smooth muscle cells.

7.2 Paracrine and Autocrine growth factor production by asthmatic (myo) fibroblasts

The observation that growth factors secreted by asthmatic fibroblasts have distinct roles in driving either proliferation or differentiation is different from what has been described before. Previously such distinctions were not made, and factors secreted by fibroblasts were simply described as pro-fibrotic. The categorization of growth factors in this manner presents the opportunity to target different aspects of the fibrotic response therapeutically. Furthermore, not only do the results indicate that the growth factors can be categorized in such ways, but that different levels of growth factor expression and secretion arise from different compartments of the mesenchyme.

The general postulated sequence of events that have resulted from the results of this thesis are summarized in figure 7.2. In the first instance, asthmatic airway fibroblasts secrete TGFβ1, which acts in an *autocrine* fashion resulting in expression of HB-EGF, which drives their proliferation. The asthmatic fibroblasts also express elevated or higher than normal levels of CTGF, which is independent of TGFβ signaling. The CTGF acts in an *autocrine* fashion to 'prime' the phenotypic differentiation from fibroblasts to myofibroblasts, such that a lesser TGFβ2 stimulus from the damaged bronchial epithelium is required to trigger the response. Once transformed, the myofibroblasts themselves secrete higher levels of the same growth factors, i.e. TGFβ1, CTGF but in addition, the levels of ET-1 expression and secretion are significantly higher. As is the case with the unstimulated fibroblasts, *autocrine* TGFβ1 results in the expression of HB-EGF. However, the HB-EGF produced by the myofibroblasts may not only be contributing to their proliferation, but may also have a role in acting as a highly mitogenic *paracrine* growth factor for smooth muscle cells. The data presented in chapter 5 indicated that ET-1 was unlikely to have a role in asthmatic fibroblast proliferation. ET-1 is also highly mitogenic for smooth muscle cells and may represent another *paracrine* growth factor for smooth muscle cells in asthma. Connective tissue growth factor, has been previously shown to be important in TGFβ stimulated collagen production in fibroblastic cells (Duncan *et al.*, 1999), and the up regulation observed in the asthmatic fibroblasts, may be responsible for the increased subepithelial fibrosis.

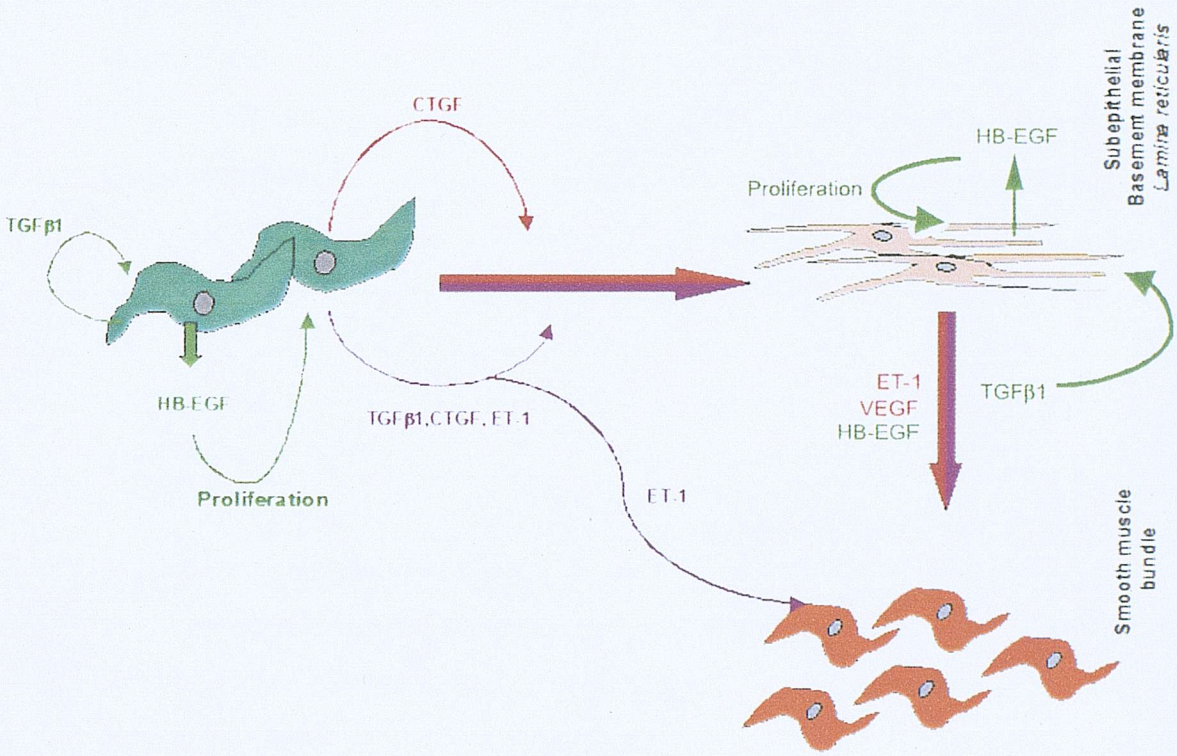


Figure 7.2: Summary of results obtained in these studies. In this model, asthmatic fibroblasts do not necessarily require TGFβ2 activation from the damaged epithelium to proliferate and produce growth factors mitogenic for smooth muscle cells. Due to autocrine production of TGFβ1, CTGF and ET-1, less TGFβ2 stimulus is required for myofibroblasts transformation. Phenotypic transformation mediated by TGFβ2 occurs via the production of CTGF. Myofibroblasts are able to proliferate by a mechansim similar to the proliferation of fibroblasts, however, the HB-EGF release by these cells may be more important in stimulating proliferation of smooth muscle cells.

7.3 Context of these results in asthmatic airway remodelling

Evidence from immunochemical analysis of bronchial biopsies from children before the onset of asthma symptoms, show a thickened *lamina reticularis*, due to interstitial collagen deposition, indicating increased myofibroblast activity (Warner *et al.*, 2000), suggesting that remodeling has the potential to occur independently of inflammation. The evidence presented in the present study is consistent with such a proposal. The autocrine TGFβ1 induced proliferation of asthmatic bronchial fibroblasts would result more cells which, independent of any further stimulation, have the ability to secrete growth factors such as HBEGF, CTGF and ET-1. These observations would indicate a low level of remodeling that occurs independently of inflammation. However, in the presence of inflammation and structural damage to the airway epithelium, there is the potential for these fibroblasts to be further activated and to differentiate into myofibroblasts as a consequence of the effects of TGFβ2 secreted by damaged epithelium and TGFβ1 produced by inflammatory cells e.g. eosinophils. This would augment the downstream remodeling changes.

Evidence from the obstructed rabbit urinary bladder (Buoro *et al.*, 1993) suggests that fibroblasts that are differentiated into myofibroblasts may undergo to a further phenotypic differentiation to smooth muscle cells. In light of this evidence, it may be possible for airway myofibroblasts to undergo further transformation to airway smooth muscle cells. If indeed this was the case, an increase in the number of fibroblasts due to autocrine proliferation, could potentially directly contribute to increased smooth muscle cell volume through phenotypic transformation.

Although TGFβ1 induced HBEGF has been implicated in driving asthmatic fibroblast proliferation, it is not clear from the present studies, whether or not this is due to transactivation of the EGF receptor. Reduction of total asthmatic fibroblast mass (when compared to asthmatic fibroblasts grown in serum free medium) with the broad spectrum MMP inhibitor GM6001, would suggest that a metalloproteinase is involved in the proliferation cascade. As outlined in section 1.6.2.2, G-protein coupled receptors, have the ability to transactivate the EGF receptor, with a critical role for zinc-dependent metalloproteinases. Such transactivation resulting in fibroblast proliferation has been described in left ventricular cardiac hypertrophy (Asakura *et al.*, 2002). In this model ET-1

was shown to activate a G-coupled receptor, which resulted in activation of ADAM-12, which resulted in the shedding of HB-EGF from the pro-form, which subsequently activated the EGF receptor, resulting in myocyte proliferation. It is unlikely from the results obtained that ET-1, which is up regulated in asthmatic fibroblasts, transactivates the EGF receptor to result in proliferation, since addition of ET-1 antagonists did not suppress the hyperproliferation. The possibility of other G-protein coupled receptor ligands may result in transactivation has remained unexplored in this thesis.

The finding first described by Boxall and co-workers (Personal communication) that TGFβ1 induces EGF ligands, poses a novel concept in TGFβ being able to result in activation the EGF receptor (although not in the traditional sense of transactivation). The observation in this thesis is that autocrine TGFβ1 has the ability to up regulate the induction of HB-EGF, which can then be subsequently cleaved to stimulate the EGF receptor. Classically, TGFβ stimulated pathways antagonize those pathways stimulated by EGFR activation. An example of this is that pathways activated by EGFR activation result in cellular proliferation, whereas pathways stimulated by TGFβ receptor activation result in growth inhibition. Another example is the differentiation of fibroblasts to myofibroblasts, which is induced by TGFβ stimulation (Desmouliere *et al.* 1993), but can be inhibited by addition of EGF (Kirkland *et al.* 1998). The results of the studies in this thesis would suggest that both TGFβ activation and EGFR activation both work along the same mechanistic pathway resulting in the autocrine proliferation of asthmatic fibroblasts.

There have been some reported studies, which suggest that the proliferative ability of TGFβ1 depends on concentration, so that low concentrations are proliferative for fibroblasts and high concentrations are anti-proliferative (McAnulty *et al.* 1998).

A possible explanation of this could be the differential induction of $p21^{waf}$. Recent studies published by Puddicombe and co-workers (2003) have shown that in epithelial cells, TGFβ at concentrations of 200pM or greater, result in the induction of $p21^{waf}$ which is a cyclin-dependent kinase inhibitor, and inhibits EGFR activated proliferation. It has also been shown that cell differentiation results in expression of $p21^{waf}$ and subsequent inhibition of cell proliferation (Puddicombe *et al.,* 2003, Gartel *et al.,* 1996). The levels of autocrine TGFβ1 found in the supernatants of the fibroblasts were much lower than those that have been used in the present study to induce differentiation and those shown by Puddicombe and co-workers shown to induce $p21^{waf}$. It may therefore be hypothesized,

that at low concentrations of TGFβ, the equilibrium between proliferation and differentiation is shifted towards proliferation, whereas at higher concentrations, the equilibrium is shifted towards differentiation, hence resulting in $p21^{waf}$ expression and an inhibition of proliferation. It may be fruitful to investigate this further by measuring the expression of $p21^{waf}$ in asthmatic and non-asthmatic fibroblasts over a range of TGFβ concentrations, although it should be noted that the effects of TGFβ are highly cell dependent, and the induction of $p21^{waf}$ by higher concentrations of TGFβ may be epithelial cell specific. This hypothesis may explain why, even though fibroblasts produce autocrine TGFβ1, they are not differentiated into myofibroblasts under basal conditions.

7.3.1 Novel therapeutic targets for asthmatic airway wall remodelling

The categorisation of growth factors into those that are proliferative or those that are responsible for phenotypic transformation presents the opportunity to target different aspects of the fibrotic response therapeutically.

The role of the myofibroblasts in transducing asthmatic epithelial damage into structural changes in the airway wall through phenotypic differentiation, presents a potential therapeutic target. The use of Fluvastatin in these studies in inhibiting CTGF gene expression and subsequent myofibroblast differentiation represents the potential of treatment using an existing compound. These preliminary studies using Fluvastatin as a means for inhibiting myofibroblast transformation would require further study, to determine if similar findings are found *in vivo* and if the doses required for therapeutic benefit are toxic or cause adverse side effects. It is also important to note that systemic administration of a compound that inhibits myofibroblast differentiation may interfere in processes other than fibrosis, where myofibroblast differentiation is necessary e.g. wound healing. In light of this local administration may be beneficial e.g. through inhaler devices. Local administration could also be achieved by the targeting of CTGF anti-sense transfection into bronchial fibroblasts, using adenoviruses as transfection vehicles.

Targeting the differentiation would however not reduce the overall number of fibroblasts present. Since asthmatic fibroblasts secrete more ET-1 than non-asthmatic fibroblasts, an increased number of fibroblasts may then still promote proliferation of smooth muscle cells. The localised antagonism of ET-1 may be of benefit, not so much in

effecting phenotypic transformation of fibroblasts to myofibroblasts, but in inhibiting the mitogenic effects on smooth muscle cells.

Targeting of the hyperproliferation of asthmatic fibroblasts may be a target by which the whole remodelling process may be dampened. The *in vitro* studies in section 5.6.3 show that use of a specific neutralising antibody against TGFβ1, resulted in a decrease in total asthmatic fibroblast cell mass, when compared to cells grown in serum free medium. It is unclear as to whether or not anti-TGFβ1 neutralising antibody may be administered locally into the airways. Presently, anti-TGFβ1 neutralising antibody (provided by Cambridge antibody technologies) has entered phase I/II clinical trials for use in diffuse systemic sclerosis, which is a form of scleroderma. The reports of this study have not been published, it would be interesting to know if any of the 36 patients in the trial has asthma, and if their symptoms were improved. Scleroderma is a life threatening disorder, with 40% dying within 10 years of being diagnosed. Any deleterious effects which may result from the inhibition of systemic TGFβ would be outweighed by potential life-saving benefits. However, although some deaths result from asthma every year, generally mortality is not a problem, hence any systemic side effects would have to be balanced against benefits. Furthermore, TGFβ1 is anti-inflammatory, and its blockade may result in the exacerbations of inflammation, which is a very distinct and important part of asthma.

Since, it has been postulated in the present thesis, that autocrine proliferation is mediated by HB-EGF which is induced by autocrine TGFβ1, targeting of HB-EGF may be a novel therapeutic target. CRM-197, a non-toxic diphtheria toxin analogue was used in the present studies to bind HB-EGF and thus act as an antagonist. Local administration of CRM-197 may reduce the HB-EGF levels available for HB-EGF driven increase fibroblast cell mass. CRM-197 protein has been used clinically as a vaccine against Pneumococcal-7 infection (marketed as Prevnar™) in young children, and presented side effects in a very small number of cases, such as fever and stomach disturbances, which are commonly encountered after any vaccination.

Results in chapter 6, showed that GM6001, a broad spectrum MMP inhibitor was able to inhibit asthmatic fibroblast hyperproliferation, presumably via inhibition of an MMP, which cleaves pro-HB-EGF to active HB-EGF. Studies by Asakura and colleagues (2002) have shown that ADAM-12 is responsible for cleavage of HB-EGF in cardiac

myocyte proliferation. Whether ADAM-12 is responsible in the present case, or whether the closely related protein ADAM-33 (polymorphims in which have recently been shown to have a high association with asthmatic bronchial hyper-responsiveness) needs to be determined, and may present another therapeutic target to inhibit proliferation of asthmatic fibroblasts.

7.4 Future work and prospects

From the results obtained, it is clear to see that TGFβ is an important driving molecule in both the hyperproliferation and differentiation of asthmatic fibroblasts. The results observed in the present studies suggest that the asthmatic fibroblasts exhibit an abnormal response to TGFβ, whereby asthmatic fibroblasts secrete higher levels of growth factors in response to TGFβ stimulation. There is no significant difference between the levels of TGFβ1 expressed by non-asthmatic and asthmatic fibroblasts, however the asthmatic fibroblasts respond by expressing significantly higher levels of HB-EGF, which is postulated to be important in their hyperproliferation. In future experiments, it may be important to determine the exact mechanisms underlying the apparent abnormal response to TGFβ exhibited by the asthmatic fibroblasts. Abnormal TGFβ responses have been observed in inflammatory bowel disease, where TGFβ1 cannot inhibit pro-inflammatory cytokine production in isolated *lamina propria* mononuclear cells from patients with Crohn's disease (Monteleone *et al.*, 2001). This abnormality was shown to be due to the overexpression of Smad7, an inhibitory Smad protein, which regulates TGFβ signalling. In their study, Monteleone and colleagues (2001) used Smad7 anti-sense oligonucleotides to block the overexpression, and restored the TGFβ1 induced inhibition of pro-inflammatory cytokines from isolated mononuclear cells. It may be possible that a Smad7 regulatory mechanism is absent or abnormal in the asthmatic fibroblasts resulting in an enhanced response to TGFβ. Therefore, experiments to measure levels of Smad7 in both the asthmatic and non-asthmatic fibroblasts should be conducted. Initial experiments could include measuring Smad7 gene expression in both cell types using Taqman™ quantitative RT-PCR. The abnormality may not be at a genetic level, but may be in protein translation, and so investigation of protein levels by western blotting may prove to be useful. In order

to prove (or disprove) that Smad7 down-regulation is responsible for the abnormal responses of asthmatic fibroblasts to TGFβ, it may possible to transfect Smad7 anti-sense into non-asthmatic fibroblasts, and determine if their behaviour is comparable to that of asthmatic fibroblasts.

The investigation of the TGFβ receptor repertoire on both non-asthmatic and asthmatic fibroblasts may yield differences that result in asthmatic fibroblasts exhibiting greater responses to TGFβ than the non-asthmatic fibroblasts. As discussed above, classically, EGFR signalling pathways antagonise the effects of TGFβ receptor signalling pathways. It therefore may be fruitful to investigate the TGFβ receptor:EGFR ratios on both non-asthmatic and asthmatic fibroblasts. It may be the case that there is a shift in the equilibrium between EGF receptor and TGFβ receptor signaling towards TGFβ receptor signaling (possibly due to a reduced EGF signaling) resulting in the abnormal responses.

Recent studies by Chambers and colleagues (2003) have shown using GeneChip hybridization studies, that TGFβ is able to up-regulate genes involved in human lung fibroblast proliferation and survival. It may be fruitful to conduct similar experiments on mRNA from non-asthmatic and asthmatic fibroblasts and compare the genes that are up or down regulated both at baseline and in response to TGFβ.

In order for work to progress in this area of remodeling, it would be necessary to see if the *in vitro* observations translate into the *in vivo* situation. Clearly, *in vivo* there are some control mechanisms which have the ability to control the proliferation and activation of asthmatic fibroblasts, otherwise uncontrolled proliferation and activation of the (myo)fibroblasts would result in complete closure of the airway lumen. Clearly, to fully understand mechanisms surrounding asthmatic airway remodeling, it is necessary to translate *in vitro* results into an *in vivo* model. Such experiments may be possible by the use of transgenic knockout mice models, in which specific genes could be knocked out e.g. a gene for a growth factor, and the progression of pathology could be investigated.

Mouse models of asthma may provide useful data to help understand the *in vivo* processes that control asthmatic fibroblast proliferation. It has been recently reported that antagonists against cysteinyl leukotrienes inhibit collagen and lung fibrosis in mouse models of asthma (Henderson *et al,* 2002). It may be possible to develop a TGFβ1 knockout mouse and cross it with the mouse model that demonstrates lung fibrosis, to see

if TGFβ1 has a role in asthmatic fibroblast proliferation. However, the issue of species specificity will always remain when using mouse models.

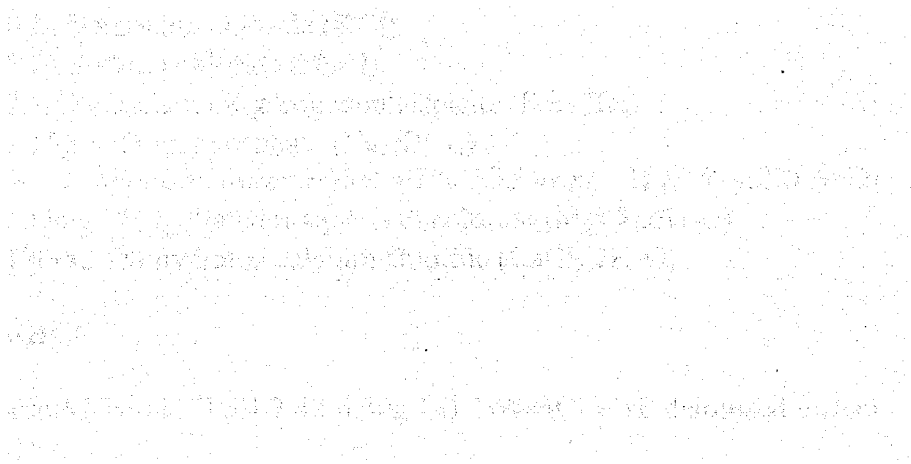
Recently, it has been reported that polymorphisms in the ADAM-33 gene have a close association with bronchial hyper-responsiveness. Since ADAM-33 is closely related to ADAM-12, which has been shown to cleave HB-EGF, it may be fruitful to investigate fibroblasts derived from ADAM-33 transgenic mice. If knocking out ADAM-33 results in the inhibition of asthmatic fibroblast hyperproliferation, it may be possible that it is involved in the processing of a growth factor as it the case with ADAM-12. However, there are a large number of polymorphisms associated with ADAM-33, and it would be necessary to determine which polymorphisms would be necessary to model in transgenic mice. Since, it would be postulated that polymorphisms in the metalloproteinase domain maybe important in HB-EGF cleavage it might be fruitful to visit these first. It may also be possible to develop anti-sense oligonucleotides against the polymorphisms, and transfect them into primary fibroblasts. Those that may be seen to affect proliferation may then be transferred into transgenic mouse models.

7.5 Conclusions

In conclusion, the results presented in these studies have the potential to change the traditional view that fibroblastic cells only contribute to airway remodeling through transduction of epithelial damage into permanent structural changes. Asthmatic fibroblasts have an intrinsic abnormality which allow them to secrete growth factors, which can act in an autocrine fashion to promote proliferation and 'prime' fibroblasts to exogenous stimulation (i.e. from a damaged epithelium) and in a paracrine fashion on underlying smooth muscle cells. The nature of this intrinsic ability is unclear, and may be the result of genetic variability (i.e. polymorphisms) or memory of the microenvironment the cells are exposed to *in vivo*. A number of chromosomal regions contain genes which are thought to influence asthma (Cookson 2002), whether any of these regions have an association with abnormal fibroblast proliferation has not been investigated, but does remain a possibility for future investigation.

The hypothesis that was initially addressed by these studies was that the increased number of myofibroblasts found in the *lamina reticularis* of asthmatic fibroblasts is a result of hyperproliferation driven by production of autocrine ligands. Overall the results in this thesis are in agreement with the hypothesis. The results suggest that asthmatic fibroblasts do exhibit an increased intrinsic ability to proliferate through the production and subsequent utilisation of autocrine growth factors. The second half of the hypothesis was that asthmatic fibroblasts exhibit an exaggerated response to TGFβ2 when compared to non-asthmatic fibroblasts. Generally, the asthmatic fibroblasts expressed higher levels of growth factors in response to TGFβ2. From the results presented, it is unclear if this is due to the 'priming' effect of some of the autocrine growth factors, or due to the lack of regulatory mechanisms in the asthmatic fibroblasts.

These studies indicate that a combination of: 1) increased number of fibroblasts through autocrine proliferation, 2) an increased capacity to differentiate into myofibroblasts that secrete paracrine smooth muscle mitogens and 3) the potential for fibroblasts to undergo phenotypic differentiation to smooth muscle cells, can result in increased asthmatic airway remodelling, which results in long term morbidity associated with chronic asthma.



APPENDIX A

Buffer and Reaction mix compositions

1. TISSUE CULTURE MEDIA

i. Tissue culture medium

For all types of culture medium: *RPMI 1640, UltraCulture*

Component	**Serum Free**	**1% FBS**	**5% FBS**	**10% FBS**
Culture medium	490ml	485ml	470ml	440ml
Penicillin / streptomycin (100X)	5ml	5ml	5ml	5ml
L-Glutamine (200mM)	5ml	5ml	5ml	5ml
Foetal Bovine Serum	0ml	5ml	20ml	50ml

ii. Collagen I *(for cell culture surface coating)*

- Vitrogen 100 collagen I solution (100µl)
- Sterile H_2O (9.9ml)

2. GENERAL PURPOSE BUFFERS

i. Dulbecco's phosphate buffered saline (DPBS)

- 0.2g Potassium chloride (KCl)
- 8.0g Sodium chloride (NaCl)
- 0.2g Potassium dihydrogenophosphate (KH_2PO_4)
- 1.15g Sodium phosphate (Na_2HPO_4)

add to 1L deionised water adjust pH to 7.35 using 1M HCl or 1M NaOH

- 100mg 1M hydrated magnesium chloride ($MgCl_2.6H_2O$)
- 130mg 1M hydrated calcium chloride ($CaCl_2.2H_2O$)

ii. Tris-HCl

- 40mM Tris-HCl pH 7.4 (6.30g 1M Tris-HCl + 1L deionised water)

iii. Tris-buffered saline (TBS)

- 0.05M Tris base (6.06g Trizma™ base + 1L deionised water)
- 0.14M NaCl (8.47g NaCl + 1L deionised water)

Adjust pH to 7.6 (with 1M HCl)

3. IMMUNOCYTOCHEMISTRY

i. Inhibitor for endogenous peroxidase

- 15ml Tris Buffered Saline
- 500µl hydrogen peroxide (30%)
- 150µl sodium azide (NaN_3)

ii. Streptavidin- Biotin solution

- 5ml Tris Buffered Saline (0.05M Tris/HCl, pH 7.6)
- 45µl reagent A (Streptavidin, in 0.01M phosphate buffer, 0.15M NaCl, 15mM NaN_3, pH 7.2)
- 45µl reagent B (Biotinylated horseradish peroxidase, in 0.01M phosphate buffer, 0.15M NaCl, 15mM NaN_3, pH 7.2)

iii. 3,3'-diaminobenzidine chromogen (DAB)

- 500µl 10X substrate buffer
- 4.5ml deionised water
- 300µl 3,3'-diaminobenzidine chromogen solution

mix thoroughly

- 150µl hydrogen peroxide substrate solution
- 10µl NaN_3

incubate for 10 minutes at room temperature

4. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

i. Carbonate coating buffer

- 0.025M sodium bicarbonate (1.05g $CHNaO_3$ + 500ml dH_2O)
- 0.025M sodium carbonate (1.33g CNa_2O_3 + 500ml dH_2O)

mix together thoroughly to achieve 1L carbonate coating buffer

ii 1M HCl

- 82.7ml concentrated HCl
- 917.3ml deionised water

iii. Tris Buffered Saline + Tween® (TBST) (1 litre)

- 20mM Tris-HCL (3.15g + 999.5ml deionised water)
- 150mM NaCl (8.77g)
- 0.05% (w/v) Tween® (500µl)

adjust pH to 7.6

5. WESTERN BLOTTING

i. Separation gel stock solution

Component	7.5%	10%	12.5%	15%
30% (w/v) acrylamide / 0.8% (w/v) bis acrylamide	22.5ml	30ml	37.5ml	45.0ml
1.5M Tris-HCl, pH 8.8	22.5ml	22.5ml	22.5ml	22.5ml
dH_2O	44.6ml	37.1ml	29.6ml	22.1ml
20% (w/v) SDS	0.45ml	0.45ml	0.45ml	0.45ml

ii. Stacking gel stock solution

Component	
30% (w/v) acrylamide / 0.8% (w/v) bis acrylamide	12.5ml
0.5M Tris-HCl, pH 6.8	25.0ml
dH_2O	62.0ml
20% (w/v) SDS	0.50ml

iii. Ammonium persulphate

- 10% (w/v) ammonium persulphate (0.1g + 1ml dH_2O)

iv. Separation gel mix (for two 1mm thick mini gels)

- Separation gel stock (10ml)
- 10% (w/v) ammonium persulphate (33µl)
- N,N,N,N',N'-tetramethylenediamine (TEMED; 5µl)

v. Stacking gel mix (for two gels)

- Stacking gel stock (5ml)
- 10% (w/v) ammonium persulphate (16.7µl)
- N,N,N,N',N'-tetramethylenediamine (TEMED; 3.8µl)

vi. 5 x sample buffer

- 0.3M Tris-HCl pH 6.8 (10.4ml)
- 50% glycerol (25ml)
- 25% 2-mercaptoethanol (12.5ml)
- 10% SDS (5g)
- 0.01% bromophenol blue

vii. 5x sample buffer + protease inhibitors

- 5x sample buffer (2ml)
- 1M sodium vanadate, Na_3VO_4 (10µl; final conc. 1mM)
- 1M sodium fluoride, NaF (10µl; final conc. 1mM)
- 70mM PMSF (143µl, final conc. 70µM)
- 200mM EGTA (50µl, final conc. 1mM)
- 1 Complete inhibitor cocktail tablet (Boehringer Mannheim) + 200µl EDTA

viii. 1x running buffer (5 litres)

- 0.025M Tris-HCl (15.15g)
- 0.192M glycine (72g)
- 20% (w/v) SDS (25ml, final conc. 0.1%)

adjust pH to 8.3 with 1M HCl and make up to 5 litres with distilled H_2O

ix. Transfer buffer (5 litres)

- 25mM Tris-HCl (15.15g)
- 192mM glycine (72g)
- 20% (v/v) methanol (1000ml)

make up to 5 litres with distilled H_2O and check pH is 8.3

x. Wash buffer (2 litres)

- 5 x PBS (200ml, final conc. 1xPBS)
- 200mM EDTA (10ml, final conc. 1mM)
- 200mM EGTA (10ml, final conc. 1mM)
- 1M NaF (2ml, final conc. 1mM)
- 1M Na_3VO_4 (2ml, final conc. 1mM)
- 70mM PMSF (2ml, final conc. 70µM)
- Tween 20® (1ml, final conc. 0.05%)

Make up to 2 litres with distilled H_2O

xi. Coomassie Brilliant Blue R-250

- 0.00125% (w/v) Coomassie Brilliant Blue R-250
- 45% (v/v) methanol
- 45% (v/v) dH_20
- 10% (v/v) glacial acetic acid

6. RT-PCR AND QUANTITATIVE PCR

i. 20µl RT-reactions (25 reactions)

Component	**1X**	**25X**
Random hexamers (50ng/µl)	1µl	25µl
DNTP mix (10mM each dATP, dCTP DGTP, dTTP)	1µl	25µl
DEPC-treated water	7µl	175µl
mRNA	1µl	(1µl /sample)

Incubate at 65 ℃ for 5 minutes, followed by quenching in ice/ethanol mix for 3 minutes

10X RT buffer (200mM Tris-HCl (pH 8.4), 500mM KCl)	2µl	50µl
25mM $MgCl_2$	4µl	100µl
0.1M DTT	2µl	50µl
RNASEOUT™ Recombinant ribonuclease Inhibitor (40units/µl)	1µl	25µl

Incubate at room temperature for 2 minutes

SUPERSCRIPT™ II reverse transcriptase (50units/µl)	1µl	(1µl / sample)

Incubate at 42 ℃ for 50 minutes

Incubate at 70 ℃ for 15 minutes

Store at -20 ℃ until ready to use

ii. Real-Time qPCR Mastermix

Produces 19 000µl (19ml) mastermix, and stored at -20 ℃ in 850µl aliquots

Component	
10X qPCR buffer	2500µl
25mM $MgCl_2$	5000µl
2.5mM dNTP mix	2000µl
Hot GoldStar (5U/µl)	125µl
dH_2O	9000µl
18S (20x ribosomal 18S RNA primer and probe mix)	375µl

iv. Real Time RT PCR reaction mix

Component	**1x**	**100x (1 plate)**
Target gene F primer	0.225µl	22.5µl
Target gene R primer	0.225µl	22.5µl
Target gene probe	(optimised for each target)	
Water	(0.55µl – probe vol.)	(55µl – probe vol.)
Real-Time qPCR mastermix	19µl	1900µl
Test cDNA	**5µl**	**5µl / reaction**

v. Volume of Taqman probe for 100 reactions

Target gene	Vol. of probe in Taqman reaction mix
α-SMA	22μl
TGFβ1	18μl
Endothelin-1	40μl
CTGF	40μl
HBEGF	3.8μl
ADAM-12	12μl
ADAM-33	38μl

APPENDIX B

Determining viable cell number by direct cell counts, using a haemocytometer

The following is a protocol for using a haemocytometer to determine the number of viable cells in a cell suspension.

The haemocytometer and coverslip were thoroughly cleaned using 70% ethanol followed by a rinse with distilled water. The coverslip was then placed on the rest supports of the haemocytometer (see figure B1) and pressed until the coverslip adhered to the haemocytometer.

The cells in the 75cm^2 culture flask were detached with Trypsin (as described in section 2.2.3) and resuspended in 1ml supplemented RPMI 1640.

Next, the cell suspension was agitated by repetitive aspirations with a 200µl pipette to ensure thorough mixing and no cell aggregation. 20µl of the cell suspension were added to 80µl of Trypan blue (Sigma, Dorset, UK) and thoroughly mixed. The Trypan blue, which is a dye that is taken up by non-viable cells, was diluted from stock in distilled water (1:10). Next, 20µl of this cell suspension in Trypan blue, were loaded into each of the two counting chambers of the haemocytometer (figure B1), and viewed under a phase contrast microscope (x100). Non-blue cells were counted according to the convention described in figure B1. The number of cells in five 1mm^2 squares was counted and the mean number determined.

In order to determine the total number of cells in the sample (i.e. in the 1ml cell suspension) the following calculation was performed:

Cells/ml = mean number of cells x dilution factor (5) x 10^4

where the dilution factor of 5 arises from the dilution in Trypan blue.

Once the number of cells in the cell suspension was determined it was possible to dilute it accordingly to achieve a desired final cell concentration.

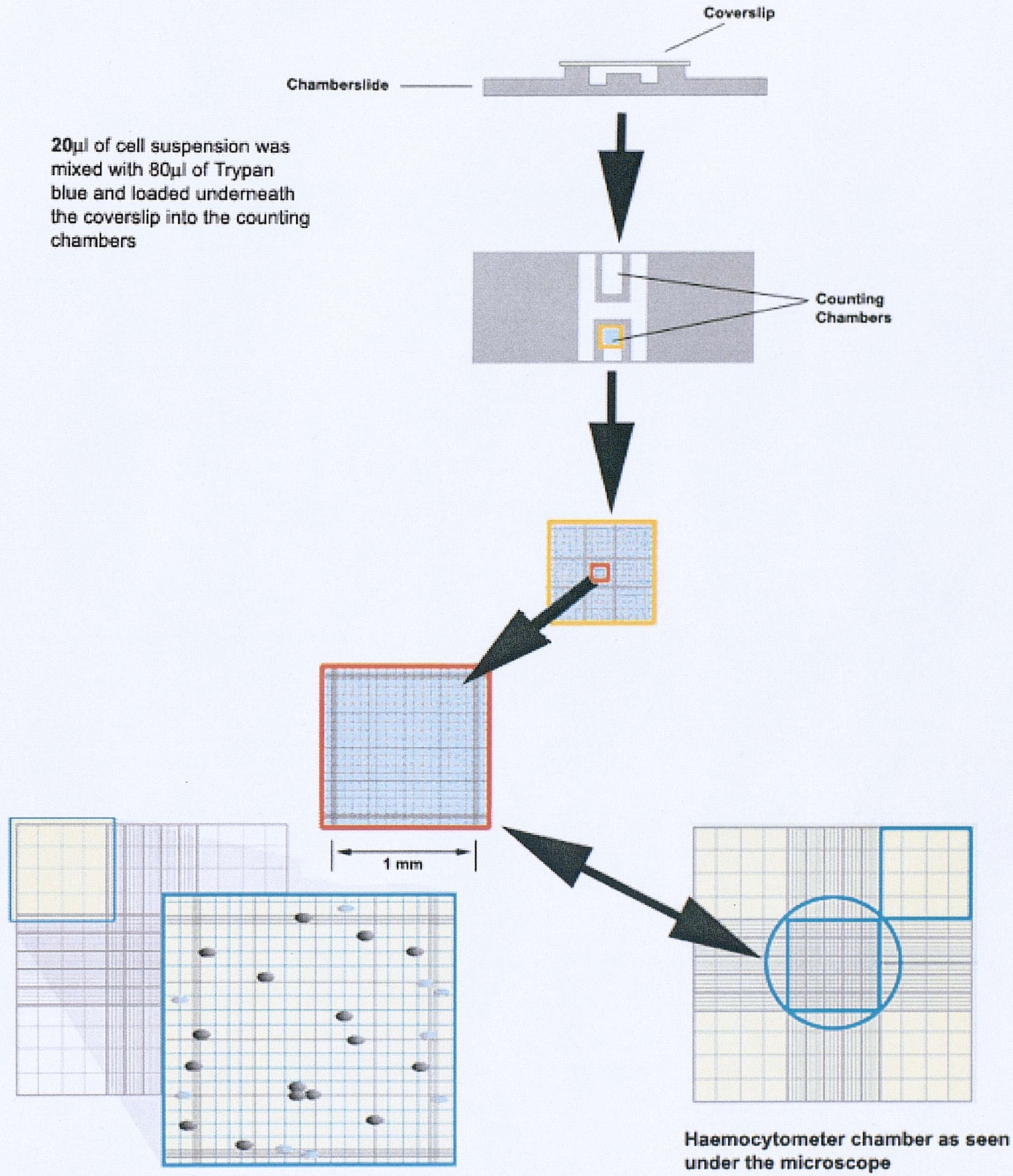


Figure B1: Determining cell number by use of a Haemocytometer
(adapted from www.bhtafe.edu.au/bhi2/toolbox4/_TEST501/task33.htm)

APPENDIX C

Plate layouts for ^{3}H Thymidine incorporation mitogenesis assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
C	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
D	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
E	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
F	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
G	Medium	NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		NR6/HER 5×10^3 cells		Medium
H	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium

Figure C1: Diagram to show the culture of NR6/HER fibroblasts. The cells were seeded at a density of 0.5×10^3 cells/well in 10% FBS/DMEM for 2 days, after which the medium was removed and replaced with 80µl 1% FBS/DMEM and incubated overnight.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium 40µl	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium	EGF (40ng/ml) 160 µl		120µl Ultraculture		Conditioned medium 150µl		Conditioned medium 150µl		Conditioned medium 150µl		Medium
C	Medium	120µl Ultraculture		120µl Ultraculture		Ultraculture 75µl		Ultraculture 75µl		Ultraculture 75µl		Medium
D	Medium	120µl Ultraculture		120µl Ultraculture		Ultraculture 75µl		Ultraculture 75µl		Ultraculture 75µl		Medium
E	Medium	120µl Ultraculture		120µl Ultraculture		Ultraculture 75µl		Ultraculture 75µl		Ultraculture 75µl		Medium
F	Medium	120µl Ultraculture		Ultraculture Negative control		Ultraculture 75µl		Ultraculture 75µl		Ultraculture 75µl		Medium
G	Medium	120µl Ultraculture		Ultraculture Negative control		Ultraculture 75µl		Ultraculture 75µl		Ultraculture 75µl		Medium
H	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium

Figure C2: Diagram to show the serial dilution (1:4) of human recombinant EGF used to construct a standard curve and the serial dilution (1:2) of conditioned medium.
Following dilution, 80µl of reagent from each well was added to the corresponding well in the culture tray (figure C1)

APPENDIX D

Optimal plating density and stimulation time for NR6/HER mitogenesis assays

Before the mitogenesis assays were carried out, it was necessary to determine the optimal plating density, i.e. the one that would result in the greatest ^{3}H thymidine incorporation by the NR6/HER fibroblasts. On determining the optimal plating density, it was then necessary to establish the time at which the cell cycles of most of the cells were synchronised. This was achieved by determining the stimulation time after which the greatest ^{3}H thymidine incorporation was achieved. It was necessary to determine these two parameters every time a new stock of cells were resuscitated from storage in liquid nitrogen, since culture conditions can alter the timing of their cell cycles. As a guideline, an incubation time of 26 hours (as determined in previous studies within the group using NR6/HER fibroblasts (Dr. Audrey Richter, personal communication)) was used for establishing the optimal plating density.

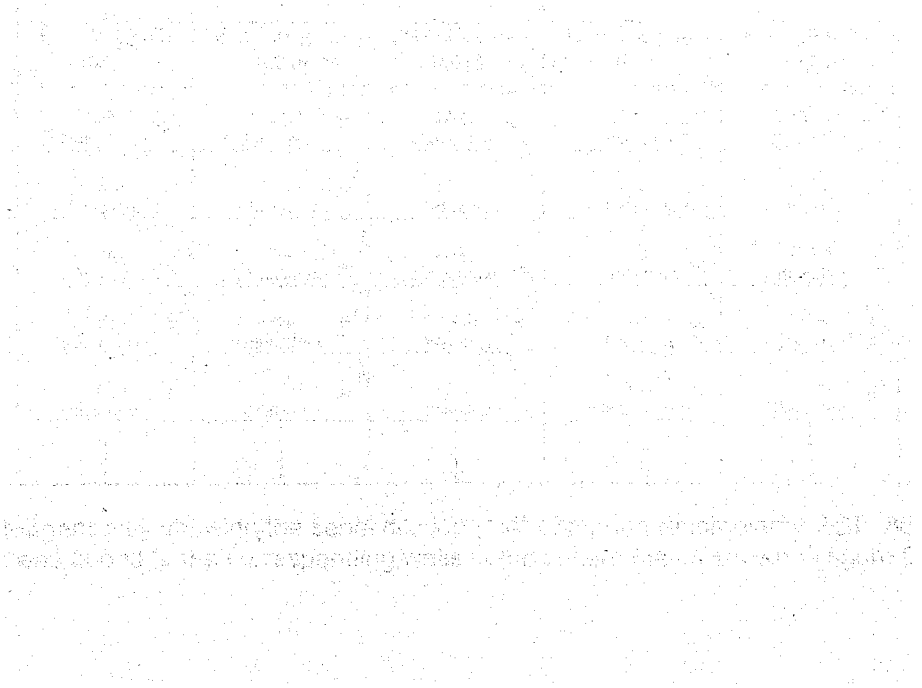
D1. Determining the optimal plating density

NR6/HER fibroblasts were seeded at 5 different densities into a 96 well tray, at the beginning of the experiment (figure D1). The cells were incubated (37°C, 5% CO_2) in 10% FBS/DMEM for 48 hours, after which time the medium was removed and replaced with 80µl 1% FBS/DMEM and incubated overnight. To stimulate the cells, human recombinant EGF was used and serially diluted in a reagent tray (as shown in figure D2). 80µl of stimuli from the reagent tray were then added to the corresponding wells in the culture tray and incubated for 26 hours (37°C, 5% CO_2). Next, 30µl of ^{3}H Thymidine and 3µl FUDR were diluted in 1470µl UltraCulture and 25µl of this were added to each well, and incubated for 2 hours. The cells were processed for DNA precipitation and the ^{3}H Thymidine uptake was measured as described in section 2.4.2. The results obtained are shown in figure D3.

From the figure it can be seen that the highest ^{3}H Thymidine incorporation was achieved by the cultures in which the starting seeding density was $1x10^3$ cells/well.

D2: Determining the time at which the majority of the cells were in S phase

Once the optimal plating density was determined, the NR6/HER fibroblasts were seeded at $1x10^3$ cells/well. As described above, the cells were incubated for two days and then the medium changed to 1% FBS/DMEM. In order to determine the optimal stimulation time, the cells were exposed to the stimuli for different lengths of time, ranging from 22hours to 30 hours as illustrated in figure D4. Cells were stimulated with human recombinant EGF, which was serially diluted in a reagent tray (figure D2) before addition to the cells. After stimulation, cells were pulsed and processed for ^3H Thymdine incorporation as described in section 2.4.3. The cultures with the highest ^3H incorporation were considered to be the one subjected to the optimal stimulation time. The results obtained are shown in figure D5.



	1	2	3	4	5	6	7	8	9	10	11	12
A	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium
B	medium	1×10^3 cells/well		2.5×10^3 cells /well		5×10^3 cell s/well		7.5×10^3 cells /well		1.0×10^4 cells /well		medium
C	medium											medium
D	medium											medium
E	medium											medium
F	medium											medium
G	medium											medium
H	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium	medium

Figure D1: Culture plate layout for optimisation of initial seeding density. NR6/HER fibroblasts were seeded at cell densities ranging from $1x10^3$ to $1x10^4$ cells/well. Cells were stimulated with EGF from the corresponding wells in the reagent tray (figure D2), and processed for ^3H Thymidine incorporation as described in section 2.4.3

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		EGF (20ng/ml) 160 µl		EGF (20ng/ml) 160 µl		EGF (20ng/ml) 160 µl		EGF (20ng/ml) 160 µl		EGF (20ng/ml) 160 µl		
C		40µl → 120µl Ultraculture		40µl → 120µl Ultraculture		40µl → 120µl Ultraculture		40µl → 120µl Ultraculture		40µl → 120µl Ultraculture		
D		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		
E		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		
F		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		
G		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		120µl Ultraculture		
H												

Figure D2: Reagent tray showing the serial dilution (1:4) of human recombinant EGF. 80µl of each reagent was added to the corresponding wells in the culture plates shown in figure D1.

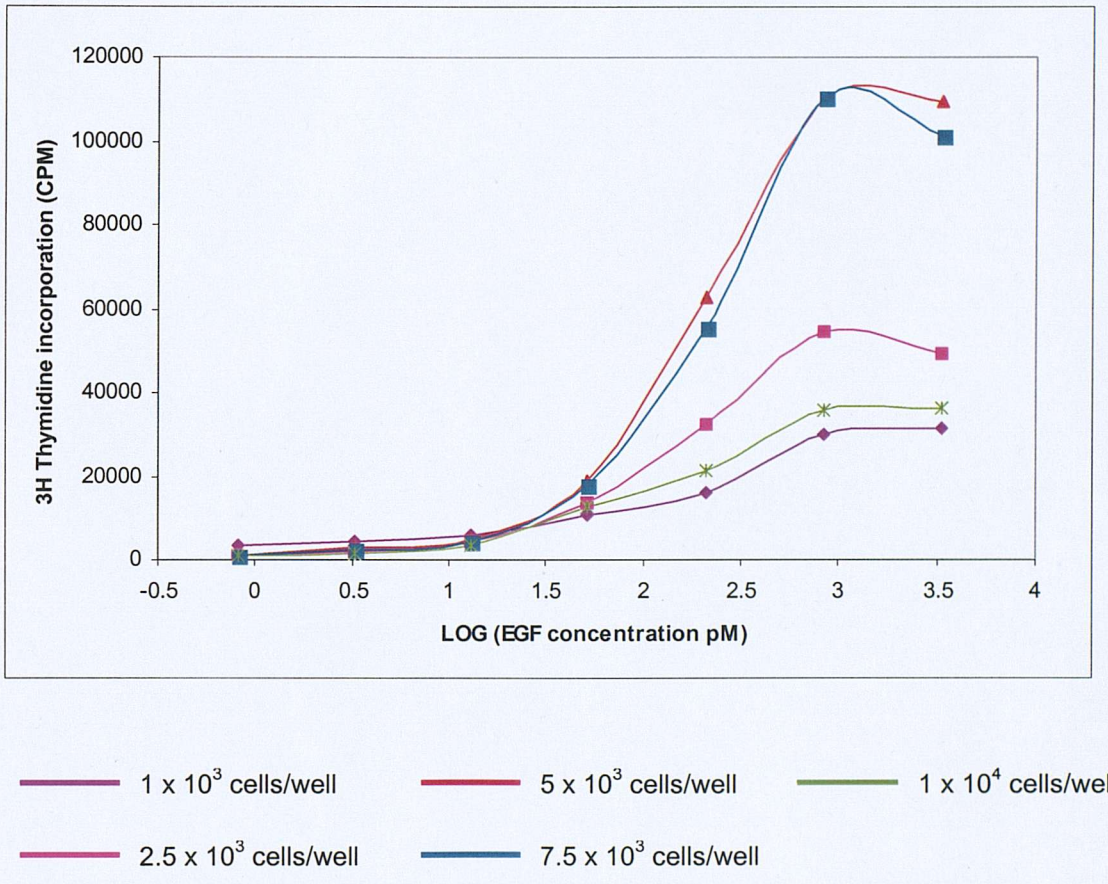


Figure D3: The ^{3}H Thymidine incorporation according to the initial seeding density of NR6HER fibroblasts. The ^{3}H thymidine incorporation generally increased with increasing starting cell density, except when the starting cell density was $1x10^4$ cells/well (green) in which case the ^{3}H Thymidine incorporation was drastically reduced. This may be due to the cells becoming over-confluent and detaching. The optimal starting density was determined to be $5x10^3$ cells/well (red).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium	Stimulate at **10:00am** Pulse at 4:00pm (+1 day) Total incubation time = **30H**		Stimulate at **12:00pm** Pulse at 4:00pm (+1 day) Total incubation time = **28H**		Stimulate at **2:00pm** Pulse at 4:00pm (+1 day) Total incubation time = **26H**		Stimulate at **4:00pm** Pulse at 4:00pm (+1 day) Total incubation time = **24H**		Stimulate at **6:00pm** Pulse at 4:00pm (+1 day) Total incubation time = **22H**		Medium
C	Medium											Medium
D	Medium											Medium
E	Medium											Medium
F	Medium											Medium
G	Medium											Medium
H	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium

Figure D4: Culture plate layout for stimulatory time optimisation. The NR6/HER fibroblasts were seeded at a cell density of $1x10^3$ cells/well. The cells were stimulated with EGF from the corresponding wells in the reagent tray (figure D2) at the times given. The following day at 4:00pm, the cells were pulsed with ^3H Thymidine and processed to determine ^3H Thymdine incorporation.

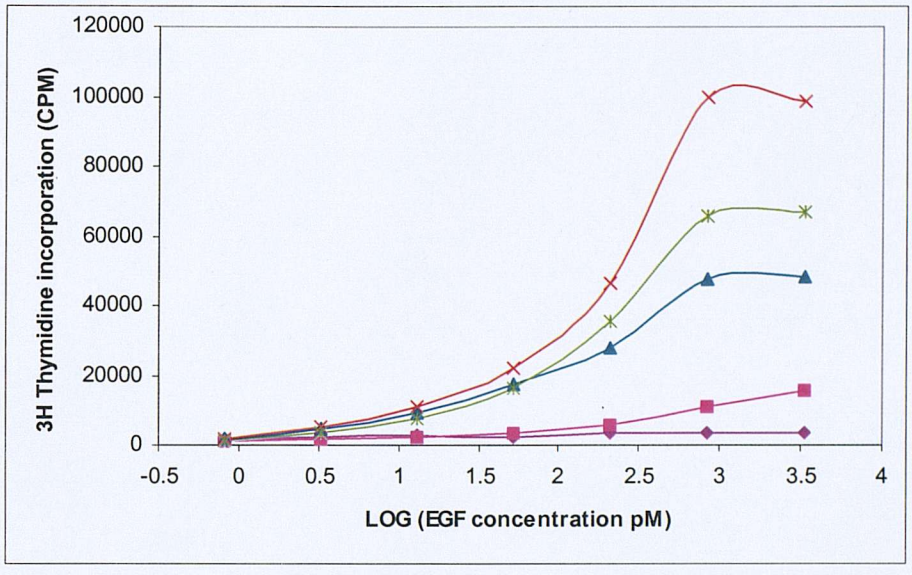


Figure D5: The ^{3}H Thymidine incorporation according to stimulation time. Successful mitogenesis assays depend on the cell cycle phase of the cells being synchronised. It is during S phase than new DNA is synthesised and therefore incorporates ^{3}H Thymidine. From the results, it can be seen that at 28 hours (red) after stimulation, most cells are synchronised (as indicated by the increased ^{3}H Thymidine incorporation). This was therefore the stimulatory time used in these studies.

APPENDIX E

Investigating Gene expression in primary asthmatic and non-asthmatic fibroblasts.

Gene expression was assessed following *in vitro* culture for up to 144 hours. The method by which this was measured was by real-time quantitative RT-PCR. RT-PCR uses RNA as it's template and produces a DNA copy by a reverse transcriptase enzyme. The following sections are a detailed account of the precautions and background to the techniques that were employed, starting with the successful isolation of mRNA from primary cells to the relative quantification of gene expression using Taqman™ real time technology.

E.1 Reverse Transcription PCR

E.1.1 RNA isolation

One of the most important factors for the synthesis of cDNA is the isolation of intact RNA. It is important that the quality of the RNA is high, since this dictates the maximum amount of sequence that can be converted into cDNA. It is therefore necessary to prevent adventitious introduction of RNases into the RNA preparation. RNA is more susceptible to degradation than DNA, due to the 2' hydroxyl groups adjacent to the phosphodiester linkages in RNA able to act as intramolecular nucleophiles in base and enzyme catalysed hydrolysis. In contrast DNases require metal ions for activity and can be inactivated with chelating agents e.g. EDTA. RNases are plentiful, and contamination is possible from a number of sources. It is essential to ensure all apparatus is sterile, and that all solutions are RNase free. Autoclaving of solutions will kill bacteria, but RNases liberated from bacterial death will still be active. Solutions were therefore prepared using DPEC. It is also possible to protect from RNases by the use to RNase inhibitors.

E.1.2 Reverse Transcriptase Polymerase Chain Reaction

This is a method by which PCR is used to determine levels of gene expression.

RT-PCR uses mRNA as it's template, and produces a DNA copy by the enzyme revere transcriptase. Reverse transcriptase is encoded by retrovirus RNA, and extends the newly synthesized cDNA molecule in the 3' to 5' direction.

Essentially, the reverse transcription generates cDNA which can then be amplified using standard PCR.

E.1.3 Priming first strand cDNA synthesis

A first strand cDNA synthesis reaction may be primed in one of three main ways. The first method is the most non-specific of the three techniques. Random hexamer primers are used typically when a particular mRNA is difficult to copy in it's entirety (Gerard et al. 1992). With this method, all RNAs in a population are templates for first strand cDNA synthesis.

The second method is a more specific primary method. An oligo(dT) primer is used to hybridise to the 3' poly(A) tails which are found at the end of eukaryotic mRNA. However, since poly(A) RNA constitutes approximately 1% to 2% of total RNA, the amount and complexity of cDNA is considerably less than when random hexamers are used.

The third method, is the most specific and uses a gene-specific primer, which contains sequence information of the target cDNA. First stand synthesis can be primed with the anti-sense PCR primer (i.e. the primer that hybridises nearest to the 3' terminus of the mRNA.

E.1.4 The effect of magnesium ions of the efficiency of the RT reaction

Mg^{2+} ions form a soluble complex with dNTPs which is essential for dNTP incorporation. Mg^{2+} ions are also required for the polymerase activity of the RT enzyme. The concentration of Mg^{2+} ions has a great effect on the efficiency and specificity of the reaction. High concentrations of Mg^{2+} improves the efficiency of the amplification and reduces specificity. The converse is true for low concentrations of Mg^{2+}.

E.1.5 The Reverse Transcriptase enzyme

Reverse transcriptase is used to synthesise first strand cDNA from RNA. Reverse transcriptase can be purified from several sources, the most common being avian myeloblastosis (AMV) and Moloney murine leukaemia VIRUS (MMLV). AMV mreverse transcriptase is an RNA-dependant DNA polymerase that uses single stranded RNA as a template and can synthesise cDNA in a 3' to 5' direction, if a primer is present. As well as DNA polymerase activity, this enzyme also exhibits ribonuclease H activity , which denatures the mRNA strand once copied.

MMLV reverse transcriptase works in a similar fashion, although it lacks the DNA endonuclease activity, an has a lower RNase H activity. It therefore has a greater chance of producing full-length copies of large mRNA molecules. A modified form of the MMLV reverse transcriptase, Superscript II (GIBCO-BRL) is also available. This enzyme is genetically engineered to eliminate the RNase H activity. Use of such an enzyme results in greater full-length cDNA synthesis and higher yields of first strand cDNA.

E.1.6 Cycling parameters for RT reaction

The RT reaction consists of four main stages. *1)Denaturation:* typically is carried out at 70°C. A denaturation step is necessary to remove any secondary structures, which may have formed in the RNA molecules. Such structures can be formed due to complementary base pair interactions within the RNA strand. This step ensures such structures are removed. *2) Annealing:* This step ensures the primer anneals to it's complementary sequence of the target DNA. *3) cDNA synthesis:* This is normally carried out at 40°C and is the optimal temperature for the reverse transcriptase enzyme. During this stage, a cDNA strand complementary to the RNA molecule is synthesized.

E.2 Real time quantitative PCR

E.2.1 Optimisation of primer and probe concentration

The RT-reaction does not include any amplification steps, and hence it is assumed that the amount of cDNA synthesised is equal to the amount of mRNA template.

One µl of each cDNA sample was taken and mixed together to prepare a mixture of all the cDNA's, required to produce a standard curve. 20µl of cDNA (5ng/µl) mix was then added to a fresh eppendorf and serial 10-fold dilutions (with DPEC-treated RNase free water) were conducted to produce a dilution series ranging from 5ng/µl to5pg/µl.

Primers were supplied at 100µM after re-solubilisation. Primers for Taqman reactions are required at 10µM, hence 35µl of sense and antisense primers were diluted 1:10 (in RNase free DPEC-treated water.) The sense to antisense primer concentration ratios investigated were 50nM:50nM, 300nM:300nM and 900nM:900nM. The volumes used to determine optimal primer concentrations are shown in table E1 and a representative Taqman trace obtained from the primer optimisation experiments are shown in figure E1.

5µl of cDNA was plated into a well to which 20µl of prepared mastermix was added, using a pipette and with sufficient force to ensure mixing. Each sample was loaded in duplicate. The plate was then sealed and briefly spun in a centrifuge (1200 rpm 60 secs) to ensure all components were at the bottom of the wells and that no air bubbles were present. After which the plate was placed in the Thermal cycler (ABI Prism™ 7700 sequence detection system) under the cycling conditions indicated in table E2. On determination of the optimum sense:antisense primer ratio, the PCR was repeated using the optimal concentrations. As before 5µl of cDNA was added to each well. As well as the cDNA, 5µl of the corresponding –RT reaction products were included on the plate (see figure E2). A standard curve was also included on all plates (figure E3).

Sense:Antisense primer ratio	Component	For 1 reaction	For 16 reactions (Standard curve)
50nM : 50nM	Taqman reaction mix	12.5µl	200µl
	Sense primer	0.13µl	2.08µl
	Antisense primer	0.13µl	2.08µl
	Probe	0.5µl	8µl
	Water	6.74µl	107.84µl
300nM : 300nM	Taqman reaction mix	12.5µl	200µl
	Sense primer	0.75µl	12µl
	Antisense primer	0.75µl	12µl
	Probe	0.5µl	8µl
	Water	5.5µl	88µl
900nM : 900nM	Taqman reaction mix	12.5µl	200µl
	Sense primer	2.25µl	36µl
	Antisense primer	2.25µl	36µl
	Probe	0.5µl	8µl
	Water	2.5µl	40µl

Table E1: The volume of Taqman PCR components for endothelin-1 primer optimisation

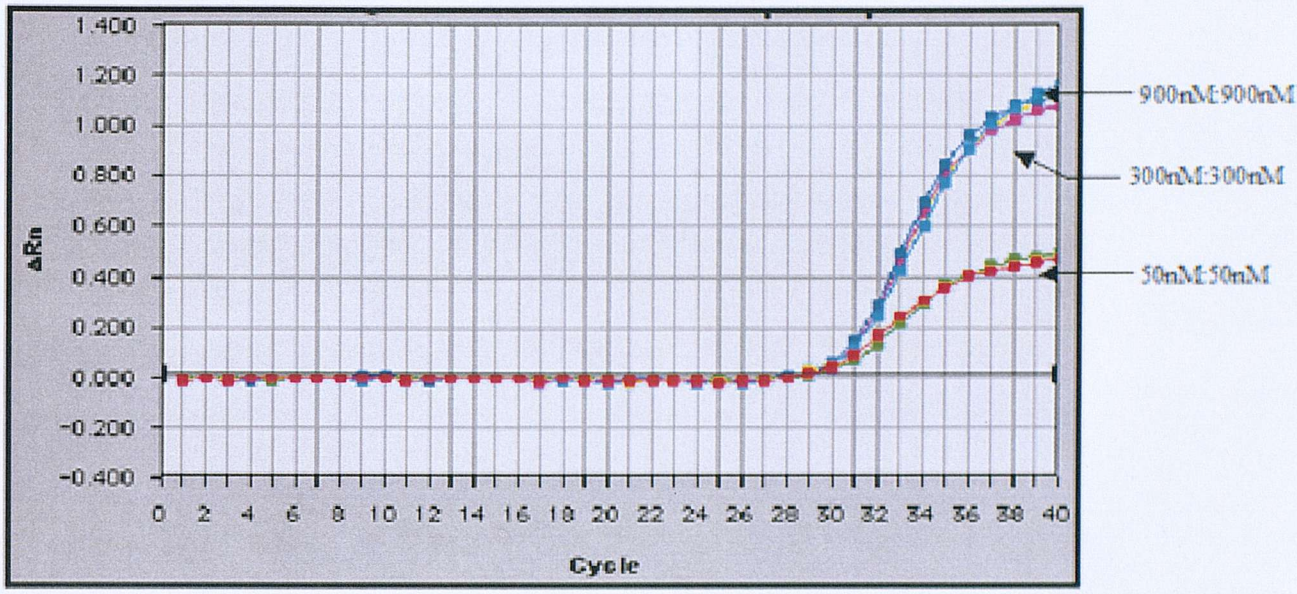


Figure E1: Taqman traces for the primer optimisation experiments. As can be seen the amplitude of the curve is dependant on the contration of primers used. There is no further increase in fluroscence with 900nM of forward and reverse primers over 300nM. In such a case, a concentration of 300nM would be used. When analysing data, the actual level of fluorescence does not effect the result, since the cycel number at which the curve crosses the threshold is considered. Howvever, a higher fluorscence for a given cycle number is indicative of a more efficient reaction.

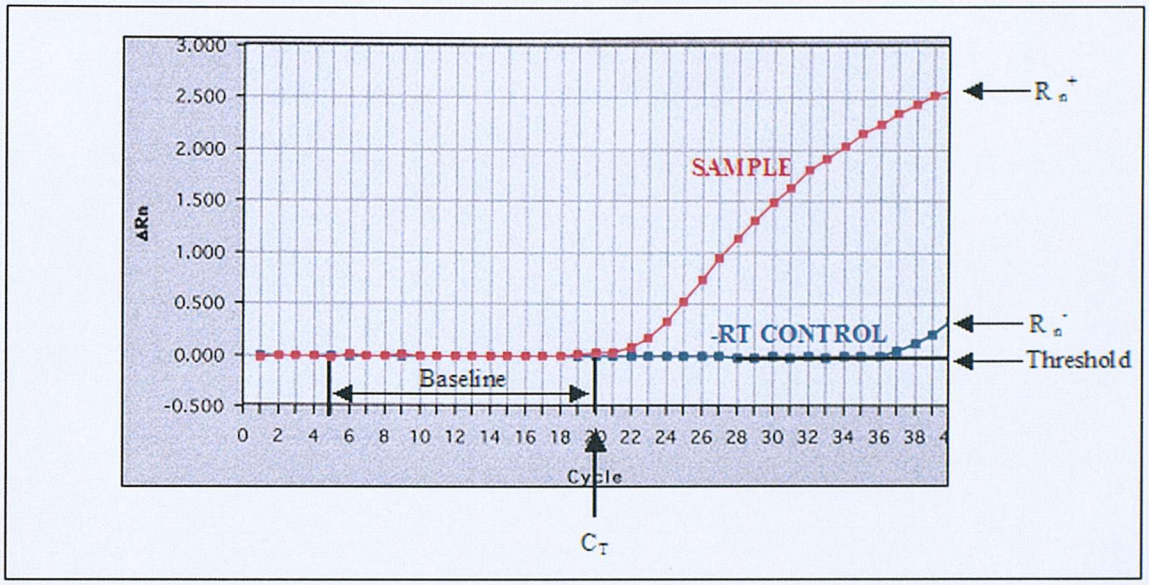


Figure E2: Characteristics of the Taqman Amplification plot illustrating the signal from a template which has undergone reverse transcription and a negative reverse transcription control

Time and temperature for thermal cycling			
Initial steps		Each of 40 cycles	
		Melt	Anneal/Extend
HOLD	HOLD	CYCLE	
2 min	10 min	15 sec	1 min
50°C	95°C	95°C	62°C

Table E2: The thermal cycling conditions required for Taqman quantitative PCR

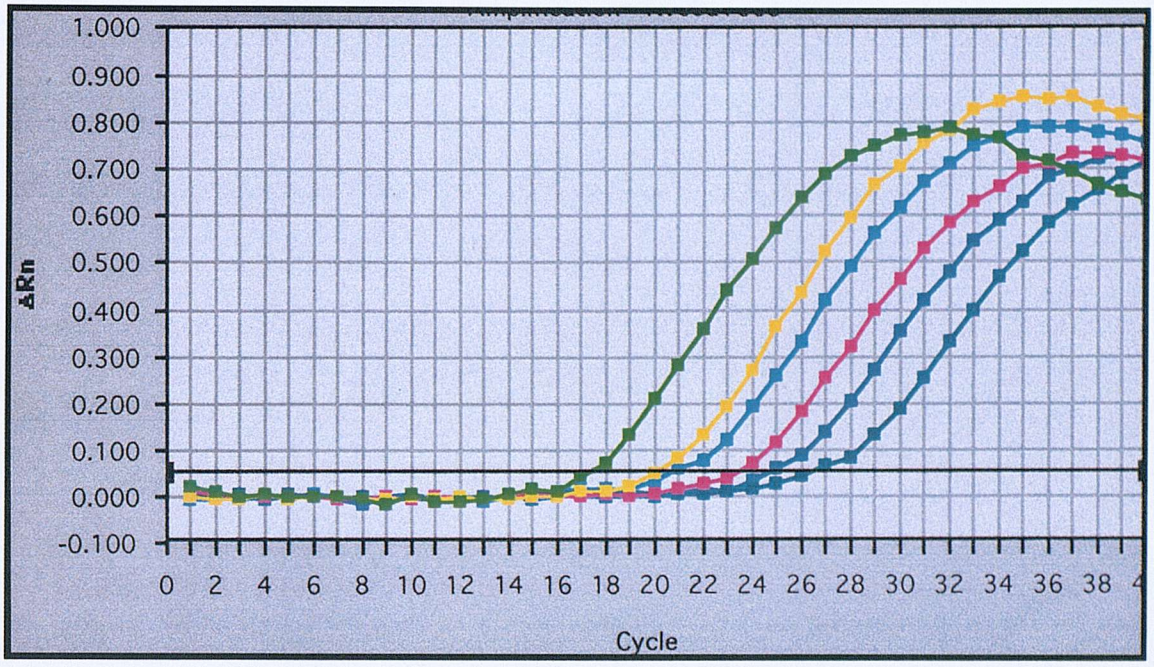


Figure E3: Taqman trace illustrating a dilution series of pooled cDNA samples.
One ml of each of the cDNA samples were harvested and pooled to make a cDNA standard. The cDNA standard was then diluted and included on each plate. The trace illustrates the 18S rRNA expression diminishing over the concentration series. The undiluted sample (light green line) crosses the threshold at an earlier cycle number than the more diluted sample due to the presence of more template. The most diluted sample (1:1000) represented by the dark green line, which crosses the threshold at the latest cycle number indicating that this sample has the least amount of starting template.

E.2.2 Worked example to illustrate the use of a standard curve for relative quantitation of gene expression

Figure E4 shows that plate setups for the relative quantitation of CTGF mRNA where the target and endogenous refernce are amplified in the same tube. Columns 1 to 6 contain cDNA from the samples under test and each sample was loaded in triplicate. Dilutions of a cDNA 'standard' prepared from the combination of all samples were loaded in columns 7 to 9. The C_T values obtained from the 'standards' were used to construct the standard curves for the amplification of CTGF and 18S rRNA shown in figure E4. A dilution series of cDNA was used to construct the standard curves, and their corresponding C_T values obtained from the amplification plots.

In order to determine the input amount of template for the particular gene (i.e. a determination of the amount of gene expressed) the calculations shown below were carried out.

i) As can be seen from figure E5, the standard curves exhibit linear proportionality and can be represented by the general equation:

$$y = mx + c$$

where in this case: $y =$ C_T value

$m =$ gradient of the standard line

$c =$ ordinal intercept

for the 18S rRNA standard curve: $y = -3.44x + 16.979$

for the CTGF standard curve: $y = -3.309x + 27.019$

ii) Using the corresponding above equations and the C_T values shown in table E3, the Log (cDNA input) is determined:

Since $y = mx + c$ it follows that: $x = \frac{(y - c)}{m}$

For the 18S rRNA primers and sample 33 ($C_T = 17.21$ – see table E3):

$$x = \text{Log (cDNA input)} = \frac{(y - c)}{m} = \frac{17.21 - 16.979}{-3.44}$$

$$= -0.120$$

$$\text{therefore Log (cDNA input)} = -0.120$$

iii) Determine input cDNA (ng): ANTILOG –0.120 = 0.758

therefore 0.758 dilution of neat sample.

Neat sample contained 25ng (see table A2) ∴ 0.758 x 25ng = 18.95ng

iv) The same steps were repeated to determine the ng cDNA input for the reaction with the CTGF primers.

For sample 33, $C_T = 31.92$ ∴ Log (cDNA input) = -1.48

∴ cDNA input = 0.03311

that is 0.012 dilution of neat sample. 0.03311 x 25ng = 0.83ng cDNA

∴ Normalised amount of CTGF ($CTGF_N$) =

$$\frac{\text{ng cDNA input for CTGF}}{\text{ng cDNA input for 18 rRna}} = \frac{0.83}{18.95} = \mathbf{0.04}$$

Sample	18 rRNA (mean C_T)	CTGF (mean C_T)	Normalised
3-33	17.21	31.93	0.038
3-34	16.62	29.87	0.108
3-35	15.72	28.75	0.129
3-36	16.12	33.31	0.007
3-37	16.04	31.12	0.031
3-38	16.31	28.83	0.181
3-39	16.49	29.43	0.135
3-40	18.17	33.74	0.021
3-41	15.88	30.09	0.057
3-42	17.49	33.11	0.020
3-43	22.70	34.96	0.183
3-44	16.38	30.29	0.069
3-45	15.99	31.47	0.023
3-46	16.01	31.1	0.031
3-47	17.11	30.03	0.134
3-48	14.74	28.63	0.072

Table E3: A table to show the C values for the 18S rRNA and CTGF primers for each of the Samples from a representative tray. The normalised gene expression is also shown

Input amount of RNA	Dilution factor	LOG (Dilution factor)	CTGF (Mean C_T)	18S rRNA (Mean C_T)
25	1	0	27.41	17.20
2.5	0.1	-1	30.01	20.53
1.255	0.05	-1.301	31.09	21.39
0.5	0.02	-1.699	32.83	22.55
0.25	0.01	-2	33.52	23.49
0.025	0.001	-3	37.21	27.72

Table E4: Data used to construct standard curves for the CTGF and 18S rRNA primers

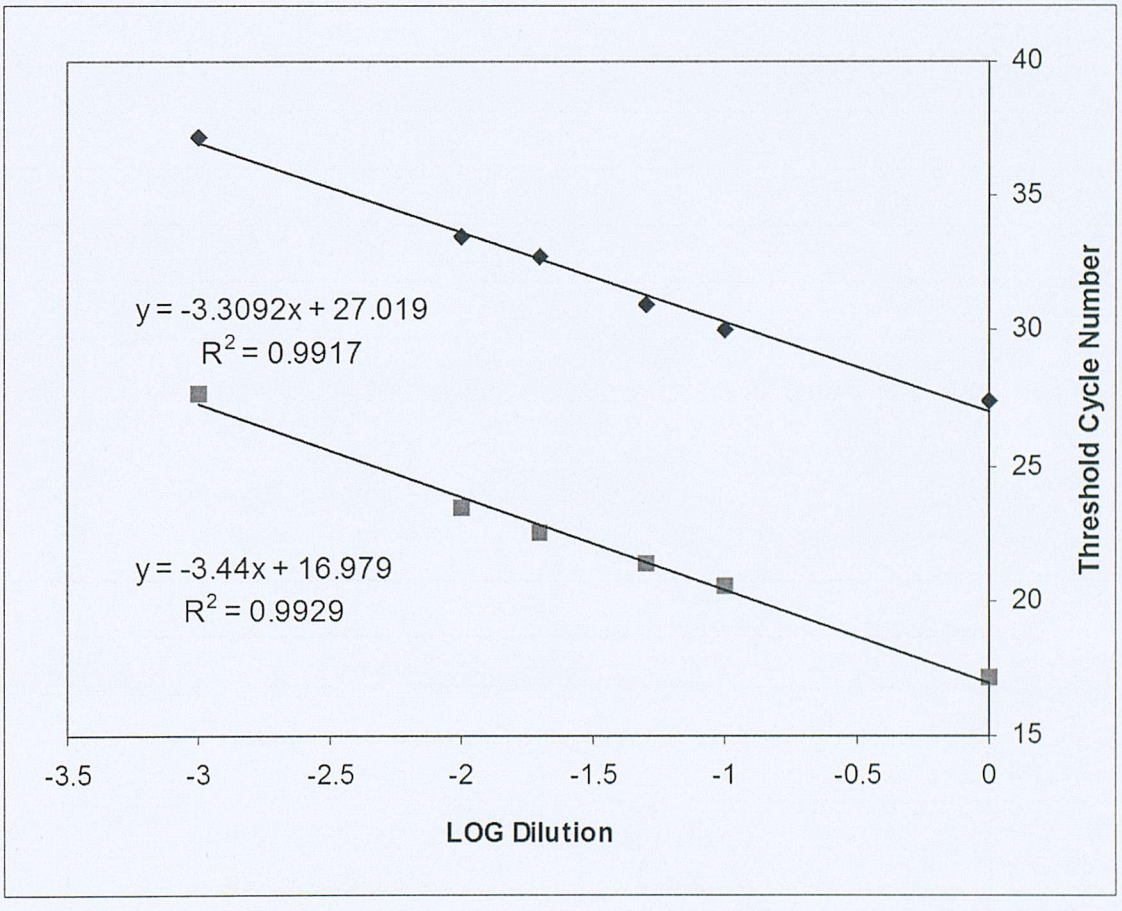


Figure E4: Standard curves for CTGF (blue) and 18S rRNA (purple) constructed from table E4, used to convert C_T values to LOG dilution.

APPENDIX F

Taqman Primer and probe binding locations

α-smooth muscle actin

Forward: 5'-GAC AGC TAC GTG GGT GAC GAA-3'

Reverse: 5'-TTT TCC ATG TCG TCC CAG TTG-3'

Probe: 5'-TGA CCC TGA AGT ACC CGA TAG AAC ATG GC-3'

Connective tissue growth factor

Forward: 5'-GCG GCT TAC CGA CTG GAA-3'

Reverse: 5'-GGA CCA GGC AGT TGG CTC TA-3'

Probe: 5'-CAC GTT TGG CCC AGA CCC AAC TAT GA-3'

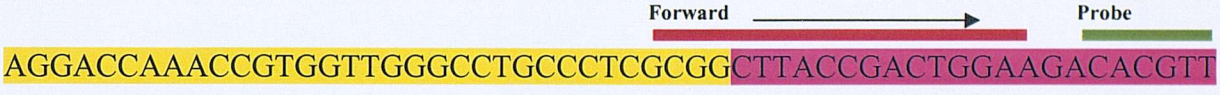
CCAAAGATGGTGCTCCCTGCATCTTCGGTGGTACGGTGTACCGCAGCGGAGAGTC

EXON 4

CTTCCAGAGCAGCTGCAAGTACCAGTGCACGTGCCTGGACGGGGCGGTGGGCTG

CATGCCCCTGTGCAGCATGGACGTTCGTCTGCCCAGCCCTGACTGCCCCTTCCCGA

GGAGGGTCAAGCTGCCCGGGAAATGCTGCGAGGAGTGGGTGTGTGACGAGCCCA



AGGACCAAACCGTGGTTGGGCCTGCCCTCGCGGCTTACCGACTGGAAGACACGTT

EXON 5

TGGCCCAGACCCAACTATGATTAGAGCCAACTGCCTGGTCCAGACCACAGAGTGG

AGCGCCTGTTCCAAGACCTGTGGGATGGGCATCTCCACCCGGGTTACCAATGACA

ACGCCTCCTGCAGGCTAGAGAAGCAGAGCCGCCTGTGCATGGTCAGGCCTTGCGA

AGCTGACCTGGAAGAGAACATTAAG

Endothelin-1

Forward: 5'-CGT CCC TGA TGG ATA AAG AGT GT-3'

Reverse: 5'-ACG TGC TCG GGA GTG TTG A-3'

Probe: 5'-TCT ACT TCT GCC ACC TGG ACA TCA TTT GG-3'

TTTTTCAGAATGGATTATTTGCTCATGATTTTCTCTCTGCTGTTTGTGGCTTGCCAA

EXON 1

GGAGCTCCAGAAACAGCAGTCTTAGGCGCTGAGCTCAGCGCGGTGGGTGAGAAC

EXON 2

GGCGGGGAGAAACCCACTCCCAGTCCACCCTGGCGGCTCCGCCGGTCCAAGCGCT

Forward → Probe

GCTCCTGCTCGTCCCTGATGGATAAAGAGTGTGTCTACTTCTGCCACCTGGACATC

← Reverse

ATTTGGGTCAACACTCCCGAGCACGTTGTTCCGTATGGACTTGGAAGCCCTAG

EXON 3

Transforming Growth Factor β1

Forward: 5'-TGG ACA TCA ACG GGT TCA CTA C-3'

Reverse: 5'-AAG CAG GAA AGG CCG GTT-3'

Probe: 5'-CGA GGT GAC CTG GCC ACC ATT CAT T-3'

Heparin binding epidermal-like growth factor

Forward: 5'-GAT CTG GAC CTT TTG AGA GTC ACT T-3'

Reverse: 5'-TCC CGT GCT CC CCT TGT T-3'

Probe: 5'-AGC CAC AAG CAC TGG CCA CAC CA-3'

TCGAAAGTGACTGGTGCCTCGCCGCCTCCTCTCGGTGCGGGACCATGAAGCTGCT

EXON 1

GCCGTCGGTGGTGCTGAAGCTCTTTCTGGCTGCAGTTCTCTCGGCACTGGTGACTG

EXON 2

GCGAGAGCCTGGAGCGGCTTCGGAGAGGGCTAGCTGCTGGAACCAGCAACCCGG

ACCCTCCCACTGTATCCACGGACCAGCTGCTACCCCTAGGAGGCGGCCGGGACCG

Forward

GAAAGTCCGTGACTTGCAAGAGGCAGATCTGGACCTTTTGAGAGTCACTTTATCC

EXON 3

Probe

Reverse

TCCAAGCCACAAGCACTGGCCACACCAAACAAGGAGGAGCACGGGAAAAGAAA

GAAGAAAGGCAAGGGGCTAGGGAAGAAGAGGGACCCATGTCTTCGGAAATACA

AGGACTTCTGCATCCATGGAGAATGCAAATATGTGAAGGAGCTCCGGGCTCCCTC

CTGCATCTGCCACCCGGGTTACCATGGAGAGAGGTGTCATGGGCTGA

EXON 4

A Disintegrin and A Metallaproteinase (ADAM) 12

Forward: 5'-AGC TAT GTC TTA GAA CCA ATG AAA AGT G-3'

Reverse: 5'-CCC CGG ACG CTT TTC AG-3'

Probe: 5'-ACC AAC AGA TAC AAA CTC TTC CCA GCG AAG AT-3'

AGGTCTCATTGCCAGCAGTTTCACGGAAACCCACTATCTGCAAGACGGTACTGAT

EXON 4

GTCTCCCTCGCTCGAAATTACACGGTAATTCTGGGTCACTGTTACTACCATGGACA

EXON 5

TGTACGGGGATATTCTGATTCAGCAGTCAGTCTCAGCACGTGTTCTGGTCTCAGG

Forward

GGACTTATTGTGTTTGAAAATGAAAGCTATGTCTTAGAACCAATGAAAAGTGCAA

EXON 6

Probe

Reverse

CCAACAGATACAAACTCTTCCCAGCGAAGAAGCTGAAAAGCGTCCGGGGATCAT

GTGGATCACATCACAACACACCAAACCTCGCTGCAAAGAATGTGTTTCCACCACC

CTCTCAGACATGGGCAAGAAGGCA

A Disintegrin and A Metallaproteinase (ADAM) 33

Forward: 5'-CCT GGA ACT GTA CAT TGT GGC A-3'

Reverse: 5'-GTC CAC GTA GTT GGC GAC TTC-3'

Probe: 5'-CCA CAC CCT GTT CTT GAC TCG GCA C-3'

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