Mechanisms of Transforming Growth Factor-β Activation in Airway Smooth Muscle Cells and its Role in Asthma

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<u>Abstract</u>

Asthma is a chronic inflammatory disease of the airways characterised by airway hyper-responsiveness (AHR), inflammation of the airways and reversible airway obstruction. Airway remodelling is a feature of asthma, especially in cases of severe and fatal asthma, and includes structural changes such as increased airway smooth muscle (ASM) mass, mucous gland hyperplasia, subepithelial fibrosis and angiogenesis. TGF-β is a pleiotropic cytokine that has been implicated in the development of many of these changes. However, TGF-β is released from cells in a latent complex, associated with its pro-peptide the latency associated peptide (LAP). Extracellular activation of latent TGF-β is the rate limiting step in TGF-β bioavailability. Although TGF-β activation has been investigated in airway epithelial cells, to date, no studies have investigated TGF-β activation by airway smooth muscle cells. The hypothesis of this thesis is therefore that human airway smooth muscle cells can activate TGF-β in vitro.

The hypothesis of this thesis has been tested by investigating effects of the serine protease mast cell tryptase, mechanical wounding of cell monolayers and the phospholipid lysophosphatidic acid (LPA) on TGF-β activation by primary airway smooth muscle cells *in vitro*. We have utilised transformed mink lung epithelial cells, a reporter cell that express a TGF-β responsive promoter driving a luciferase gene, and quantitative PCR for the TGF-β-inducible gene plasminogen activator inhibitor-1 (PAI1) to investigate TGF-β activation. Moreover, we show for the first time that TGF-β activation can be assessed *in vitro* by detecting the translocation of Smad 2 and 3 from the cytoplasm to the nucleus by western blotting.

The results presented in this thesis provide evidence that airway smooth muscle cells are capable of activating TGF-β *in vitro*. These data show that the serine protease tryptase, released from activated mast cells, can proteolytically activate TGF-β via a mechanism that is independent of the tryptase receptor protease activated receptor-2 (PAR2). This effect is not accompanied by increased expression of the latent TGF-β complex. Furthermore, these data provide evidence that airway smooth muscle cells can activate TGF-β via the integrin αVβ5 in response to LPA

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stimulation. We have found that cells from asthmatic patients activate more TGF-β in response to LPA than cells from non-asthmatic individuals and this is not due to a difference in cell surface expression levels of the αVβ5 integrin. LPA-induced TGF-β activation can be inhibited by the β2 adrenoreceptor agonist formoterol, which is a commonly used asthma therapy, and the muscarinic receptor agonist methacholine, which causes cell contraction, also causes TGF-β activation by airway smooth muscle cells. Furthermore, the data presented here show that the cytoplasmic domain of the integrin β5 subunit interacts with the cytoskeletal protein talin to mediate TGF-β activation.

Together, these data highlight two previously unreported, biologically relevant, mechanisms of TGF-β activation employed by airway smooth muscle cells *in vitro*, both of which could contribute to the development of airway remodelling in asthma *in vivo*. Data concerning αVβ5-mediated TGF-β activation has led us to hypothesise that contraction of airway smooth muscle leads to TGF-β activation *in vivo*. If correct, this could be vital to our understanding of how airway remodelling is initiated in asthma, and could lead to the development of new therapies aimed at inhibiting contraction-induced TGF-β activation, for the treatment of asthma.

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Publications

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Tryptase Activates TGF-β in Human Airway Smooth Muscle Cells via Direct Proteolysis

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Lysophosphatidic Acid-Induced αVβ5-Mediated TGF-β Activation by Airway Smooth Muscle Cells

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Abstracts

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αVβ5-Mediated TGF-β Activation by Airway Smooth Muscle Cells in Asthma

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Abbreviations

α-granules	Alpha granules
AHR	Airway hyper-responsiveness
ALK	Activin receptor-like kinase
APC	Antigen presenting cells
β_2-agonists	β_2-adrenergic receptor agonists
BALF	Bronchoalveolar fluid
B cells	B lymphocytes
BCR	B cell receptor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
C3a	Complement component 3a
CCL3	Macrophage inflammatory protein-1α
CCL5	RANTES
CCL11	Eotaxin-1
CCR3	Receptor for RANTES, eotaxin-1, -2 and -3, MCP-2, -3 and -4 and MIP-1α and β
CoSmads	Common partner Smads
Cα	Constant region immunoglobulin A heavy chain
Cε	Constant region immunoglobulin E heavy chain
Cγ	Constant region immunoglobulin G heavy chain
Cμ	Constant region immunoglobulin M heavy chain
CXCR1	Interleukin 8 (CXCL8) receptor
CSCL8	Interleukin 8
DMSO	Dimethyl sulfoxide
EAR	Early asthmatic response
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMTU	Epithelial-mesenchymal trophic unit
EPO	Eosinophil peroxidase
FEV_1	Forced expiratory volume in 1 second
GM-CSF	Granulocyte macrophage - colony stimulating factor
HASM	Human airway smooth muscle cells
HRP	Horse-radish peroxidase
ICAM-1	Intercellular adhesion molecule 1
I-domain	Insertion domain
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN-γ	Interferon-γ
IGF	Insulin growth factor
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4

IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-13	Interleukin 13
I-Smads	Inhibitory Smads
LAR	Late asthmatic response
LAP	Latency associated protein
LLC	Large latent complex
LTC_4	Leukotriene C4
LTBP	Latent transforming growth factor-β binding protein
L-TGF-β	Latent transforming growth factor-β
Mab	Monoclonal antibody
MBP	Major basic protein
MC	Mast cells
MCP-1	Monocyte chemoattractant protein-1
MHC	Major Histocompatibility complex
MIDAS	Metal ion-dependant adhesion site
mIg	Membrane bound immunoglobulin
MIP-1α	Macrophage inflammatory protein-1α
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP-3	Matrix metalloprotease 3
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PDGF	Platelet derived growth factor
PGD_2	Prostaglandin D_2
R-Smads	Receptor Smads
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLC	Small latent complex
T cells	T lymphocytes
T_C	Cytotoxic T cells
T_H	Helper T cells
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TNF-β	Tumour necrosis factor-β
T_{reg}	Regulatory T cells
TCR	T cell receptor
TSP1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular cell adhesion molecule-1
VDJ	Variable region immunoglobulin heavy chain

Introduction

1   Introduction

1.1   Asthma

Asthma is a chronic inflammatory condition characterised by reversible airway obstruction, airway hyper-responsiveness (AHR) and infiltration of the airway mucosa with inflammatory cells, notably eosinophils. Its clinical symptoms include intermittent episodes of coughing, dyspnoea, wheezing and a sensation of chest tightness. It is a very common disorder affecting 5.2 million people in the UK alone (Census 2001), of which 1.1 million are children (Asthma-UK 2001b). Worldwide asthma prevalence varies greatly between countries; however, it has a tendency to be higher in developed countries such as the UK and Australia (ISAAC 1998). The prevalence of both adult and childhood asthma in the UK appears to be still increasing (Asthma-UK 2001a; Burr 2006), a trend that is also seen in other allergic conditions such as hayfever (allergic rhinitis).

Asthma can be subdivided in to two broad subtypes; atopic and non-atopic, also known as extrinsic or allergic and intrinsic or non-allergic respectively. Atopy refers to the genetic susceptibility of an individual to develop immediate hypersensitivity reactions against common environmental allergens. Clinical features of atopic asthma include increased levels of immunoglobulin E (IgE) antibodies in the serum, positive skin tests when challenged with common aeroallergens and AHR. Patients with non-atopic asthma show negative results to skin tests, serum IgE levels are within the normal range and there are no detectable IgE antibodies present in the serum directed against common aeroallergens. Higher age, a lack of family history and female sex are associated with an increased risk of non-atopic asthma, which tends to be more severe than atopic asthma (Romanet-Manent *et al.* 2002). The incidence of non-atopic asthma varies between studies but is consistently between 10 and 30% of all asthmatics.

In spite of the clinical differences, both types of asthma appear to share many common immunological mechanisms that contribute to the disease. Both atopic and non-atopic asthma

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have been shown to be characterised by increased numbers of eosinophils present in the bronchial mucosa (Amin *et al.* 2000). Furthermore, despite patients with non-atopic asthma having serum IgE levels within the normal range it has been shown that these patients have higher IgE levels than non-atopic people that do not suffer from asthma (Beeh *et al.* 2000), suggesting a role for IgE in non-atopic asthma as well as atopic asthma. This is supported by the finding that immunoglobulin class switching from IgM and IgG to IgE production occurs in both atopic and non-atopic asthma (Ying *et al.* 2001; Takhar *et al.* 2007). Moreover, the number of cells expressing the high affinity IgE receptor, FcɛRI, on their surface, such as mast cells, macrophages and eosinophils, is also increased in both types of asthma (Humbert *et al.* 1996b). These findings imply that non-atopic asthma is not as distinct from atopic asthma as originally thought. It is possible that non-atopic asthma is caused by a very similar mechanism to atopic asthma i.e. sensitisation towards an inhaled antigen, but that the antigen involved is yet to be identified. This would explain why non-atopic asthma patients show a negative result to skin tests and why they appear to lack antigen specific IgE in their serum. Although this hypothesis has yet to be confirmed experimentally, one study in which families were counselled on how to avoid allergen exposure in the home showed that this can significantly reduce asthma attacks in both atopic and non-atopic asthmatic children (Nishioka *et al.* 2006), implying a role for allergen exposure in non-atopic as well as atopic asthma.

1.1.1 <u>Sensitisation</u>

One of the prominent characteristics of atopic asthma, and possibly non-atopic asthma, is AHR to inhaled antigens also known as asthmatic stimuli or allergens. Such stimuli include air pollutants, house dust mites, pollen etc. However, for individuals to respond to inhaled antigens they must first become sensitised. The airway epithelium is constantly exposed to antigen from the external environment through the inhalation of air. At the epithelial surface antigen presenting cells (APC), such as macrophages and dendritic cells, phagocytose the antigen and become activated. They migrate to the local lymph tissue whilst processing and presenting fragments of the antigen

on their cell surface in association with major histocompatibility complex (MHC). In the lymph tissue the APC present the antigen-MHC complex to naïve T cells, which recognise the antigen via their T cell receptor (TCR). This interaction activates the T cells, which then proliferate and differentiate into a form capable of producing effector functions such as cytokine secretion. Under the influence of cytokines such as IL-4 activated T cells preferentially differentiate into T_H2 cells over T_H1 cells (Seder *et al.* 1992) driving an antibody mediated immune response. Antigen-specific IgE antibodies are produced by activated B lymphocytes (B cells) and bind to high affinity FcεRI receptors present on mast cells and basophils and low affinity FcεRII receptors present on B cells and eosinophils. This creates antigen specific receptors present on the surface of these cells and enables them to become activated following interaction with the specific antigen.

APC   e.g.   Ag-MHC   TCR   TH cell

AG specific IgE   IL-4 GM-CSF   IL-13   IL-5   B cell

Mast cell   FcεRI
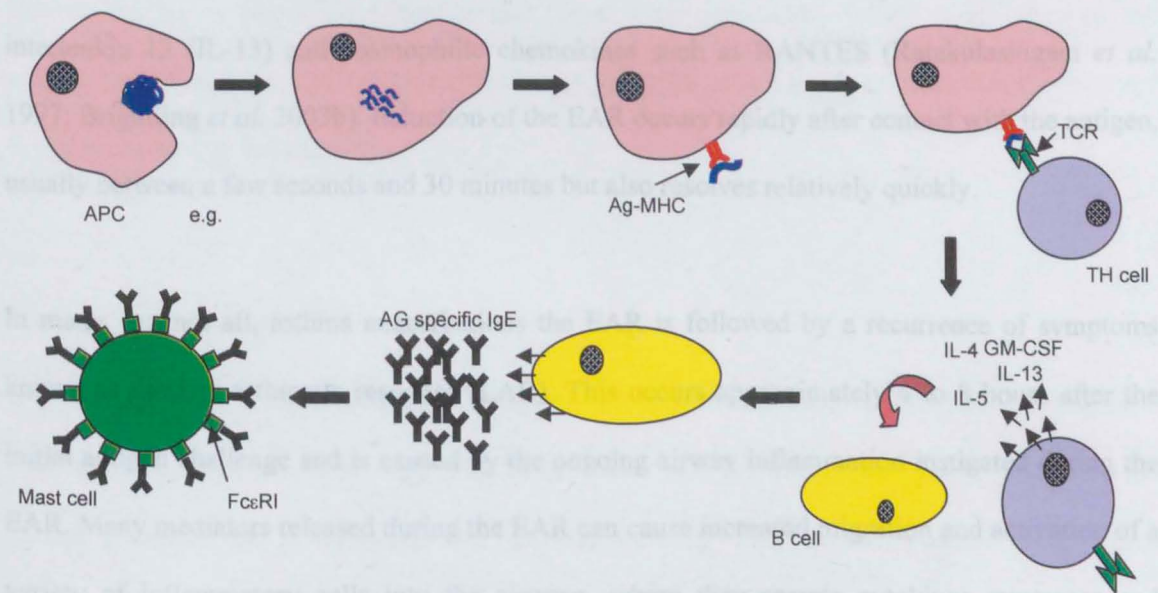
Figure 1.1: Sensitisation

Foreign antigens are engulfed by APC such as macrophages and dendritic cells. The antigen is processed in to peptide fragments, which are presented on the APC surface in a complex with MHC. T_H cells recognise this complex via their TCR and become activated. They are then able to secrete a variety of cytokines, which aid B cell production of antigen specific IgE antibodies.

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1.1.2   Early and Late Asthmatic Responses

The early asthmatic response (EAR) occurs when a sensitised individual comes into contact with the antigen to which they are sensitised. It is associated with airway obstruction and an instant fall in forced expiratory volume in 1 second (FEV$_1$). The antigen binds to the IgE antibodies present on the surface of mast cells and basophils resulting in cross linking of the FcεRI and FcRεII receptors, which initiates cell activation and signalling. Cell signalling events initiated by the cross linking of FcεRI receptors cause the release of intracellular granules containing preformed mediators such as histamine, tryptases, chymases and heparin, and the de novo synthesis of a variety of lipid mediators such as leukotrienes and prostaglandins. These mediators cause constriction of the bronchial airways (bronchoconstriction), dilation of pulmonary blood vessels, they stimulate mucous secretion and increase the permeability of the blood vessels resulting in the common clinical symptoms of an "asthma attack," such as wheeze, dyspnoea and coughing. Furthermore, mast cell activation can also result in the secretion of cytokines such as IL-4, interleukin 13 (IL-13) and eosinophilic chemokines such as RANTES (Rajakulasingam *et al.* 1997; Brightling *et al.* 2003b). Induction of the EAR occurs rapidly after contact with the antigen, usually between a few seconds and 30 minutes but also resolves relatively quickly.

In many, but not all, asthma exacerbations the EAR is followed by a recurrence of symptoms known as the late asthmatic response (LAR). This occurs approximately 4 to 8 hours after the initial antigen challenge and is caused by the ongoing airway inflammation instigated during the EAR. Many mediators released during the EAR can cause increased migration and activation of a variety of inflammatory cells into the airways, where they secrete cytokines, proteases and chemokines causing a state of chronic inflammation and a recurrence of AHR and bronchoconstriction. Helper T lymphocytes and eosinophils are important in orchestrating the LAR. Infiltration of these cells into the asthmatic airway is associated with development of LAR (Nabe *et al.* 2005). Furthermore, the transfer of helper T cells from ovalbumin sensitised mice, a common murine model of asthma, into naive mice confers antigen-induced LAR (Ohtomo *et al.*

2009). The role of inflammatory cells and the mediators they secrete in the pathogenesis of asthma is discussed in more detail in the following section.

1.1.3   Airway Inflammation

Inflammation of the bronchial mucosa is a key feature of asthma. This dense inflammatory infiltrate consists of eosinophils, mast cells, basophils and T lymphocytes (Macfarlane *et al.* 2000). Although the cellular composition of the inflammatory infiltrate is very similar between the two types of asthma, it is not identical: the infiltrate of non-atopic asthmatics contains large numbers of macrophages and neutrophils whereas the infiltrate of atopic asthmatics is composed mostly of eosinophils (Bentley *et al.* 1992; Amin *et al.* 2000; Shahana *et al.* 2005). Inflammatory cells are attracted into the airways by chemokines, which are produced by structural and inflammatory cells already present. The infiltration of inflammatory cells into the bronchial mucosa results in abnormal cytokine and chemokine expression, which contributes to the pathogenesis of asthma. This chapter will discuss the types of cell and mediators that are involved in asthmatic airway inflammation.

1.1.3.1   T Lymphocytes

T lymphocytes (T cells) are the key instigators of antigen-dependent cell-mediated immunity. They develop from lymphoid stem cells in the bone marrow, mature into naïve T cells in the thymus then enter the blood stream and circulate the body. They express TCR on their surface through which they recognise foreign antigen. When T cells have recognised correctly presented foreign antigen (i.e. in a complex with MHC), they differentiate, proliferate and become activated. This enables them to perform effector functions involving the secretion of a diverse range of cytokines and inflammatory mediators.

T cells can be broadly sub-divided into four groups. Cytotoxic T cells (T_C), which express the cell surface marker CD8 are generally involved in the cell mediated immune response, and are able to

induce apoptosis in infected or damaged cells. Helper T cells (T_H) express the surface marker CD4 and have various functions including activation of T_C cells, assisting B cells in the production of antibodies and regulating immune and inflammatory responses through the release of cytokines and mediators. Memory T cells are long lasting antigen specific T cells that rapidly differentiate into effector cells upon activation allowing the immune system to respond rapidly to antigen it has encountered before. Regulatory T cells (T_{reg}), also known as suppressor T cells, are involved in regulating T cell mediated responses and suppressing T cells directed against "self" antigens. In 1986 it was shown that T_H cells could be further sub-divided on the basis of the panel of cytokines and chemokines that they secrete (Mosmann *et al.* 1986). Although some cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α) are produced by both subsets of T_H cells, the predominant cytokines produced by T_H1 cells are interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β). In contrast, T_H2 cells secrete IL-4, IL-5 and IL-13 (Cherwinski *et al.* 1987). T_H1 cells are generally considered to be involved in cell mediated immune responses and T_H2 cells are involved in humoural or antibody-mediated immune responses. Moreover, each subset down-regulates the responses of the other subset, so that only one type of response is used to deal with a particular antigen.

Asthma, like many other allergic diseases, is considered to be driven by T_H2 responses, an idea that is supported by many observations. It has been shown that T_H2 cells but not T_H1 cells are capable of inducing AHR in sensitised mice following challenge with antigen (Cohn *et al.* 1998; Iwamura *et al.* 2007). Another prominent feature of asthma is goblet cell hyperplasia and increased mucous secretion, which is promoted by T_H2 cells in mice (Cohn *et al.* 1997; Hoshino *et al.* 2004). Furthermore, the expression of IL-4 and IL-5 by T cells and other inflammatory cells is increased in the bronchial mucosa of both atopic and non-atopic asthmatics (Humbert *et al.* 1996a; Ying *et al.* 1997a; Brightling *et al.* 2003b). These features imply a T_H2 mediated rather than T_H1 mediated response.

1.1.3.2 T$_H$2 Cytokines in Asthma

IL-4 has a diverse range of functions relevant to asthma and other allergic diseases. As mentioned in section 1.1.1, IL-4 facilitates B cells in their production of antigen specific IgE molecules (Del Prete *et al.* 1988; Pene *et al.* 1988; Yamada *et al.* 2002). Furthermore, it contributes to the recruitment of T cells and other inflammatory cells into the airways by increasing vascular permeability and increasing expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) by the endothelium (Ying *et al.* 1997b; Kotowicz *et al.* 2004). Studies using transgenic mice constitutively expressing IL-4 in the lungs have shown that these mice suffer from an intense pulmonary inflammatory response, with increased numbers of macrophages, eosinophils, lymphocytes and neutrophils. Furthermore, they show a marked increase in baseline airway resistance (Rankin *et al.* 1996).

The role of IL-5, another T$_H$2 cytokine, in asthma pathogenesis has been confirmed using studies with IL-5-deficient transgenic mice, which lack blood and tissue eosinophilia and airway hyperreactivity to methacholine, both of which are characteristic of asthma (Foster *et al.* 1996; Kopf *et al.* 1996). Furthermore, anti-IL-5 monoclonal antibodies have been developed for use as an asthma therapy. However, the efficacy of such treatment is still unclear. The first clinical trial found that although a monoclonal anti-IL-5 antibody caused a reduction in blood and sputum eosinophil numbers, there was no discernible effect on AHR or either the EAR or LAR (Leckie *et al.* 2000). However, a more recent study found that anti-IL5 treatment decreased the frequency of asthma exacerbations in severe asthmatics as well as reported decreased eosinophil counts in sputum and blood (Haldar *et al.* 2009). The clinical benefit of such a treatment to asthma patients is therefore still uncertain but may depend upon the severity of asthma.

IL-13, also a T$_H$2 cytokine, is closely related to IL-4 and like IL-4 and IL-5 has been implicated in asthma. A study using transgenic mice that over express IL-13 in the lung showed that this T$_H$2 cytokine is involved in several of the features of asthma including eosinophilia, hyper-secretion of mucous and goblet cell hyperplasia, obstruction of the airways and AHR (Zhu *et al.* 1999;

Kuperman *et al.* 2002). Moreover, IL-13 is a potent inducer of eotaxin from airway epithelial cells (Li *et al.* 1999; Matsukura *et al.* 2001), which may contribute to airway eosinophilia in asthma.

In conclusion, T_H2 cells have a fundamental role in the orchestration of the inflammatory response in an asthmatic setting. After becoming activated by a specific antigen, they are able to interact with many other cells such as B cells, eosinophils and mast cells through the secretion of a variety of mediators and cytokines. These cytokines augment the inflammatory response by activating other inflammatory cells and by acting back on T_H2 cells in a positive feedback mechanism. The various interactions of T_H2 cells and their cytokines with other inflammatory cells are summarised in Figure 1.2.
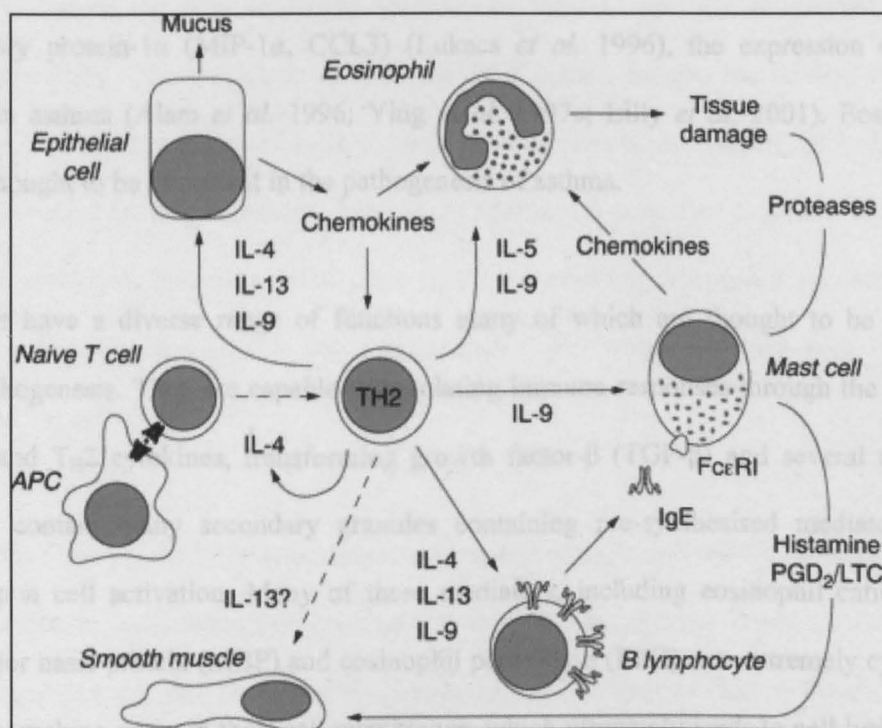
Figure 1.2: Role of T_H2 Cells and Their Cytokines in Asthma

This figure gives an overview of the role of TH2 cells and the cytokines they produce in asthma. TH2 cytokines, such as IL-4, IL-5 and IL-13, are responsible for the development and/or activation of several cell types involved in the pathogenesis of asthma. TH2 cells are therefore thought of as orchestrators of asthmatic airway inflammation.

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1.1.3.3 Eosinophils

Eosinophils are a type of leukocyte that develop and mature in the bone marrow from a myeloid precursor, and are so named due to the fact that they bind to the dye eosin. Eosinophilia is the term given to the presence of abnormally high numbers of eosinophils. Immature eosinophil precursors differentiate and mature through the influence of the cytokines IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-5, with IL-5 being responsible for terminal differentiation and therefore eosinophilia. Transgenic mice that lack IL-5 are unable to develop eosinophilia (Foster *et al.* 1996; Kopf *et al.* 1996) and administration of IL-5 causes blood eosinophilia (van Rensen *et al.* 2001). Eosinophilia of the peripheral blood and tissues is an important and prominent characteristic of asthma. Expression of IL-5 is increased in the bronchial mucosa in asthma (Humbert *et al.* 1996a; Ying *et al.* 1997a), which may contribute to pulmonary eosinophilia. Furthermore, eosinophils are attracted to sites of inflammation by several chemotactic mediators, including RANTES (CCL5), eotaxin (CCL11) and macrophage inflammatory protein-1α (MIP-1α, CCL3) (Lukacs *et al.* 1996), the expression of which is increased in asthma (Alam *et al.* 1996; Ying *et al.* 1997c; Lilly *et al.* 2001). Eosinophils are therefore thought to be important in the pathogenesis of asthma.

Eosinophils have a diverse range of functions many of which are thought to be involved in asthma pathogenesis. They are capable of regulating immune responses through the secretion of both T_H1 and T_H2 cytokines, transforming growth factor-β (TGF-β) and several chemokines. They also contain many secondary granules containing pre-synthesised mediators that are secreted upon cell activation. Many of these mediators, including eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil peroxidase (EPO), are extremely cytotoxic and kill cells by making pores in their cell membranes, which ultimately leads to cell lysis (Young *et al.* 1986). This effect is augmented by the release of reactive oxygen intermediates from eosinophils, such as hydrogen peroxide, which are also very damaging to cells. Release of these mediators and compounds in the lungs causes damage to the airway epithelium resulting in shedding of cells from the epithelial layer. In addition to their cytotoxic effects, MBP and ECP

are able to activate mast cells and cause release of histamine, even following an initial activation of the mast cells by allergen challenge (Piliponsky *et al.* 2001), suggesting a role for these mediators in LAR. Furthermore, MBP is capable of inducing AHR (Gundel *et al.* 1991; Coyle *et al.* 1995; Homma *et al.* 2005). These studies indicate that pulmonary eosinophilia, which occurs in asthma, leads to the release of many mediators from eosinophils that are capable of contributing to asthma pathogenesis.

1.1.3.4 B Lymphocytes

B lymphocytes (B cells) are the primary cell type involved in antibody-mediated, or humoural, immune responses. They are produced in the bone marrow from lymphoid stem cells, but unlike T cells they do not migrate to the thymus to develop and instead undergo full development in the bone marrow. B cell development involves multiple, random rearrangements of the genes encoding the heavy and light chains of antibody molecules, resulting in massive diversity in antibody specificity. The specificity of a B cell for a certain antigen is achieved via its B cell receptor (BCR), which consists of antigen specific membrane-bound immunoglobulin (mIg) and a disulphide-linked heterodimer called Igα/Igβ. In addition, B cells also express a wide variety of cell surface molecules including MHC class II, the complement receptors CR1 and CR2, B220 which is commonly used as a B cell marker and CD40 which interacts with CD40 ligand (CD40L) on TH cells.

Naïve B cells circulate the body in an inactive form and must be activated before they are able to produce antibodies. Activation occurs when the BCR becomes cross linked by the binding of antigen. For some antigen this is sufficient for B cell activation and antibody production, however, the majority of antigens are thymus dependent and are unable to cause full activation of B cells by this process alone. B cells specific for such antigen also require a second activation signal from interaction with T_H cells before they are able to produce and secrete antibodies directed against the antigen.

B cells have an important role in the pathogenesis of asthma. Under the influence of cytokines such as IL-4 released from activated TH2 cells B cells switch to preferentially producing class E antibodies (IgE) (Del Prete *et al.* 1988; Pene *et al.* 1988; Yamada *et al.* 2002). Once secreted these antibodies "coat" the surface of mast cells, basophils and other inflammatory cells rendering them able to respond immediately to encounters with that antigen, producing an immediate hypersensitivity reaction (see chapter 1.1.1). In order for B cells to express IgE over other isoforms of immunoglobulins class switch recombination of the immunoglobulin heavy chain locus is needed. The gene is rearranged so that the variable region (VDJ) is linked to the constant region of immunoglobulin E (Cε) rather than to the constant region of another immunoglobulin class (Cα, Cγ or Cμ). The resulting mRNA is then translated into IgE proteins. Class switch recombination can be induced by the actions of certain TH2 cytokines, such as IL-4 (Yamada *et al.* 2002). There is much evidence to show B cells undergo class switch recombination to the IgE isoform in the bronchial mucosa of both atopic and nonatopic asthmatics (Ying *et al.* 2001; Takhar *et al.* 2007), suggesting that antigen specific IgE molecules are synthesised and secreted locally in both forms of asthma. The finding that antigen specific IgE may be produced locally may help to explain how the levels of IgE in the serum of nonatopic asthmatics are within the normal range despite the apparent allergic nature of this form of asthma.

1.1.3.5 Mast Cells

Mast cells (MC) appear to play a fundamental role in the orchestration of airway inflammation. They are highly granular, tissue resident, cells that develop from CD34+ progenitors in the bone marrow. After leaving the bone marrow they circulate in an immature form, lacking the characteristic granules of mature MC, until they reach a mucosal or connective tissue site where they undergo final differentiation. They are attracted into tissues by a variety of mediators. Supernatants collected from HASM cells stimulated with T_H2 cytokines are highly chemotactic for MC, an effect found to be mediated in part through the IL-8 receptor, CXCR1, as well as

CCR3 the receptor for a variety of chemokines such as CCL2, CCL3, CCL4, CCL5, CCL11, CCL24, CCL26 (Sutcliffe *et al.* 2006). Moreover, all three isoforms of TGF-β can induce MC chemotaxis (Olsson *et al.* 2000). The cytoplasm of mature MC contains large numbers of granules containing preformed mediators and enzymes. MC can be subdivided into two groups on the basis of some of the proteases that their granules contain; MC containing tryptase are designated MCT, and MC containing tryptase and chymase are designated MCTC. Both subtypes can be found in the lung but the predominant type is MCT (Brightling *et al.* 2003a). MC granules also contain a wide variety of different preformed mediators and proteases including histamine, serine proteases including tryptase and chymase, proteoglycans such as heparin and cytokines such as TNF-α, certain interleukins and chemokines. Furthermore, MC activation also results in the synthesis and secretion of many lipid mediators and cytokines (summarised in Table 1.1).

In addition to many other receptors, MC express FcεRI, a type of high affinity IgE receptor on their surface, which binds antigen specific IgE molecules. MC can be activated by cross-linking of FcεRI-IgE complexes on their surface by specific antigen. Furthermore, they may also be activated through the binding of other ligands to their cell surface receptors, independently of FcεRI cross-linking. Such ligands include complement component 3a (C3a) and pathogen associated molecular patterns (PAMPs) from various bacteria and viruses, however, several other ligands are able to influence FcεRI-dependant activation either negatively or positively. MC activation results in release of their cytoplasmic granules, which contain a vast array of enzymes and mediators in a process known as degranulation. This causes a classic type I hypersensitivity reaction that in asthma is known as EAR (discussed in Introduction section 1.1.2).

The secretion of preformed mediators from activated MC induces the EAR and the synthesis and release of new mediators contributes to LAR, (see Table 1.1) resulting in the characteristic symptoms of an asthma exacerbation. Histamine is strongly implicated in type I hypersensitivity reactions and therefore the induction of EAR. In addition to increasing the permeability of blood vessels leading to increased chemotaxis of inflammatory cells, which could later contribute to

LAR, it also causes contraction of HASM through activation of H_1 histamine receptors causing

severe shortness of breath and wheezing. Furthermore, the most abundant MC protease, tryptase,

which is released with histamine, can potentiate histamine induced HASM contraction (Johnson

et al. 1997a). Leukotrienes are also heavily involved, contributing to LAR by causing

bronchoconstriction, increased mucous production and chemotaxis of inflammatory cells in to the

lungs.

Mediators in granules	Histamine, tryptase, chymase (MCTC only), heparin, carboxypeptidase A, matrix metalloprotease 3 (MMP-3)
Cytokines – in granules and newly synthesised	Interleukin 3 (IL-3), IL-4 (MCTC only), IL-5 (MCT only), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 9 (IL-9), interleukin 10 (IL-10), IL-13 (MCTC only), TNF-α
Chemokines – in granules and newly synthesised	CCL5, CCL11, MCP-1, -3 and-4, IL-8
Growth factors – in granules and newly synthesised	Stem cell factor (SCF), GM-CSF, TGF-β, platelet activating factor (PAF), platelet derived growth factor (PDGF)
Newly formed mediators	Prostaglandin D2 (PGD2), leukotriene C4 (LTC4)

Table 1.1: Mast Cell Mediators

This table summarises the different types of mediator released from mast cells. Some mediators, such as histamine and tryptase, are stored in cytoplasmic granules and released immediately upon cell activation. Other mediators are synthesised following MC activation and are therefore not released immediately.

Adapted from Brightling et al, 2003

A role for MC in asthma has been suggested for many years but their relative importance to asthma pathogenesis has been debated. It was observed over 20 years ago that increased numbers of MC are evident in the bronchoalveolar fluid (BALF) of asthmatics compared with non-asthmatics (Tomioka et al. 1984). Recently several studies have confirmed that they have an important role to play. Studies using transgenic mice deficient for MC, have shown that MC have an important role airway inflammation and hyper-responsiveness (Williams et al. 2000). These mice exhibit less airway hyper-reactivity and less airway infiltration by eosinophils than wild-type mice following ovalbumin challenge. Furthermore, the fundamental difference between biopsies from patients with asthma and eosinophilic bronchitis is increased MC infiltration of the ASM layer in asthmatics (Brightling et al. 2002). Eosinophilic bronchitis is a chronic inflammatory disease of the airways which differs from asthma in that patients do not present with AHR or reversible airway obstruction. This suggests that the presence of MC contributes to AHR. Studies have shown that the numbers of MC present in the airways is increased in asthmatics compared with non-asthmatics (Carroll et al. 2002) and within the ASM layer of sensitised bronchial tissue sections compared with non-sensitised sections (Ammit et al. 1997). The degree of MC degranulation is positively associated with asthma severity with fatal severe asthmatics showing the most MC degranulation in their airways (Carroll et al. 2002) supporting an important role for MC and their products in asthma severity. Interestingly, infiltration of the airways by MC is a feature of non-atopic asthma as well as atopic asthma (Humbert et al. 1996b) implying that MC are involved in the pathogenesis of both types of asthma, adding further support to the hypothesis that they are not as distinct from one another as initially thought. The EAR and LAR can be suppressed by inhibiting the binding of IgE molecules to MC using an anti-IgE monoclonal antibody (Fahy et al. 1997) confirming the role of MC activation in these processes. Furthermore, the magnitude of the LAR can also be reduced by treatment of patients with an inhaled inhibitor of mast cell tryptase (Krishna et al. 2001).

In summary, MC numbers are increased within asthmatic airways, they localise mainly within the ASM layer and they play an important role in the orchestration of airway inflammation and hyper-responsiveness. This makes the MC an attractive candidate when studying asthma pathogenesis and disease mechanisms, and for the development of drugs to treat and prevent asthma.

1.1.4 Mild, Moderate and Severe Asthma

Asthma is a heterogeneous disease with huge variability in both the nature and severity of symptoms between patients. Broadly, asthma can be subdivided into 3 groups, mild, moderate and severe, depending upon symptom severity and efficacy of treatment methods. The majority of asthma patients have either mild or moderate forms of the disease. Mild asthma refers to patients who suffer from sporadic symptoms of disease which respond extremely well to treatment. Mild asthmatics generally have normal lung function when they are not suffering from an asthma exacerbation. Moderate asthmatics suffer from symptoms more regularly than mild asthmatics and tend to require treatment of their symptoms on a daily basis. Treatment with a short-acting medication is usually effective. Unlike mild asthmatics, these patients may have abnormal lung function when not suffering from an exacerbation. Approximately 5-10% of asthmatics suffer from severe asthma (Holgate et al. 2006). The clinical features of this subgroup of asthma vary greatly between patients (ATS 2000); Some patients have intermittent profound airway obstruction whereas in others it may be persistent. Similarly some severe asthma patients, but not all, suffer from excessive mucous production resulting in mucous plugs in the airways, which could contribute to persistent airway obstruction. Severe asthmatics frequently suffer debilitating exacerbations of their symptoms and often exhibit significantly diminished lung function compared with patients suffering from milder disease (Mascia et al. 2005). They require treatment with high dose corticosteroids and long-acting β2-adrenergic receptor agonists (β2-agonists) on a daily basis (Holgate et al. 2006). In many cases the disease does not respond well, if at all, to treatment resulting in a higher risk of hospital admission, mortality and morbidity than

the other subgroups of asthma. For these reasons, patients with severe asthma account for a disproportionate use of NHS resources.

1.2 Airway Remodelling

Airway remodelling is the collective term given to the structural and cell phenotypical changes seen in the asthmatic airway, especially in cases of severe and fatal asthma. The common features of airway remodelling are summarised in Figure 1.3. At present there is still much to learn about how airway remodelling contributes to asthma pathogenesis. There is currently a lack of non-invasive procedures available to accurately measure airway remodelling, which means that it is difficult to make associations between remodelling events and changes in airway function. However, the rate of decline in lung function associated with increased age is much faster in asthmatics than in non-asthmatics and diminished lung function over time is associated with asthma severity (Lange *et al.* 1998; Bai *et al.* 2000; Mascia *et al.* 2005). Most studies to date suggest that airway remodelling is worse in patients suffering from severe asthma than in those suffering from moderate asthma (Benayoun *et al.* 2003; Pepe *et al.* 2005). Furthermore, computer models have shown that asthmatics have increased airway wall thickness, associated with irreversible airway obstruction (Kasahara *et al.* 2002), which would contribute to decreased lung function. These studies suggest that decreased lung function over time in chronic asthma is caused by airway remodelling. It is still not clear, however, whether airway remodelling is an early event in asthma pathogenesis or whether it occurs in response to chronic inflammatory events. This chapter will describe the common features of airway remodelling, the mechanisms driving the changes and the implications of these changes for the disease.

Increased deposition ECM glycoproteins in airway wall and smooth muscle layer

Mucous gland hyperplasia

Epithelial damage

Airway Remodelling

Angiogenesis

ASM hypertrophy + hyperplasia

Subepithelial fibrosis

Increased numbers of fibroblasts + myofibroblasts
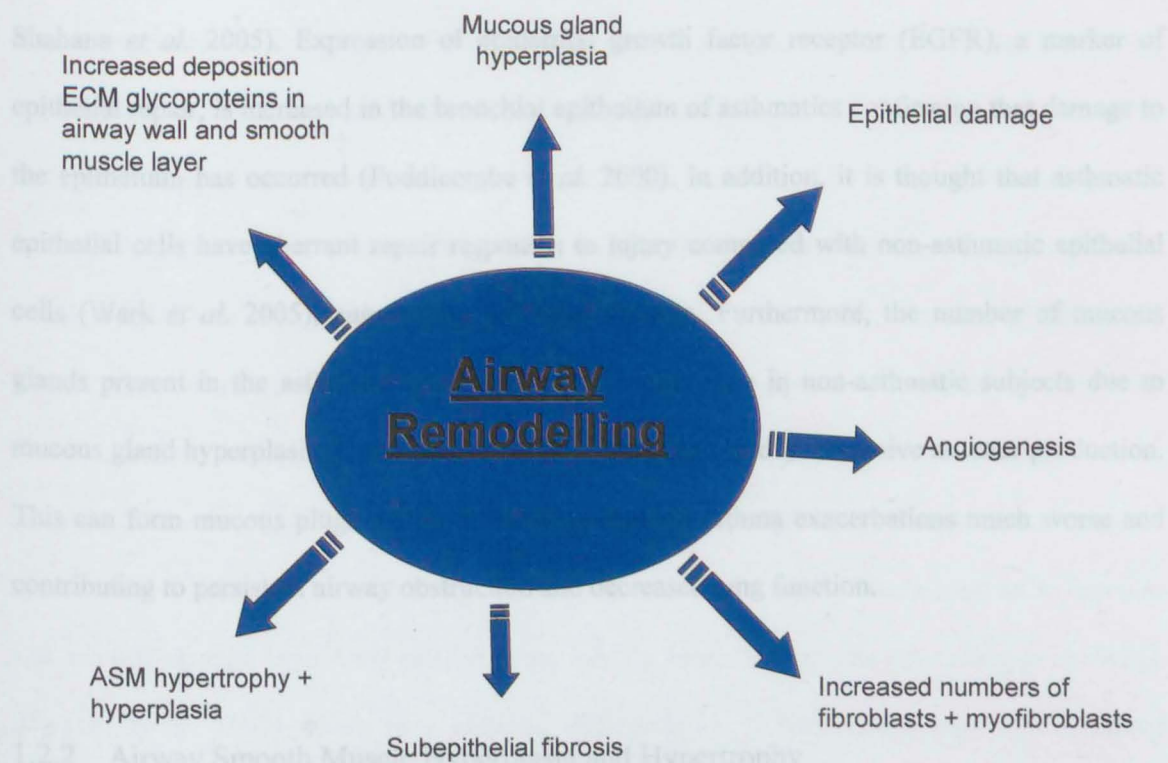
Figure 1.3: Features of Airway Remodelling

This figure summarises the key changes to the airways of asthmatics that are collectively termed airway remodelling. These changes include mucous gland hyperplasia, damage to the epithelial layer, angiogenesis, increased numbers of fibroblasts and myofibroblasts, subepithelial fibrosis and increased ASM mass. These changes could contribute to the increased loss of lung function over time observed in asthmatics.

1.2.1 Epithelial Changes

The airway epithelium of both asthmatics and non-asthmatics is constantly exposed to potentially damaging agents from the external environment, such as pollution, allergens and viruses. In normal individuals, these are dealt with adequately via the epithelial barrier and cells of the innate immune system and therefore do not cause any problems. However, evidence suggests that there is a fundamental difference between the epithelium of asthmatics compared with non-asthmatics; asthmatic epithelium becomes damaged more easily, resulting in apoptosis and excessive

shedding of the epithelial layer (Laitinen *et al.* 1985; Jeffery *et al.* 1989; Amin *et al.* 2000; Shahana *et al.* 2005). Expression of epidermal growth factor receptor (EGFR), a marker of epithelial repair, is increased in the bronchial epithelium of asthmatics confirming that damage to the epithelium has occurred (Puddicombe *et al.* 2000). In addition, it is thought that asthmatic epithelial cells have aberrant repair responses to injury compared with non-asthmatic epithelial cells (Wark *et al.* 2005), augmenting epithelial damage. Furthermore, the number of mucous glands present in the asthmatic epithelium is far higher than in non-asthmatic subjects due to mucous gland hyperplasia (Ordonez *et al.* 2001), which can lead to excessive mucous production. This can form mucous plugs within the airways making asthma exacerbations much worse and contributing to persistent airway obstruction and decreased lung function.

1.2.2   Airway Smooth Muscle Hyperplasia and Hypertrophy

The ASM layer of asthmatic airways is also fundamentally different to normal airways. Hyperplasia and hypertrophy result in an overall increase in smooth muscle mass in both the large and small airways (Ebina *et al.* 1993; Woodruff *et al.* 2004; Bentley *et al.* 2009). This contributes to narrowing of the airways and therefore persistent airway obstruction, increasing the stiffness of the airway wall which, in turn, increases the resistance of the airways. Greater muscle mass also means that the ASM layer can contract with more force during an exacerbation. Studies comparing the degree of ASM hyperplasia and hypertrophy between asthmatics have found that the ASM layer of severe asthmatics is significantly larger than both control subjects and subjects with mild or moderate forms of the disease (Benayoun *et al.* 2003) suggesting an important role for increased ASM mass in asthma pathogenesis. This is supported by the finding that persistent airway obstruction is associated with thickening of the bronchial wall (Bumbacea *et al.* 2004).

Hyperplasia and hypertrophy of the ASM layer arises due to several factors. Hypertrophy of smooth muscle occurs as a normal homeostatic response to increased workload and so the bronchoconstriction observed during an asthma exacerbation may cause ASM hypertrophy.

Furthermore, the presence of a vast array of inflammatory cells within asthmatic airways causes altered expression of cytokines, inflammatory mediators and growth factors many of which can contribute to ASM cell hypertrophy, including angiotensin and TGF-β (McKay *et al.* 1998; Goldsmith *et al.* 2006). Several factors and mediators, including TGF-β and epidermal growth factor (EGF), are capable of stimulating ASM proliferation and therefore contribute to ASM cell hyperplasia (Black *et al.* 1996; Berger *et al.* 2001; Chen *et al.* 2006). The rate of ASM cell proliferation certainly appears to differ between asthmatic subjects and normal controls with ASM cells from asthmatic patients proliferating at a much faster rate than those from normal controls (Johnson *et al.* 2001). Migration of ASM cells is also likely to contribute to ASM hyperplasia. It has been shown that myofibroblasts, which themselves have a contractile function and can differentiate in to ASM cells, migrate into the bronchial mucosa after allergen challenge (Gizycki *et al.* 1997) where they undergo differentiation. Fibrocytes, a type of circulating myofibroblast progenitor cell, have also been shown to migrate in to the bronchial mucosa following allergen challenge (Schmidt *et al.* 2003) where they too may begin expressing contractile proteins such as α-smooth muscle actin (α-SMA). These studies highlight that several contributing processes, including hypertrophy, proliferation and migration, are responsible for the increased mass of the ASM layer observed in asthmatics.

1.2.3   Subepithelial Fibrosis

Subepithelial fibrosis refers to the thickening of the lamina reticularis, which lies beneath the basement membrane, by the deposition of ECM proteins such as collagens and fibronectin. In addition to being observed in human asthmatic subjects (Roche *et al.* 1989; Boulet *et al.* 1997; Tomkowicz *et al.* 2008), it has also been documented in murine and rat models of asthma (Palmans *et al.* 2000; Silva *et al.* 2008). Moreover, myofibroblasts migrate into the bronchial mucosa during the LAR following allergen challenge (Gizycki *et al.* 1997), which may contribute to subepithelial fibrosis.   Subepithelial fibrosis may contribute to persistent airway obstruction and alter airway function by increasing the thickness and stiffness of the airway wall, which

increases the resistance of the airways. Using a murine model of severe asthma it has been found that increased airway resistance is associated with deposition of collagen (Silva *et al.* 2008). The relationship between subepithelial fibrosis and asthma symptoms is still not entirely clear, however, it has been shown that the degree of subepithelial fibrosis is significantly correlated with AHR (Boulet *et al.* 1997) and a decrease in lung function (Kasahara *et al.* 2002).

1.2.4 Angiogenesis

Airway remodelling also involves the bronchial and pulmonary vasculature. The bronchial mucosa of asthmatic patients has many more blood vessels, which are also larger in size, than that of non-asthmatics (Li *et al.* 1997; Vrugt *et al.* 2000). Angiogenesis occurs in response to many inflammatory mediators and growth factors including vascular endothelial growth factor (VEGF), angiotensin and plasminogen activator inhibitor-1 (PAI1). Many of these mediators are increased in the asthmatic airway. A study by Hirst and colleagues found that BALF from asthmatic patients had significantly higher concentrations of many pro-angiogenic mediators, including VEGF and MCP-1, compared with BALF from non-asthmatics (Simcock *et al.* 2007). Furthermore, levels of VEGF and angiotensin are increased in the sputum of asthmatics (Kristan *et al.* 2009). These findings are supported by data which show that human ASM cells from asthmatic patients are able to promote angiogenesis in vitro via increased release of VEGF in to culture media (Simcock *et al.* 2008).

In summary, airway remodelling is a collective term used to describe the many, diverse structural changes that occur within the airways of asthmatics. It involves most types of structural cell present in the airways. At present, it is not known whether airway remodelling results from the chronic inflammation that characterises asthma, or whether it occurs prior to disease onset and actually contributes to the development of an asthma phenotype. However, airway remodelling is detrimental to both baseline lung function and asthma symptoms, especially in patients suffering

from severe asthma. It is therefore an important process to study and develop new treatment strategies for.

1.2.5 Epithelial-Mesenchymal Trophic Unit (EMTU)

The airway wall contains many different cell types. Below the airway epithelium and its associated basement membrane lies a layer of mesenchymal cells, mainly fibroblasts and myofibroblasts, which has been termed the attenuated fibroblast sheath. This layer is in very close proximity to the epithelial layer above it and the ASM layer below it. The epithelial-mesenchymal trophic unit (EMTU) refers to communication between the airway epithelium and the underlying mesenchymal layer via cell-cell and cell-ECM interactions and also the paracrine and autocrine secretion of mediators involved in cell proliferation and fibrosis. This allows metabolic changes in one cell to directly influence the response of other cell types present within the airway wall. EMTU has a fundamental role in promoting branch formation during foetal lung development and is thought to play a role in epithelial repair responses to injury since the attenuated fibroblast sheath is able to respond quickly to signals and stimuli received from the epithelial layer above.

Recently it has been proposed that the EMTU is reactivated in the asthmatic airway (Holgate *et al.* 2004). It is thought that damage to the epithelium and epithelial shedding, which occurs in asthma, causes the epithelium to release a vast array of mediators and growth factors as part of its repair response. These mediators act in a paracrine manner on the attenuated fibroblast sheath regulating processes such as cell proliferation, ECM protein deposition and chemotaxis of inflammatory cells. This form of communication between the airway epithelium and underlying fibroblasts has been observed in an in vitro model. Airway epithelial cells were cultured in air-liquid interface (ALI) with normal lung fibroblasts cultured on the basal side of the ALI insert. The ALI insert only, and therefore only the epithelial cells, was subjected to mechanical stress by increasing atmospheric pressure. Soluble signals released from the stressed epithelial cells caused

increased expression of collagens by the unstressed fibroblasts and increased production of fibronectin by the epithelial cells, confirming communication between the two cell types (Swartz *et al.* 2001). Another study using a similar model found that epithelial cell injury caused fibroblast proliferation and secretion of mediators and growth factors such as TGF-β2, bFGF, PDGF and endothelin-1 in to culture medium (Zhang *et al.* 1999).

Although reactivation of the EMTU is a normal epithelial response to injury, the epithelium of asthmatics is more prone to injury than the epithelium of non-asthmatics (Bucchieri *et al.* 2002) and shows increased expression of markers of cell injury and stress such as epidermal growth factor receptor (EGFR) (Puddicombe *et al.* 2000). Moreover, there is evidence of increased apoptosis of the epithelial layer of asthmatics (Benayoun *et al.* 2001). This leads to increased reactivation of EMTU and aberrant repair responses, which contribute to chronic inflammation and airway remodelling. For many years it was thought that reactivation of the EMTU occurs mainly as a response to the chronic inflammation observed in asthmatics. However, it has recently been described that there is evidence of epithelial damage and airway remodelling within the airways of asthmatic children without any associated increase in eosinophil numbers (Fedorov *et al.* 2005). This suggests a role for epithelial abnormalities and reactivation of the EMTU very early on in the pathogenesis of asthma.

1.3   Airway Smooth Muscle

ASM cells, like all smooth muscle, are structural cells derived from the mesenchyme. Individually, the cells are spindle shaped with a centrally located, oval nucleus but collectively they form large sheets or bundles. In culture, confluent ASM cells have a "hill and valley" appearance (Pang *et al.* 1997). ASM cells have a fundamental role in the pathogenesis of asthma since it is the ASM layer that contracts in response to asthmatic stimuli, resulting in bronchoconstriction. In addition to this fairly obvious role for ASM cells in asthma pathogenesis,

they are also capable of synthesising and secreting a vast array of inflammatory mediators, cytokines and growth factors. ASM cells, like vascular smooth muscle cells, are capable of switching from a contractile phenotype to a synthetic phenotype and vice versa via a process known as phenotypic plasticity (Chamley-Campbell *et al.* 1979; Halayko *et al.* 1996; Johnson *et al.* 1997b).

A. Cytokines and Chemokines

Inflam cell

T$_H$2 cell

IFN-γ

TNF-α

TNF-α
IL-1β

IL-4
IL-10
IL-13

RANTES

TNF-α

ICAM

VCAM

CD44

C. Adhesion molecules

B. Bronchoprotective factors

Cholinergic nerve

Inflam cell

Ach

Bradykinin
IL-1β

IL-6
IL-8

PGE$_2$

NO   LPS

iNOS

IGF

IGFBP-2

MMP1

PDGF-BB

PDGF-R

IGF-R

LTD$_4$

IL-1β

D. Growth factors
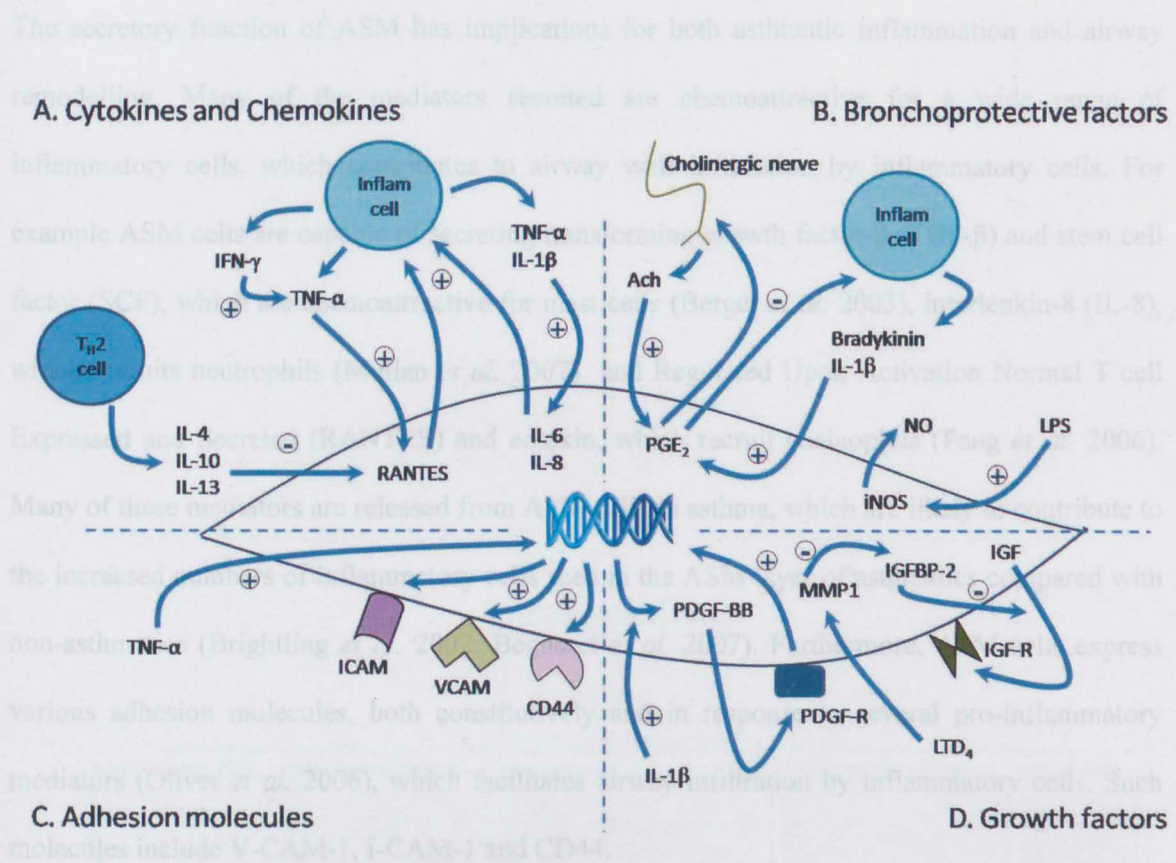
This diagram illustrates the diverse synthetic functions of ASM cells in asthma. Airway smooth muscle cells are capable of synthesising and releasing a vast array of cytokines, chemokines, inflammatory mediators, adhesion molecules, growth factors and mediators involved in regulation of wound repairs. A) Mediators released from inflammatory cells present in the asthmatic airway regulate the production of cytokines and chemokines from ASM cells. For example IFN-γ and TNF-α upregulate the chemokine RANTES but IL-4 , IL-13 and IL-10 downregulate it. TNF-α and IL-1β increase the cytokines IL-6 and IL-8, which contribute to increased migration and activation of other inflammatory cells. B) PGE2, which relaxes smooth muscle cells, is produced by ASM cells in response to inflammatory mediators such as bradykinin and IL-1β, and acetylcholine released from cholinergic nerves. C) The pro-inflammatory mediator TNF-α upregulates expression of the adhesion molecules ICAM, VCAM and CD44 by ASM cells, which contributes to migration and adhesion of inflammatory cells and lymphocytes. D) IL-1β stimulates the production of PDGF, a mitogen for ASM cells. ASM cells produce IGF. Following stimulation of ASM cells with LTD$_4$, IGF activity is increased due to increased production of MMP-1.

(Adapted from Johnson *et al.* 1997b)

The secretory function of ASM has implications for both asthmatic inflammation and airway remodelling. Many of the mediators secreted are chemoattractive for a wide range of inflammatory cells, which contributes to airway wall infiltration by inflammatory cells. For example ASM cells are capable of secreting transforming growth factor-β (TGF-β) and stem cell factor (SCF), which are chemoattractive for mast cells (Berger *et al.* 2003), interleukin-8 (IL-8), which recruits neutrophils (Mullan *et al.* 2007) and Regulated Upon Activation Normal T cell Expressed and Secreted (RANTES) and eotaxin, which recruit eosinophils (Pang *et al.* 2006). Many of these mediators are released from ASM cells in asthma, which are likely to contribute to the increased numbers of inflammatory cells seen in the ASM layer of asthmatics compared with non-asthmatics (Brightling *et al.* 2002; Begueret *et al.* 2007). Furthermore, ASM cells express various adhesion molecules, both constitutively and in response to several pro-inflammatory mediators (Oliver *et al.* 2006), which facilitates airway infiltration by inflammatory cells. Such molecules include V-CAM-1, I-CAM-1 and CD44.

Several ASM derived mediators are also involved in processes of airway remodelling, most notably TGFβ. As will be discussed in the following chapters, TGF-β, which can be secreted by ASM cells (Berger *et al.* 2003), has been implicated in several different remodelling events including ASM cell hypertrophy and hyperplasia. Moreover, ASM cells are capable of secreting a vast array of ECM proteins (Johnson *et al.* 2000; Coutts *et al.* 2001; Moir *et al.* 2008), which contribute to subepithelial fibrosis.

1.4   Transforming Growth Factor-β

The TGF-β superfamily of genes, of which TGF-β1 is the prototype, includes a diverse group of 45 different proteins including TGF-β, inhibins, activins and bone-morphogenetic proteins (BMPs) (all members of the superfamily are shown in Figure 1.5). Members of this superfamily regulate fundamental developmental and physiological processes such as cell proliferation, survival and differentiation. The carboxyl terminal domain of all TGF-β superfamily members

contains seven highly conserved cysteine residues. Six of these cysteine residues form a characteristic fold known as a cysteine knot in the tertiary structure of TGF-β superfamily members. Some members, including the TGF-β isoforms and certain inhibins, also contain a further two cysteine residues within this domain. Members of TGF-β superfamily can be sub-divided into smaller families based upon sequence homology outside of the cysteine knot region. The TGF-β isoforms form one such family.

TGF-β
Superfamily

TGF-β Family	BMP Family	Activin/Inhibin Family	GDNF Family	Modulators
TGF-β1	BMP-1	Activin A	GDF-1	LEFTY-B
TGF-β2	BMP-2	Activin B	GDF-2	Anti-mullerian hormone
TGF-β3	BMP-3	Activin C	GDF-3	Dorsalin
	BMP-3b	Activin AB	GDF-5	NODAL
	BMP-4	Activin E	GDF-5	
	BMP-5		GDF-6	
	BMP-6		GDF-7	
	BMP7		GDF-8	
	BMP-8		GDF-9	
	BMP-8b		GDF-10	
	BMP-9		GDF-11	
	BMP-10		GDF-15	
	BMP-11		GDNF	
	BMP-12		Artemin	
	BMP-13		Neurturin	
	BMP14		Persephin	
	BMP-15			

Figure 1.5: TGF-β Superfamily

This figure described all members of the TGF-β superfamily of proteins, which includes TGF-β, activins and BMPs. Members of this superfamily are subdivided into smaller families. The three isoforms of TGF-β form the TGF-β family.

1.4.1 Structure and Expression of TGF-β

At present three mammalian isoforms of TGF-β have been identified, designated TGF-β1, TGF-β2, TGF-β3, following the discovery and isolation of TGF-β1 in the 1980s (Assoian *et al.* 1983; Derynck *et al.* 1985; Cheifetz *et al.* 1987). All three of these isoforms (TGF-β1, TGF-β2 and TGF-β3) are expressed in humans, sharing 64-85% sequence homology between one another (Frank *et al.* 1996). All three isoforms are expressed by most cell types in the body, however, TGF-β1 is the most abundant isoform found in humans. The genes for these 3 isoforms are located on separate chromosomes: TGF-β1 is found on chromosome 19q13 (Fujii *et al.* 1986), TGF-β2 on chromosome 1q41 (Nishimura *et al.* 1993) and TGF-β3 is located on chromosome 14q23-24 (ten Dijke *et al.* 1988). The protein products of these genes are large precursors of the mature proteins containing an amino terminal signal peptide, a pro-domain and the mature protein located at the carboxyl terminal.

TGF-β's are subject to considerable post-transcriptional modifications. The endopeptidase, furin initially cleaves the precursor protein in two leaving a mature TGF-β domain that is 390 amino acids in length and the N terminal propeptide (Dubois *et al.* 1995). Mature TGF-β domains then associate with one another to form homodimers, resulting in biologically active TGF-β molecules of 25kD. The remaining N terminal propeptide domains form disulphide-bonded homodimers known as latency associated proteins (LAP). These associate non-covalently with mature TGF-β homodimers forming a functionally inactive complex called latent-TGF-β (L-TGF-β), which is secreted. This form is also known as the small latent complex (SLC) (see Figure 1.6).

TGFβ precursor

Propeptide   TGFβ

LAP   Mature TGF-β

Small latent complex

Figure 1.6: Processing of TGF-β by Furin

Schematic diagram showing the post-transcriptional processing of TGF-β precursors by the endopeptidase furin. The TGF-β precursor contains an N-terminal propeptide and TGF-β. This precursor protein is cleaved by furin within the Golgi complex. Both the propeptide and TGF-β form homodimers resulting in mature TGF-β molecules of 25kDa. Homodimers of the propeptide are known as lantency associated protein (LAP). LAP proteins non-covalently associate with mature TGF-β homodimers forming an inactive complex known as the small latent complex (SLC).

It is thought that SLC is secreted from cells very slowly and that for efficient and proper secretion of L-TGF-β it must be bound to the protein product of a separate gene called latent TGF-β binding protein (LTBP) (Miyazono *et al.* 1991). LTBP is covalently bound to the SLC via a disulphide bond between cysteine residues within the amino terminal of TGF-β and cysteine residues in LTBP (Gleizes *et al.* 1996). There are 4 different isoforms of LTBP and despite being structurally similar they bind to LAP with varying affinities; LTBP-2 is not capable of binding LAP at all and LTBP-4 binds LAP relatively poorly, whereas the remaining two isoforms (LTBP-

1 and LTBP-3) readily bind to LAP (Saharinen *et al.* 2000). A key function of LTBP appears to be in the tethering of L-TGF-β to the ECM via a covalent interaction between amino acids within the N-terminal of LTBP and proteins of the ECM (Saharinen *et al.* 1996). This allows cells to accumulate stores of L-TGF-β in their ECM, which require activation before it can bind to cell surface TGF-β receptors.
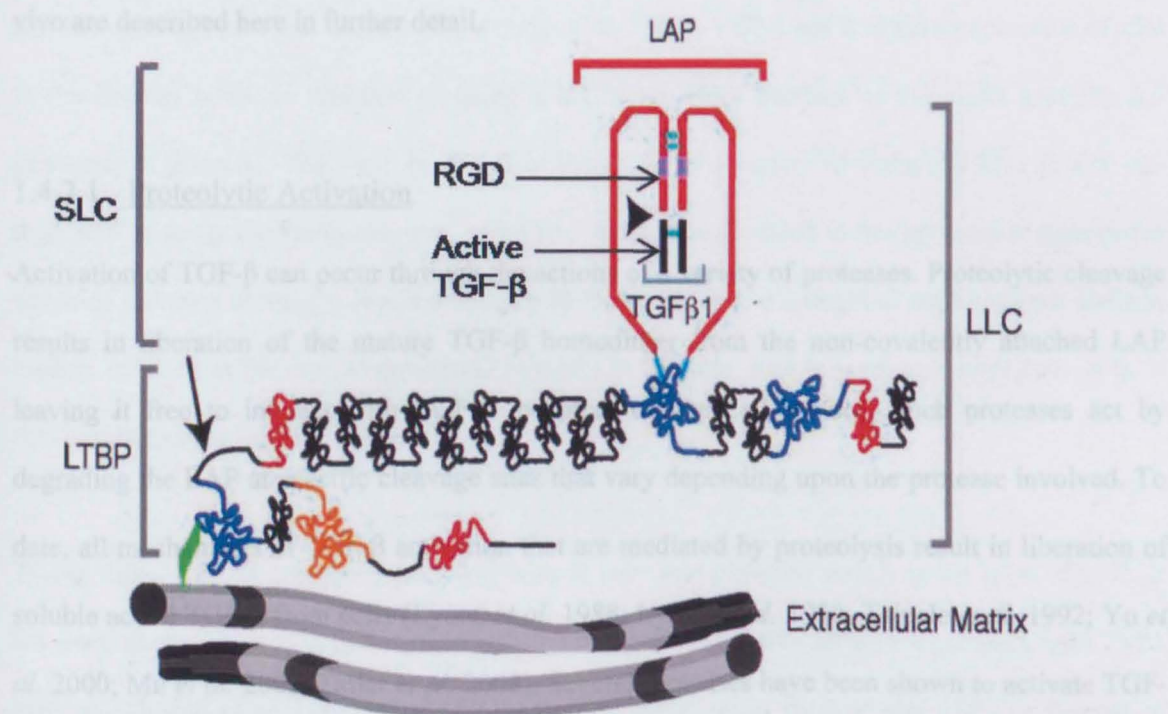
Figure 1.7: Structure of Latent TGF-β

This schematic diagram, adapted from (Annes *et al.* 2003), illustrates the structure of both the small latent complex and large complex of TGF-β. The LAP contains an RGD integrin binding domain. The active TGF-β molecule is secreted non-covalently associated with latency associated peptide (LAP). This complex is called the small latent complex (SLC). However, LAP can also bind to latent TGF-β binding proteins (LTBP) forming the large latent complex (LLC). LTBP serves to tether the latent TGF-β complex to proteins of the ECM.

1.4.2   Transforming Growth Factor-β Activation

Secretion of TGF-β in a latent form allows cells and tissues to build up large stores in their ECM and activation of this TGF-β is the main rate-limiting step in TGF-β bioavailability (Annes *et al.* 2003). Various physical mechanisms of activation have been described in vitro including acidification, alkalization, high temperature and reactive oxygen species (ROS) (Jullien *et al.* 1989; Brown *et al.* 1990; Barcellos-Hoff *et al.* 1994). In addition, many biological mechanisms of activation have been described in vitro but considerably fewer mechanisms have been shown to function in vivo. The various biological mechanisms of TGF-β activation and their relevance in vivo are described here in further detail.

1.4.2.1   Proteolytic Activation

Activation of TGF-β can occur through the actions of a variety of proteases. Proteolytic cleavage results in liberation of the mature TGF-β homodimer from the non-covalently attached LAP leaving it free to interact with TGF-β receptors on the cell surface. Such proteases act by degrading the LAP at specific cleavage sites that vary depending upon the protease involved. To date, all mechanisms of TGF-β activation that are mediated by proteolysis result in liberation of soluble active TGF-β from cells (Lyons *et al.* 1988; Lyons *et al.* 1990; Taipale *et al.* 1992; Yu *et al.* 2000; Mu *et al.* 2002; Tatler *et al.* 2008). Several proteases have been shown to activate TGF-β in this manner in vitro including the serine protease plasmin (Lyons *et al.* 1990), urokinase-type (uPA) and tissue-type plasminogen activators (tPA) (Chu *et al.* 1998), the ECM proteases matrix metalloproteinase-2 (MMP-2), MMP-9 (Yu *et al.* 2000) and MMP-13 (D'Angelo *et al.* 2001). Many of these proteases are normally involved in processes of ECM degradation and so could provide an important link in vivo between ECM degradation events and the release of a mediator involved in the production of ECM components. This would allow homeostatic replacement of ECM when it has been degraded. However, at present none of the above proteases capable of activating TGF-β in vitro have been shown to be in vivo TGF-β activators.

1.4.2.2 Activation by Thrombospondin-1 (TSP1)

The thrombospondin (TSP) family consists of 5 members, termed TSP1, TSP2, TSP3, TSP4 and TSP5. The thrombospondins are large, multifunctional, glycoproteins involved in processes such as cell adhesion, platelet aggregation, angiogenesis and cell proliferation. They can be divided into two groups: TSP-1 and TSP-2 are homotrimers and make up group A, and TSP-3, TSP-4 are homopentamers and make up group B. Thrombospondin-1 is a large (420 kDa) disulphide bonded homotrimer (see Figure 1.8), in which each of the three subunits contains 6 domains; the amino-terminal domain, a procollagen homology domain, type 1 repeats, type 2 repeats, type 3 repeats and the carboxyl-terminal domain (Esemuede *et al.* 2004). TSP-1 has a limited expression profile in the healthy adult. In addition to being a major secretory product of the alpha granules (α-granules) of platelets, TSP1 can be found in the ECM of a variety of tissues. TSP-1 is also up-regulated in many tissues in response to injury. It has been located in the peritubular connective tissue of kidneys, along the luminal surface of the aorta, in the interstitial area between skeletal muscle bundles, at the dermal-epidermal junction in the skin, and beneath epithelial cells in both the dermis and the lung (Wight *et al.* 1985).

TGF-β, like TSP1, is released from α-granules of activated platelets mainly in the form of LLC. It has been shown, however, that TGF-β can be released in its active form in a complex with TSP1 (Murphy-Ullrich *et al.* 1992). Further work by the same group showed that TSP1 is capable of activating latent TGF-β secreted from endothelial cells (Schultz-Cherry *et al.* 1993) and in cell free systems (Schultz-Cherry *et al.* 1994b) and this occurs independently of proteases. The TSP1 domain involved in TGF-β activation has been identified as the K412RFK415 sequence located between the first and second type 1 repeats (Schultz-Cherry *et al.* 1994a; Schultz-Cherry *et al.* 1995). This sequence interacts with the sequence L54SKL57 found at the amino terminus of LAP causing a rearrangement in the conformation of LAP, which leaves the active domain of TGF-β free to interact with its cell surface receptors despite the ongoing association of LAP (Ribeiro *et al.* 1999). Activation of TGF-β by TSP1 could represent a positive feedback loop in certain cells since treatment of endothelial cells with TGF-β results in increased expression of TSP1

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(RayChaudhury *et al.* 1994). TSP-1-mediated TGF-β activation appears to be relevant in vivo because mice null for either TGF-β or TSP1 share similar phenotypes (Crawford *et al.* 1998), including hyperplasia of vascular smooth muscle, airway epithelium and pancreatic islet cells, and hypoplasia of exocrine pancreas. TSP-1 null mice did not completely recapitulate the phenotype of TGF-β1 null mice; inflammation was not as extensive in the TSP-1 null mice and they survived past week four, unlike TGF-β1 null mice. TSP-1-mediated TGF-β1 activation is thought to contribute to several inflammatory and/or fibrotic diseases including fibrotic renal disease (Daniel *et al.* 2004). However, TSP1 is not solely responsible for TGF-β activation *in vivo*. Residual staining for active TGF-β was still observed in TSP1 null mice (Crawford *et al.* 1998) and not all available latent TGF-β was activated by TSP1 in vitro (Schultz-Cherry *et al.* 1993). This means that additional mechanisms of TGF-β activation must be employed in vivo.
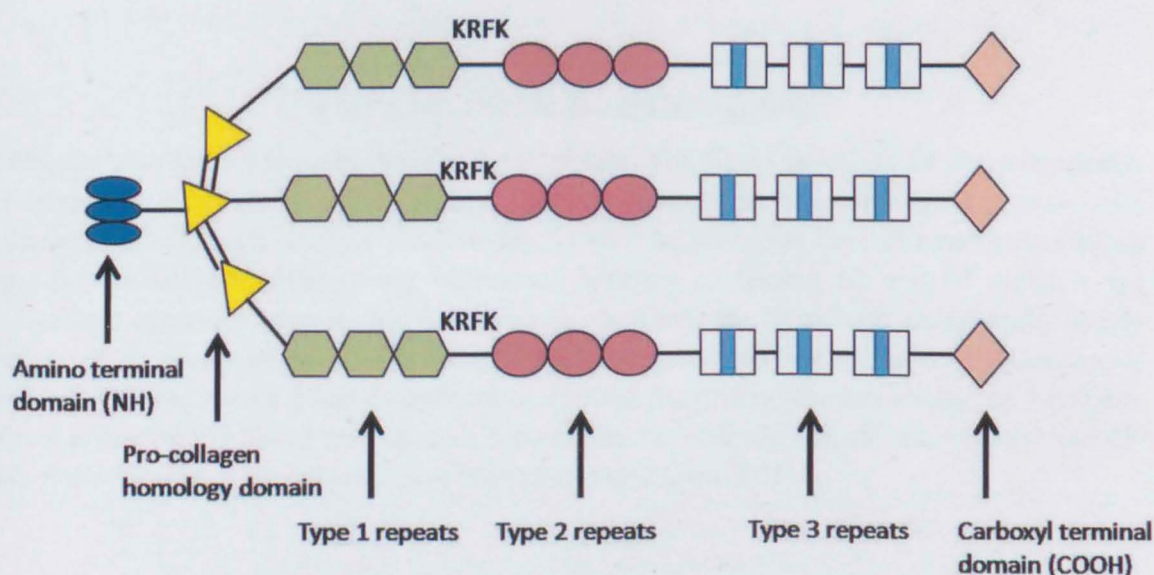
Figure 1.8: Structure of Thrombospondin-1

This schematic diagram shows the structure of TSP-1. It is a large, homotrimeric, glycoprotein of approximately 420kDa. The amino terminal domain contains a heparin binding site and is involved in platelet aggregation, and cell adhesion. The procollagen homology domain has a role in angiogenesis. The type 1 repeats contribute to cell adhesion, chemotaxis and angiogenesis. The type 2 repeats contain epidermal growth factor-like domains. The type 3 repeats contain RGD integrin binding sites and have a role in cell adhesion. The carboxyl terminal domain contains binding site for integrin associated protein (IAP) and functions in cell adhesion and chemotaxis.

1.4.2.3 Integrin Mediated Activation

Integrins are a group of transmembrane, heterodimeric, cell surface receptors that are involved in cell-cell interactions and in anchoring cells to components of the ECM. Moreover, integrins are able to transduce signals from the ECM to the cell, allowing cells to respond to changes in the extracellular environment. They consist of two distinct subunits, named α and β. There are currently 18 α and 8 β subunits identified creating 24 distinct integrin molecules (Takada *et al.* 2007). The different combinations of α and β subunits are illustrated in Figure 1.9. Both integrin subunits are type I transmembrane proteins, consisting of a short carboxy terminal cytoplasmic domain, a single-spanning transmembrane domain and a large amino terminal extracellular domain. In the intact molecule the extracellular domains of the two subunits form two "legs" extending out from the membrane on top of which is a large globular head, which is responsible for binding of ligands.
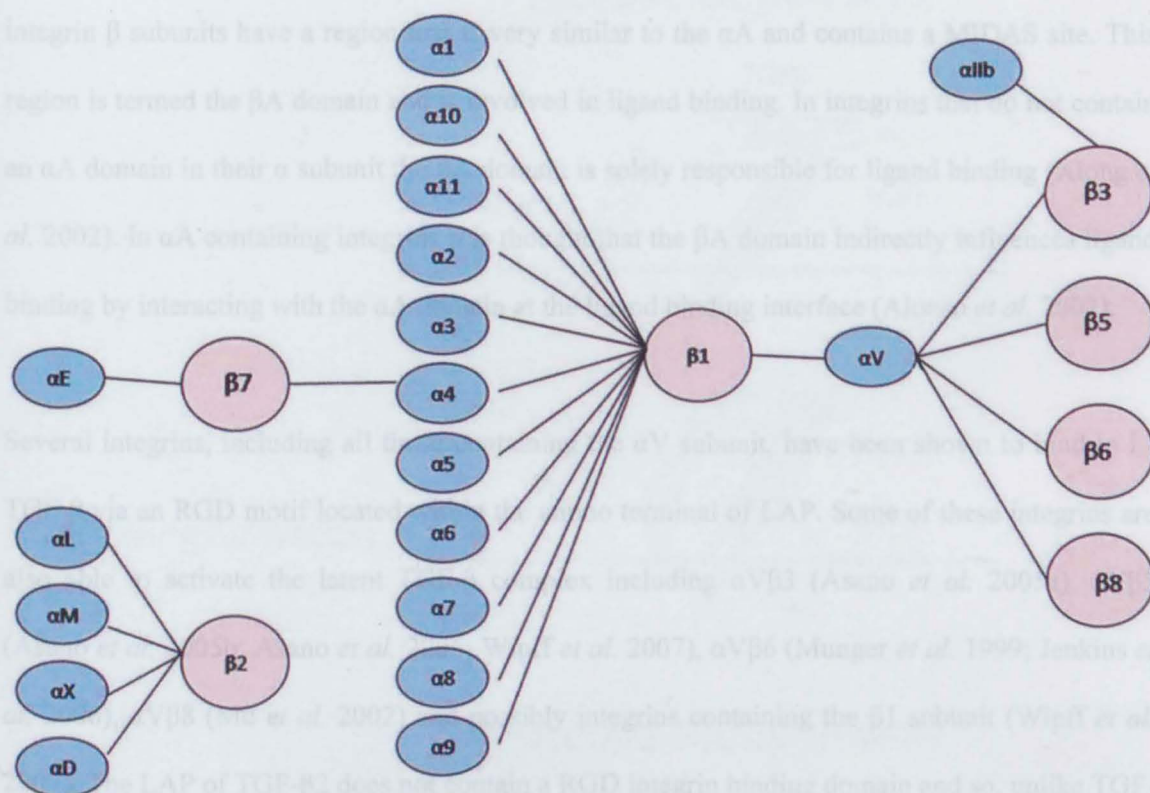
Figure 1.9: Pairing of Integrin Subunits

There are 18 α and 8 β integrin subunits in vertebrates. This figure shows the 24 possible pairings of integrin α (blue) and β (pink) subunits. Integrin subunits are inactive alone and must form heterodimers with each other for functionality. Integrin heterodimers have diverse ligand binding specificities and are differentially expressed. Integrins containing β2 and β7 subunits are exclusively expressed upon leukocytes. Integrins containing the β1 subunit are generally widely expressed on many cell types. The αIIbβ3 integrin is exclusively expressed on platelets and is rapidly activated during platelet aggregation. Half of integrin α subunits contain an I-domain, which is involved in ligand binding. This subunits are αL, αM, αX, αD, αE, α1, α10, α11 and α2. All of the αV containing integrins have been shown to activate TGF-β.

The integrin family of receptors can be grouped into 2 smaller families based upon the presence or absence of the αA domain (aka insertion domain or I-domain) within the extracellular domain of the α subunit of the integrin. There are 9 integrin α subunits that have an I-domain. This domain contains a region essential for ligand binding that is able to bind divalent metal cations

(Michishita *et al.* 1993), called the metal ion-dependant adhesion site (MIDAS). Furthermore, all integrin β subunits have a region that is very similar to the αA and contains a MIDAS site. This region is termed the βA domain and is involved in ligand binding. In integrins that do not contain an αA domain in their α subunit the βA domain is solely responsible for ligand binding (Xiong *et al.* 2002). In αA containing integrins it is thought that the βA domain indirectly influences ligand binding by interacting with the αA domain at the ligand binding interface (Alonso *et al.* 2002).

Several integrins, including all those containing the αV subunit, have been shown to bind to L-TGF-β via an RGD motif located within the amino terminal of LAP. Some of these integrins are also able to activate the latent TGF-β complex including αVβ3 (Asano *et al.* 2005a), αVβ5 (Asano *et al.* 2005b; Asano *et al.* 2006; Wipff *et al.* 2007), αVβ6 (Munger *et al.* 1999; Jenkins *et al.* 2006), αVβ8 (Mu *et al.* 2002) and possibly integrins containing the β1 subunit (Wipff *et al.* 2007). The LAP of TGF-β2 does not contain a RGD integrin binding domain and so, unlike TGF-β1 and TGF-β3, cannot be activated by integrins. At present, two main mechanisms of integrin-mediated TGF-β activation have been described. These are illustrated in Figure 1.10. Some integrins, such as αVβ8 (Mu *et al.* 2002), appear to activate TGF-β by utilising a protease and it has been suggested that these integrins facilitate proteolytic activation by localising L-TGF-β to specific areas of the cell surface allowing efficient interaction with the protease. Other integrins appear to mediate TGF-β activation independently of proteolytic activity, such as αVβ6, αVβ3 and αVβ5. The mechanism employed by integrin αVβ6 to activate TGF-β has been well elucidated, however, the mechanism employed by αVβ3 and αVβ5 is still not completely clear. Binding of αVβ6 to LAP is not sufficient for TGF-β activation (Munger *et al.* 1999) suggesting that binding to LAP and TGF-β activation are distinct processes in αVβ6-mediated TGF-β activation. Whether this applies to the other integrins remains to be determined.

Protease-Dependent

TGFβ

MMP14   αvβ8

Prior to Activation

RGD

MMP14   αvβ8

Following Activation

Protease-Independent

Latent TGFβ

LTBP   RGD   αvβ6

Prior to Activation

Following Activation

αvβ6
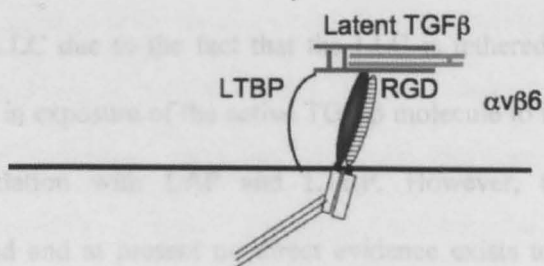
Actin
polymerisation
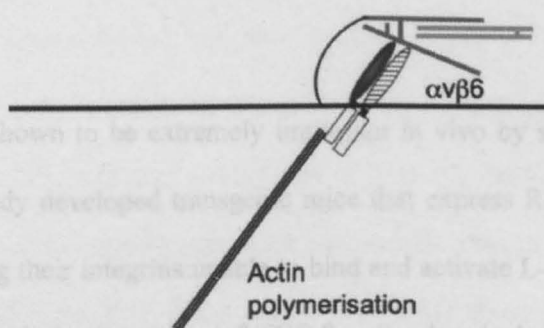
Figure 1.10: Two Mechanisms of Integrin –Mediated TGF-β Activation

These are two main mechanisms of TGF-β activation employed by integrins. One mechanism
requires proteolytic activity and the second acts independently of proteolytic activity. (Goodwin
et al. 2009)

A recurring theme between integrins that do not require proteolysis to activate TGF-β is the involvement of the cytoskeleton. For example, activation of TGF-β by the integrin αVβ5 and the β3 and β1 subunits occurs in response to contraction of myofibroblasts or myofibroblast cytoskeleton preparations (Wipff et al. 2007). Furthermore, the cytoskeleton was found to be involved in αVβ6 mediated TGF-β activation since inhibitors of RhoA, a protein involved in reorganisation of the actin cytoskeleton, and cytochalasin D, an inhibitor of cytoskeletal reorganisation, are able to inhibit activation of TGF-β mediated via this integrin (Munger et al. 1999; Jenkins et al. 2006). These findings suggest that reorganisation of the cytoskeleton is a requirement for TGF-β activation by these integrins. The ECM is also thought to be important for integrin-mediated TGF-β activation. Activation of TGF-β by αVβ5 can be increased by increasing the stiffness or tension of the ECM matrix (Wipff et al. 2007) and αVβ6 mediated TGF-β activation is dependent upon the LLC being bound to the ECM (Annes et al. 2004). These findings have led to the proposal that cytoskeletal changes are transferred via integrins into conformational changes within the LLC due to the fact that the LLC is tethered to the ECM. These conformational changes result in exposure of the active TGF-β molecule to its cell surface receptors despite continuing association with LAP and LTBP. However, this proposed mechanism has not been investigated and at present no direct evidence exists to confirm this hypothesis.

Integrin-mediated TGF-β has been shown to be extremely important in vivo by several studies using transgenic mice. One such study developed transgenic mice that express RGE instead of RGD in the LAP of TGF-β, rendering their integrins unable to bind and activate L-TGF-β. These mice display many of the fundamental characteristics of TGF-β null mice, including extensive inflammation of the lungs, heart and liver and defective vasculogenesis, despite being able to produce and secrete L-TGF-β normally (Yang et al. 2007). In support of these observations, the lungs of integrin β6 knockout mice develop normally but after approximately 21 days show signs of inflammation reminiscent of TGF-β null mice (Huang et al. 1996). These mice also develop emphysema of the lungs as they age (Morris et al. 2003). Deletion of the αV integrin subunit

results in mice that lack many of the TGF-β activating integrins. A very large percentage of these mice die during gestation despite appearing to have normal organ development. Those that survive develop extensive haemorrhaging and cleft palates (Bader *et al.* 1998), similar to TGF-β3 and TGF-β2 null mice (Proetzel *et al.* 1995; Sanford *et al.* 1997). A role for αVβ8-mediated TGF-β activation in vivo has been identified since loss of this integrin on leukocytes causes autoimmunity in mice (Travis *et al.* 2007). Furthermore, a recent study has highlighted the fundamental role of αVβ6 and αVβ8 in activation of TGF-β1 and TGF-β3, since mice lacking these integrins develop cleft palates and autoimmunity similar to TGF-β1 and TGFβ3 knockout mice (Aluwihare *et al.* 2009). Taken together, these studies show that integrin-mediated TGF-β activation is essential in vivo.

1.4.3   Receptors and Signal Transduction

Members of the TGF-β superfamily signal to cells through interaction with two types of specific cell surface receptors, termed type I and type II, both of which have intracellular serine/threonine kinase domains. There are seven different type I receptors, which are also known as Activin-receptor-like kinases (ALKs), and five different type II receptors. Each member of the TGF-β super family binds to a certain combination of the two types. The 3 TGF-β isoforms bind to type II TGF-β receptor (TGF-βRII) and several type I receptors, including type I TGF-β receptor (TGF-βRI), ALK-5 and ALK1, but most commonly to ALK5. Both type I and type II receptors form homodimers on the cell surface. There is also a third type of TGF-β receptor (TGF-βRIII) called betaglycan. This is a transmembrane proteoglycan with a molecular mass of 250-350 kDa. It has a role in presentation of TGF-β to TGF-βRII, rather than being directly involved in signalling. Although both TGF-β1 and TGF-β3 bind TGF-βRII receptors with high affinity, TGF-β2 only binds with low affinity. Betaglycan interacts with TGF-β2 and increases its affinity for TGF-βRII, therefore aiding TGF-β2 signalling (Esparza-Lopez *et al.* 2001).

The first step in initiation of TGF-β signalling is the binding of active TGF-β to TGF-βRII. This interaction causes recruitment of a type I receptor forming a large hetero-tetrameric complex. The serine/threonine kinase domain of TGF-βRII phosphorylates TGF-βRI causing activation of its kinase domain. A specific family of transcription factors called Smads are involved in propagating TGF-β signals from the activated receptor to the cell nucleus. There are three subsets of Smads within the family; receptor-regulated Smads (R-Smads), common partner Smads (Co-Smads) and inhibitory Smads (I-Smads). Following phosphorylation of TGF-βRI, its cytoplasmic serine/threonine kinase domain phosphorylates two of the R-Smads, Smad2 and Smad3. This is aided by interaction of the Smad Anchor for Receptor Activation (SARA) with TGF-βRI, Smad2 and Smad3. The R-Smads then form heterotrimeric or heterodimeric complexes with Smad4, a Co-Smad, which then migrates to the nucleus to regulate gene transcription. There are several other R-Smads (Smads-1, -5 and -8) but these are involved in the signalling of other members of the TGF-β super family, such as BMPs. The I-Smads (e.g. Smad 7), as the name implies, have an inhibitory effect on TGF-β signalling and serve to regulate and terminate signalling. Smad7 recruits Smurf1 and Smurf2 and moves to the plasma membrane where it is able to inhibit phosphorylation of Smad2 and Smad3 by competing for the receptor binding site (Hayashi *et al.* 1997). Furthermore, the Smad7/Smurf1 and 2 complex can target the TGF-β receptor for degradation (Kavsak *et al.* 2000). An overview of the TGF-β Smad signalling pathway is illustrated in Figure 1.11.
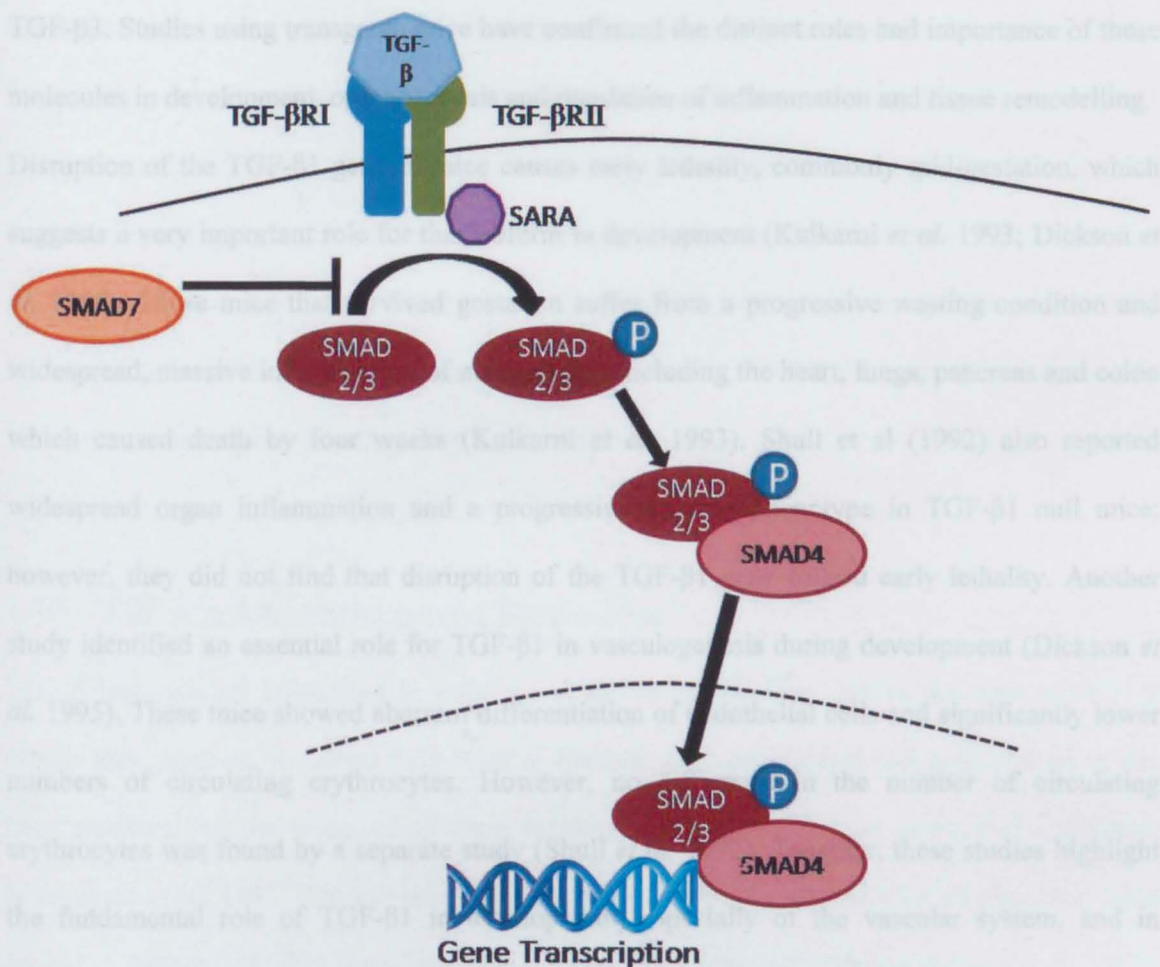
Figure 1.11: Overview of Smad Signalling Pathway

The Smad signalling pathway is initiated following the binding of active TGF-β to its cell surface receptors. Activation of the TGF-β receptor leads to phosphorylation, and therefore activation of Smad2/3, which then associate with Smad4. This complex then translocates to the nucleus to initiate gene transcription.

1.4.4 Biological Functions of TGF-β

The TGF-β isoforms are widely distributed multifunctional proteins that are able to induce multiple effects in the same cell and have different effects on different cell types. They are able to influence and regulate many processes that are fundamental to development, homeostasis and repair including cell proliferation, differentiation, wound repair and fibrosis, angiogenesis and inflammation. TGF-β1 is the most abundant of the 3 human isoforms followed by TGF-β2 then

TGF-β3. Studies using transgenic mice have confirmed the distinct roles and importance of these molecules in development, organogenesis and regulation of inflammation and tissue remodelling. Disruption of the TGF-β1 gene in mice causes early lethality, commonly mid-gestation, which suggests a very important role for this isoform in development (Kulkarni *et al.* 1993; Dickson *et al.* 1995). Those mice that survived gestation suffer from a progressive wasting condition and widespread, massive inflammation of many organs including the heart, lungs, pancreas and colon which caused death by four weeks (Kulkarni *et al.* 1993). Shull et al (1992) also reported widespread organ inflammation and a progressive wasting phenotype in TGF-β1 null mice; however, they did not find that disruption of the TGF-β1 gene caused early lethality. Another study identified an essential role for TGF-β1 in vasculogenesis during development (Dickson *et al.* 1995). These mice showed aberrant differentiation of endothelial cells and significantly lower numbers of circulating erythrocytes. However, no difference in the number of circulating erythrocytes was found by a separate study (Shull *et al.* 1992). Together, these studies highlight the fundamental role of TGF-β1 in development, especially of the vascular system, and in regulating immune responses.

Development of TGF-β2 knockout out mice has suggested that this isoform has a very important role in development and organogenesis, however, the phenotype of these mice shared no similarities with TGF-β1 null mice (Sanford *et al.* 1997). Two thirds of mice homozygous for the TGF-β2 null allele die before or during birth and the survivors have widespread developmental defects of multiple organs including the heart and kidney, and skeletal abnormalities (Sanford *et al.* 1997). The heart of TGF-β2 null mice had many structural abnormalities including a hypoplastic aorta wall and abnormal pulmonary valves. Although the development of the lungs did not appear to be affected, widespread collapse of the conducting airways occurred in some cases following birth.

Disruption of the TGF-β3 gene in mice causes early but not prenatal lethality, with death occurring within 24 hours of birth. Unlike the TGF-β2 mice, these mice have severe

developmental defects of the lungs, which results in respiratory failure (Kaartinen *et al.* 1995). These mice also have a cleft palate suggesting disruption of normal epithelial-mesenchymal interactions during development (Kaartinen *et al.* 1995; Proetzel *et al.* 1995). The findings described here highlight the differences in function between the three isoforms of TGF-β. TGF-β1 is primarily involved in regulation of inflammatory and immune responses. The role of TGF-β2 appears to be primarily in development of multiple organs, notably the heart. TGF-β3 seems to have an important role in development, especially in the lungs.

1.4.5   TGF-β in Asthma and Airway Remodelling

TGF-β isoforms are implicated in asthma and airway remodelling due to the wide variety of effects they produce, including their roles in fibrosis, angiogenesis and cell proliferation. It has been reported that the levels of all three isoforms are increased in the airways of asthmatics (Redington *et al.* 1997; Batra *et al.* 2004; Torrego *et al.* 2007) and the level of phosphorylated Smad2, a marker of TGF-β signalling, is also increased in asthmatic airways (Sagara *et al.* 2002). Increased levels of TGF-β have been detected in BALF taken from asthmatics after allergen challenge (Batra *et al.* 2004). Furthermore, several inflammatory mediators that are implicated in asthma pathogenesis have been shown to increase expression of TGF-β, including IL-1β, IL-4 and IL-13 (Offner *et al.* 1996; Wen *et al.* 2002; Lee *et al.* 2006). ECP released from activated eosinophils present in the asthmatic airway is able to stimulate TGF-β release from lung fibroblasts (Zagai *et al.* 2007). Furthermore, studies concentrating on the identification of gene polymorphisms in disease have also found that a polymorphism in the TGF-β gene is associated with asthma, which may result in increased TGF-β transcription in asthmatics (Silverman *et al.* 2004).

Most studies to date have focused on the role of TGF-β1 in asthma. Such studies have shown that TGF-β1 has a very important role in asthma pathogenesis. It is chemoattractive for mast cells (Olsson *et al.* 2000; Berger *et al.* 2003) and induces expression of various genes involved in

asthma pathogenesis and airway remodelling, including PAI1, fibronectin, VEGF and collagen (Laiho *et al.* 1987; Kucich *et al.* 2000; Kenyon *et al.* 2003; Kobayashi *et al.* 2005; Moir *et al.* 2008). However, recently more attention has been paid to the role of TGF-β2 in asthma. Following allergen challenge large increases in the number of eosinophils expressing TGF-β2 but not TGF-β1 have been described (Torrego *et al.* 2007). Increased expression of TGF-β2 but not TGF-β1 has been described in the bronchial mucosa of asthmatics (Chu *et al.* 2004) and IL-4 and IL-13, T$_H$2 cytokines abundant in asthma, increase expression of TGF-β2 but not TGF-β1 (Wen *et al.* 2002). Furthermore, TGF-β2, but not TGF-β1, can induce mucin expression, a component of mucous (Chu *et al.* 2004). These data suggest that both TGF-β1 and TGF-β2 could be important for asthma pathogenesis. There is very little data showing a role for TGF-β3 in asthma and airway remodelling.
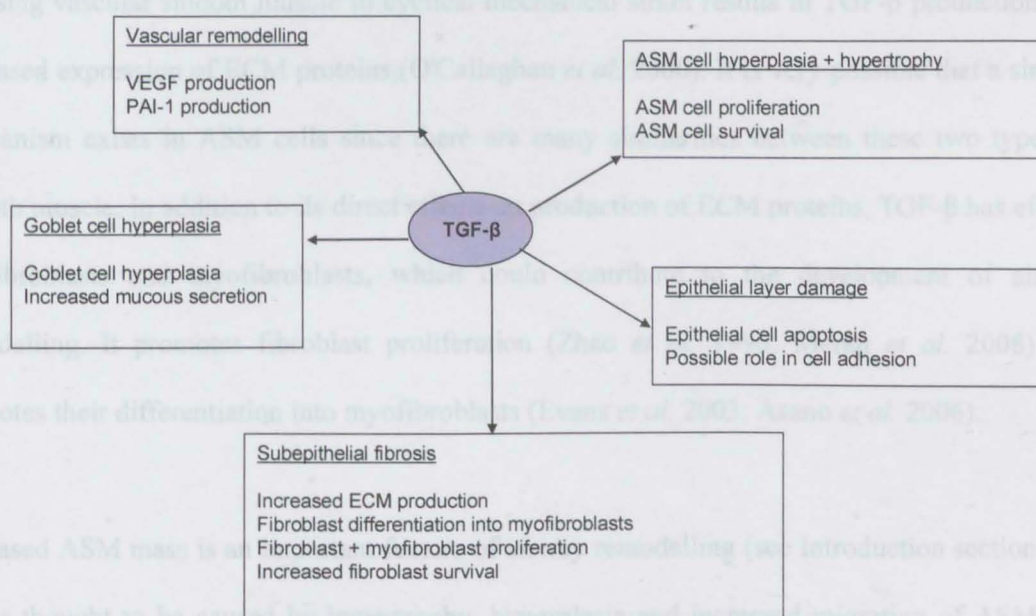
Figure 1.12: Role of TGF-β in Airway Remodelling

This figure summarises the effects that TGF-β has on various cell types in the lung. These responses contribute to airway remodelling.

(Makinde *et al.* 2007)

As discussed in Introduction section 1.2, the term airway remodelling refers to the structural changes that occur in asthmatic airways and includes changes such as subepithelial fibrosis, hyperplasia and hypertrophy of ASM cells and angiogenesis. TGF-β has been implicated in many of these changes (for summary see Figure 1.12). Arguably the most important function of TGF-β in the context of airway remodelling and asthma is its effects on ECM production. TGF-β potently induces the expression of many matrix proteins, which can contribute to subepithelial fibrosis. Administration of TGF-β1 into the lungs of experimental mice results in increased deposition of collagen in the ECM and increased expression of collagen I and III mRNA (Kenyon et al. 2003). Furthermore, proteolytic activation of TGF-β from ASM cells stimulates ASM cells to synthesise collagen I (Coutts et al. 2001). Stimulating ASM cells with exogenous TGF-β causes increased production of glycosaminoglycans (Black et al. 1996), from which ECM proteoglycans can be produced, and fibronectin (Moir et al. 2008). It has also been shown that exposing vascular smooth muscle to cyclical mechanical strain results in TGF-β production and increased expression of ECM proteins (O'Callaghan et al. 2000). It is very possible that a similar mechanism exists in ASM cells since there are many similarities between these two types of smooth muscle. In addition to its direct effects on production of ECM proteins, TGF-β has effects on fibroblasts and myofibroblasts, which could contribute to the development of airway remodelling. It promotes fibroblast proliferation (Zhao et al. 1996; Meran et al. 2008) and promotes their differentiation into myofibroblasts (Evans et al. 2003; Asano et al. 2006).

Increased ASM mass is an important feature of airway remodelling (see Introduction section 1.2) and is thought to be caused by hypertrophy, hyperplasia and increased migration of ASM cell precursors. TGF-β1 is thought to be involved in these processes. It is able to induce both hypertrophy and hyperplasia of ASM cells (Black et al. 1996; Chen et al. 2006; Goldsmith et al. 2006; Xie et al. 2007) and inhibition of TGF-β in a rat allergen-challenge model of asthma decreases both airway inflammation and remodelling events such as cell proliferation (Leung et al. 2006). In addition to its effects on ASM hyperplasia and hypertrophy, TGF-β1 can stimulate migration of ASM cells (Howat et al. 2002), which may also contribute to increased ASM mass.

TGF-β is thought to contribute to epithelial shedding, which occurs in asthma. It promotes apoptosis of epithelial cells and potentiates the effects of apoptosis inducing stimuli (Solovyan *et al.* 2006; Cheng *et al.* 2007). Furthermore, TGF-β inhibits the proliferation of epithelial cells (Bogdanowicz *et al.* 2000), which could affect repair of the epithelium in response to injury. Indeed, TGF-β delays epithelial wound closure in a wounding model of cellular injury (Neurohr *et al.* 2006). In addition, it is very likely that TGF-β is involved in the angiogenesis observed in asthmatic airway remodelling. TGF-β up-regulates expression of the potent pro-angiogenic mediator VEGF (Stocks *et al.* 2005; Clifford *et al.* 2008) and PAI1 (Laiho *et al.* 1987; Kutz *et al.* 2001).

In conclusion, TGF-β is released from various cells in the asthmatic airway as part of the chronic inflammatory response. Once released and activated it can exhibit both pro-inflammatory and anti-inflammatory functions depending upon the cell type it is stimulating. Although it may be involved in both amplifying and inhibiting the inflammatory response, TGF-β has a fundamental role in the structural changes collectively known as airway remodelling, causing cell proliferation and differentiation, fibrosis and angiogenesis.

1.5 Summary

Asthma is an extremely common inflammatory disease of the airways, in which airway remodelling is an important feature, especially in cases of severe asthma. Airway remodelling includes structural changes such as ASM cell hyperplasia and hypertrophy, subepithelial fibrosis, shedding of the epithelial layer, angiogenesis and goblet cell hyperplasia, which contribute to decreased lung function. The mechanisms responsible for the development of airway remodelling in asthma are not well understood. However, the widely distributed, pluripotent cytokine TGF-β has been implicated.

TGF-β is secreted by most cell types present in the asthmatic airways including inflammatory cells such as eosinophils and structural cells such as fibroblasts and ASM cells. TGF-β is potently pro-fibrotic and mitogenic and could contribute to many of the changes associated with airway remodelling. However, TGF-β is released from cells and sequestered in the ECM as a latent complex. Activation of latent TGF-β is therefore a fundamental step in TGF-β bioavailability. Various methods of TGF-β activation have been described in vitro, however, the mechanism of TGF-β activation employed in vitro has still not been confirmed. It is possible that the mechanism differs between cell types and during different processes. Whether HASM cells can activate TGF-β in vitro is not known, and the mechanism by which this might be done has not been investigated. Furthermore, whether HASM cell activation of TGF-β is involved in asthma pathogenesis has not been studied.

1.6   Aims

The overall aim of this thesis was to test the hypothesis that HASM cells can activate TGF-β *in vitro*. This was achieved by addressing three more specific aims

1) Investigate the effect of mast cell tryptase on TGF-β activation by HASM cells.

2) Investigate the effect of mechanical wounding of HASM cell monolayers on TGF-β activation.

3) Investigate the effect of lysophosphatidic acid on TGF-β activation by HASM cells.

If HASM cells can activate TGF-β in response to any of these stimuli the mechanism of activation will be further investigated.

Methods and Materials

2   Methods and Materials

2.1   Introduction

This chapter outlines the general methods employed in this thesis. The source of human tissue and cells is described. All primer sequences, buffer and media recipes and reagents are listed in the Appendix.

2.2   Cell Culture

2.2.1   Human Airway Smooth Muscle Cells

Human airway smooth muscle (HASM) cells from non-asthmatic donors were supplied by either, Professor Chris Brightling, Institute for Lung Health, University of Leicester, UK or Professor Maria Belvisi, Imperial College London, UK. HASM cells from asthmatic patients were supplied by Professor Chris Brightling, Institute for Lung Health, University of Leicester. They were cultured in serum plus Dulbecco's modified Eagles medium (DMEM) (see Appendix) at 37^0C, 5% $CO2$ in a humidified atmosphere. The medium was changed every 2 to 3 days. Experiments were performed on 100% confluent cells. The cells were growth arrested for 24 hours prior to all experiments in serum free DMEM (see Appendix) and experiments were performed in this medium. All cells were classified as being free from mycoplasma infection prior to experiments.

2.2.2   Normal Human Bronchial Epithelial Cells

Normal human bronchial epithelial (NHBE) cells were purchased from Cambrex Biosciences, Walkersville Inc at passage number 2. These cells had been certified as free from HIV-1, mycoplasma, hepatitis-B, hepatitis-C, bacteria, yeast and fungi by the supplier prior to shipping. The cells were delivered to the lab packed in dry ice and transferred to liquid nitrogen on arrival. The cells were cultured in the supplier's own Bronchial Epithelial Cell Medium containing the supplied supplements (hydrocortisone, epinephrine, transferrin, insulin, retinoic acid,

triiodothyronine, human epidermal growth factor) at 37^0C, 5% CO2 in a humidified atmosphere. NHBE cells were used in experiments at passage 4-5 when 100% confluent. The cells were growth arrested for 24 hours prior to experiments in the suppliers Bronchial Epithelial Cell Medium without the addition of the supplements. Cells were classified as being free from mycoplasma infection prior to experiments.

2.2.3 Transformed Mink Lung Epithelial Cells

Transformed mink lung epithelial cells (TMLC) were a kind gift from Professor Dan Rifkin, University of New York, USA. TMLC were used as a reporter cell to detect TGF-β activity (Xu *et al.* 2009). They stably express a TGF-β responsive element of the PAI1 promoter driving a luciferase gene and a neomycin resistance gene. TMLC were cultured in specific growth media containing the antibiotic G418 (see Appendix) at 37^0C, 5% CO2 in a humidified atmosphere. Experiments were performed in serum free DMEM. Cells were certified as free from mycoplasma infection prior to experiments.

2.2.4 CS-1 Cells

CS-1 cells are a hamster melanoma suspension cell line and were kindly supplied by Professor Dean Sheppard, University of California, San Francisco. Prior to transfection they were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% serum (see Appendix) at 37^0C, 5% CO2 in a humidified atmosphere. Following transfection with plasmids containing the neomycin resistance gene CS-1 cells were cultured as before with the addition of 500μg/ml of the antibiotic G418 to the culture medium. Cells were growth arrested prior to experiments for 24 hours in serum free RPMI (see Appendix). Cells were certified as being free from mycoplasma infection prior to experiments.

2.2.5 A549 Cells

A549 cells are a lung cancer derived human alveolar epithelial cell line. These cells were used as a positive control for protease-activated receptor (PAR) expression in Results section 3.3.9. They were cultured in serum plus RPMI (see Appendix). Cells were growth arrested for 24 hours in serum free RPMI and experiments were performed in this medium. Cells were certified as being free from mycoplasma infection prior to experiments.

2.2.6 Freezing Cells

Cells were cultured in $150cm^3$ flasks until fully confluent and trypsinised. After centrifugation (1500 rpm, 5 minutes) the pellet was resuspended in 15ml 90% foetal calf serum (FCS) plus 10% dimethyl sulfoxide (DMSO). The suspension was aliquoted in 1ml cryovials and placed in a Nunc Cryo 1°C Freezing Container. This contains 100% isopropan-2-ol, which allows the cells to be frozen at a rate of 1°C/min. This was placed in -80°C freezer overnight. The following day the cells were transferred to liquid nitrogen and stored until required.

2.2.7 Cell Counting

The number of cells in a cell suspension was determined using an Improved Neubauer haemocytometer and a light microscope. Cells were diluted 1:10 with Trypan blue to distinguish between live and dead cells. 10μl of cells was pipetted under the cover slip on the haemocytometer. The cells were observed under the microscope using the x40 magnification lens and the number of cells in each of the large squares in the corners of the grid was counted. Cells lying on the margins of the grid were counted. The total number of cells was divided by 4, multiplied by 10 (dilution factor) then multiplied by 10^4 to give the number of cells per ml. To determine the total number of cells, this number was multiplied by the total volume of the suspension.

2.3   Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.3.1   Principles

RT-PCR is a semi-quantitative technique used to assess gene expression within a cell or tissue by measuring messenger RNA (mRNA) levels. mRNA makes up approximately 1-5% of the total RNA present in mammalian cells and is the product of gene transcription, which is translated into protein. The first step of RT-PCR is the isolation of RNA from cells or tissues. Due to the unstable nature of RNA and its susceptibility to digestion by RNase enzymes, experimental work with RNA is always performed on ice. RNA is commonly isolated using the phenol-chloroform extraction method originally described by Chromczyski and Sacchi (Chomczynski *et al.* 1987). Cells are digested in a solution containing guanidinium thiocyanate, which disrupts the cell membranes, denatures intracellular proteins including endogenous RNases, and solubilises the cell contents. Phenol and chloroform are then added. Centrifugation of the mixture results in phase separation of its components, with proteins in the bottom phase, RNA in the top aqueous phase and DNA at the interface of the two. The top phase containing RNA is collected and isopropanol added to precipitate the RNA, which can then be collected by centrifugation.

The second step of RT-PCR is reverse transcription of the collected RNA into complementary DNA (cDNA). Most mRNAs have a polyA tail at their 3' end composed of multiple adenosine residues. Primers composed of multiple tyrosine residues, known as Oligo dT primers, are used as templates from which reverse transcriptase polymerises DNA. The Oligo dT primers associate with the 3' polyA tails of the mRNA strands and reverse transcriptase polymerises a DNA strand complementary to the mRNA strand.

The final step of RT-PCR is the polymerase chain reaction (PCR) itself. PCR involves the amplification of DNA using the enzyme DNA polymerase. PCR reaction mixtures contain several essential elements: themostable DNA polymerase, commonly from *Thermus aquaticus (Taq)*, a pair of synthetic oligonucleotide primers complementary to sequences located at the two ends of

the DNA to be amplified, deoxynucleoside triphosphates (dNTPs) from which the polymerase amplifies the DNA and template DNA i.e. cDNA produced by reverse transcription. Furthermore, thermostable DNA polymerases require free divalent cations to catalyse DNA polymerisation. This is generally supplied as Mg^{2+}. The PCR reaction consists of three steps performed at different temperatures. The first step is denaturation of the DNA. Denaturation serves to separate the two template DNA strands to allow the primers and DNA polymerase to bind to a single template DNA strand. This is performed at around 90-95℃. The second step involved allowing the sequence specific primers to anneal to the single stranded template DNA. The temperature required for annealing is dependent upon many factors, including the length of the primers and the percentage of guanine and cytosine residues present. Usually the annealing temperature used is around 3-5℃ below the melting temperature of the primers. Because of this primer pairs with similar melting temperatures are generally used. The final step of PCR is DNA elongation, in which the thermostable DNA polymerase polymerises DNA from the primers and template DNA. This step is performed at the temperature at which the DNA polymerase optimally synthesises DNA, which is usually around 72℃. These three steps are performed sequentially over a number of cycles, with production of the target DNA occurring at an exponential rate until one or more of the reagents becomes limiting. The DNA products can then be separated by gel electrophoresis and visualised by staining the gel with a fluorescent DNA intercalating agent such as ethidium bromide.

Denaturation

95°C for 1 minute

~ 30 cycles

Annealing

? °C for 1 minute

Extension

72°C for 1 minute
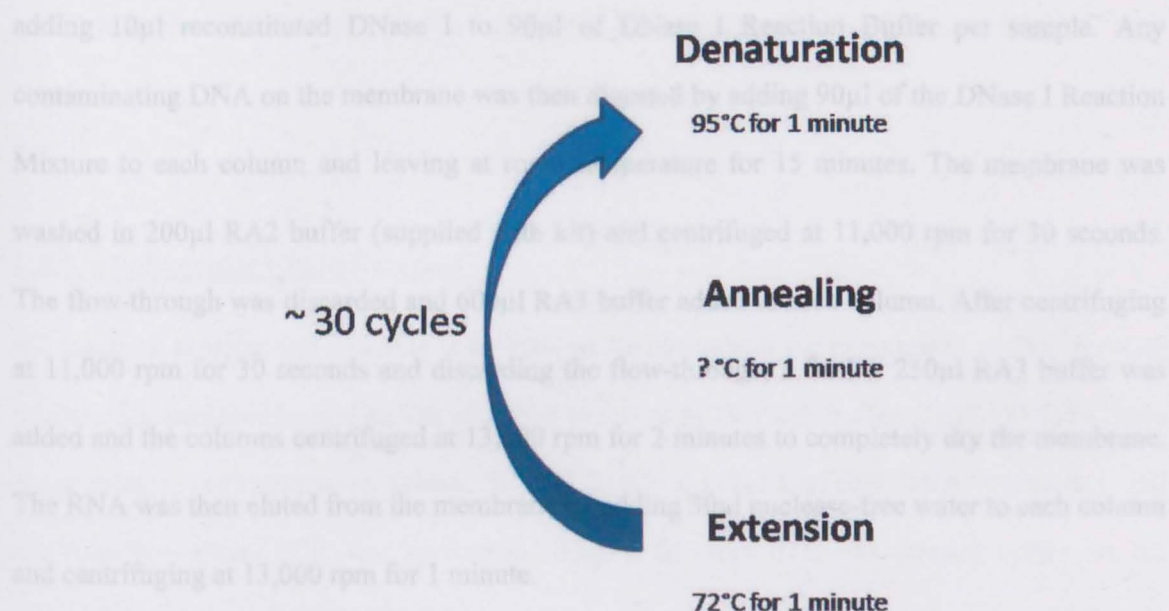
Figure 2.1: Typical PCR Thermal Profile

This figure gives an example of the typical thermal profile used for a PCR reaction including the three steps denaturation, annealing and DNA extension. Individual PCR thermal profiles for specific primer sets are described in the Appendix.

2.3.2 Isolation of RNA

To increase the speed of RNA isolation and to make isolation of multiple samples easier the NucleoSpin RNA II kit (Macherey Nagel, Germany), which uses silica membrane technology, was used. All columns and buffers used were supplied with the kit. Following treatment, cells were lysed in 350µl of RA1 lysis buffer supplemented with 10µl/ml β-mercaptoethanol and stored at -80°C until required. RNA was thawed at room temperature then added to the homogenisation column (pink) and centrifuged at 11,000 rpm for 30 seconds. The column was discarded and 350µl 70% ethanol added to each tube and mixed thoroughly. The RNA was then added to the NucleoSpin Filter column (blue) and centrifuged at 11,000 rpm for 30 seconds to collect the RNA on the membrane inside the column. The waste in the tube was discarded and the membrane washed in 500µl Membrane Desalting buffer (MDB), to remove contaminating salts, and centrifuged at 11,000 rpm for 30 seconds. The DNase I Reaction Mixture was prepared by

adding 10μl reconstituted DNase I to 90μl of DNase I Reaction Buffer per sample. Any contaminating DNA on the membrane was then digested by adding 90μl of the DNase I Reaction Mixture to each column and leaving at room temperature for 15 minutes. The membrane was washed in 200μl RA2 buffer (supplied with kit) and centrifuged at 11,000 rpm for 30 seconds. The flow-through was discarded and 600μl RA3 buffer added to each column. After centrifuging at 11,000 rpm for 30 seconds and discarding the flow-through, a further 250μl RA3 buffer was added and the columns centrifuged at 13,000 rpm for 2 minutes to completely dry the membrane. The RNA was then eluted from the membrane by adding 30μl nuclease-free water to each column and centrifuging at 13,000 rpm for 1 minute.

2.3.3   Determination of RNA Concentration

RNA concentrations were determined using a spectrophotometer. Absorbance was measured at 260 and 280nm. The spectrophotometer was calibrated against a distilled water zero. The RNA concentration of the eluate was calculated using the following equation:

[RNA] μg/ml = OD @ 260nm x 38 (extinction coefficient of RNA) x dilution factor

Purity of the preparation was assessed using the ratio of OD at 260nm: OD at 280nm. Purity ratios of 1.8-2 were deemed to be acceptable for use in PCR reactions.

2.3.4   Reverse Transcription

All reagents and RNA were kept on ice for the duration of the experiment. After determining the RNA concentration 1μg of RNA was added to 0.5μg of oligo (DT) primers, 2mM of each dNTP, 200 units Moloney murine leukaemia virus (MMLV) reverse transcriptase, 25 units RNAsin, 5μl RT buffer (Promega, supplied with MMLV reverse transcriptase). The volume of the reaction mixture was made up to 25μl with nuclease-free water. The reaction mixtures were mixed thoroughly and placed in a BioRad PCR cycler on the following cycle: 72^{0}C for 5 minutes, 42°C

for 90 minutes followed by 90°C for 5 minutes. The resulting cDNA was stored at -20°C until required.

2.3.5 Polymerase Chain Reaction

All reagents, samples and enzymes were kept on ice throughout the experiment. A 25µl reaction mixture was prepared by adding 1µl of cDNA to 5µl of 5x GoTaq® buffer, 200µM DNTPs, 1µM sense primer, 1µM anti-sense primer, 1.5mM MgCl, 0.125µl GoTaq® DNA polymerase and nuclease-free water. PCR was performed using a Bio-Rad PTC-100 thermal cycler on the appropriate program for the primers being used (see Appendix). DNA products were subjected to electrophoresis on a 2% agarose gel stained with 0.5µg/ml ethidium bromide in 0.5 x TBE buffer. PCR products were visualised by illuminating the gel with ultraviolet (UV) light and photographed using GeneGenius gel documentation and analysis system (Syngene).

2.4 Quantitative Polymerase Chain Reaction

2.4.1 Principles

Quantitative polymerase chain reaction (Q-PCR), also known as real-time polymerase chain reaction, is used to amplify and quantify DNA within a tissue or cell sample using a fluorescence-detecting thermocycler. Fluorescent DNA binding dyes, such as ethidium bromide or SYBR Green I, are added to the PCR reaction mixture. These intercalate into the double stranded DNA PCR product. These dyes do not bind to the primer DNA as primers are only single stranded. The intensity of the fluorescent signal is directly proportional to the mass of DNA present and is determined after each cycle of amplification. This allows the accumulation of PCR product to be determined in real time throughout the course of the reaction. To minimise variability between samples an endogenous standard is used. These are DNA sequences (often referred to as a housekeeping gene) whose expression level is expected to be constant between samples. These sequences are amplified in addition to the target DNA sequence. Quantification of the expression

of target DNA is calculated by comparing the amount of housekeeping gene DNA to the target DNA.

At the end of the reaction, the PCR products are subjected to dissociation curve analysis to ensure that a single amplification product has been produced and no primer dimers have formed. The thermocycler is used to generate a thermal denaturation curve (dissociation curve) of the PCR product. Presence of a single peak, and therefore single melting temperature, indicates the presence of a single DNA product. The melting temperature gives an indication that the correct product has been amplified. Presence of multiple peaks in the dissociation curve indicates that multiple products are present. This may be the result of non-specific binding of the primer pairs to DNA or by the primers binding to one another to form primer dimers. The presence of primer dimers in the product is confirmed by peaks in the dissociation curve at relatively low melting temperatures.

2.4.2   Q-PCR Method

QPCR analysis of cDNA was performed using primers and thermal cycling conditions described in the Appendix 9.7. 2µl cDNA was subjected to QPCR using Takara SYBR Premix Ex Taq mastermix containing SYBR green and an Mx3000P® QPCR system (Stratagene). Amplification was performed in 10µl Takara SYBR Premix Ex Taq in the presence of 200nM of both sense and antisense primers and nuclease-free water to achieve a final volume of 20µl. A no-template control containing no cDNA was performed for every experiment. All samples and controls were assayed in duplicate. Integration of SYBR green into the product was recorded after each annealing step. The cycle threshold (Ct), which is the point at which PCR product is detectable above a fixed threshold, was determined for both the target gene and the housekeeping gene. Expression of target gene was determined as previously described (Weglarz *et al.* 2006). Data was normalised to the housekeeping gene β2-microglobulin (β2-M) by subtracting the Ct for β2-M from the Ct for the target gene. Data for control samples (0 hour time point) was then

subtracted from all data sets resulting in values for ΔΔCt. The relative expression of the target gene compared with control was then calculated using the following equation:

$$\text{Relative expression} = 2\text{-}\Delta\Delta Ct$$
$$\text{(Fold increase)}$$

Confirmation that amplification of a single product had occurred was obtained by dissociation curve analysis. Presence of a single dissociation peak confirmed the presence of a single PCR product.

2.5   TGF-β1 ELISA

Levels of total TGF-β1 in cell culture supernatants were determined by ELISA assay as previously described (Tatler *et al.* 2008) according to the manufacturer's instructions. Cell culture supernatants were collected following stimulation of cells and stored at -20°C. Prior to loading of samples on to ELISA plate each sample was acid treated with 20% v/v 1N HCl acid for 10 minutes to activate all TGF-β present. The samples were neutralised by adding 20% v/v 1.2N NaCl. They were loaded on to an ELISA plate coated the previous day with anti-TGF-β1 with a standard curve of known TGF-β1 concentrations. Following addition of a secondary antibody conjugated to horseradish peroxidase (HRP) a colorimetric HRP substrate was added and the optical density at 450nm was determined.

2.6   Reporter Cell Assay

2.6.1   Principles of Assay

The reporter cell assay utilises transformed mink lung epithelial cells (TMLC), which stably express a TGF-β-sensitive portion of the PAI1 promoter driving luciferase (Abe *et al.* 1994) and can measure TGF-β activity in cell supernatants (Tatler *et al.* 2008). The production of luciferase by these cells increases concentration-dependently upon stimulation with TGF-β. This is a very

sensitive assay to detect all three isoforms of TGF-β, and is also relatively specific for TGF-β since other inducers of PAI1 have a negligible effect on these cells (Abe *et al.* 1994). Addition of a pan-TGF-β blocking antibody allows any TGF-β-independent luciferase activity to be determined. Cell supernatants are removed from stimulated cells and used to stimulate TMLCs. After 16 hours the cells are lysed and the luciferase activity of the cell lysates is measured using a luminometer (Berthold Technologies, UK). The raw luciferase data can be converted into TGF-β activity using a TGF-β1 standard curve of known TGF-β concentrations against luciferase activity. The TMLCs can only detect active TGF-β. This gives the opportunity to investigate the levels of active and active plus latent TGF-β in cell supernatants by utilising the property of latent TGF-β to become activated in extremes of heat; heating samples to 80°C for 10 minutes prior to stimulation of TMLCs activates all TGF-β present (Brown *et al.* 1990). TMLCs are therefore a powerful tool for investigating TGF-β activation in vitro.

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2.6.2   Method

TMLCs were cultured as described in Methods section 2.2.3. TMLC were counted and resuspended at a density of 0.5×10^6 cells/ml in serum free DMEM (see Appendix). To an aliquot of TMLC a pan TGF-β neutralising antibody (clone 1D11) at 20µg/ml was added (achieving final concentration of 10µg/ml following stimulation). The cells were plated at 100µl/well in a 96 well plate and left to adhere to the plate for 1 hour. Cell supernatants from HASM cells were used to stimulate the TMLC (100µl/well). All stimulations were performed in duplicate, ensuring that each sample was used to stimulate both TMLC alone and TMLC plus anti-TGF-β antibody. To measure the levels of total TGF-β an aliquot of the cell supernatants were heated to 80°C for 10 minutes and allowed to cool prior to stimulation of TMLCs (Brown *et al.* 1990). After 16 hours, the medium was removed and 50µl luciferase lysis buffer (supplied with Luciferase reporter assay kit) was added to each well to lyse the TMLCs. The plate was frozen at -20°C for at least 1 hour to aid cell lysis. After thawing the luminescence was measured using a luminometer (Berthold Technologies). Each well was exposed to the light detector in the luminometer for 10

seconds and the relative light units (RLU) were recorded. Mean data from wells containing the pan TGF-β blocking antibody was subtracted from the data obtained from the wells treated in the same manner but without the antibody. This removes any TGF-β-independent luciferase activity from all data.

2.7   Co-culture Assay

2.7.1   Principles of Assay

The TMLC reporter cells described in Methods section 2.6.1 can also be used in a co-culture assay to detect TGF-β activation that requires direct cell-cell contact, for example integrin-mediated TGF-β activation (Xu *et al.* 2009). TMLC are seeded directly on top of the experimental cells, in this case HASM cells. The co-culture is then stimulated according to the experimental requirements and incubated for 16 hours. The cells are lysed and the luminescence measured as described in Methods section 2.6.1. The raw luciferase data can be converted into TGF-β activity using a TGF-β1 standard curve of known TGF-β concentrations against luciferase activity. TMLCs can be influenced directly by agonists used in experiments and so the effect of agonists on the response of TMLCs must be investigated.

2.7.2   Method

Following growth arrest of HASM cells, TMLC were plated directly on top of HASM cells at 0.5×10^6 cells/ml, 100µl/well in a 96 well plate. To an aliquot of TMLC, a pan TGF-β blocking antibody was added at 20µg/ml (to achieve final concentration of 10µg/ml following stimulation), before the cells were seeded on to the HASM cells. The co-culture was stimulated in duplicate as required for each individual experiment, ensuring that each stimulation was performed on TMLC and TMLC plus anti-TGF-β. Certain wells of the co-culture were stimulated with 0, 250, 500 and 1000 pg/ml recombinant TGF-β1 to obtain a TGF-β1 standard curve. The cells were incubated for 16 hours and the cells were lysed as in Methods section 2.6.2. The luminescence was measured

using a luminometer (Berthold Technologies) as in Methods section 2.6.2. Mean data from wells containing the pan TGF-β blocking antibody was subtracted from the data obtained from the wells treated in the same manner but without the antibody. This removes any TGF-β-independent luciferase activity from all data. This data was then converted into concentrations of TGF-β activity using the TGF-β1 standard curve. The co-culture assay was also used to measure levels of total TGF-β sequestered in the ECM by unstimulated HASM cells. Unstimulated, growth arrested HASM cells were treated with 20% v/v 1N HCL acid directly in to the culture medium for 10 minutes. The acid was neutralised by addition of an equal volume of 1.2N NaCl. TMLCs were seeded directly on top of these HASM as described above and culture for 16 hours. A TGF-β1 standard curve using 0, 250, 500 and 1000 pg/ml TGF-β was obtained as described above to allow the raw luciferase data to be converted into concentrations of TGF-β activity. The cells were lysed and the luminescence measured as in section Methods 2.6.2.

2.8 Cell Titer Glow Assay

2.8.1 Principles of Assay

Due to reported differences in cell size of HASM cells isolated from asthmatics and non-asthmatics (Ebina *et al.* 1993), it was necessary to normalise all data from asthmatic vs non-asthmatic co-culture experiments to cell number. Cell Titer-Glow® luminescent cell viability assay (Promega, UK) determines the number of viable cells by quantifying the amount of ATP present. Its 96 well plate format makes it ideal for quantifying the number of cells in the co-culture assay, which is also performed in a 96 well plate. Both the co-culture assay and the Cell Titer-Glow® assay require cell lysis to obtain the data. Therefore, when calculation of cell number was required additional wells of the co-culture were treated exactly the same as the experimental wells. The experimental wells were lysed according to Methods section 2.6.2 and the additional wells were used to quantify cell number according to the protocol below.

2.8.2   Method

Wells to be used for quantifying cell number were treated in exactly the same manner as the experimental wells. Additional HASM cells were trypsinised and counted then seeded in duplicate at 0, 3.38, 6.75, 12.5, 25 and 50 $\times 10^4$ cells/ml, 100µl/well to produce a standard curve of known cell densities against luminescence. After the 16 hour co-culture assay incubation period the experimental co-culture wells were lysed according to Methods section 2.6.2. The plate containing the wells for cell number quantification was removed from the incubator and the temperature allowed to adjust to room temperature for approximately 10 minutes. 100µl Cell Titer-Glow® reagent was added directly to the culture medium of each well. The plate was placed on a rocker for 30 minutes at room temperature to allow efficient cell lysis and stabilisation of the luminescent signal. The signal remains stable for up to 6 hours. The luminescence was then measured using a luminometer (Berthold Technologies, UK), exposing each well to the light detector for 0.1 seconds. The luminescence data was converted into actual cell numbers using the standard curve of known cell numbers against luminescence.

2.9   Isolation of Nuclear and Cytoplasmic Protein Fractions

2.9.1   Principles of Assay

Once activated, many transcription factors translocate from the cytoplasm into the nucleus to interact with DNA promoter regions and initiate gene transcription. By isolating cytoplasmic and nuclear fractions of cell protein separately it is possible to detect this translocation by western blotting (see Methods section 2.11). This has been shown for numerous transcription factors (Clarke *et al.* 2008; Clifford *et al.* 2008; John *et al.* 2009). Since Smad2 and Smad3 are transcription factors that translocate to the nucleus it is possible to detect this translocation as an additional method of measuring TGF-β activity.

Isolation of nuclear and cytoplasmic fractions of cell protein is achieved by manipulating the difference in osmotic pressure between the cytoplasmic and nuclear compartments of cells. Since proteins are very susceptible to damage from heat and proteases, nuclear and cytoplasmic protein isolations are performed on ice in the presence of several protease inhibitors. Cells are collected and lysed in a hypotonic lysis buffer, which causes the cells to swell and lyse leaving the nuclei intact. The nuclei are collected by centrifugation, storing the supernatant which contains the cytoplasmic proteins, and then lysed in a high-salt lysis buffer (hypertonic) to collect the nuclear proteins. Insoluble proteins are removed by centrifugation and the two fractions of cell protein stored at -80°C to minimise degradation of the proteins.

2.9.2 Method

Isolation of nuclear and cytoplasmic proteins was performed using a NXtract CellLytic nuclear extraction kit as previously described (Clifford *et al.* 2008) according to the manufacturer's instructions. Following stimulation cells were washed with ice cold PBS, scraped into sterile eppendorfs and centrifuged for 5 minutes at 450xg at 4°C. After discarding the supernatant the cell pellet was lysed in 250μl 1x lysis buffer plus 1mM DTT and 1% protease inhibitor cocktail on ice for 15 minutes. 0.06% v/v IGEPAL CA-360 detergent was added and the samples vortexed for 10 seconds, avoiding foaming of the sample. Samples were centrifuged at 11000xg for 30 seconds at 4°C. The supernatant containing the cytoplasmic fraction of cell proteins was collected and stored at -80°C. The remaining pellet was washed in lysis buffer plus 1mM DTT and 1% protease inhibitor cocktail and centrifuged to minimise contamination of the nuclear fraction with cytoplasmic proteins. The cell pellet was lysed in 50μl Extraction buffer plus 1% protease inhibitor cocktail and 1mM DTT by vortexing for 15 minutes. The samples were centrifuged at 20000xg for 5 minutes at 4°C. The supernatant containing the nuclear fraction was collected and stored at -80°C.

2.10 Bicinchoninic Acid (BCA) Protein Assay

The BCA assay is a colorimetric assay for determining protein concentrations in experimental samples. The BCA protein assay kit was supplied by Pierce, UK. A standard curve of known BSA concentrations was added in duplicate to a 96 well flat bottomed plate. Samples were diluted 1:10 and 1:25 and also added in duplicate to the plate. Reagent B (contains 4% cupric sulphate, supplied with kit) was diluted 1:50 with reagent A (contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide, supplied with kit) and mixed. Then 200µl of this solution was added to each well containing standards or samples. The plate was incubated at 37°C for 30 minutes. After cooling the plate to room temperature the absorbance at 590nm was determined using a plate reader. A best-fit standard curve was constructed by the plate reader and protein concentrations of the experimental samples were calculated. Protein concentrations were determined immediately prior to separation by western blotting to avoid freeze thawing the samples, which can promote protein degradation.

2.11 Western Blotting

2.11.1 Principles of Assay

Western blotting is a commonly used semi-quantitative technique to detect specific proteins within a sample of cells or homogenised tissue. It was originally based upon the techniques Northern and Southern blotting. It utilises gel electrophoresis to separate proteins within a sample of mixed proteins according to their molecular weight. Cells or tissue are lysed on ice and in the presence of various protease inhibitors to inhibit protein degradation. The protein is then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Following separation the proteins are transferred via an electric current onto a membrane of either nitrocellulose or polyvinylidene fluoride (P.V.D.F) membrane. It is then possible to probe this membrane with an antibody directed against a protein of choice to detect expression of that protein.

2.11.2 Cell Lysis

Following removal of culture medium the cells were washed with ice-cold sPBS. Cells were lysed on ice in 100µl lysis buffer (see Appendix 9.5.2.). The cell lysates were collected into pre-chilled eppendorfs and centrifuged (10,000 rpm, 3 minutes at 4°C) to remove any insoluble protein. The protein concentration was determined by BCA assay (Methods section 2.10).

2.11.3 SDS-PAGE

The gel casting apparatus was set up according to the manufacturer's instructions (BioRad). A 10% resolving gel was prepared and poured into the gel casting apparatus. Once set the stacking gel was prepared and poured on top of the resolving gel. The comb was inserted and the gel left at room temperature to set. After removal of the comb the apparatus was carefully loaded into the tank. The middle compartment was filled with 1x running buffer (see Appendix) and the tank was filled to about 1/3 full with 1x running buffer. Rainbow™ protein marker was added to the first lane (7µl for small gels and 20µl for large gels) and the samples loaded into the subsequent wells. Gels were then ran on ice at 90mA for approximately 45 minutes.

2.11.4 Transfer

Two pieces of filter paper, two sponges and a piece of pure P.V.D.F membrane were soaked in methanol for 30 seconds. They were washed in tap water then left to soak with the sponges for the transfer apparatus in 1x transfer buffer (see Appendix 9.5.2) on a rocker for 3 minutes. The gel was removed from the running apparatus and soaked for 3 minutes in 1x transfer buffer. A piece of sponge was laid on to the cassette, followed by filter paper and then the P.V.D.F membrane. The gel was carefully placed on top of the membrane. A piece of filter paper then a piece of sponge was laid on top of the gel and any bubbles expelled by rolling a pipette across the top. The transfer cassette placed in the transfer tank with the gel closest to the negative (black)

electrode and the membrane closest to the positive electrode. After addition of transfer buffer (see Appendix), voltage was applied through the system at 100mV for 1 hour standing in ice. Once the transfer was completed the membrane was removed and stained with Ponceau's stain to ensure that protein had transferred to the membrane. The membrane was then washed for 2 x 5 minutes in Tris buffered saline plus Tween (TBST) (see Appendix 9.5.2)

2.11.5 Protein Detection

The membrane was blocked in 5% non-fat milk at room temperature for 1 hour to reduce the non-specific binding of antibodies. The membrane was washed three times in TBST. The primary antibody was applied in 5% non-fat milk at the correct dilution for the antibody and the membrane was left on a rocker at 4°C overnight. The following day the membrane was washed in TBST for 3 x 5 minutes on a rocker. The secondary antibody was then applied in 5% non-fat milk at the correct dilution for the antibody and the membrane was left on a rocker for 1 hour at room temperature. After 1 hour the membrane was washed for 3 x 5 minutes as before. Equal volumes of the two ECL™ detection reagents were mixed together and poured over the membrane ensuring the whole surface of the membrane was covered and the membrane was left for 2 minutes. It was blotted dry with tissue paper to remove excess ECL™ detection reagent and placed in a plastic wallet. The membrane was then developed in a dark room using Hyperfilm ECL™. The position of the bands was checked against the Rainbow™ protein marker to determine the molecular weight. In order to ensure equal loading of protein samples, the membranes were also probed with a monoclonal antibody against GAPDH. GAPDH is an example of a housekeeping gene that is constitutively expressed in all cells and whose expression should not vary between cell types or with stimulation.

2.12 Stable Transfections

2.12.1 Principles of Assay

Transfection involves the delivery of nucleic acids, commonly DNA, in to eukaryotic cells, which consequently translate the nucleic acids into protein. This can be achieved by biochemical or physical means. Physical means of transfection generally involve physically perforating the cell membrane using electrical pulses, which allows the delivery of nucleic acids across the cell membrane. Biochemical transfection uses a chemical agent that forms a complex with the nucleic acids and aids its transfer across the cell membrane. Several different biochemical agents can be used for transfection of nucleic acids into eukaryotic cells including calcium phosphate and cationic lipids. Calcium phosphate complexes with DNA and forms an insoluble precipitate which is taken up by the cell by endocytosis. Cationic lipids form liposomes that bind to the nucleic acids. These fuse to the phospholipid cell membrane and deliver the nucleic acid in to the cell.

Transfections of nucleic acids into eukaryotic cells can be either transient or stable. Transient transfection involves the temporary introduction of nucleic acids into a cell. The expression level of the nucleic acid will be high but will only remain for a short period of time because the introduced nucleic acids are not incorporated into the host cell genome. Stable transfection involves the generation of clonal transfected cell lines by selective cell culture. The cell lines often have the nucleic acids inserted into their genome. The vector containing the nucleic acids to be transfected carries an antibiotic resistance gene. This means that successfully transfected cells survive culture in the presence of the antibiotic allowing clonal expansion of the transfected cells. Such stable transfectants can be maintained in culture for long periods of time providing that the selection agent is present.

2.12.2 Method

DNA plasmid constructs corresponding to the common full length integrin β5 subunit and a polymorphic integrin β5 subunit in the vector pc.DNA3.1 were kindly provided by Professor Kawahara (Kanazawa University, Japan). The transfection reagent Transfast (Promega, UK), which uses cationic lipids for nucleic acid delivery, was used to stably transfect CS-1 hamster melanoma cells. Four different concentrations of DNA (1, 2, 3 and 4μg) were used to separately transfect CS-1 cells with a ratio of DNA: Transfast of 1:2. This was the recommended ratio outlined in the manufacturer's instructions. Cells were transfected in 75cm² tissue culture flasks in a total volume of 6ml. The appropriate amount of DNA and Transfast reagent was added to transfection media to make a final volume of 6ml. After vortexing the mixture, it was left for 15 minutes at room temperature to allow the DNA to complex with the Transfast. The DNA: Transfast complex was then added to cells cultured in transfection medium (see Appendix 9.6). The cells were incubated overnight at 37°C in a humidified atmosphere. The following day, the media was removed and fresh transfection medium applied. Expression of β5 was evident after 2 days when CS-1 cells began to adhere to the plastic. At this point the cells were cultured in CS-1 media containing 500μg/ml G418 to select for transfected cells.

2.13 Co-Immunoprecipitation

2.13.1 Principles of Assay

Immunoprecipitation refers to the precipitation of proteins out of a mixture of proteins using specific antibodies directed against the protein of choice. Co-Immunoprecipitation (Co-Ip) is used in molecular biology to investigate protein interactions within the cell. Cells are lysed on ice in the presence of protease, phosphatase and deacetylase inhibitors, to prevent dissociation of any protein complexes within the cell. The cell protein is incubated with an antibody directed against a target protein. Magnetic beads that bind to antibodies are used to immunoprecipitate the target protein. Any proteins that are associated with the target protein are "captured" simultaneously.

Proteins that are suspected to be associated with the target protein can then be detected by western blot. Co-Ip is a powerful experimental tool for measuring protein-protein interactions.

2.13.2 Method

The interaction of the integrin β5 subunit with the cytoskeletal protein talin was investigated using a Universal Magnetic Co-IP kit (Active Motif, USA) as previously shown for different proteins (Clifford *et al.* 2008). A rabbit polyclonal antibody directed against integrin β5 and a mouse monoclonal directed against both talin 1 and 2 (clone 8D4) were used (both from Abcam, UK). 500μg protein was incubated with 5μg antibody for 4 hours. 20μl magnetic beads coated with protein G, which binds with high affinity to IgG antibodies, were added and the tubes incubated at 4°C on a rocker for 1 hour. Using a magnet the beads were washed in 1ml Co-Ip wash buffer (supplied with kit) 5 times then resuspended in 20μl 2x reducing loading buffer (130mM Tris pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 100mM DTT) and stored at -20°C. Protein was separated by SDS-PAGE (see Methods section 2.11.3). 10μg of protein prior to immunoprecipitation was also loaded on to the gel as a loading control. The resulting membrane was probed with the second antibody (10μg/ml) and developed as previously described (Methods section 2.11.5).

2.14 Flow Cytometry

2.14.1 Principles of Assay

Flow cytometry is a powerful technique used to accurately quantify and examine cells or other particles within a suspension. A flow cytometer can provide information about individual cell size, the internal complexity or granularity of a cell and the relative fluorescence intensity of a cell using a combination of fluid mechanics and optics. Cell suspensions are hydrodynamically focused within a sheath fluid through a laserbeam, and optical detectors detect the scatter of light. Forward scatter (FSc) is detected directly in front of the passing cells and laser beam. FSc refers

to light defracted from the cell surface as it passes the laser beam. Forward scatter gives information about the cell size. Side scatter (SSc) is detected at a 90° angle to the laser beam and refers to light reflected and refracted from the cell surface. SSc provides information about the internal complexity of the cells.

Flow cytometry can also be used to assess expression of proteins by cells. The target protein, either intracellular or cell surface, is labelled with a specific antibody conjugated to a fluorochrome. The flow cytometer is set up so that the laser beam excites the fluorochrome at its specific excitation wavelength and the emitted light is detected. The emitted fluorescence intensity is directly proportional to the number of binding sites for the antibody allowing accurate determination of protein expression levels. Many different fluorochromes exist allowing the simultaneous detection of more than one protein on a cell.

2.14.2 Method

Cell surface expression of $\alpha V \beta 5$ by HASM cells was assessed by flow cytometry using a method previously used to assess expression of $\alpha V \beta 6$ by epithelial cells. Cells were trypsinised using 0.25% trypsin/EDTA solution and counted (see Methods section 2.2.7). 0.5×10^6 cells were added to FACs tubes. The cells were centrifuged and the media discarded. They were then washed in PBS, centrifuged and the PBS discarded. The cells were blocked in 200µl goat serum for 20 minutes at 4°C to inhibit non-specific binding of the antibodies. The goat serum was removed by centrifugation and the cells washed in PBS. After removing the PBS the primary antibody was added at 25µg/ml for 20 minutes at 4°C. After removal of the primary antibody by centrifugation the cells were washed and the PBS removed. The cells were incubated with the secondary phycoerythrin (PE) labelled antibody 1:200 dilution for 20 minutes at 4°C in the dark to prevent light bleaching of PE. The antibody was removed, the cells washed in PBS the resuspended in 500µl PBS. The cells were analysed using a BD FACSCalibur flow cytometer.

2.15 Statistics

Data is expressed as mean ± SEM for all experiments unless otherwise stated. Statistical analysis was performed using GraphPad Prism 4. When a comparison of only two data sets was needed an unpaired Student's t test was performed. When comparing more than 2 data sets a one-way analysis of variance (ANOVA) was performed, with either a Bonferroni's post test to compare 2 specific data sets, or a Dunnet post test to compare all data sets with a control data set. When comparing two treatment groups, each with multiple data sets, a two-way ANOVA was performed. This determines if means were significantly different for a) data sets within a treatment group, or b) between the treatment groups. P values less than 0.05 were accepted as significant.

Proteolytic Activation of TGF-β from HASM Cells by Mast Cell Tryptase

3 Proteolytic Activation of TGF-β from HASM Cells by Mast Cell Tryptase

3.1 Rationale

Mast cells (MC) play a fundamental role in orchestration of airway inflammation in asthma (discussed in Introduction section 1.1.3.5). The EAR, and therefore brochoconstriction, is instigated when FcεRI receptors on the surface of MC become cross-linked by antigen, causing MC degranulation and the release of a vast array of inflammatory mediators. The number of MC present in the airways is much higher in asthmatics compared with non-asthmatics (Carroll et al. 2002) and infiltration of the ASM layer by MC is an important characteristic of asthma (Brightling et al. 2002). Furthermore, the degree of MC degranulation correlates positively with asthma severity (Carroll et al. 2002).

Tryptase is the most abundant protease released from activated MC. It is a serine protease with trypsin-like activity, which is unique to the secretory granules of MC. Several isoforms have been identified and can be divided in to four groups designated α-tryptases, β-tryptases, γ-tryptases and ε-tryptases. β tryptases is one of the most abundant proteases present in MC. α tryptases, however, are predominantly found in the secretory granules of basophils. γ tryptases are expressed in a variety of tissues but, unlike α and β tryptases, they have a hydrophobic region at their carboxyl terminal, which is important for tethering the protease to the cell membrane. ε tryptases are widely expressed in the lungs, especially in the epithelium but are not expressed by mast cells. For the purposes of this thesis I will concentrate upon β-tryptase since this is the predominant form released from MC. β-tryptase will now be referred to as tryptase.

Tryptase is stored as proteolytically active tetramers in MC granules. The monomers form a square ring with the catalytic domains of each subunit pointing towards a central pore, which forms the active site (Pereira et al. 1998). The tetramers are complexed with a proteoglycan,

usually heparin, which stabilises the tetramer complexes and is essential for tryptase activity (Schwartz et al. 1986). Our group has shown that tryptase can cleave and inactivate the chemokines CCL5 and CCL11 *in vitro* (Pang et al. 2006). However, in vivo a substrate for tryptase still needs to be identified. It is commonly thought that tryptase mediates many of its effects via cleavage and activation of proteinase-activated receptor-2 (PAR2). Members of this family of receptors are activated by a novel proteolytic mechanism. The receptor is recognised by its activating protease, which cleaves the receptor at a specific site within the amino terminal domain. This exposes a tethered ligand which interacts with the second extracellular loop of the receptor to initiate cell signalling in a similar manner to other G protein coupled receptors (see Figure 3.1).
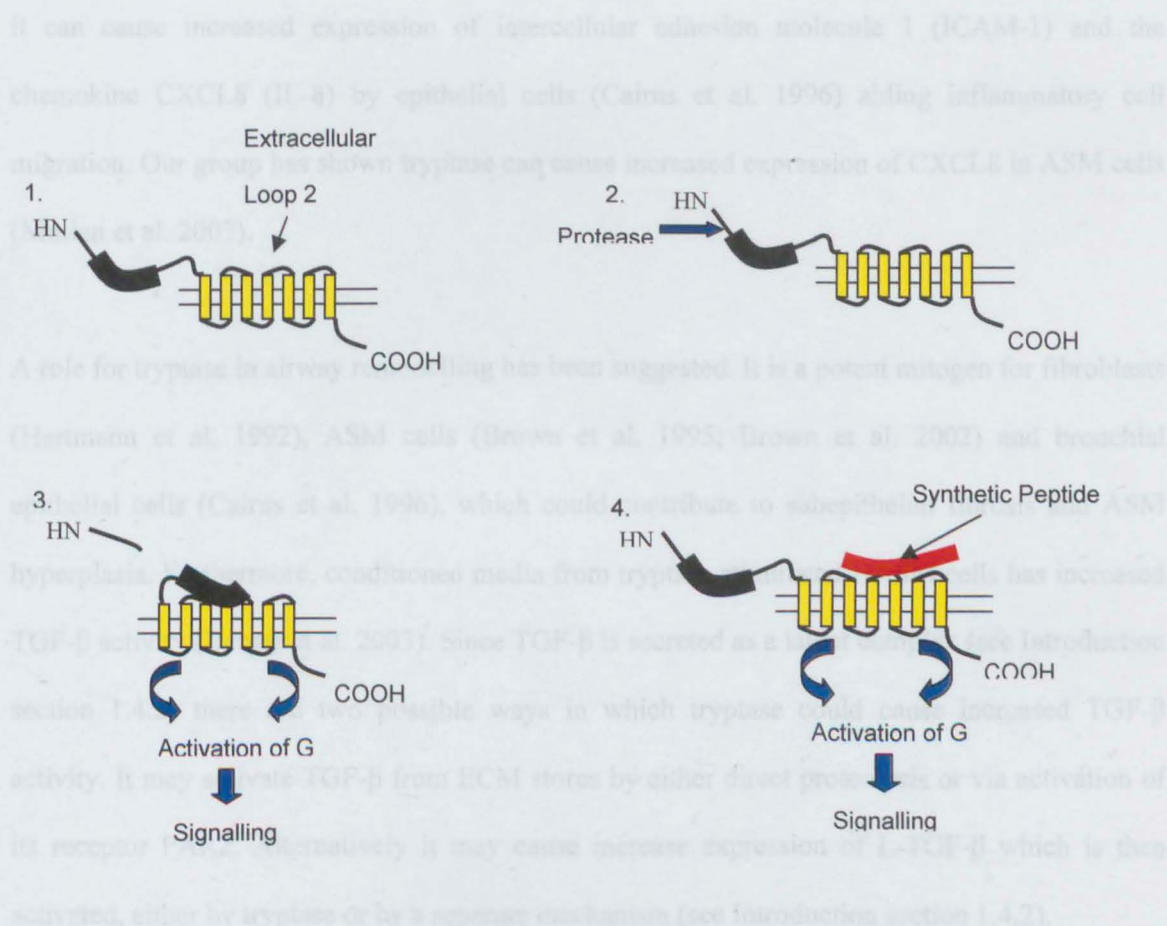
Figure 3.1: Mechanism of Activation of PARs

Proteolytic activators of PARs cleave the extracellular domain of the receptor at a specific site (2). This liberates a tethered ligand, which is now able to interact with the second extracellular loop of the receptor causing activation of G proteins and cell signalling (3). Synthetic peptides corresponding to the tethered ligand domain are able to activate the receptor in a proteolysis independent mechanism by interacting directly with the second extracellular loop of the receptor.
Adapted from (Macfarlane et al. 2001).

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Tryptase has been implicated in asthma pathogenesis. The level of tryptase in BALF from atopic asthmatic is increased (Wenzel et al. 1988), and increases following allergen challenge have been reported (Flint et al. 1985). The immediate effects of tryptase following its release are associated with EAR (see Introduction section 1.1.2). It can potentiate histamine-induced bronchoconstriction (Johnson et al. 1997), activate MC causing degranulation (Molinari et al. 1996) and has been implicated in AHR (Schmidlin et al. 2002; Ebeling et al. 2005). A role for tryptase in the LAR has also been suggested since inhalation of the tryptase inhibitor APC366 can diminish allergen-induced LAR (Krishna et al. 2001). It also contributes to infiltration of the airways by inflammatory cells. It increases microvascular permeability (He et al. 1997) and can stimulate migration of eosinophils and neutrophils (Walls et al. 1995; He et al. 1997). Moreover, it can cause increased expression of intercellular adhesion molecule 1 (ICAM-1) and the chemokine CXCL8 (IL-8) by epithelial cells (Cairns et al. 1996) aiding inflammatory cell migration. Our group has shown tryptase can cause increased expression of CXCL8 in ASM cells (Mullan et al. 2007).

A role for tryptase in airway remodelling has been suggested. It is a potent mitogen for fibroblasts (Hartmann et al. 1992), ASM cells (Brown et al. 1995; Brown et al. 2002) and bronchial epithelial cells (Cairns et al. 1996), which could contribute to subepithelial fibrosis and ASM hyperplasia. Furthermore, conditioned media from tryptase stimulated HASM cells has increased TGF-β activity (Berger et al. 2003). Since TGF-β is secreted as a latent complex (see Introduction section 1.4.2) there are two possible ways in which tryptase could cause increased TGF-β activity. It may activate TGF-β from ECM stores by either direct proteolysis or via activation of its receptor PAR2. Alternatively it may cause increase expression of L-TGF-β which is then activated, either by tryptase or by a separate mechanism (see Introduction section 1.4.2).

3.2 Aims

The aims of this chapter were to investigate whether tryptase causes increased TGF-β activity in HASM cell supernatants by increasing total TGF-β expression or by activating existing stores of L-TGF-β, or by a combination of both mechanisms. The effects of tryptase's proteolytic activity on TGF-β activity will be investigated. The role of the tryptase receptor PAR2 in tryptase-mediated effects on TGF-β expression and activity will be determined using a synthetic peptide that has the same amino acid sequence as the tethered ligand of PAR2.

3.3 Results

3.3.1 Concentration-Response of Tryptase on Levels of Active and Total TGF-β in Cell Supernatants

HASM cells were treated in duplicate with 0, 3.75, 15 and 60 mU/ml tryptase. Cell culture supernatants were collected after 4 or 24 hours and the amount of active and total TGF-β determined by reporter cell assay (see Methods section 2.6). The TGF-β-independent luciferase activity was subtracted from all data sets as described in Methods section 2.6 and the data expressed as fold increase in TGF-β activity. Stimulation of HASM cells with tryptase caused a concentration-dependent increase in active TGF-β released in to cell supernatants after 4 hours (Figure 3.2). The lowest concentration tested was 3.75mU/ml which caused a 2.2 fold increase in TGF-β activity ($p < 0.05$) rising to a 4.84 fold increase when stimulated with 60mU/ml tryptase ($p < 0.01$). The levels of total TGF-β released in to cell supernatants after 4 hours did not change with increasing tryptase concentration.

The tryptase-induced increase in active TGF-β observed after 4 hours of stimulation was not seen when cell supernatants were collected after 24 hours of stimulation. None of the tryptase concentrations tested caused an increase in active TGF-β in cell supernatants as shown in Figure 3.3. The highest concentration of tryptase tested (60mU/ml) only caused a 1.28 ± 0.25 fold increase in active TGF-β over basal. Furthermore, none of the concentrations tested had any effect on levels of total TGF-β present in the supernatants after 24 hours of stimulation.

Figure 3.2: Concentration-Response of Tryptase on Levels of Active and Total TGF-β in Cell Supernatants After 4 Hours

HASM cells were stimulated with 0, 3.75, 15 or 60mU/ml tryptase. The cell supernatants were collected after four hours and used in a reporter cell activity to detect active and total TGF-β. Tryptase induced a concentration-dependent increase in active TGF-β present in cell supernatants after 4 hours. Levels of total TGF-β remained unchanged at all concentrations tested. This figure shows the amalgamation of data from 3 separate experiments expressed as the mean fold increase over basal ± SEM.
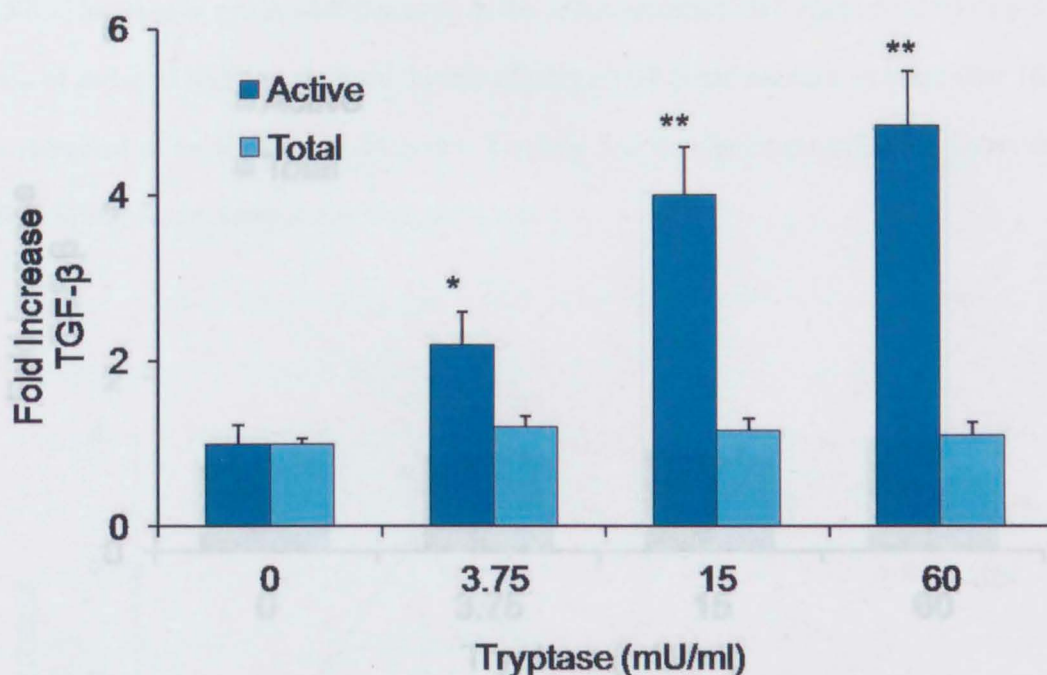
$* P<0.05$ $** P<0.01$

Figure 3.3: Concentration-Response of Tryptase on Levels of Active and Total TGF-β in Cell Supernatants After 24 Hours

HASM cells were stimulated in duplicate with 0, 3.75, 15 and 60mU/ml tryptase. Cell supernatants were collected after 24 hours and assayed for active and total TGF-β using a reporter cell assay. Tryptase had no effect on levels of active of total TGF-β present in cell supernatants collected after 24 hours of stimulation. This figure represents data from 3 separate experiments expressed as the mean fold increase over basal ± SEM.

3.3.2 Effect of Tryptase Stimulation Time on Levels of Active and Total TGF-β in Conditioned Media

Results from the previous experiment showed that stimulation of HASM cells with 15mU/ml tryptase causes a statistically significant increase in active TGF-β present in cell supernatants after four hours of stimulation (see Figure 3.2). The response appeared to plateaux at this

concentration as stimulation with 60mU/ml tryptase did not induce a much greater increase in

active TGF-β. All future experiments were therefore performed using 15mU/ml tryptase. HASM

cells were treated in duplicate with 15mU/ml purified human lung tryptase. Cell culture

supernatants were collected after 0, 4, 8, 16 and 24 hours and assayed for active and total TGF-β

using the reporter cell assay (Methods section 2.6). As Figure 3.4 shows, tryptase caused a 3.61 ±
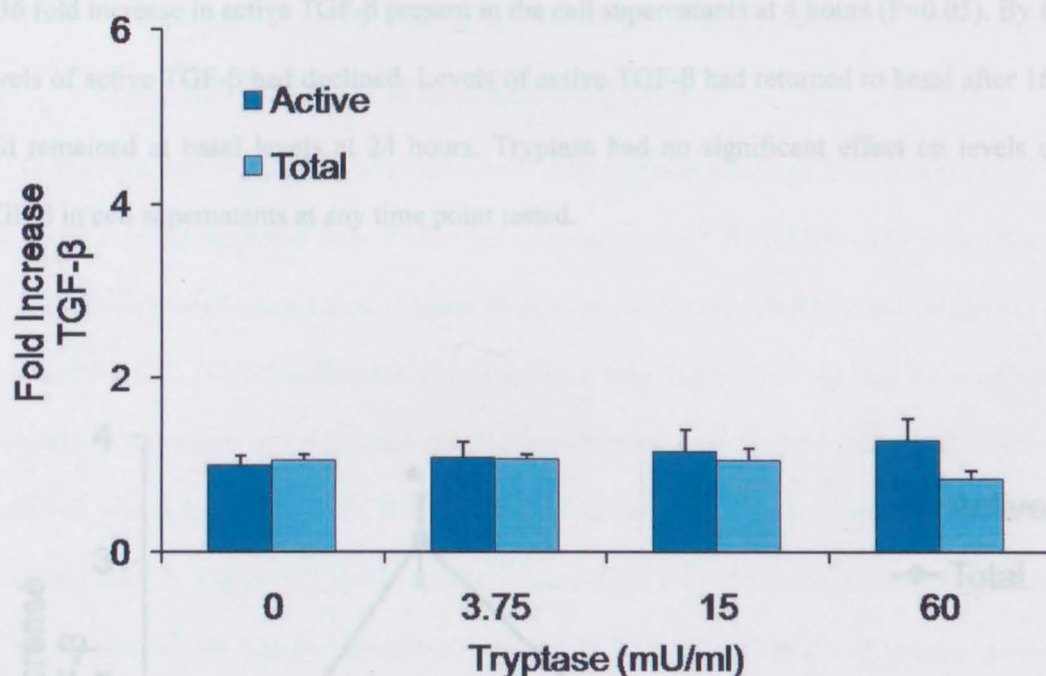
0.36 fold increase in active TGF-β present in the cell supernatants at 4 hours (P<0.05). By 8 hours

levels of active TGF-β had declined. Levels of active TGF-β had returned to basal after 16 hours

and remained at basal levels at 24 hours. Tryptase had no significant effect on levels of total

TGF-β in cell supernatants at any time point tested.
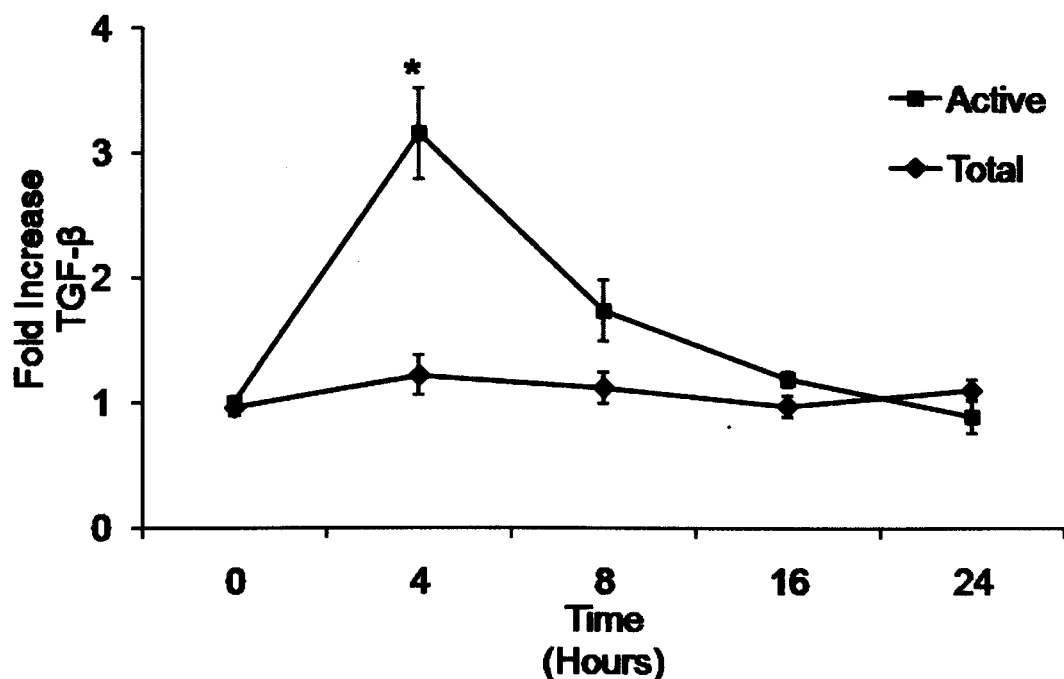
Figure 3.4: Effect of Tryptase Stimulation Time on Levels of Active and Total TGF-β in Cell Supernatants

HASM cells were stimulated in duplicate with 15mU/ml tryptase and cell supernatants assayed for active and total TGF-β after 0, 4, 8, 16 and 24 hours. Stimulation of HASM cells with 15mU/ml tryptase caused an increase in active TGF-β present in cell supernatants after 4 hours, after which time levels declined returning to basal at 16 hours. Levels of total TGF-β were unaffected. Data was expressed as fold increase in TGF-β activity. This figure represents an amalgamation of data from 3 separate experiments.

* P<0.05

3.3.3 Effect of Tryptase on Expression of TGF-β-Inducible Gene Plasminogen Activator Inhibitor-1 (PAI1) by HASM Cells

To confirm that stimulation of HASM cells with tryptase results in TGF-β activation, expression of the TGF-β-responsive gene PAI1 was assessed by Q-PCR. HASM cells were stimulated with 15mU/ml purified human lung tryptase in duplicate and total cell RNA was collected after 0, 1, 2, 4 and 8 hours. HASM cells were also stimulated with 2ng/ml TGF-β1 and RNA collected after 4 hours as a positive control. After reverse transcription (see Methods section 2.3.4) the resulting cDNA was subjected to Q-PCR analysis for PAI1 and the housekeeping gene β2-microglobulin (β2m) mRNA. Figure 3.5 shows that tryptase caused a time dependant increase in PAI1 mRNA expression. There was no significant increase in PAI1 after 2 hours of tryptase stimulation but after 4 hours tryptase induced an 8.56 ± 3.15 fold increase in PAI1 mRNA. Expression of PAI1 mRNA had increased further after 8 hours where a 17.33 ± 4.76 fold increase was recorded. Stimulation of HASM cells with 2ng/ml TGF-β caused a 24.71 ± 14.39 fold increase in PAI1 mRNA. Tryptase-induced expression of PAI1 mRNA was statistically significant at both 4 and 8 hours ($P < 0.05$).
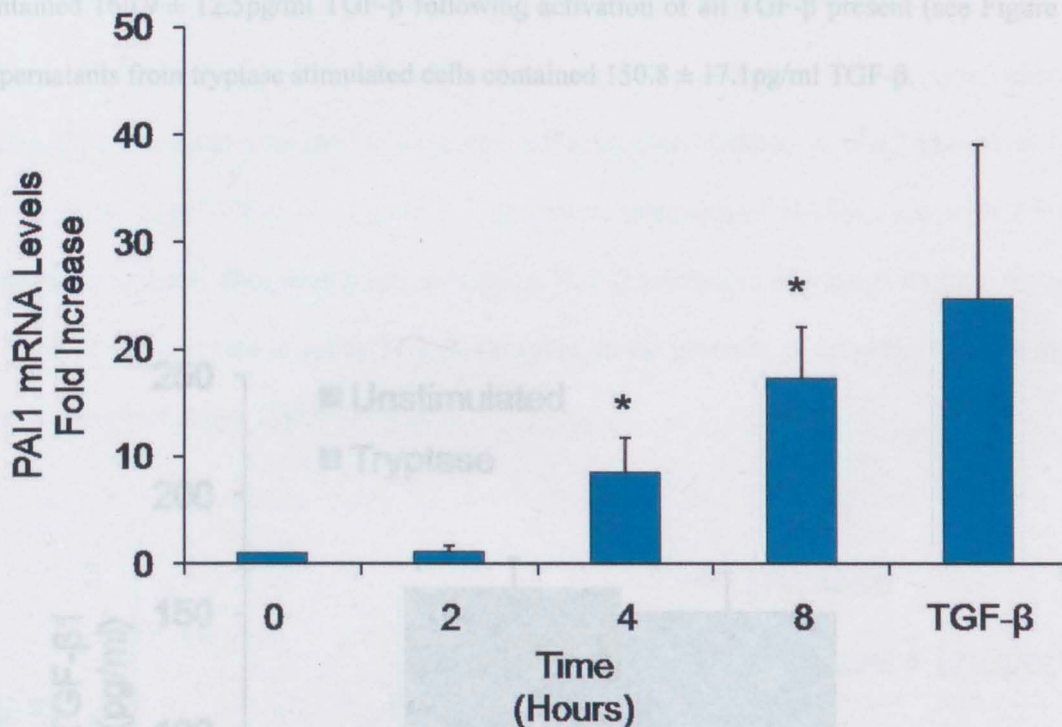
Figure 3.5: Effect of Tryptase on Expression of PAI1 mRNA

HASM cells were stimulated with 15mU/ml tryptase and total cell RNA was collected after 0, 2, 4 and 8 hours. After reverse transcription expression of PAI1 And β2M was determined by Q-PCR. Stimulation of HASM cells with tryptase caused a time dependant increase in PAI1 mRNA expression. This figure shows the data from 3 separate experiments. Data is expressed as fold increase over basal.

* $P<0.05$

3.3.4   Effect of Tryptase on Total TGF-β Present in Cell Supernatants

To confirm that tryptase had no effect on levels of total TGF-β present in cell supernatants an ELISA was used. HASM cells were stimulated with 15mU/ml tryptase in duplicate and cell supernatants collected after 24 hours. The amount of total TGF-β present in the supernatants was measured by ELISA (Methods section 2.5) and TGF-β concentrations were calculated from a standard curve of recombinant active TGF-β. As shown by Figure 3.6, tryptase had no effect on the amount of total TGF-β present in cell supernatants. Cell supernatants from unstimulated cells

contained 160.9 ± 12.5pg/ml TGF-β following activation of all TGF-β present (see Figure 3.6).

Supernatants from tryptase stimulated cells contained 150.8 ± 17.1pg/ml TGF-β.

Figure 3.6: Effect of Tryptase on Total TGF-β Present in Cell Supernatants

HASM cells were stimulated in duplicate with 15mU/ml tryptase and cell supernatants assayed after 24 hours by ELISA for the amount of total TGF-β. Stimulation of HASM cells with 15mU/ml tryptase did not affect the amount of total TGF-β present in cell supernatants after 24 hours of stimulation. This figure represents data from 3 separate experiments expressed as mean $\pm$ SEM.

3.3.5   Effect of Leupeptin on Tryptase-Induced Activation of TGF-β

To determine if tryptase-induced TGF-β activation occurred due to proteolysis leupeptin, a non-selective protease inhibitor was used in a reporter cell assay. HASM cells were either left untreated, treated with 15mU/ml tryptase or 15mU/ml tryptase plus 2.5µg/ml leupeptin. Cells to

be treated with leupeptin were pre-incubated for 30 minutes with leupeptin prior to addition of tryptase. All stimulations were performed in duplicate. Cell supernatants were collected after 0, 4, 8, 16 and 24 hours and were used in a reporter cell assay (see Methods section 2.6) to assay levels of active and total TGF-β. As Figure 3.7 illustrates, treatment of HASM cells with 2.5μg/ml leupeptin completely abrogated tryptase-induced TGF-β activation. At 4 hours tryptase induced a 2.23 ± 0.12 fold increase in active TGF-β. However, in the presence of leupeptin tryptase did not increase levels of active TGF-β (P<0.005).
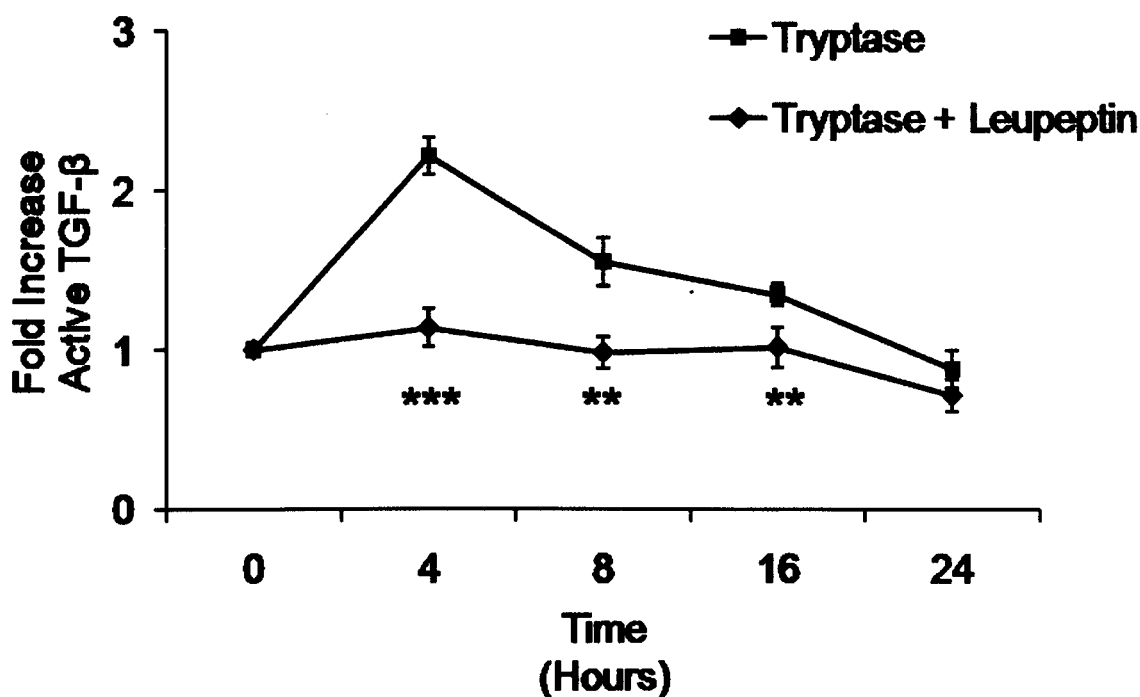
Figure 3.7: Effect of Leupeptin on Tryptase-Induced TGF-β Activation

HASM cells were either left untreated, treated with 15mU/ml tryptase or treated with 15mU/ml tryptase plus 2.5μg/ml leupeptin. Addition of leupeptin completely abolished tryptase-induced TGF-β activation. This figure shows data from 3 separate experiments expressed as mean fold increase over basal ± SEM.

** P<0.01   *** P<0.005

3.3.6 Effect of Selective Tryptase Inhibitor on Tryptase-Induced TGF-β Activation

To confirm that activation of TGF-β is due to the proteolytic activity of tryptase and not of a contaminating protease in the purified tryptase preparation, the selective tryptase inhibitor nafamostat mesilate (FUT175) was used. HASM cells were either left untreated, treated with 15mU/ml tryptase or 15mU/ml tryptase plus 0.2nM selective tryptase inhibitor FUT175. Cells to be treated with FUT175 were pre-incubated for 30 minutes with the inhibitor. All stimulations were performed in duplicate. Cell supernatants were collected after 0, 4, 8, 16 and 24 hours and were used in a reporter cell assay (see Methods section 2.6). FUT175 completely abolished tryptase-induced TGF-β activation (Figure 3.8). After 4 hours of stimulation tryptase induced a 5.53 ± 0.53 fold increase in active TGF-β present in cell supernatants. After 8 hours, levels of active TGF-β had returned to basal. In the presence of FUT175 tryptase-induced TGF-β activation was inhibited (P<0.005).
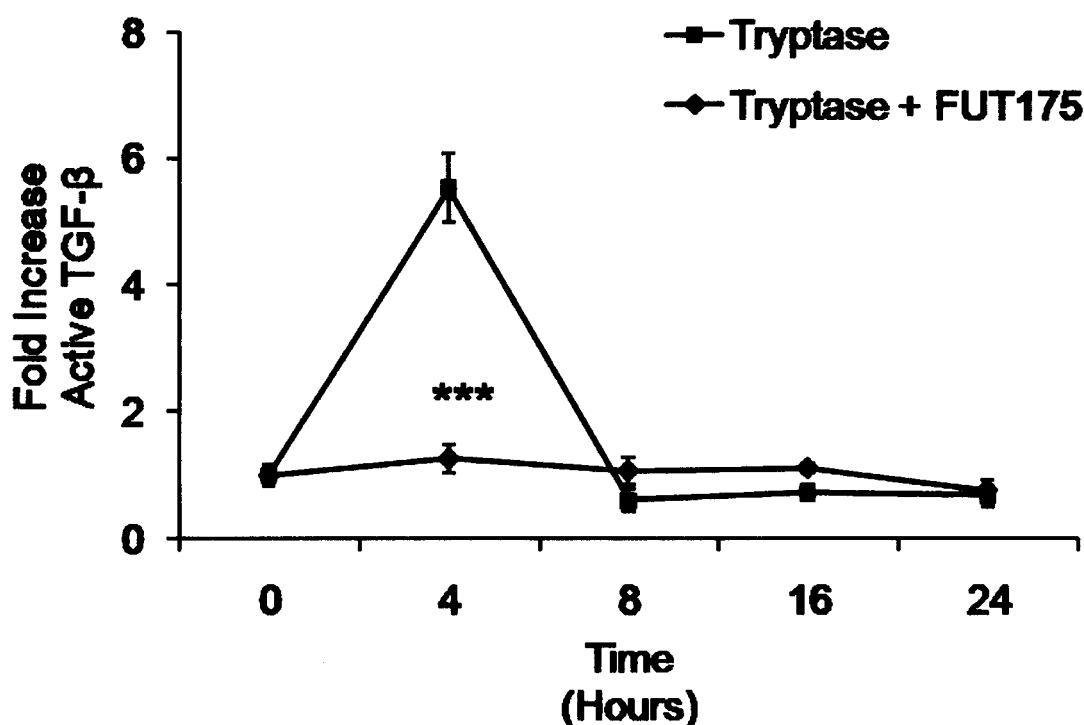
Figure 3.8: Effect of Selective Tryptase Inhibitor on Tryptase-Induced TGF-β Activation

HASM cells were either left untreated, treated with 15mU/ml tryptase or treated with 15mU/ml tryptase plus 0.2nM FUT175. Cell supernatants were assayed for active TGF-β using a reporter cell assay after 0, 4, 8, 16 and 24 hours. All treatments were performed in duplicate. Addition of FUT175 completely abolished tryptase-induced activation of TGF-β. This figure shows the data from 3 separate experiments expressed as mean fold increase over basal ± SEM.

*** $P<0.005$

3.3.7 Effect of PAR2 Activating Peptide (PAR2-AP) on TGF-β Activation

Tryptase-induced TGF-β activation occurs via proteolysis as shown by Figure 3.7 and Figure 3.8. As mentioned in Results section 3.1 tryptase mediates many of its effects through proteolytic cleavage and therefore activation of PAR2 on the cell surface. The involvement of PAR2 in tryptase-induced TGF-β activation was assessed using a synthetic peptide (PAR2-AP) corresponding to the tethered ligand of PAR2 (SLIGKV-NH₂). HASM cells were either left unstimulated, stimulated with tryptase (15mU/ml) or with a synthetic PAR2 activating peptide, SLIGKV-NH₂ (500μM). All stimulations were performed in duplicate. Cell supernatants were collected after 0, 4, 8, 16 and 24 hours and assayed for active TGF-β and total TGF-β using a

reporter cell assay (Methods section 2.6). Figure 3.9 illustrates that tryptase caused a 3.36 ± 0.28 fold increase in active TGF-β present in cell supernatants after 4 hours, after which levels returned to basal. However, stimulation of HASM cells with the PAR2-AP (SLIGKV-NH$_2$) did not result in an increase in active TGF-β present in cell supernatants at any time point tested. Furthermore, neither SLIGKV-NH$_2$ nor tryptase had any effect on levels of total TGF-β present in cell supernatants at any time point tested (Figure 3.10).
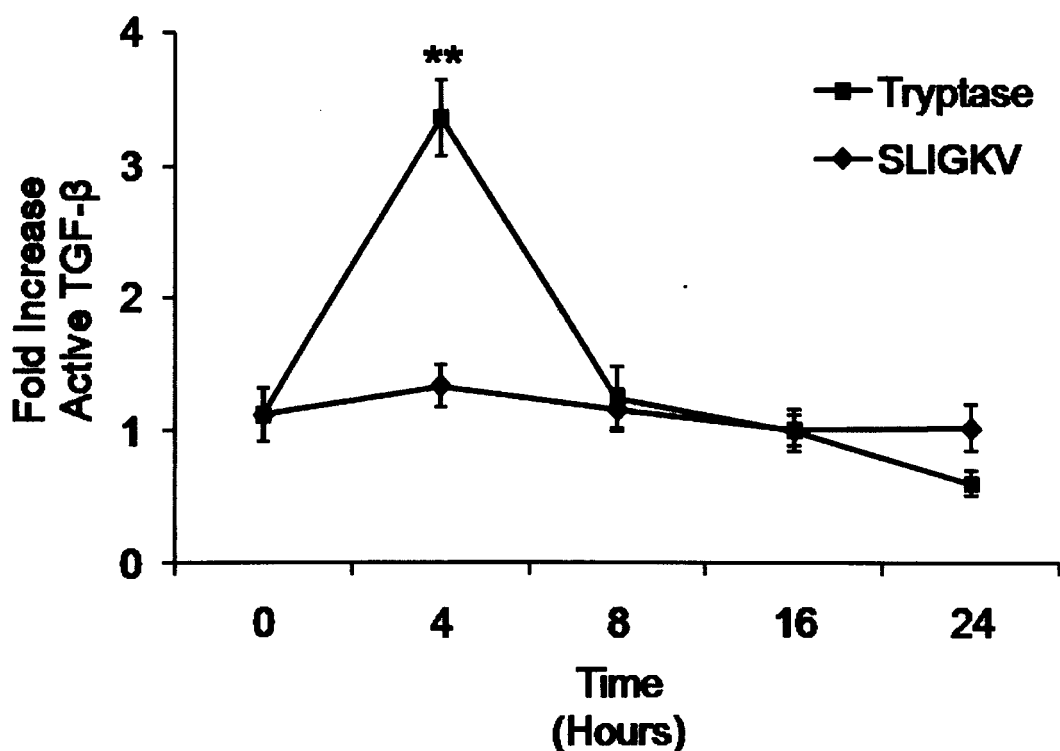
Figure 3.9: Effect of PAR2-AP on Levels of Active TGF-β in Cell Supernatants

HASM cells were treated in duplicate with either media alone, 15mU/ml tryptase or 500μM SLIGKV-NH$_2$. Cell supernatants were assayed for active TGF-β using a reporter cell assay after 0, 4, 8, 16 and 24 hours. Tryptase caused an increase in active TGF-β at 4 hours after which levels returned to basal. SLIGKV-NH2 had no effect on levels of active TGF-β at any time point tested. This figure shows the amalgamation of data from 3 separate experiments and is expressed as mean fold increase over basal ± SEM.

** $P<0.01$
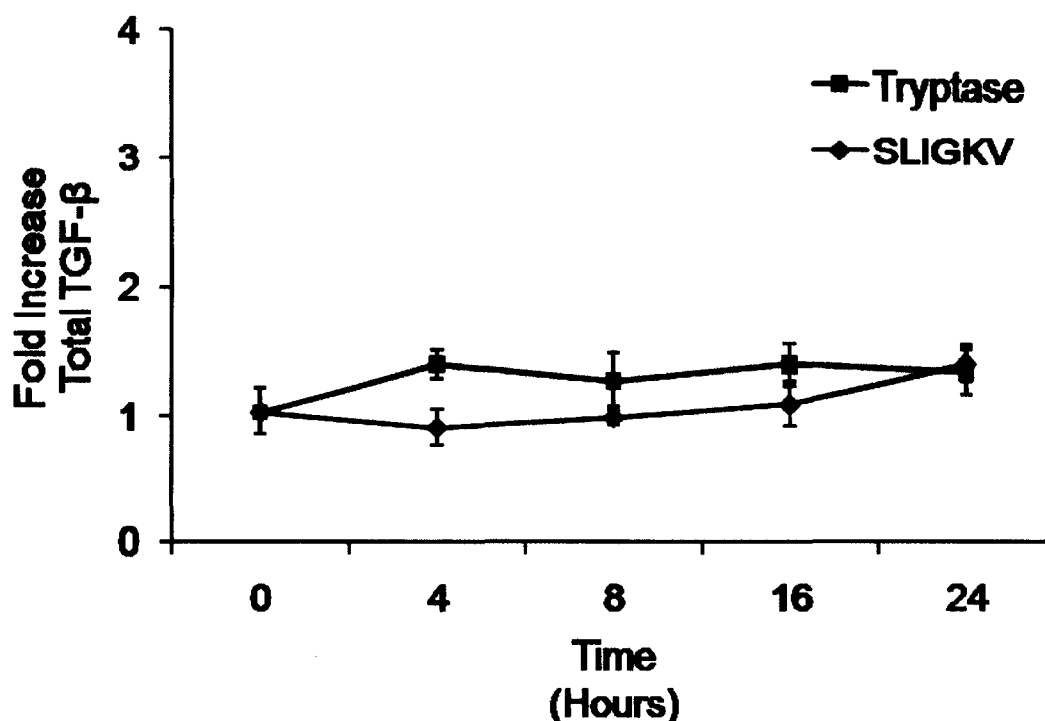
Figure 3.10: Effect of PAR2-AP on Levels of Total TGF-β in Cell Supernatants

HASM cells were treated in duplicate with either media alone, 15mU/ml tryptase or 500μM SLIGKV-NH$_2$. Cell supernatants were assayed for total TGF-β using a reporter cell assay after 0, 4, 8, 16 and 24 hours. Neither tryptase nor the PAR2-AP SLIGKV-NH2 altered the level of total TGF-β present in cell supernatants at any time point tested. This figure shows data from 3 separate experiments expressed as fold increase over basal ± SEM.

3.3.8   Expression of PAR2 Protein by HASM Cells

Data presented in Figure 3.9 show that the PAR2-AP SLIGKV-NH2 had no effect on TGF-β

activation at any time point tested, despite tryptase inducing an increase in active TGF-β after 4

hours. This may be because tryptase-induced TGF-β activation occurs independently of PAR2,

because the cells used did not express PAR2, or because PAR2 is expressed but not functional.

The expression of PAR2 protein by HASM cells was therefore assessed using western blotting

(Methods section 2.11). 30μg of total protein from unstimulated HASM and NHBE cells was

separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed

with a 1:1000 dilution of anti-PAR2 monoclonal antibody (clone SAM11) and a 1:10,000 dilution

of anti-GAPDH monoclonal antibody as a loading control. NHBE cells constitutively express PAR2 protein and were used as a positive control. Figure 3.11 shows that NHBE cells express PAR2 protein illustrated by a band at 50kD. However, no band corresponding to PAR2 was observed in total cell protein collected from HASM cells. GAPDH was used as a loading control and bands representing GAPDH can be seen in both NHBE and HASM cells at 35kD.

NHBE Cells   **HASM Cells**

50kD PAR2 →

35kD GAPDH →
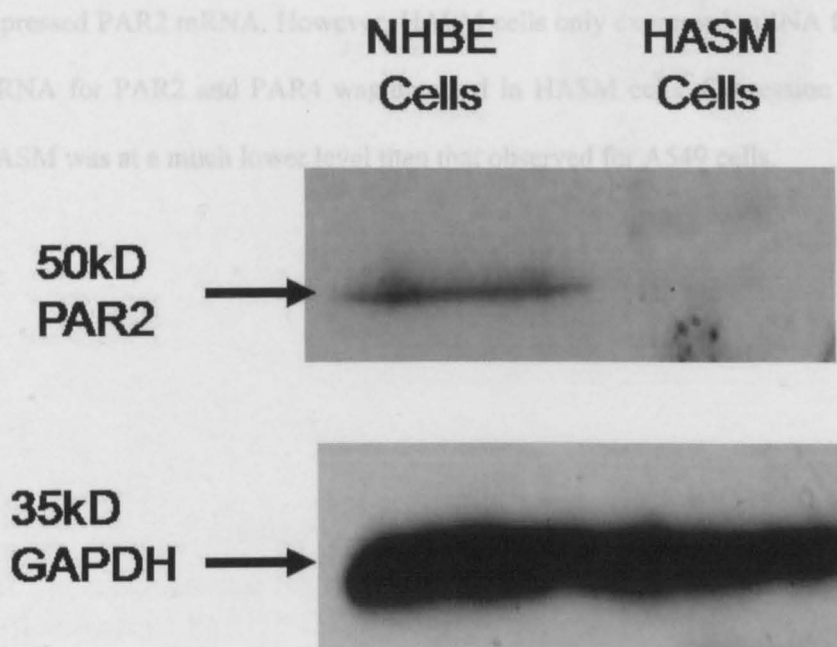
Figure 3.11: Expression of PAR2 Protein by HASM Cells

Western blotting was used to assess expression of PAR2 protein in unstimulated HASM cells using NHBE cells as a positive control. 30µg of total cell protein was loaded per lane. This is a representative blot from 3 separate experiments.

3.3.9 Expression of PAR mRNA by HASM Cells

As Figure 3.11 shows, PAR2 protein was not expressed by HASM cells. In order to determine whether PAR2, and the other three members of the PAR family, were expressed at the mRNA level, their expression was assessed using RT-PCR (chapter 2.3.5). Total cell RNA was collected from unstimulated, growth arrested HASM cells. RNA from NHBE and A549 cells was used as positive controls. NHBE cells were used as a positive control for PAR2 and A549 cells were used as a positive control for PAR1, PAR3 and PAR4. The primers used are outlined in Appendix 9.7. As shown by Figure 3.12, A549 cells expressed mRNA for PAR1, PAR3 and PAR4. NHBE cells expressed PAR2 mRNA. However, HASM cells only expressed mRNA for PAR1 and PAR3. No mRNA for PAR2 and PAR4 was detected in HASM cells. Expression of PAR1 and PAR3 by HASM was at a much lower level than that observed for A549 cells.

PAR1

PAR2

PAR3

PAR4

HASM

+ve control

Figure 3.12: Expression of PAR mRNA by HASM cells

RNA from unstimulated HASM, A549 and NHBE cells was reverse transcribed and subjected to PCR analysis for PAR1, PAR2, PAR3 and PAR4 to determine expression of these four receptors at the mRNA level. HASM cells were shown to express both PAR1 and PAR3 at a very low level, as shown by faint bands in these lanes. However, PAR2 and PAR4 were not expressed by HASM cells. DNA bands were present in the positive control lane for each receptor confirming that the primers were functional. This is a representative figure from 3 separate experiments.

3.3.10 Effect of PAR2-AP on NHBE Cells

These data show that tryptase can activate TGF-β independently of PAR2 as the HASM cells used did not express PAR2. However, it has previously been shown that activation of PAR2 by tryptase causes increased expression of TGF-β (Berger *et al.* 2003). In order to investigate the effect of PAR2 activation on levels of TGF-β released NHBE cells, which express PAR2 (see

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Figure 3.11), were used. NHBE cells were stimulated in duplicate with either 15mU/ml tryptase

or 500μM PAR2-AP (SLIGKV-NH$_2$). Cell supernatants were collected after 0, 4, 8, 16 and 24

hours and used in a reporter cell assay to determine levels of total TGF-β. Neither tryptase nor

PAR2-AP had an effect on levels of total TGF-β in cell supernatants at any time point tested (see

Figure 3.13).
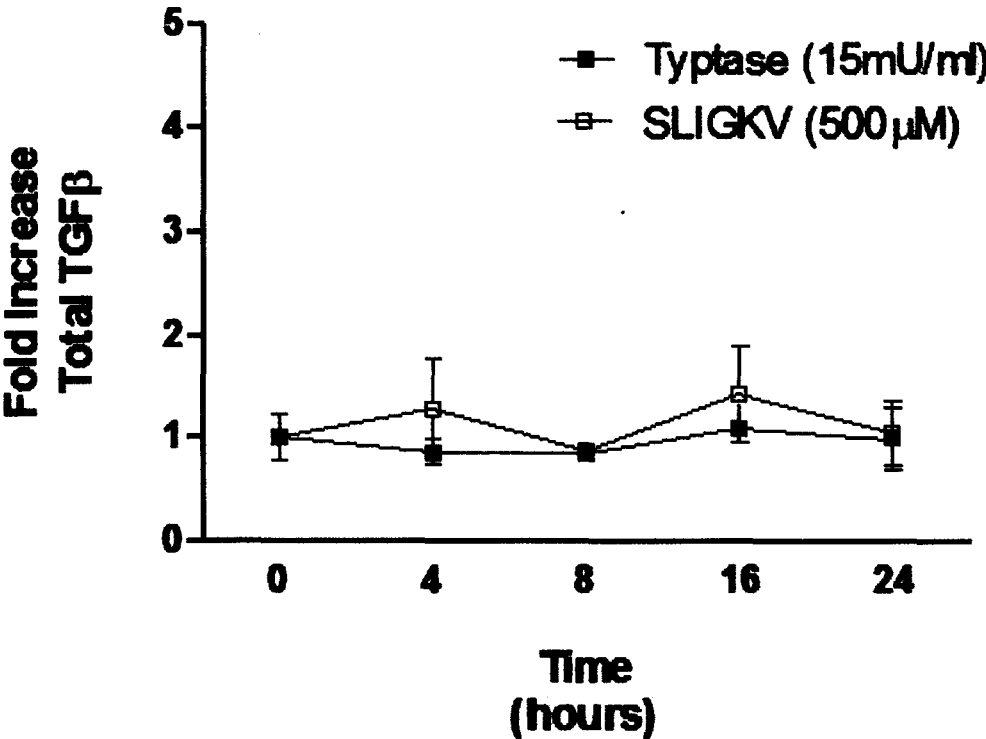
Figure 3.13: Effect of Tryptase and PAR2-AP on NHBE Cells

NHBE cells were stimulated in duplicate with either 15mU/ml or 500μM PAR2-AP. Cells supernatants were collected after 0, 4, 8, 16 and 24 hours and assayed for TGF-β using reporter cell assay. Neither tryptase nor PAR2-AP had any effect on levels of total TGF-β released in to the cell supernatants. This figure is representative of 3 independent experiments. Data is expressed as mean fold increase ± SEM.

3.4   Discussion

The aim of this thesis was to determine the mechanisms of TGF-β activation employed by HASM cells in vitro. It has previously been shown that stimulation of HASM cells with tryptase can affect TGF-β function (Berger et al. 2003), as shown by increased migration of mast cells towards conditioned media from tryptase-stimulated HASM cells. I found that mast cell tryptase released active TGF-β from HASM cells without affecting the overall levels of TGF-β. Tryptase increased active TGF-β via proteolysis, however, this effect was not mediated through PAR2 because the HASM cells used did not express PAR2 at either the protein or mRNA level.

These data are consistent with a mechanism of proteolytic cleavage of latent TGF-β by tryptase because overall levels of TGF-β (latent plus active) remained unchanged. Tissues generally contain large stores of latent TGF-β awaiting activation. These stores are sequestered in the ECM by interaction between LTBP and proteins in the ECM (Annes et al. 2003). A similar mechanism of TGF-β activation has been described for the serine proteases plasmin and thrombin (Lyons *et al.* 1990; Taipale *et al.* 1992), which proteolytically cleave latent TGF-β sequestered in the extracellular matrix without affecting overall expression of the latent complex.

Addition of leupeptin to the reporter cell assay completely abrogated tryptase-induced TGF-β activation confirming that the mechanism of TGF-β activation was proteolytic. Since tryptase, like plasmin, is a serine protease we performed experiments to determine if activation of TGF-β was due to the proteolytic activity of tryptase and not any contaminating proteases within the human lung tryptase preparation. We used the serine protease inhibitor FUT175 which is a potent competitive inhibitor of tryptase (Ki=0.095nM) (Mori et al. 2003). FUT175 also inhibits other serine proteases such as plasmin and trypsin but the Ki for inhibition of these proteases is much higher than the Ki for inhibition of tryptase. For example, the Ki of FUT175 for human thrombin is 840nM, human factor B is 60μM, plasmin is 310nM and human factor Xa is 4.1μM (Matsuoka *et al.* 1989; Mori *et al.* 2003). We found that FUT175, when used at a concentration that specifically inhibits tryptase, completely abrogated TGF-β activation confirming that tryptase-

induced TGF-β activation is mediated by the proteolytic effect of tryptase rather than any contaminating proteases that may be present.

The tryptase-induced increase in active TGF-β present in cell supernatants was transient, peaking at 4 hours. It is unlikely that the amount of latent TGF-β available is the limiting factor in this system since large stores of latent TGF-β are sequestered in the ECM and subsequent experiments showed that tryptase had no effect on levels of total TGF-β (see Results section 3.3.4). The transient increase in active TGF-β observed was probably due to the half-life of tryptase, which is approximately 2.5 hours (Schwartz et al. 1986). After 2.5 hours the levels of tryptase would begin to decrease resulting in a slower rate of TGF-β activation. Decreased tryptase activity over time has been previously reported (Berger *et al.* 2003). It is likely that after 8 hours of stimulation only negligible amounts of tryptase remained and so very little or no TGF-β activation occurred, despite there being large amounts of latent TGF-β still sequestered in the ECM.

It is possible that tryptase activates PAR2 (Akers et al. 2000; Berger et al. 2001), a G protein coupled receptor. PAR2 is up-regulated in the respiratory epithelium of asthmatics (Knight et al. 2001) implying that PAR2 may be involved in asthma pathogenesis. In an ovalbumin mouse model of asthma, activation of PAR2 using a PAR2-AP enhances inflammation and AHR (Ebeling et al. 2005). Furthermore, activation of PAR2 on eosinophils can cause release of cysteinyl leukotrienes which may contribute to asthma (Bolton et al. 2003). In the present study, however, it was found that PAR2-AP did not mimic the effects of tryptase on HASM TGF-β activation suggesting the PAR2 is not involved in tryptase-induced TGF-β activation. The peptide was functional as we have shown previously that it can cause increased release of IL-8 from NHBE cells (Mullan et al. 2007). Furthermore, neither tryptase nor PAR2-AP activated TGF-β in NHBE cells. These data contradict the findings of Berger *et al* (2003) who found that stimulation of HASM cells with PAR2-AP caused the cell supernatants to have increased chemotactic activity for mast cells, which the authors attributed to increased TGF-β due to the ability of a

TGF-β neutralising antibody to inhibit the chemotaxis. However, the authors did not directly measure TGF-β activity in the cell supernatants. In contrast, I have used two direct bioassays to measure TGF-β activity in response to both tryptase and PAR2-AP. This may suggest that PAR2 mediated effects lead to downstream TGF-β synthesis and activation in cells that express this receptor.

As the PAR2-AP had no effect on TGF-β activation in HASM cells, we decided to assess the expression of PAR2 by HASM cells. We applied western blotting and RT-PCR to determine the expression level of PAR2 protein and mRNA respectively and found that neither was expressed in HASM cells. The antibodies and primer sets were functional as expression was detected in NHBE cells. There are several published reports demonstrating expression of PAR2 in ASM cells (Chow *et al.* 2000; Schmidlin *et al.* 2001; Ebeling *et al.* 2005; Freund-Michel *et al.* 2006; Mullan *et al.* 2007), which the findings presented here contradict.· It is possible that the cells used in this study have lost their expression of PAR2 as an artefact of cell culture, as all HASM cells were used at passage 6. Variability in expression of PAR2 by HASM cells is evident. Some studies have reported high expression in HASM (Schmidlin *et al.* 2001) whereas other have described very low level expression (Mullan *et al.* 2007). However, this confirms that tryptase-induced activation of TGF-β can occur independently of PAR2 cleavage and activation.

Although it is commonly thought that tryptase mediates many of its effects via cleavage and activation of PAR2 it is also known to have several PAR2 independent effects. It is able to activate human eosinophils causing the release of eosinophil peroxidise (EPO) from the secretory granules (Vliagoftis et al. 2004) and induce proliferation of dog airway smooth muscle cells (Brown et al. 2006), all of which are PAR2-independent effects. It has also been shown to stimulate proliferation of HASM cells via a completely non-proteolytic mechanism (Brown et al. 2002). Furthermore, tryptase can cleave and inactivate eotaxin and RANTES in a PAR2 independent manner (Pang et al. 2006).

These data show that tryptase can activate TGF-β without affecting levels of total TGF-β present in cell supernatants. This contradicts data from a separate group who found that both tryptase and the PAR2-AP (SLIGKV-NH2) cause increased expression of TGF-β mRNA and protein (Berger et al. 2003). NHBE cells, which I have shown express PAR2, were used to investigate the effect of tryptase and the PAR2-AP on TGF-β released from cells that express PAR2. However, release of TGF-β from NHBE cell was unaffected by either tryptase or PAR2-AP, suggesting that activation of PAR2 does not lead to increased release of latent TGF-β in this cell type. However, NHBE cells are not very contractile thus it is possible that in HASM cells that express PAR2, agonists of PAR2 cause downstream expression and activation of TGF-β, as in the case of Berger *et al* (2003).

In conclusion we have shown that tryptase activates latent TGF-β from HASM cells in vitro via a proteolytic but PAR2 independent manner. It is possible that tryptase is released from activated mast cells, which infiltrate the smooth muscle layer of asthmatics (Brightling et al. 2002), during an asthma exacerbation. Released tryptase could activate TGF-β sequestered in the ECM of the surrounding cells, which could contribute to the development of airway remodelling (discussed in Introduction section 1.4.5)

Activation of TGF-β by HASM Cells in Response to Mechanical Wounding

4 Activation of TGF-β by HASM Cells in Response to Mechanical Wounding

4.1 Rationale

As discussed in Introduction section 1.1.1, the airways are constantly challenged with agents from the external environment such as pollution, viruses etc, which can cause damage or injury to the airways. In non-asthmatic individuals this causes no problems and the insults from the external environment are dealt with adequately by the epithelial barrier and the innate immune system. However, there is evidence to suggest that the epithelial layer of asthmatics is abnormal compared with non-asthmatics and becomes damaged more easily resulting in shedding of epithelial cells (Laitinen *et al.* 1985; Jeffery *et al.* 1989). In vitro mechanical wounding of cell monolayers can be used to simulate the cell injury that occurs in vivo (Puddicombe *et al.* 2000; Howat *et al.* 2002), a method that has been extensively used to study the effects of epithelial damage. It has been shown previously that wounding epithelial cells causes activation of both TGF-β1 and TGF-β2 (Howat *et al.* 2002) and increased expression of TGF-β2 (Puddicombe *et al.* 2000).

The effects of *in vitro* wounding on the ASM layer are less well studied. The location of the ASM layer within the airway wall, below the epithelium and basement membrane, means that it is unlikely to become damaged to any significant degree in asthma despite extensive damage to the epithelium. However, one study has shown that conditioned media from wounded bovine ASM cells has increased TGF-β activity (Coutts *et al.* 2001), and a further study found that expression of type I and type II TGF-β receptors by bovine ASM cells is increased following mechanical wounding (Chen *et al.* 2002).

In addition to its use as a model of cell injury, mechanical wounding of cells can also stimulate cell migration (Savani *et al.* 1995; Li *et al.* 2003; Wadsworth *et al.* 2006). Migration of ASM

cells is thought to contribute to the increased ASM mass evident in the remodelled airways of asthmatic patients. It is possible that stimulating ASM cells to migrate results in endogenous activation of TGF-β since cell supernatants from wounded ASM cells has increased TGF-β activity (Coutts *et al.* 2001), however, the mechanism of activation has not been investigated.

4.2 Aims

The effects of mechanical wounding on TGF-β activation by HASM cells have not been investigated so it is the aim of this work to establish if HASM cells are capable of activating TGF-β in response to wounding using an *in vitro* wound model. If activation occurs neutralising antibodies will be used to determine which of the three TGF-β isoforms are activated. The mechanism of TGF-β activation (see chapter 1.4.2) will also be investigated.

4.3   Results

4.3.1   Basal Expression of Active and Total TGF-β by HASM Cells

The basal expression of both active and latent TGF-β by HASM cells was determined using TMLC reporter cells and the ability to activate L-TGF-β by acid treatment (see Methods section 2.7). Following acidification and neutralisation of unstimulated HASM cells, which activates all available latent TGF-β in the ECM and culture media (Brown *et al.* 1990), TMLC reporter cells were added to the HASM cells and the luciferase activity measured after 16 hours. TMLC were also added to HASM cells that had not been acidified to measure basal levels of active TGF-β. Under basal conditions HASM cells activate latent TGF-β as shown by 33.1 ± 32.1 pg/ml TGF-β activity in cells that were not acid treated (see Figure 4.1). However, following acidification, and therefore activation of all latent TGF-β, 1478 ± 891 pg/ml TGF-β activity was detected. This means that under basal conditions HASM cells activate only around 2% of the available latent TGF-β stored intracellularly and in their ECM.
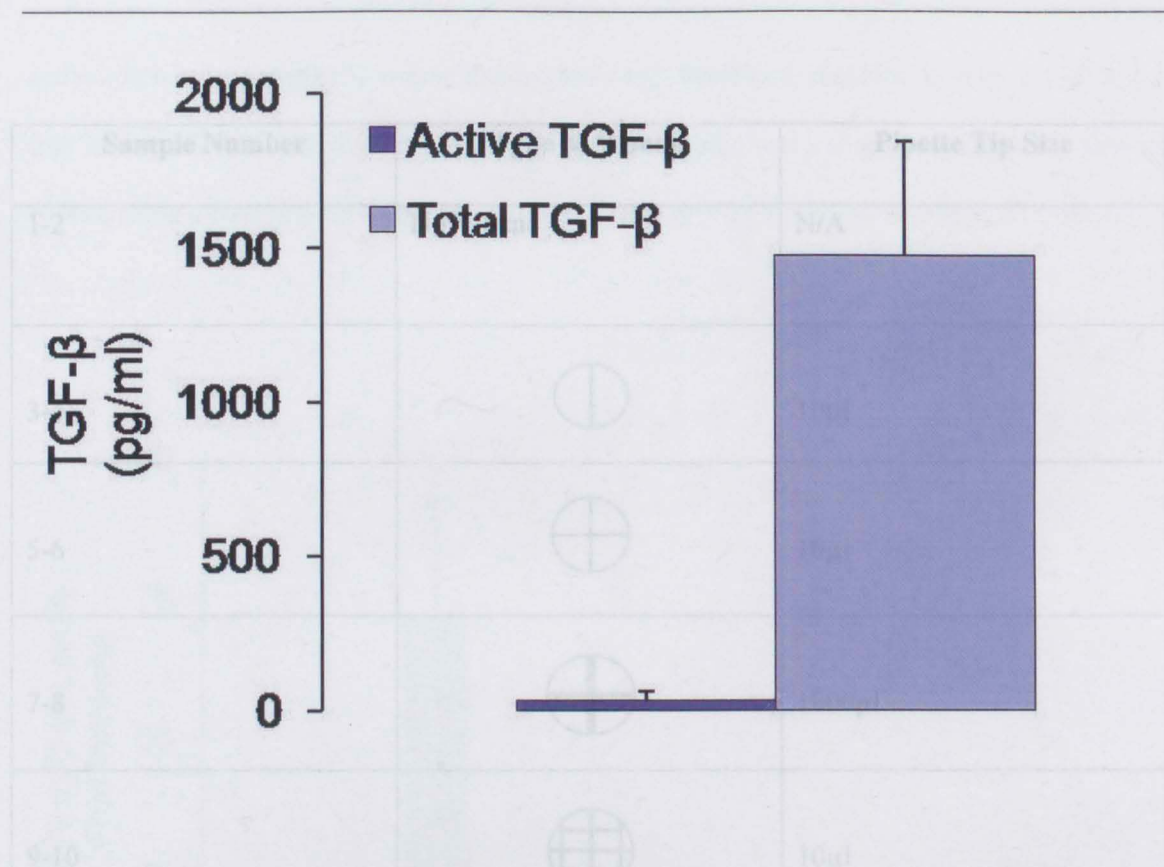
Figure 4.1: Basal Expression of Active and Total TGF-β by HASM Cells

The expression of both active and total TGF-β by HASM cells was determined using TMLC reporter cells. TMLC were added to HASM cells or HASM cells following acidification. The figure shows the means ± SEM from 2 separate experiments each for 3 HASM cell lines.

4.3.2 Optimising Wound Model

Several different methods of wounding cell monolayers have been described using both pipette tips and rubber policemen (Savani *et al.* 1995; Chen *et al.* 2002; Howat *et al.* 2002). In order to optimise the wound model for the purposes of this study HASM cells were wounded by a variety of pipette tips and to varying degrees. The types of wound used are illustrated in Table 4.1. Following wounding, the cells were washed twice in PBS to remove any cell debris. RNA was collected after 8 hours in order to allow any TGF-β activated to bind to its receptor and initiate gene transcription. Following reverse transcription (Methods section 2.3.4) the cDNA was subjected to QPCR analysis (see Methods section 2.4) to assess expression of the TGF-β-inducible gene PAI1. The primers are outlined in Appendix 9.7.

Sample Number	Type of Wound	Pipette Tip Size
1-2	No Wound	N/A
3-4	(circle with single vertical line)	10µl
5-6	(circle with cross)	10µl
7-8	(circle with cross)	1000µl
9-10	(circle with grid)	10µl
11-12	(circle with single vertical line)	1000µl

Table 4.1: Overview of Types of Wound

This table outlines the different types of wound used to optimise the wounding model. Each line represents a single scratch with a pipette tip.

As shown by Figure 4.2, the type of wound that produced the largest increase in PAI1 expression after 8 hours was a single scratch across diameter of well with a 10µl pipette tip, which gave a 71.99 ± 17.33 fold increase in PAI1 expression. Scratching a criss-cross in the well using a 10µl pipette tip was the second most effective technique resulting in an 11.04 ± 3.7 fold increase in PAI1 mRNA. However, a criss-cross scratch with a 1000µl pipette tip resulted in no increase in PAI1 mRNA. Scratching a grid of 3 vertical and 3 horizontal lines in the wells caused TGF-β

activation but the magnitude was less than previously described, resulting in only a 4.07 ± 2.93

fold increase in PAI1 mRNA. The final type of wound tested was a single scratch across diameter

of well using a 1000μl pipette tip. This resulted in a 6.29 ± 0.69 fold increase in PAI1 mRNA.

Figure 4.2: Optimising Wound Model

The wound model was optimised using different techniques of wounding and measuring PAI1
expression in response. HASM cells were wounded by various methods using either a 10μl or a
1000μl pipette tip (see Table 4.1). Cells were washed with sterile PBS to remove any cell debris
and RNA was collected after eight hours. Data was normalised to the housekeeping gene β-2M
and relative PAI1 expression calculated using the delta CT equation. This figure shows data from
2 separate experiments expressed as mean fold increase ± SEM.
** P<0.01

4.3.3 Effect of Wounding on Expression of the TGF-β-Inducible Gene PAI1

The data presented in Figure 4.2 shows that wounding HASM cells with a single scratch using a

10μl pipette tip produced the largest increase in PAI1 gene expression. This method of wounding

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was therefore used for all future experiments. HASM cells were wounded and RNA collected after 0, 4, 8 and 24 hours. After reverse transcription (see Methods section 2.3.4) the resulting cDNA was subjected to QPCR analysis (see chapter 2.4) for the TGF-β-inducible gene PAI1 and the housekeeping gene β-2M. Figure 4.3 shows that expression of PAI1 mRNA increased following wounding by 6.9 ± 3.7 fold after 4 hours implying activation of TGF-β. PAI1 mRNA increased further after 8 hours (21.9 ± 18.5 fold increase). The increase observed did not reach statistical significance (P value at 4 hours was 0.198 and at 8 hours it was 0.279).
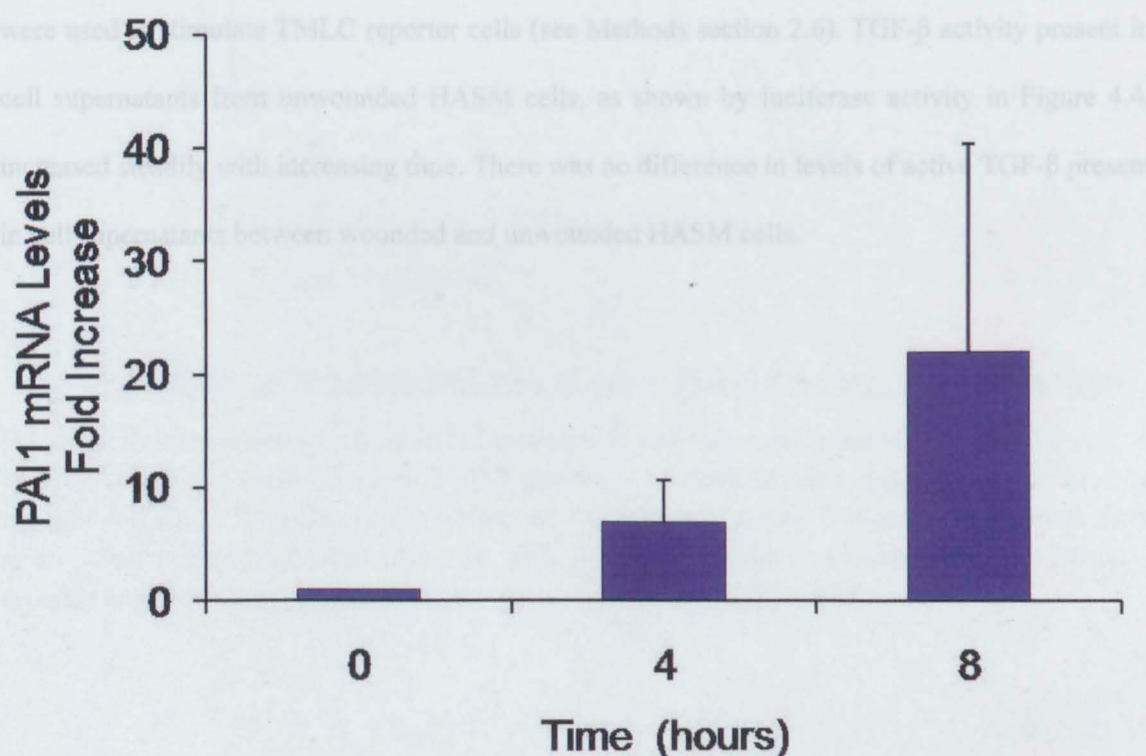
Figure 4.3: Effect of Wounding on PAI1 Expression

HASM cells were wounded as previously described and RNA collected after 0, 4 and 8 hours. cDNA was subjected to QPCR analysis for the TGF-β inducible gene PAI1. Wounding caused a time-dependent increase in PAI1 gene expression. The figure shows the means ± SEM from 8 separate experiments.

4.3.4 Effect of Wounding on Levels of Active TGF-β Present in Cell Supernatants

Proteolytic activation of TGF-β results in the release of active TGF-β into cells supernatants (Lyons *et al.* 1988; Lyons *et al.* 1990; Taipale *et al.* 1992; Yu *et al.* 2000; Mu *et al.* 2002; Tatler *et al.* 2008). Presence of active TGF-β in cell supernatants following wounding would suggest that a proteolytic mechanism of TGF-β activation. To investigate whether active TGF-β is released into cell supernatants following wounding levels of active TGF-β in cell supernatants was determined using a reporter cell assay (see Methods section 2.6). Cells were either wounded or left unwounded and cell supernatants collected after 0, 4, 8, 16 and 24 hours. The supernatants were used to stimulate TMLC reporter cells (see Methods section 2.6). TGF-β activity present in cell supernatants from unwounded HASM cells, as shown by luciferase activity in Figure 4.4, increased steadily with increasing time. There was no difference in levels of active TGF-β present in cell supernatants between wounded and unwounded HASM cells.
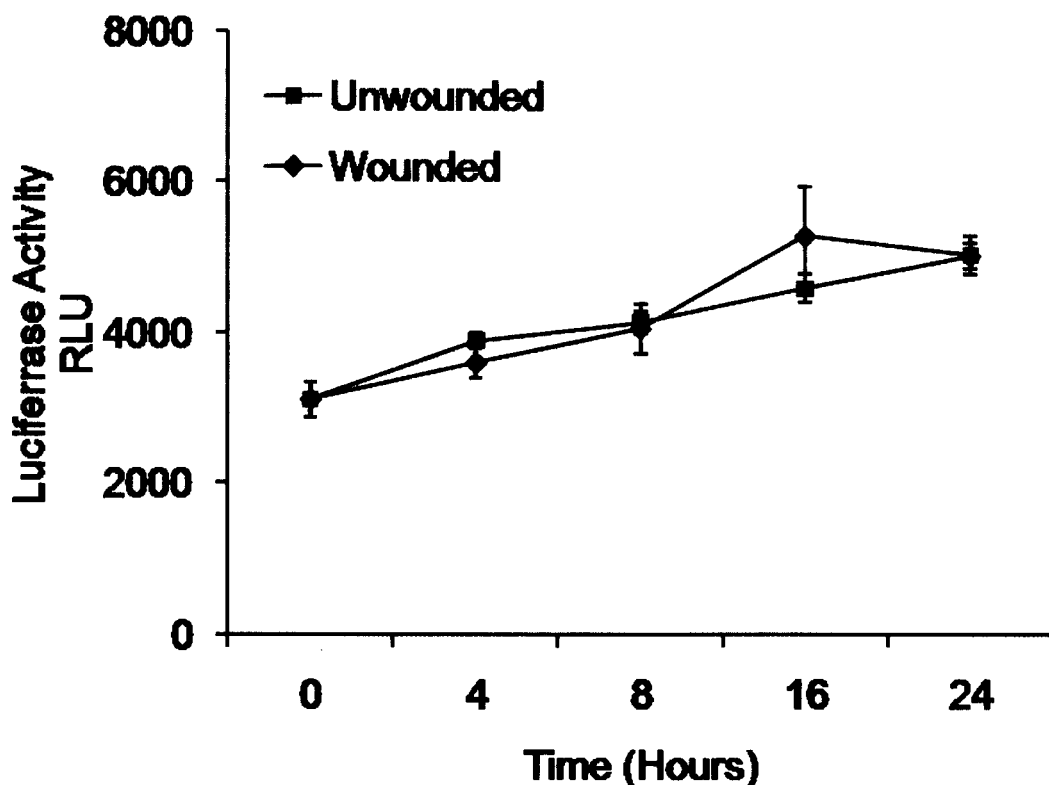
Figure 4.4: Effect of Wounding on Levels of Active TGF-β Present in Cell Supernatants

HASM cells were either wounded or left unwounded and cell supernatants collected after 0, 4, 8, 16 and 24 hours. Levels of active TGF-β present in the cell supernatants were determined by reporter cell assay. Wounding had no effect on the release of active TGF-β into cell supernatants at any time point tested over 24 hours. This figure shows the amalgamation of data from 2 separate experiments expressed as mean relative light units (RLU) ± SEM.

4.3.5   Effect of Anti-TGF-β1 and Anti-TGF-β2 Antibodies on Wound-Induced PAI1 Expression

Data presented in Figure 4.3 suggests that wounding results in activation of TGF-β by HASM cells, as expression of the TGF-β-inducible gene PAI1 increased in response to wounding. To investigate which isoform of TGF-β is activated in response to wounding neutralising antibodies directed against TGF-β1 and TGF-β2 were used. HASM cells were pre-treated for 30 minutes with anti-TGF-β1 (200ng/ml), anti-TGF-β2 (200ng/ml) or an anti-IgG1 isotype control (200ng/ml) antibodies then wounded in the presence of antibody. RNA was collected after 0, 4

and 8 hours, reverse transcribed and analysed by QPCR. Figure 4.5 shows that addition of an anti-TGF-β1 neutralising antibody to the wound model appeared to abrogate wound-induced increases in PAI1 mRNA. Wounding caused an increase in PAI1 mRNA after 4 hours, which was blocked by an anti-TGF-β1 antibody. There was a minimal increase in PAI1 expression at 8 hours but it was again blocked by the anti-TGF-β1 antibody. The anti-TGF-β1 had no effect on basal PAI1 expression by HASM cells. The large error bars illustrated by Figure 4.5 show that there was large variability between experiments resulting in large values for SEM and statistical significance was not reached.
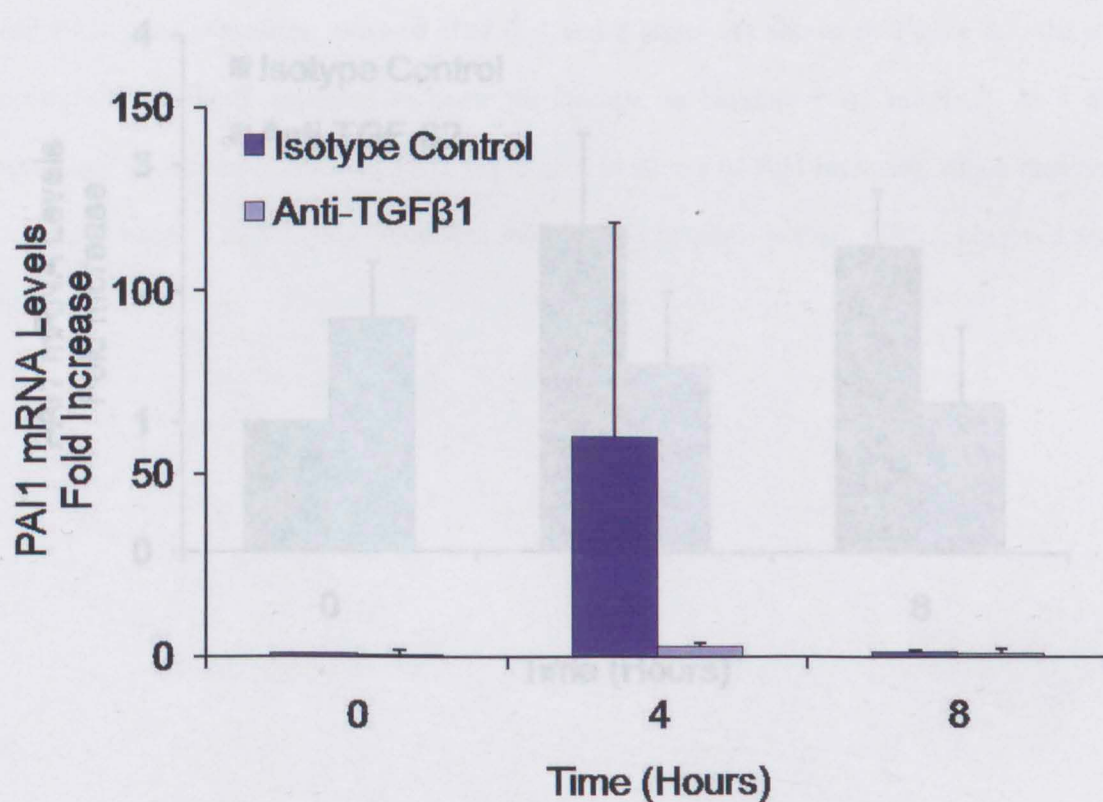
Figure 4.5: Effect of Anti-TGF-β1 on Wound-Induced PAI1 Expression
HASM cells were wounded in the presence of either 10μg/ml anti-TGF-β1 (dark bars) or an equivalent amount of an IgG isotype control antibody(light bars). PAI1 gene expression was assessed after 0, 4 and 8 hours by QPCR using the primers described in the Appendix. Addition of a TGF-β1 blocking antibody appeared to abrogate wound-induced increases in PAI1 expression. This figure shows data from 6 separate experiments amalgamated and expressed as mean fold increase in PAI1 expression ± SEM. P values less than 0.05 were accepted as significant.

Addition of an anti-TGF-β2 neutralising antibody also appeared to inhibit wound-induced increases in PAI1 mRNA (Figure 4.6). A 2.51 ± 0.73 fold increase in PAI1 mRNA was recorded 4 hours after wounding. This was partially inhibited by addition of an anti-TGF-β2 antibody. This was also observed 8 hours after wounding. Inhibition of wound-induced increases in PAI1 mRNA by anti-TGF-β2 did not reach statistical significance. However, the anti-TGF-β2 antibody appeared to increase baseline PAI1 expression.
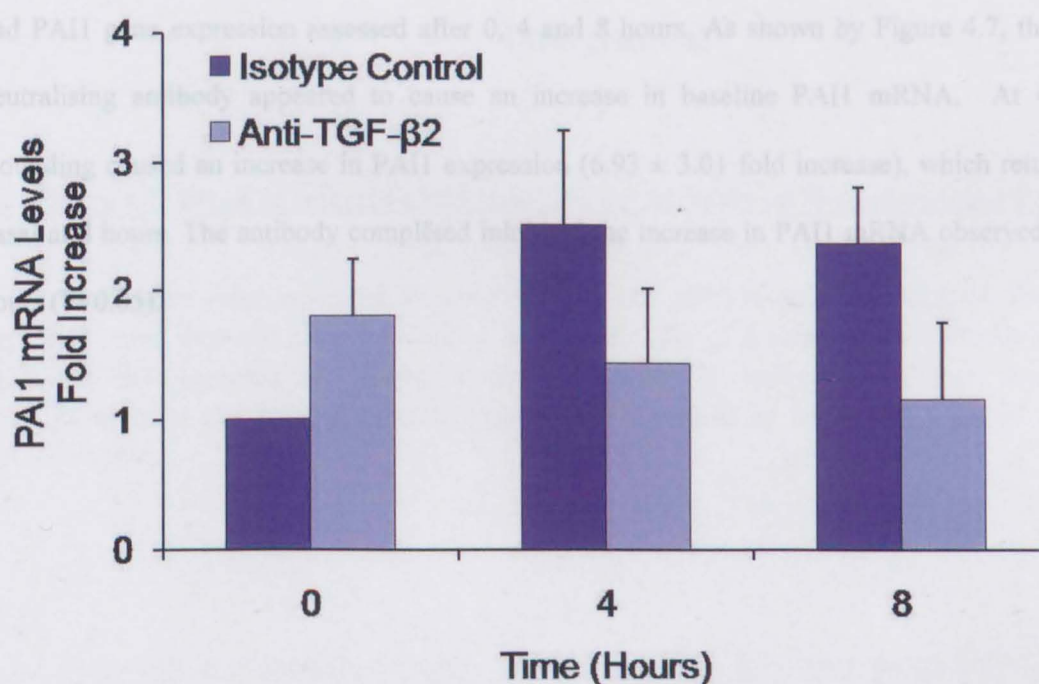
Figure 4.6: Effect of Anti-TGF-β2 on Wound-Induced PAI1 Expression

HASM cells were wounded in the presence of either 10μg/ml anti-TGF-β2 (dark bars) or an equivalent amount of an IgG isotype control antibody (light bars). PAI1 gene expression was assessed after 0, 4 and 8 hours by QPCR using the primers described in the Appendix. Addition of TGF-β2 blocking antibody appeared to inhibit wound-induced PAI1 expression at both 4 and 8 hours. This figure shows the amalgamation of data from 4 separate experiments expressed as mean fold increase in PAI1 mRNA ± SEM. P values less than 0.05 were accepted as significant.

4.3.6 Effect of Integrin αVβ5 Neutralising Antibody on Wound-Induced PAI1 Expression

Wound-induced activation of TGF-β by HASM cells does not result in the release of active TGF-β in to cell supernatants, suggesting that the mechanism is not mediated by proteolysis. Integrin-mediated TGF-β activation is another possible mechanism. The integrin αVβ5 has been shown to activate TGF-β in other mesenchymal cell types such as myofibroblasts and fibroblasts (Asano *et al.* 2005b; Wipff *et al.* 2007; Scotton *et al.* 2009). The role of this integrin in wound-induced TGF-β activation was investigated. HASM cells were pre-treated with an anti-αVβ5 blocking antibody (10μg/ml) or an IgG1 isotype control (10μg/ml) for 30 minutes. Cells were wounded and PAI1 gene expression assessed after 0, 4 and 8 hours. As shown by Figure 4.7, the αVβ5 neutralising antibody appeared to cause an increase in baseline PAI1 mRNA. At 4 hours wounding caused an increase in PAI1 expression (6.93 ± 3.01 fold increase), which returned to basal at 8 hours. The antibody completed inhibited the increase in PAI1 mRNA observed after 4 hours ($P < 0.05$).
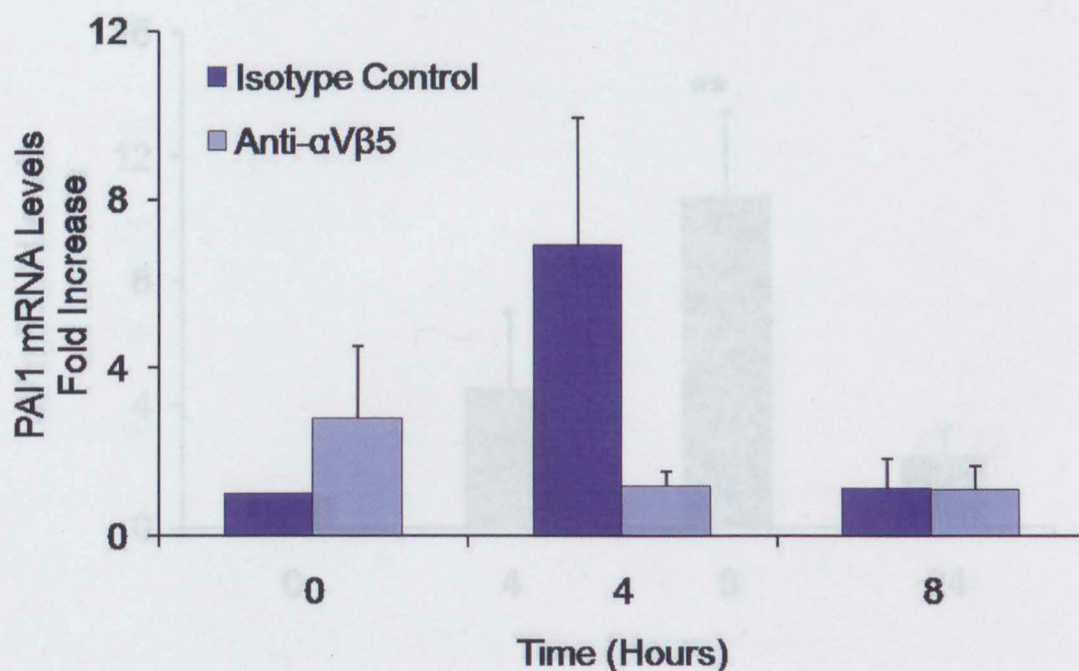
Figure 4.7: Effect of Integrin αVβ5 Neutralising Antibody on Wound-Induced PAI1 Expression

HASM cells were wounded in the presence of either anti-αVβ5 or an isotype control. PAI1 gene expression was assessed after 0, 4 and 8 hours. Addition of a neutralising antibody directed against αVβ5 appeared to inhibit wound-induced PAI1 expression. Figure shows the amalgamation of data from 4 separate experiments expressed as mean fold increase in PAI1 mRNA ± SEM.

4.3.7   Expression of Integrin Subunits β5 and αV mRNA following Wounding

QPCR analysis was applied to mRNA collected from wounded HASM cells to determine the effect wounding has on the expression of the integrin subunits αV and β5 using the primers described in Appendix 9.7. Figure 4.8 demonstrates that wounding HASM cells causes a time dependent increase in expression of mRNA for the αV integrin subunit. The increase in αV mRNA was observed after 4 hours of wounding and is increased further after 8 hours. By 24 hours levels of αV mRNA had returned almost to basal.
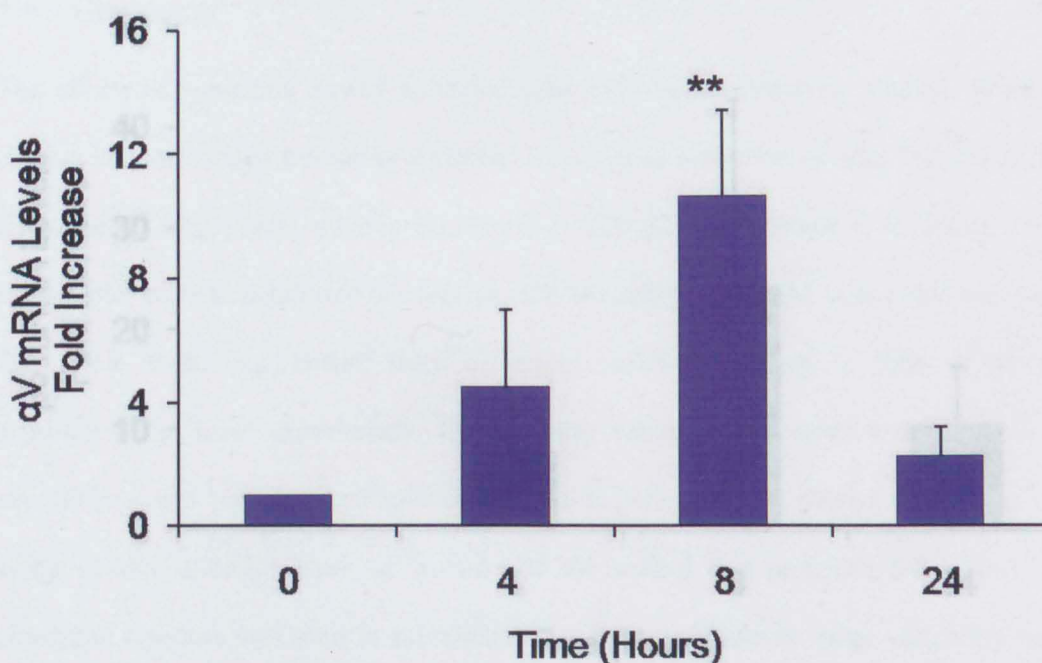
Figure 4.8: Effect of Wounding on Expression of αV Integrin Subunit

HASM cells were wounded and expression of αV integrin subunit mRNA was assessed by QPCR after 0, 4, 8 and 24 hours. Wounding HASM cells causes increased expression of the αV integrin subunit at mRNA level. This figure shows the amalgamated data from 3 separate experiments expressed as fold increase over basal ± SEM. Statistical analysis was performed using ANOVA comparing all data sets to the 0 hour data set.

** $p < 0.01$

Similar levels of increased expression of mRNA for the β5 integrin subunit were observed following wounding (Figure 4.9). Like αV, β5 mRNA was increased after 4 hours of wounding and increased further after 8 hours (24.09 ± 18.49). However, unlike the expression of αV mRNA, levels of β5 mRNA remained elevated at 24 hours although reduced from 8 hours. However, none of these data were statistically significant.
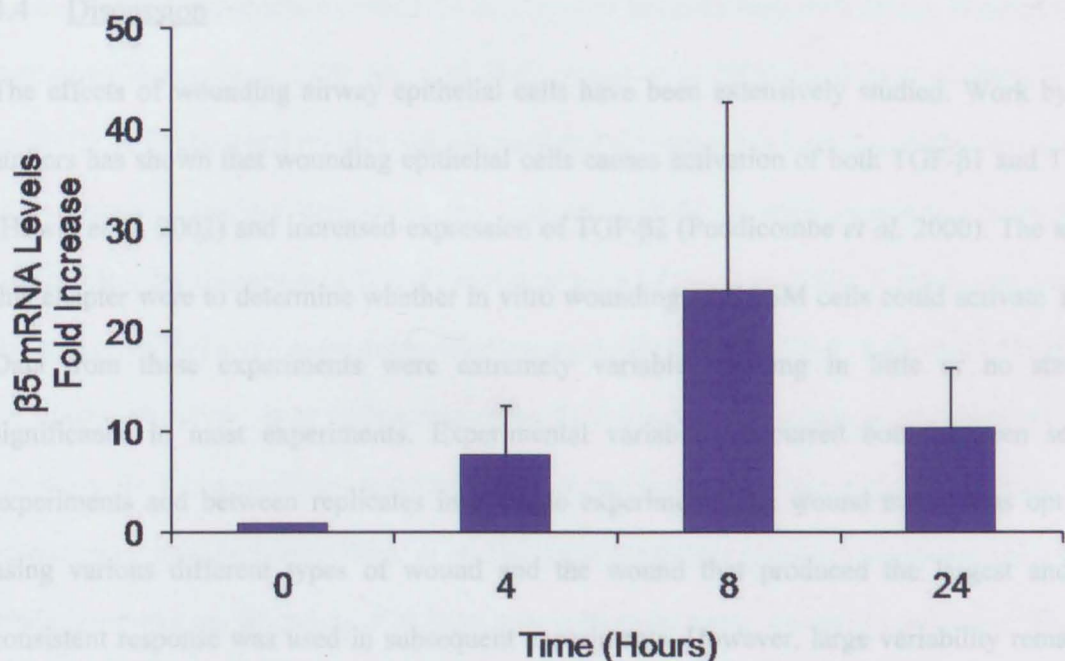
Figure 4.9: Effect of Wounding on Expression of β5 Integrin Subunit

HASM cells were wounded and expression of β5 integrin subunit mRNA was assessed by QPCR after 0, 4, 8 and 24 hours. Wounding HASM increases expression of the β5 integrin subunit at mRNA level. This figure shows amalgamated data from 3 separate experiments expressed as fold increase over basal ± SEM.

4.4 Discussion

The effects of wounding airway epithelial cells have been extensively studied. Work by other authors has shown that wounding epithelial cells causes activation of both TGF-β1 and TGF-β2 (Howat *et al.* 2002) and increased expression of TGF-β2 (Puddicombe *et al.* 2000). The aims of this chapter were to determine whether in vitro wounding of HASM cells could activate TGF-β. Data from these experiments were extremely variable resulting in little or no statistical significance in most experiments. Experimental variability occurred both between separate experiments and between replicates in a single experiment. The wound model was optimised using various different types of wound and the wound that produced the largest and most consistent response was used in subsequent experiments. However, large variability remained a problem. Due to this variability, conclusions based on the data are difficult to make. The results described here suggest that HASM cells can activate TGF-β in response to wounding since expression of the TGF-β-inducible gene PAI1 did increase inconsistently following wounding. Experiments were performed using neutralising antibodies against TGF-β1 and TGF-β2 to confirm that the increase in PAI1 expression was caused by TGF-β activation and to determine which isoform of TGF-β is activated. Addition of anti-TGF-β2 appeared to affect wound-induced PAI1 expression by HASM cells. However, the data also suggests that TGF-β1 might be involved in the response to wounding. The data also suggests that the mechanism could be mediated by the widely expressed integrin αVβ5.

Release of active TGF-β into cell supernatants was not increased following wounding implying that if TGF-β is activated by HASM cells in response to wounding then the activated TGF-β remains bound to the cell surface. This observation excludes the possibility that proteolysis is involved in wound-induced TGF-β activation by HASM. All previous mechanisms of TGF-β activation that involve proteolysis result in the liberation of active TGF-β into cell supernatants (Lyons *et al.* 1988; Lyons *et al.* 1990; Taipale *et al.* 1992; Yu *et al.* 2000; Mu *et al.* 2002; Tatler *et al.* 2008), due to the proteolytic cleavage or degradation of LAP.

As discussed in Introduction section 1.4.2.3 , several integrins have been shown to activate TGF-β in vitro (Munger *et al.* 1999; Mu *et al.* 2002; Asano *et al.* 2005b; Jenkins *et al.* 2006; Wipff *et al.* 2007). A fundamental role for integrin mediated activation has been suggested since mutation of the integrin binding domain in the LAP of TGF-β1 in mice causes a phenotype that recapitulates the phenotype of TGF-β1 knockout mice (Yang *et al.* 2007). Furthermore, mice null for both αVβ6 and αVβ8 share many developmental defects with TGF-β1 null mice (Aluwihare *et al.* 2009). However, it is likely that other TGF-β-activating integrins are involved in the pathogenesis of disease. It has previously been shown that the integrin αVβ5 can mediate TGF-β activation in myofibroblasts (Wipff *et al.* 2007) and fibroblasts (Asano *et al.* 2005a; Scotton *et al.* 2009). Furthermore, wounding of HASM cells is likely to cause changes in cell shape. Therefore it was interesting to investigate the role of this integrin in this model. Addition of a neutralising antibody directed against the integrin αVβ5 appeared to abrogate wound-induced TGF-β activation. Contraction of myofibroblasts, which like HASM cells are mesenchymal in origin and have many similarities to HASM cells, causes TGF-β activation via αVβ5 (Wipff *et al.* 2007). Changes in cells shape and cell migration leads to reorganisation of the actin cytoskeleton, the formation of stress fibers and the presence of traction forces from the ECM. Reorganisation of the cytoskeleton has been implicated in integrin-mediated TGF-β activation (Munger *et al.* 1999; Wipff *et al.* 2007; Xu *et al.* 2009). It is therefore plausible that HASM cells are capable of activating TGF-β via the αVβ5 integrin in response to wounding.

The precise mechanism of αVβ5-mediated TGF-β activation has not yet been fully elucidated, however, it has been shown that αVβ5 interacts with both TGF-βRI and TGF-βRII receptors within clathrin-coated vesicles (Asano *et al.* 2006). This suggests that once activated, active TGF-β is internalised with αVβ5 and both types of TGF-β receptor. It has been proposed that such internalisation of active TGF-β would potentiate TGF-β signalling by sequestering TGF-β away from inhibitors of TGF-β signalling such as Smad7 and Smurf2 (Di Guglielmo *et al.* 2003). It is therefore possible that the effects of αVβ5 on TGF-β signalling are twofold; it may cause TGF-β

activation and help potentiate TGF-β signalling once it is activated by interacting with TGF-β receptors.

The results presented here also show that mechanical wounding of HASM cells causes increased expression of both αV and β5 integrin subunits at the mRNA level. This may be via autocrine induction following TGF-β activation because TGF-β causes increased expression of β5 mRNA (Sheppard *et al.* 1992; Lai *et al.* 2000; Kintscher *et al.* 2002) and the promoter of the β5 gene contains a TGF-β-responsive element (Lai *et al.* 2000). However, existing data concerning the effects of TGF-β on αV mRNA are contradictory. Expression of αV is increased in response to TGF-β in human cells (Chin *et al.* 2003) but not in guinea pig cells (Sheppard *et al.* 1992). In light of these studies, it is not surprising that mechanical wounding of HASM cells, which probably causes TGF-β activation, results in increased expression of αV and β5 mRNA. This could provide a positive feedback loop for the cells, where αVβ5-mediated TGF-β activation causes increased cell surface expression of αVβ5 integrin which in turn enhances the ability of the cells to activate TGF-β via the αVβ5 integrin. It is widely believed that expression of the αV subunit is less important with regards to cell surface expression of αV containing integrins than expression of the β subunit. It is thought that the αV subunit is synthesised in excess, while expression of the β subunits is tightly regulated, resulting in a limited number of complete integrin heterodimers being expressed on the cell surface (Sheppard *et al.* 1992). The effects of activated TGF-β on expression of the β5 subunit in this model are likely to influence cell surface expression of αVβ5 more profoundly than the effects of TGF-β on expression of the αV subunit.

The continued irregularities and variability in the data were likely to be due to the nature of the wound model used. It was impossible to completely control the size of the wound both between replicates and between experiments. Even taking utmost care, mechanically wounding a monolayer of HASM cells sometimes resulted in lifting a large section of the monolayer from the plate causing a very large wound area. Occasionally some of these cells would re-adhere to the plate but oftentimes they would become completely detached and die. Cell death results in the

release of a variety of mediators which may influence the response of the remaining cells to wounding. Due to the number of problems using the wound model and the inconsistency of the data it was concluded that the wound assay was not a suitable model of studying the mechanism of TGF-β activation in HASM cells and further experiments were not performed. However, these data raised the interesting possibility that HASM contraction could lead to TGF-β activation via the αVβ5 integrin, which could be studied using different experimental methods.

LPA-Induced αVβ5-Mediated

TGF-β Activation

5 LPA-Induced TGF-β Activation is via αVβ5 Integrin

5.1 Rationale

The results presented in the previous chapter raised the possibility that HASM cells could activate TGF-β in response to mechanical wounding via the cell surface integrin αVβ5. Activation of TGF-β by αVβ5 has been described in various other cell types including myofibroblasts in response to cellular contraction (Wipff *et al.* 2007). In addition to its use as a model of cellular injury, wounding can also be used as a stimulus of cell migration (Savani *et al.* 1995; Wadsworth *et al.* 2006). There are many similarities between the cellular processes of migration and contraction, both of which require reorganisation of the actin cytoskeleton and traction forces from the ECM.

Airway hyper-responsiveness (AHR) to asthmatic stimuli and enhanced bronchoconstriction are significant features of asthma and ASM cells are the main cell type responsible. Contraction of airway smooth muscle cells occurs due to increased levels of phosphorylated myosin light chain (MLC), which interacts with the actin filaments of the cytoskeleton to cause cell contraction. Levels of phosphorylated MLC increase due to either an increase in intracellular calcium levels, which activates myosin light chain kinase to phosphorylate MLC, or by inactivation of myosin light chain phosphatase (MLCP) by the cytoplasmic proteins RhoA/Rho kinase (Kimura *et al.* 1996), resulting in accumulation of levels of phosphorylated MLC.

There is evidence to suggest that the ASM cells themselves are fundamentally altered in asthma, contributing to enhanced bronchoconstriction and AHR. Primary cells isolated from asthmatic patients are more contractile than cells from non-asthmatic individuals in a cell culture model (Ma *et al.* 2002; Matsumoto *et al.* 2007), suggesting that it is the ASM cells that are different rather than the influence of any factors present in the environment of the asthmatic airway. Wipff and colleagues (Wipff *et al.* 2007) have shown that inducing contraction of myofibroblast results

in TGF-β activation. However, the effects of ASM cell contraction on TGF-β activation, especially in the context of asthma and airway remodelling, have not been investigated.

Lysophosphatidic acid is a soluble, bioactive phospholipid released from activated platelets and is present in serum (Eichholtz *et al.* 1993). It is a contraction agonist for smooth muscle (Chrzanowska-Wodnicka *et al.* 1996; Ainslie *et al.* 2004) and many other cell types. Furthermore, it has been shown to enhance contraction of HASM cells in response to several contraction agonists and leads to the formation of actin stress fibers in ASM cells and airway epithelial cells (Toews *et al.* 2002; Hirakawa *et al.* 2007; Xu *et al.* 2009). LPA induces cell contraction via activation of one of 4 LPA receptors (named LPA receptor 1(LPAR1), LPA receptor 2 (LPAR2) LPA receptor 3 (LPAR3) and LPA receptor 4 (LPAR4)), all of which are G protein coupled receptors (GPCRs). Following receptor activation by LPA, the $G_{12/13}$ proteins coupled to the receptor dissociate and bind to a Rho-specific guanine nucleotide exchange factor (GEF). The GTPase Rho, which is part of the Ras superfamily of proteins, is subsequently activated and in turn activates Rho kinase. Rho kinase is then able to phosphorylate both MLCK and MLC resulting in cytoskeletal changes and cell contraction. For a summary of this pathway see Figure 5.1.

In addition to its effects on cell contraction LPA has been implicated in wound repair and in asthma pathogenesis. Concentrations of LPA are increased in bronchoalveolar lavage fluid (BALF) following allergen challenge (Georas *et al.* 2007). It may contribute to airway inflammation by increasing infiltration of eosinophils and neutrophils in to BALF (Hashimoto *et al.* 2003) and to AHR (Hashimoto *et al.* 2001). There are also several studies showing that LPA can increase expression of several inflammatory cytokines that are involved in asthma pathogenesis, including IL-13 and IL-8, through activation of the transcription factor NFκB (Cummings *et al.* 2004; Rubenfeld *et al.* 2006; Klemm *et al.* 2007; Kalari *et al.* 2009). Furthermore, LPA is a potent mitogen for human ASM cells (Cerutis *et al.* 1997; Ediger *et al.* 2001) and can enhance fibronectin assembly (Olorundare *et al.* 2001) implicating it in airway

remodelling. It has previously been shown to induce αVβ6 integrin-mediated TGF-β activation

from airway epithelial cells via activation of RhoA and the G protein Gαq (Xu *et al.* 2009).

However, αVβ6 expression is restricted to epithelial cells. The effects of LPA stimulation on

TGF-β activation by HASM cells have not been investigated.
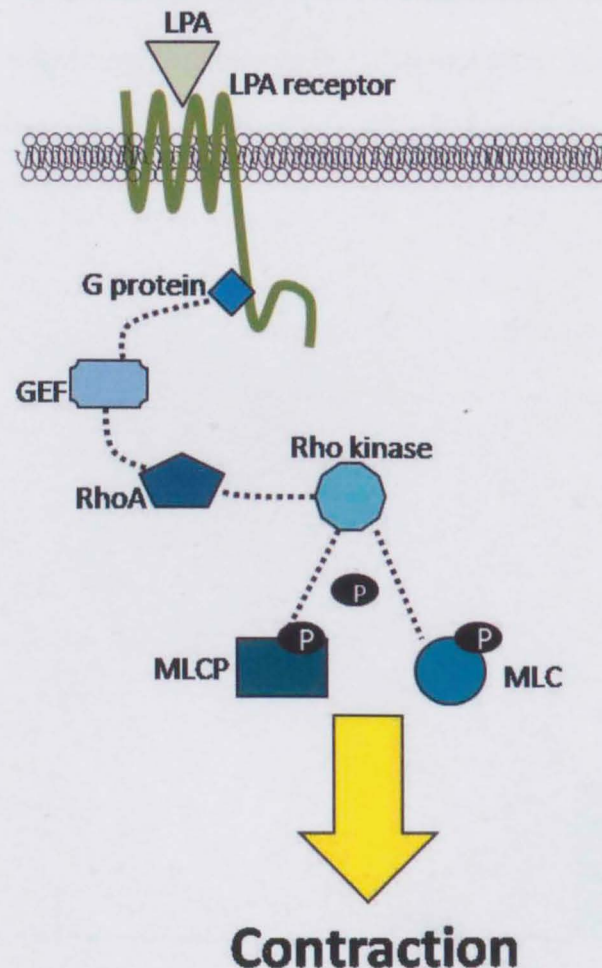
Figure 5.1: Overview of LPA-Induced Contraction

This is a schematic diagram illustrating how binding of LPA to its GPCR on the cell surface results in cell contraction. Activation of the LPA receptor causes dissociation of its associated G proteins, which following interaction with a guanine exchange factor activate RhoA. RhoA activates Rho kinase, which phosphorylates MLC and MLCP. MLC is activated upon phosphorylation and is able to initiate cell contraction. Phosphorylation of MLCP renders it unable to dephosphorylate MLC, contributing to increased intracellular levels of phosphorylated MLC.

5.2 Aims

The aims of this chapter were to determine whether LPA can cause TGF-β activation from HASM cells by inducing cytoskeletal changes. Using HASM cells isolated from asthmatic patients, any differences in TGF-β activation in response to LPA stimulation by asthmatic HASM cells compared with non-asthmatic HASM cells will be determined.

5.3 Results

5.3.1 Concentration-Response of LPA on TGF-β Activation

To assess the effect of LPA stimulation on TGF-β activation by HASM cells a co-culture of TMLCs with HASM cells was stimulated in duplicate with 0.1, 1, 10 and 100μM LPA. LPA caused a concentration-dependent increase in TGF-β activation (Figure 5.2). The lowest concentration tested (0.1μM) had no effect on TGF-β activation compared with media alone. All other concentrations tested caused an increase in TGF-β activation. Stimulation with 100μM LPA caused the largest increase in TGF-β activation (118.3 ± 32.8 pg/ml TGF-β, p<0.01).
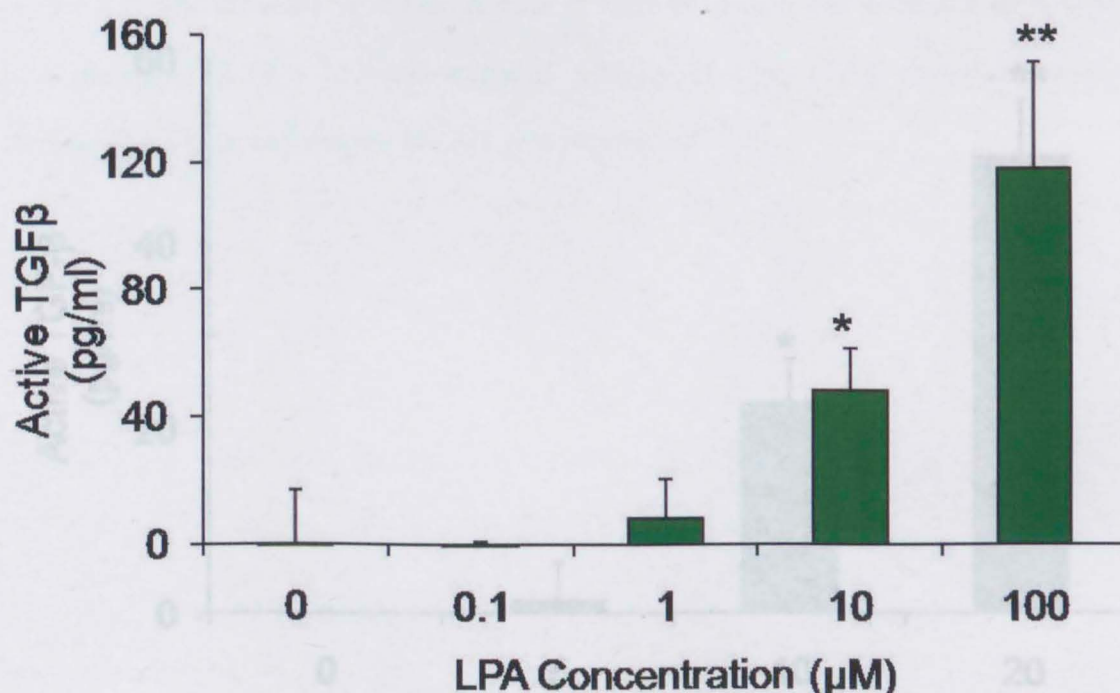
Figure 5.2: Concentration-Response of LPA on TGF-β Activation

A co-culture of HASMs and TMLCs was stimulated in duplicate with 0, 0.1, 1, 10 and 100μM LPA. LPA caused a concentration-dependent increase in TGF-β activity. This figure shows the amalgamation of data from 3 independent experiments using 3 separate HASM cell lines. Data is expressed as mean TGF-β activity ± SEM. P values less than 0.05 were accepted as significant.

* P<0.05   **P<0.01

LPA is found in varying concentrations in several bodily fluids. In serum its concentration ranges from around 1-5µM but local concentrations at sites of injury are likely to be higher (Eichholtz *et al.* 1993). In light of this another concentration-response experiment was performed using physiologically relevant LPA concentrations. Since all 3 HASM cell lines in the previous experiment responded in a similar manner to one another, one cell line was selected for further studies. A co-culture of HASM cells and TMLCs was stimulated with 0, 5, 10 and 20µM. In the absence of LPA no TGF-β activity was detected. However, as found previously a concentration-dependent increase in TGF-β activity following stimulation with LPA was observed. The highest concentration tested in this experiment was 20µM.
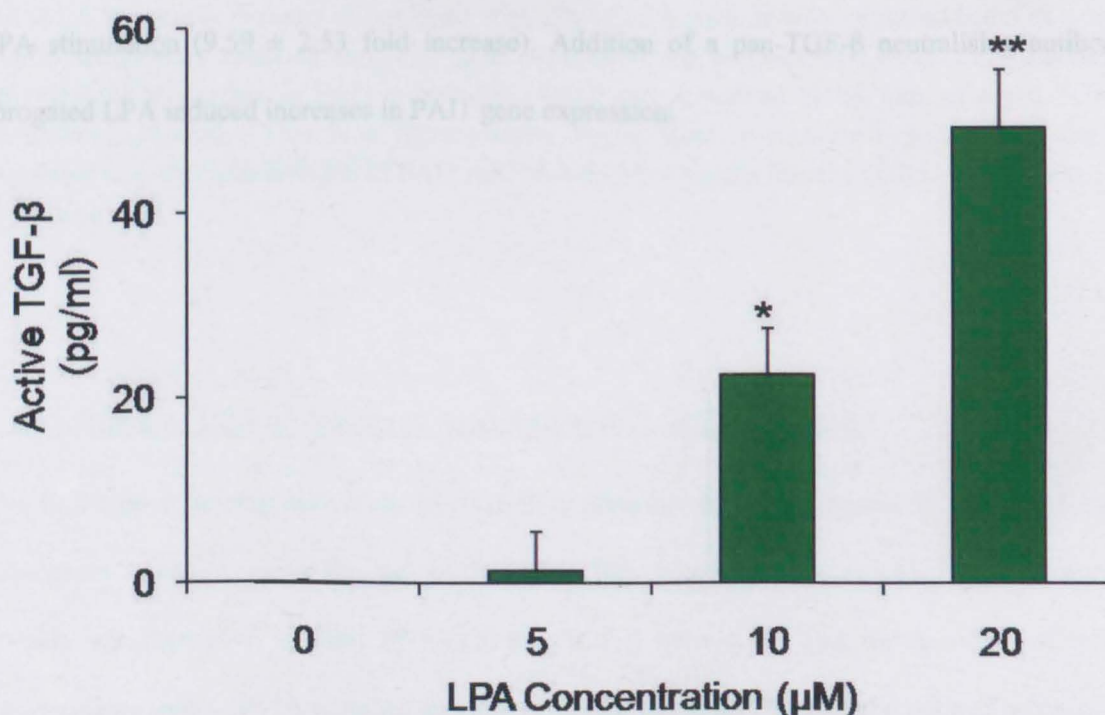
Figure 5.3: Physiological Concentration-Response of LPA on TGF-β Activation

A co-culture of HASMs and TMLCs was stimulated in duplicate with 0, 5, 10 and 20µM LPA. LPA caused a concentration-dependent increase in TGF-β activity. This figure shows the amalgamation of data from 3 independent experiments using 1 HASM cell line. Data is expressed as mean TGF-β activity ± SEM. P values less than 0.05 were accepted as significant.

* P<0.05   **P<0.01

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5.3.2 Effect of LPA Stimulation on PAI1 Expression by HASM Cells

To further investigate whether LPA causes TGF-β activation by HASM cells, expression of the TGF-β-inducible gene PAI1 was assessed following LPA stimulation using QPCR (see Methods section 2.4). HASM cells were stimulated in duplicate with 20μM LPA in the presence or absence of a pan TGF-β neutralising antibody at 10μg/ml. Total cell RNA was collected after 0, 4, 6 and 8 hours and reverse transcribed (see Methods section 2.3.4). Stimulation of HASM cells with 20μM LPA caused a time-dependent increase in PAI1 expression ($p<0.05$), which was almost completely abrogated by addition of a pan TGF-β neutralising antibody ($p<0.05$). Increased expression of PAI1 in response to LPA stimulation was observed at the earliest time point tested (4 hours). Levels of PAI1 remained elevated after 6 hours of stimulation with LPA (3.73 ± 2.21 fold increase). A further increase in PAI1 expression was detected after 8 hours of LPA stimulation (9.59 ± 2.53 fold increase). Addition of a pan-TGF-β neutralising antibody abrogated LPA induced increases in PAI1 gene expression.
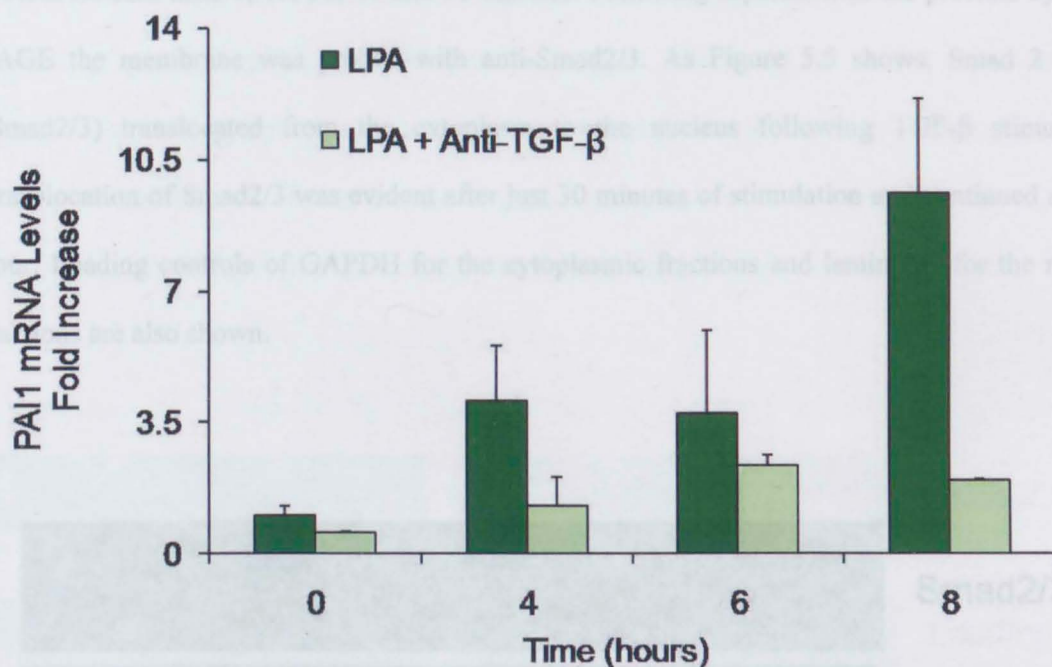
Figure 5.4: Effect of LPA Stimulation on Expression of PAI1

HASM cells were stimulated in duplicate with 20µM LPA with or without the addition of a pan-TGF-β neutralising antibody and PAI1 gene expression was assessed by QPCR. LPA induced a time-dependent increase in PAI1 expression, which was abrogated by addition of a pan TGF-β neutralising antibody. This is a representative figure from 3 separate experiments. Data is expressed as mean fold increase in PAI1 mRNA ± SEM. P values less than 0.05 were accepted as significant.

5.3.3 Effect of LPA on Nuclear Translocation of Smad2 and Smad3

The first step following activation of TGF-β is phosphorylation of Smads 2 and 3 and their subsequent translocation to the cell nucleus (see Introduction section 1.4.3). It was useful to develop an alternative method of measuring TGF-β activation. The translocation of many transcription factors can be assessed by isolating cytoplasmic and nuclear fractions of cell protein and using western blotting to detect a specific transcription factor (Clarke *et al.* 2008; Clifford *et al.* 2008). It was hypothesised that by isolating nuclear and cytoplasmic fractions of protein and using an antibody directed against Smad2 and Smad3 it would be possible to detect the translocation of these Smads from the cytoplasm into the nucleus. To investigate this, HASM cells were stimulated with 2ng/ml recombinant TGF-β1 and cytoplasmic and nuclear fractions of

protein isolated after 0, 15, 30, 45 and 60 minutes. Following separation of the proteins by SDS-PAGE the membrane was probed with anti-Smad2/3. As Figure 5.5 shows, Smad 2 and 3 (Smad2/3) translocated from the cytoplasm to the nucleus following TGF-β stimulation. Translocation of Smad2/3 was evident after just 30 minutes of stimulation and continued up to 1 hour. Loading controls of GAPDH for the cytoplasmic fractions and lamin AC for the nuclear fractions are also shown.
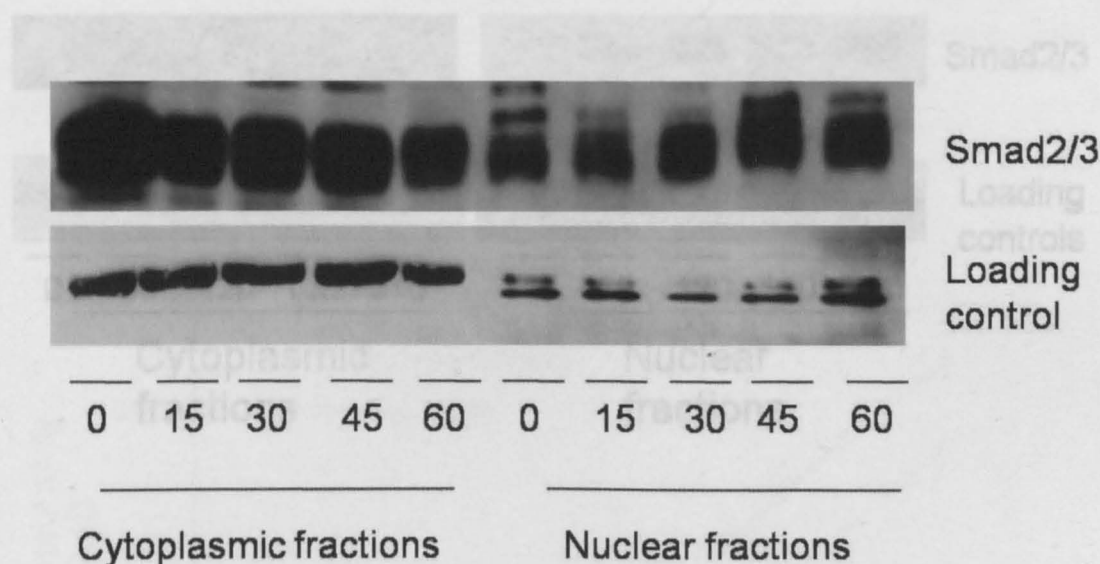
Figure 5.5: Translocation of Smad2/3 to Nucleus Following TGF-β1 Stimulation

HASM cells were stimulated with 2ng/ml TGF-β1 and nuclear and cytoplasmic fractions of cell protein were isolated. Smad2 and Smad3 translocate from the cytoplasm to the nucleus in a time-dependent manner. Time shown is minutes following TGF-β stimulation. This figure is representative of three independent experiments. Doublet Bands corresponding to Smad2/3 were observed at 52kD. This figure also shows the GAPDH and lamin AC loading controls for the cytoplasmic and nuclear fractions respectively.

5.3.4   Effect of LPA-Stimulation on Translocation of Smad2 and Smad3 to the Nucleus

In order to confirm that LPA causes TGF-β activation by HASM cells, cells were stimulated with LPA and cytoplasmic and nuclear fractions isolated after 0, 60, 120, 180 and 240 minutes. 25µg

of protein was subjected to SDS-PAGE and the resulting membrane probed with a Smad2/3 antibody. Figure 5.6 shows that stimulation of HASM cells with LPA caused translocation of Smad2/3 from the cytoplasm to the nucleus. Increased levels of Smad2/3 in the nucleus were evident after 60 minutes of stimulation and further increases seen at 120, 180 and 240 minutes. A decrease in Smad2/3 levels in the cytoplasmic fraction was most evident at 240 minutes.
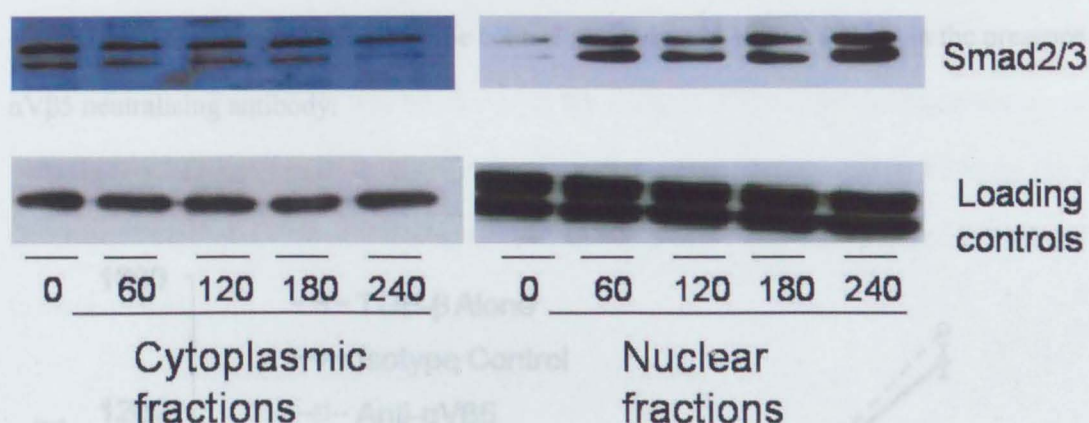
Figure 5.6: Translocation of Smad2/3 in Response to LPA Stimulation

HASM cells were stimulated with 20μM LPA and nuclear and cytoplasmic fractions of cell protein isolated after 0, 60, 120, 180 and 240 minutes. LPA caused a time-dependant translocation of Smad2 and Smad3 from the cytoplasm into the nucleus. This figure is representative of three separate experiments. Bands corresponding to Smad2/3 were observed at 52kD. This figure also shows the GAPDH loading control for the cytoplasmic fractions and the lamin AC loading control for the nuclear fractions.

5.3.5   Effect of αVβ5 Neutralising Antibody on TMLC and Co-culture

Results presented in the previous chapter suggested that HASM cells could activate TGF-β via the integrin αVβ5 in response to mechanical wounding. The role of this integrin in LPA-induced TGF-β activation was therefore investigated using a neutralising antibody against αVβ5. To determine the effect this antibody had on the response of the TMLCs, the effect of the antibody

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on a TGF-β1 standard curve was investigated. TMLCs were stimulated in duplicate with 250, 500 and 1000pg/ml recombinant TGF-β1 with or without 10µg/ml anti-αVβ5. This was repeated stimulating HASM cells co-cultured with TMLCs. The TMLC responded in a concentration-dependent manner to TGF-β1 (Figure 5.7). The response of the TMLCs to TGF-β did not significantly alter in the presence of an IgG1 isotype control antibody. In the presence of the anti-αVβ5 antibody the standard curve did shift down (P<0.05) but a concentration dependent increase in RLU was still evident: the highest concentration of 1000pg/ml TGF-β1 resulted in 1346.5 ± 49.5 RLU in the presence of an isotype control antibody and 1089 ± 0 RLU in the presence of the αVβ5 neutralising antibody.
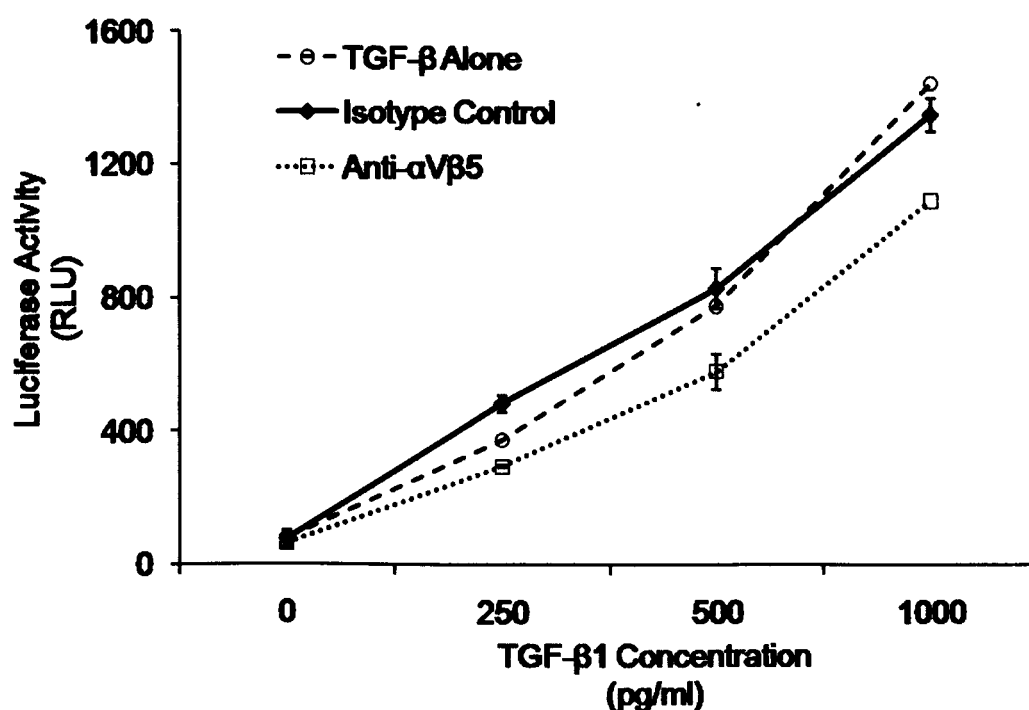
Figure 5.7: Effect of αVβ5 Neutralising Antibody on TMLC

TMLC were stimulated with 0, 250, 500 and 1000 pg/ml TGF-β alone, with 10µg/ml anti-αVβ5 or with 10µg/ml isotype control. Anti-αVβ5 antibody had an inhibitory effect on the response of the TMLCs but the gradient of the slope remained unchanged. This figure represents 1 experiment.

To determine whether the anti-αVβ5 antibody had an effect on the response of the TMLCs when in co-culture with HASM cells the experiment was repeated stimulating HASM cells in co-culture with TMLCs. The co-cultured was stimulated as before with 0, 250, 500 and 1000 pg/ml TGF-β1 with or without either 10μg/ml anti-αVβ5 or 10μg/ml isotype control. As found previously the co-culture responded in a concentration dependent manner to increasing concentrations of recombinant TGF-β1 (Figure 5.8). Addition of an isotype control antibody did not significantly affect this response. Addition of the αVβ5 neutralising antibody had an inhibitory effect on the response of the TMLC in the co-culture (P<0.005) as found previously (Figure 5.7), however, there was no change in the gradient of the slope. In the co-culture assay, it is likely that basal αVβ5 mediated TGF-β activation by the TMLCs is responsible for the shift in the curve. These data illustrate the importance of using more than one assay of TGF-β activation in these studies.
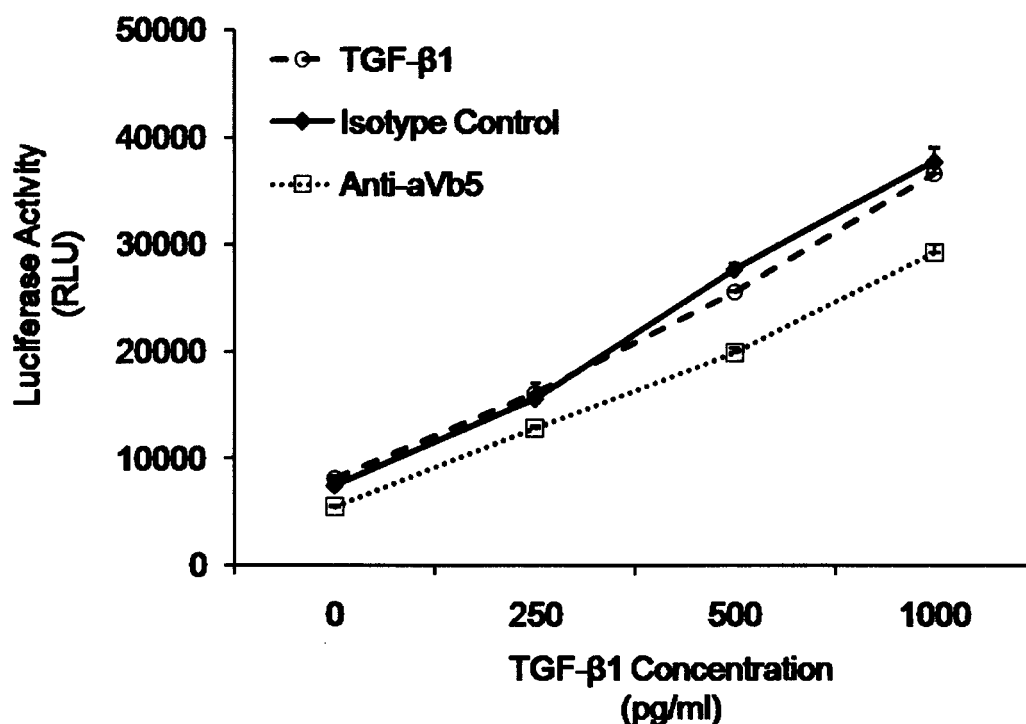
Figure 5.8: Effect of αVβ5 Antibody on Co-culture

HASM cells in co-culture with TMLC were stimulated with 0, 250, 500 and 1000 pg/ml TGF-β alone, with 10μg/ml anti-αVβ5 or with 10μg/ml isotype control. Anti-αVβ5 antibody had an inhibitory effect on the response of TMLC in co-culture with HASM to increasing concentrations of TGF-β. This figure represents 1 experiment.

5.3.6 Effect of αVβ5 Neutralising Antibody on LPA-Induced Luciferase Activity

After establishing that TMLC could still elicit a concentration-dependent response to TGF-β in the presence of anti-αVβ5, the role of integrin αVβ5 in LPA-induced TGF-β activation was investigated. A co-culture of TMLC and HASM cells was stimulated in duplicate with 20μM LPA with or without 10μg/ml anti-αVβ5 or an isotype control. Confirming previous results, 20μM LPA caused a statistically significant ($p<0.05$) increase in RLU. This increase was completely inhibited by addition of 10μg/ml αVβ5 neutralising antibody ($P<0.005$). Basal luciferase activity was also inhibited by anti-αVβ5.
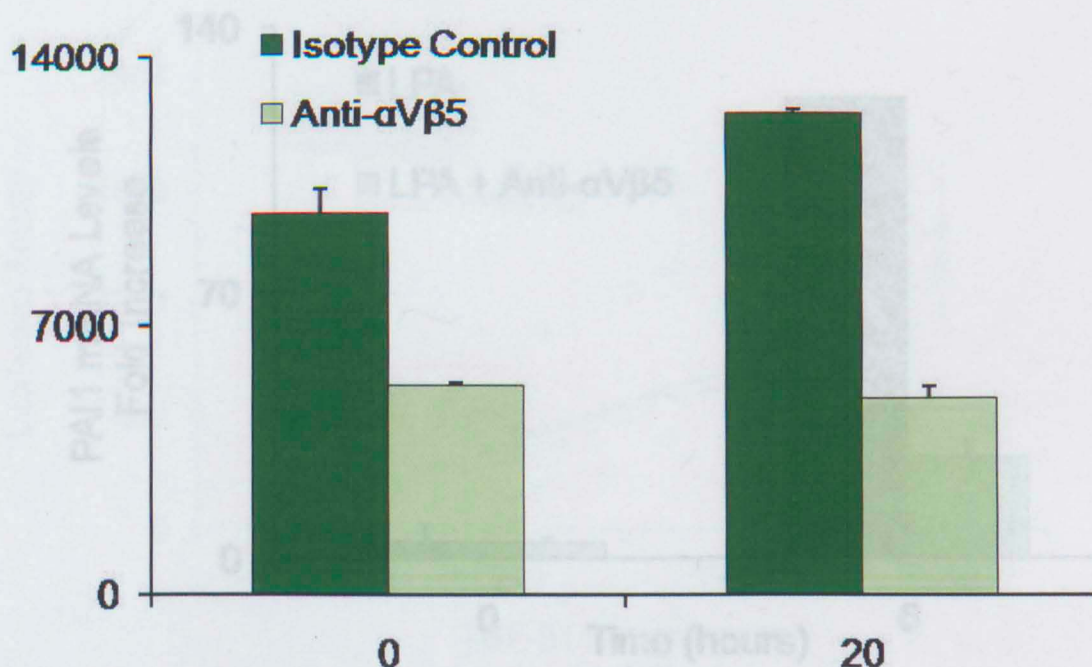
Figure 5.9: Effect of αVβ5 Neutralising Antibody on LPA-Induced RLU

HASM cells were co-cultured with TMLCs. The co-culture was stimulated with 20µM LPA with or without 10µg/ml anti-αVβ5. Addition of anti-αVβ5 completely abrogated LPA-induced increases in RLU. This is a representative figure from 3 independent experiments. Data is expressed as mean RLU ± SEM. P values less than 0.05 were accepted as significant.

5.3.7 Effect of Anti-αVβ5 on LPA-Induced PAI1 Expression

The data presented in Figure 5.9 suggests that LPA-induced TGF-β activation is mediated by the integrin αVβ5. To confirm the role of αVβ5 in LPA-induced TGF-β activation by HASM cells, HASM cells were stimulated in duplicate with 20µM LPA with or without the addition of 10µg/ml αVβ5 neutralising antibody. RNA was collected after 0 and 6 hours and reverse transcribed in to cDNA. This was subjected to QPCR analysis for the genes PAI1 and β2-M. LPA induced a 121 ± 0 fold increase in PAI1 mRNA levels (P<0.005) that was significantly, but not completely, inhibited by addition of an anti-αVβ5 antibody (P<0.01).

Figure 5.10: Effect of Anti-αVβ5 on LPA-Induced PAI1 Expression

HASM cells were stimulated in duplicate with 20µM LPA with or without anti-αVβ5. Addition of the αVβ5 neutralising antibody inhibited LPA-induced increase in PAI1 expression. This is a representative figure from 2 independent experiments. Data is expressed as mean ± SEM. P values less than 0.05 were accepted as significant.

5.3.8 Effect of Cytochalasin D on TMLC and Co-culture

To investigate the role of the cytoskeleton in LPA-induced αVβ5-mediated TGF-β activation an inhibitor of cytoskeleton reorganisation, cytochalasin D, was used. To establish whether this inhibitor had any effect on the response of the TMLCs its effects on a TGF-β1 standard curve were investigated. TMLCs were stimulated in duplicate with 250, 500 and 1000 pg/ml recombinant TGF-β1 with or without either 1µg/ml cytochalasin D or an equal volume of its vehicle DMSO. This experiment was repeated stimulating a co-culture of TMLCs with HASM cells. As Figure 5.11 shows, addition of cytochalasin D or its vehicle DMSO had no effect on the response of TMLC to increasing concentrations of recombinant TGF-β1.
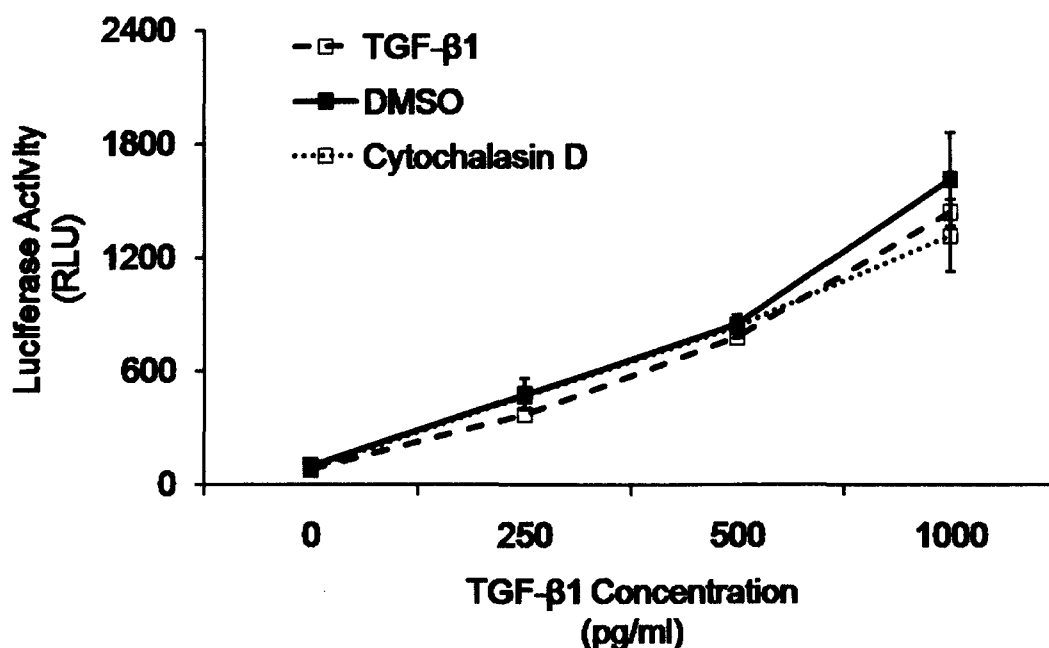
Figure 5.11: Effect of Cytochalasin D on TMLC

TMLC were stimulated in duplicate with 0, 250, 500 and 1000 pg/ml recombinant TGF-β1 with or without either 1μg/ml cytochalasin D or an equal volume of its vehicle DMSO. Cytochalasin D had no effect on the response of TMLCs to increasing concentrations of recombinant TGF-β1. This figure shows the data from 1 experiment.

In order to confirm that cytochalasin D does not affect the responsiveness of TMLCs to TGF-β when co-cultured with HASMs the experiment was repeated using TMLCs in co-culture with HASM cells. Neither cytochalasin D nor its vehicle DMSO had any effect on the response of the co-culture to increasing concentrations of TGF-β1 (Figure 5.12).
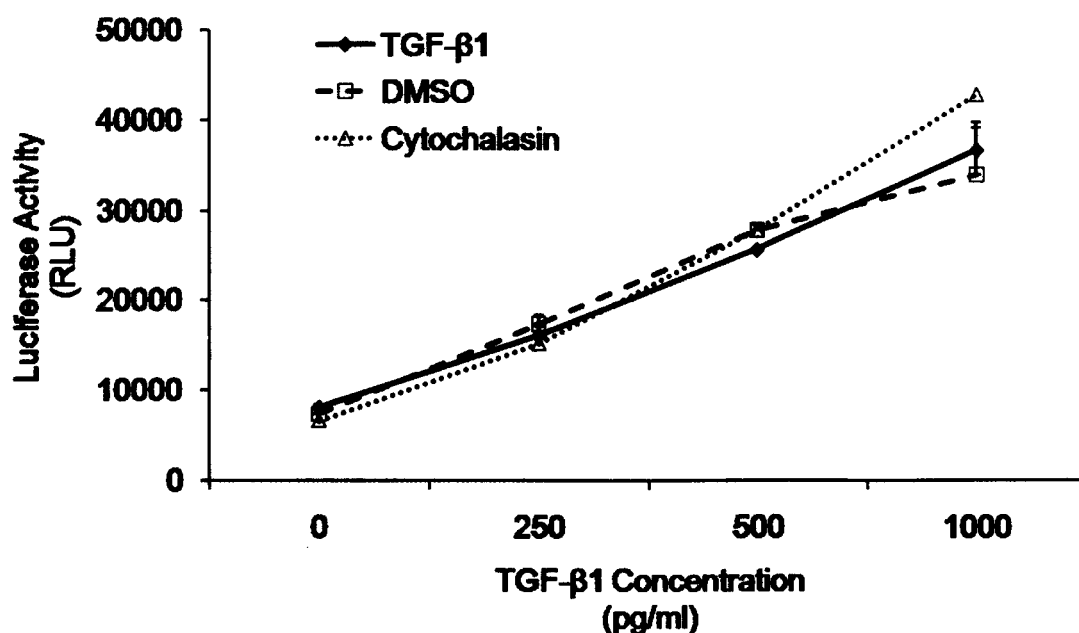
Figure 5.12: Effect of Cytochalasin D on Co-culture

A co-culture of HASM cells and TMLCs was stimulated in duplicate with 0, 250, 500 and 1000 pg/ml recombinant TGF-β1 with or without either 1μg/ml cytochalasin D or an equal volume of its vehicle DMSO. Neither cytochalasin D nor its vehicle DMSO had any effect on the response of TMLCs to increasing concentrations of recombinant TGF-β1. This figure shows the data from 1 experiment.

5.3.9   Effect of Cytochalasin D on LPA-Induced Luciferase Activity

To investigate the role of the cytoskeleton in LPA-induced αVβ5-mediated TGF-β activation by HASM cells cytochalasin D was added to a co-culture of HASMs and TMLCs stimulated with LPA. A co-culture of TMLCs with HASM cells was stimulated in duplicate with 20μM LPA with the addition of either 1μM cytochalasin D or an equal volume of its vehicle DMSO. Confirming previous findings, LPA induced a statistically significant increase in RLU (see Figure 5.13). However, when cells were pre-treated with cytochalasin D there was no increase in LPA-induced TGF-β activation.
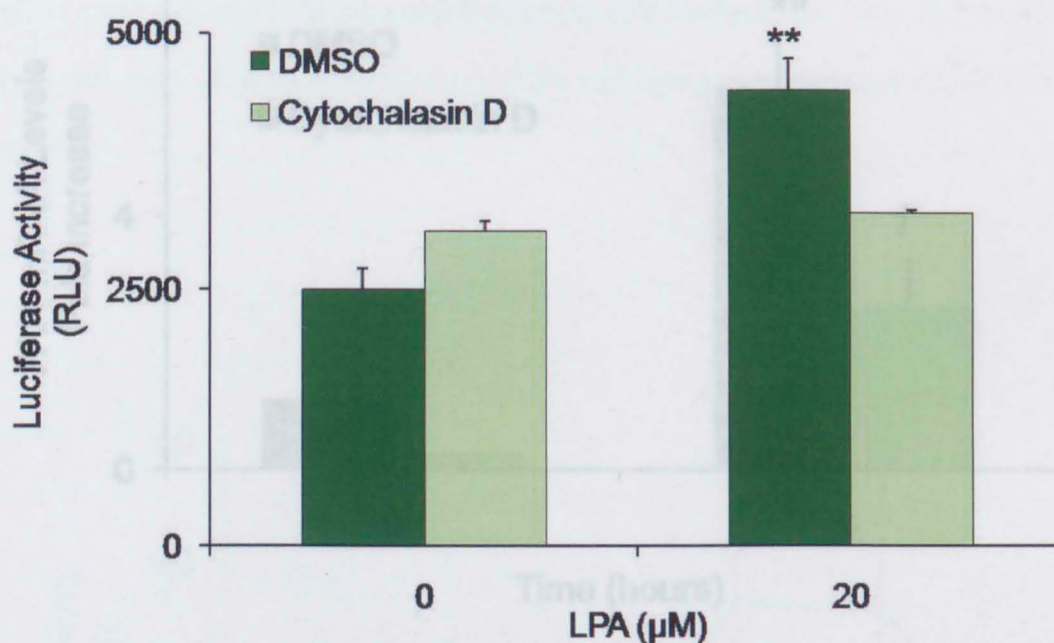
Figure 5.13: Effect of Cytochalasin D on LPA-Induced Luciferase Activity

A co-culture of HASM cells and TMLC was stimulated with 20μM LPA with or without 1μg/ml cytochalasin D. Cytochalasin D inhibited LPA-induced increases in RLU. This is a representative figure from 3 separate experiments. Data is expressed as mean RLU ± SEM.

** P<0.01 Comparing 20μM LPA with 0μM LPA

5.3.10 Effect of Cytochalasin D on LPA-Induced PAI1 Expression

To confirm the role of the cytoskeleton in LPA-induced TGF-β activation, the effect of cytochalasin D on LPA-induced PAI1 expression was investigated. HASM cells were stimulated in duplicate with 20μM LPA with or without 1μM cytochalasin D. Cells to be treated with the inhibitor were pre-treated for 30 minutes prior to the addition of LPA. RNA was collected after 0 and 6 hours and PAI1 gene expression assessed. Pre-treatment with 1μg/ml cytochalasin D partially inhibited LPA-induced TGF-β activation, P<0.05 (see Figure 5.14).
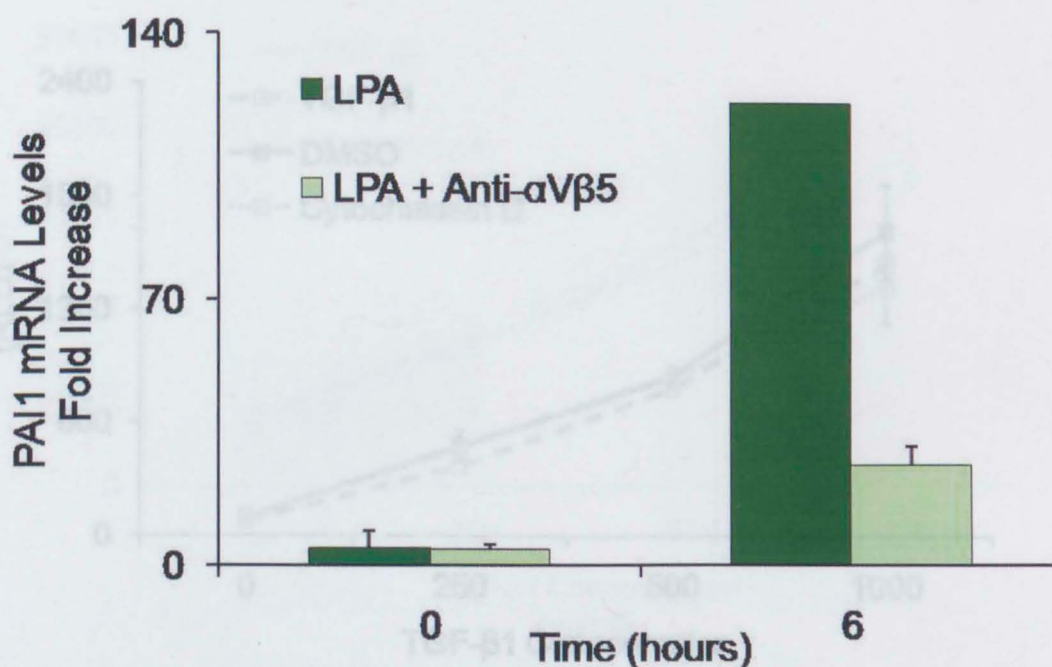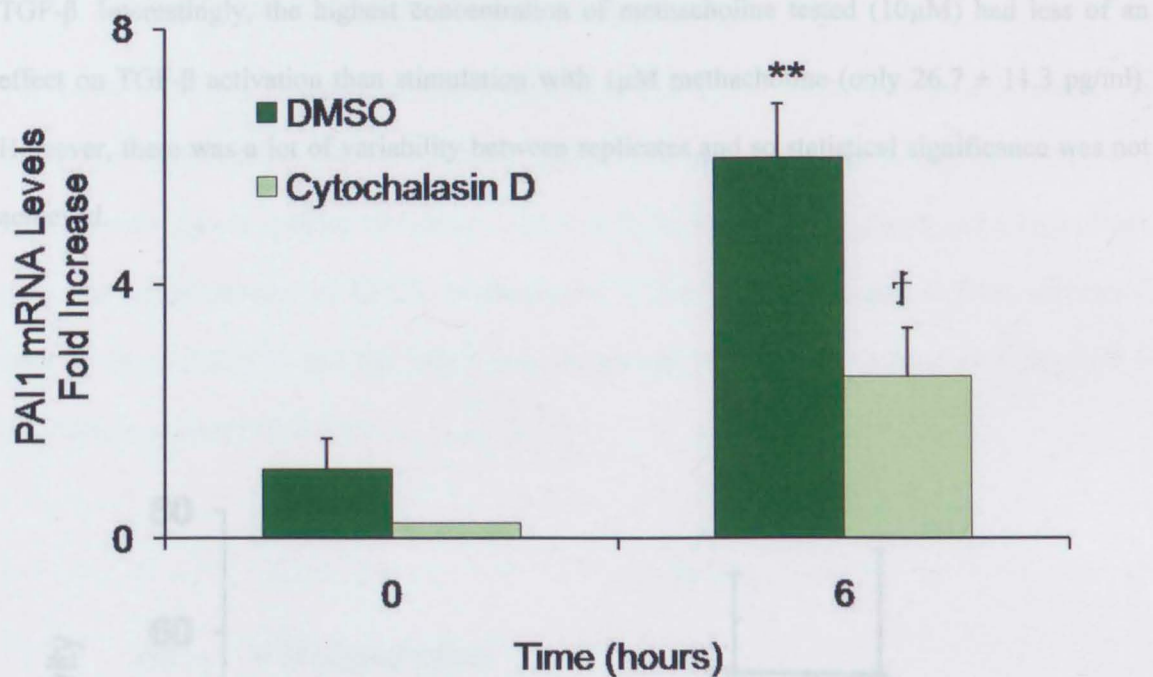
Figure 5.14: Effect of Cytochalasin D On LPA-Induced PAI1 Expression

HASM cells were stimulated in duplicate with 20µM LPA with or without 1µg//ml cytochalasin D. Cytochalasin D partially, but significantly, inhibited LPA-induced PAI1 expression after 6 hours of stimulation. This is a representative figure from 3 separate experiments. Data is expressed as mean fold increase ± SEM.

   ** P<0.01 Comparing LPA treatment at 0 hours with LPA treatment at 6 hours
   □ P<0.05 Comparing DMSO treatment with cytochalasin treatment at 6 hours

5.3.11 Concentration-Response of Methacholine on TGF-β Activation

LPA is known to cause contraction of several cells types. To investigate whether cell contraction of HASM cells per se can activate TGF-β, the muscarinic receptor agonist methacholine, which is used clinically to induce HASM contraction, was used to stimulate a co-culture of HASM cells with TMLCs. The co-culture was stimulated in duplicate with 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M methacholine in the presence or absence of 10µg/ml pan-anti-TGF-β. Methacholine appeared to induce TGF-β activation in a concentration dependent manner (see Figure 5.15). The lowest concentration tested only activated relatively small amounts of TGF-β (2.4 ± 0.1 pg/ml). Stimulation of the co-culture with 1µM methacholine caused activation of 52.5 ± 20.9 pg/ml

TGF-β. Interestingly, the highest concentration of methacholine tested (10μM) had less of an effect on TGF-β activation than stimulation with 1μM methacholine (only 26.7 ± 11.3 pg/ml). However, there was a lot of variability between replicates and so statistical significance was not achieved.
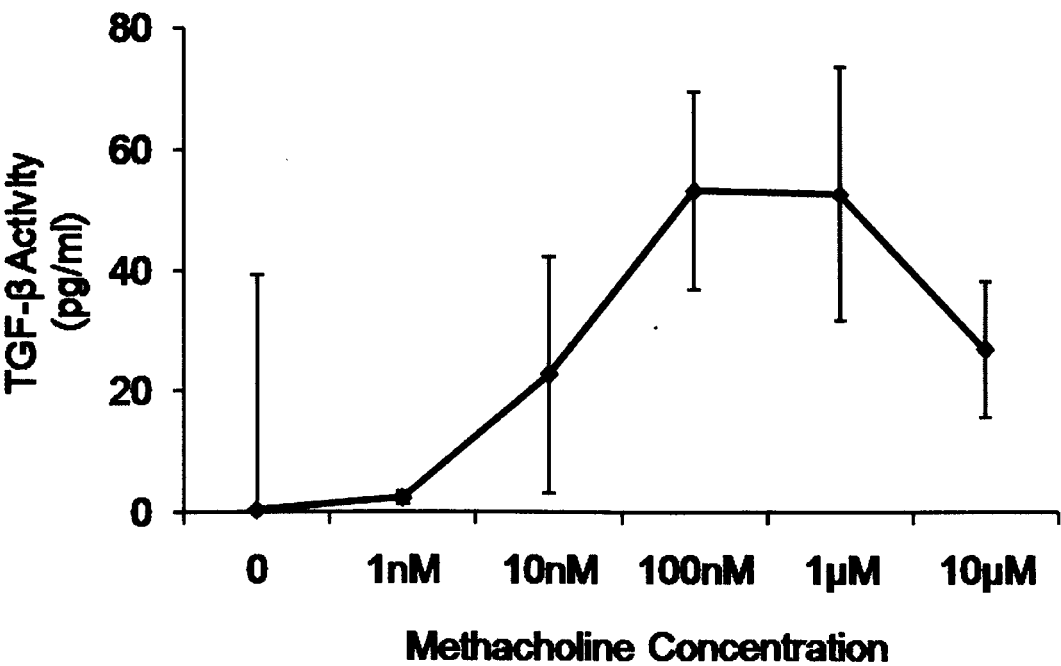
Figure 5.15: Concentration-Response of Methacholine on TGF-β Activation

HASM cells co-cultured with TMLC were stimulated with 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M methacholine with or without 10μg/ml anti-TGF-β. Methacholine caused a concentration dependent increase in TGF-β activation. This is a representative figure from 2 separate experiments. Data is expressed as mean ± SEM.

5.3.12 Effect of Methacholine on PAI1 Expression

To further investigate whether methacholine causes TGF-β activation PAI1 gene expression in response to methacholine was investigated. HASM cells were stimulated in duplicate with 1µM methacholine with or without 10µg/ml anti-TGF-β. RNA was collected after 0 and 6 hours PAI1 gene expression assessed by QPCR. Methacholine induced a large increase in PAI1 expression after 6 hours ($P<0.005$) and this effect was completely inhibited by addition of a pan-TGF-β neutralising antibody ($P<0.005$).
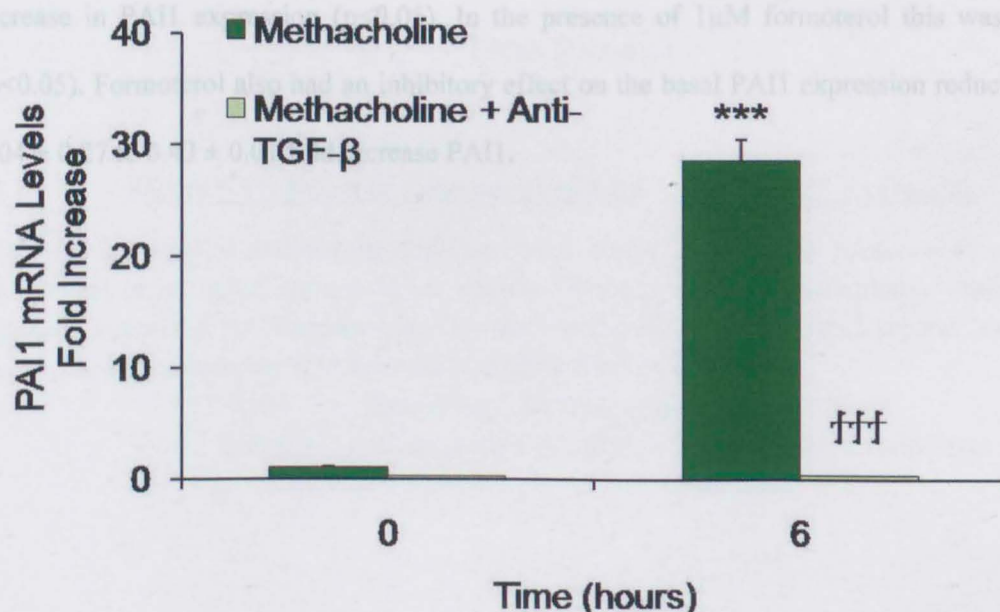
Figure 5.16: Effect of Methacholine on PAI1 Expression

HASM cells were stimulated in duplicate with 1µM methacholine with or without 10µg/ml anti-TGF-β. Stimulation of HASM cells with methacholine causes an increase in PAI1 expression, which is abrogated by a pan TGF-β neutralising antibody. This is a representative figure from 3 separate experiments. Data is expressed as mean ± SEM.

*** $P<0.005$ comparing methacholine at 0 hours with methacholine at 6 hours.

☐☐☐ $P<0.005$ comparing methacholine at 6 hours with methacholine plus anti-TGF-β at 6 hours.

5.3.13 Effect of β2 Agonist Formoterol on LPA-Induced PAI1 Expression

The data presented here implicate cytoskeletal reorganisation in LPA-induced TGF-β activation. Furthermore, the contraction agonist methacholine also stimulated TGF-β activation by HASM cells. It is therefore possible that contraction of HASM cells results in TGF-β activation. β2-agonists are a class of asthma drug that are able to cause ASM relaxation (Nials *et al.* 1994; Delmotte *et al.* 2009). The effects of the β2 agonist formoterol on LPA-induced TGF-β activation were investigated. HASM cells were stimulated in duplicate with 20µM LPA in the presence of either 1µM formoterol or an equal volume of its vehicle. RNA was collected after 0 and 6 hours and PAI1 gene expression assessed by QPCR. Confirming previous findings LPA caused an increase in PAI1 expression ($p < 0.05$). In the presence of 1µM formoterol this was abolished ($p < 0.05$). Formoterol also had an inhibitory effect on the basal PAI1 expression reducing it from 1.04 ± 0.27 to 0.43 ± 0.01 fold increase PAI1.
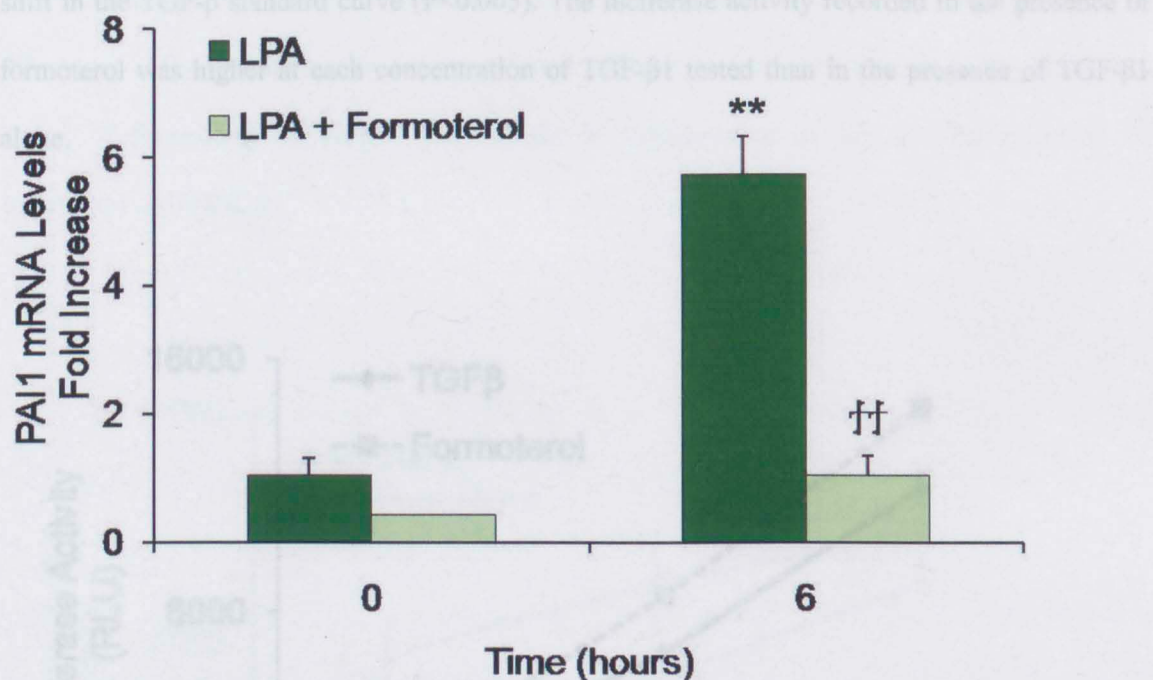
Figure 5.17: Effect of Formoterol on LPA-Induced PAI1 Expression

HASM cells were stimulated in duplicate with 20μM LPA in the presence of either 1μM Formoterol or an equal volume of its vehicle DMSO. Formoterol completely inhibited LPA-induced increases in PAI1 expression. This is a representative figure from 3 separate experiments. Data is expressed as mean fold increase in PAI1 mRNA ± SEM.

      ** P<0.01   Comparing LPA stimulation at 0 and 6 hours
      □□ P<0.01   Comparing LPA stimulation with LPA + formoterol at 6 hours

5.3.14 Effect of Formoterol on TMLC and Co-culture

The data presented in Figure 5.17 shows that the β2 agonist formoterol can inhibit LPA-induced PAI1 expression, which suggests that formoterol can inhibit LPA-induced TGF-β activation. To confirm that formoterol can abolish LPA-induced TGF-β activation its suitability for use in a co-culture assay was first determined by investigating its effects on the responsiveness of the reporter cells. TMLCs were stimulated in duplicate with 250, 500 and 1000 pg/ml recombinant TGF-β1 with or without 1μM formoterol or an equal volume of its vehicle DMSO. Addition of formoterol to TMLCs stimulated with increasing concentrations of TGF-β1 caused an upward

shift in the TGF-β standard curve (P<0.005). The luciferase activity recorded in the presence of formoterol was higher at each concentration of TGF-β1 tested than in the presence of TGF-β1 alone.
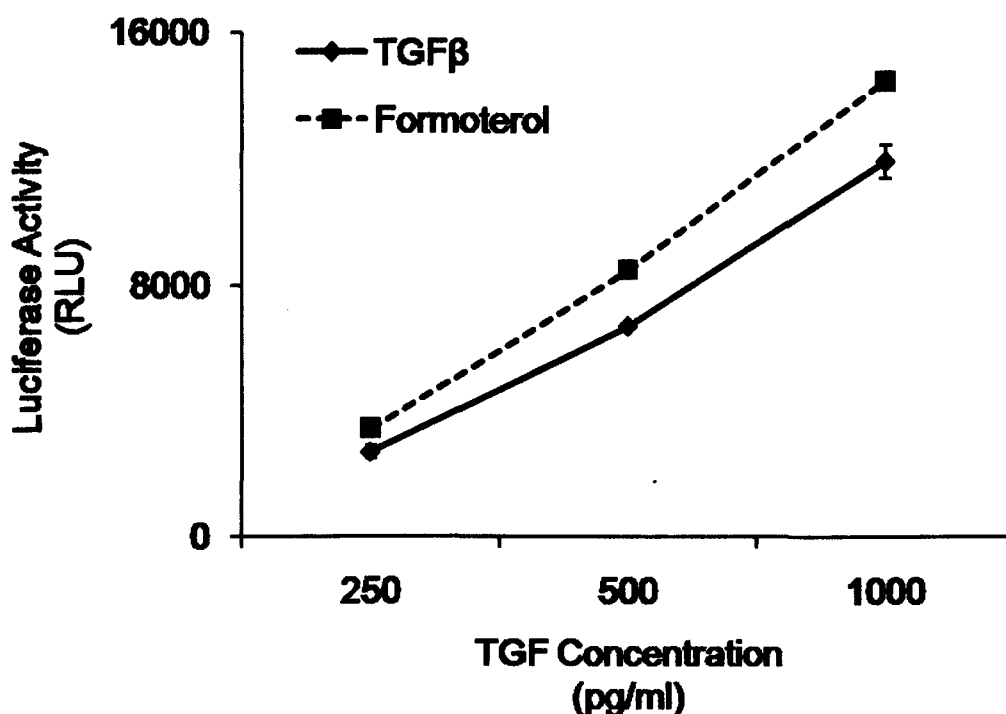
Figure 5.18: Effect of Formoterol on TMLCs

TMLCs were stimulated with 0, 250, 500 and 1000 pg/ml recombinant TGF-β with or without 1μM formoterol. Formoterol increased the response TMLCs to a TGF-β1 standard curve. This figure represents 1 independent experiment. Data is expressed as mean RLU ± SEM.

The effect of formoterol on a co-culture of TMLCs with HASM cells was also assessed by repeating the experiment on a co-culture of TMLC with HASM cells. Confirming the results of the previous experiment (see Figure 5.18), addition of formoterol to a co-culture of HASM cells and TMLCs resulted in an exaggerated response to increasing concentrations of TGF-β1, as shown by Figure 5.19 (P<0.05). The luciferase activity recorded was increased at each

concentration of TGF-β1 in the presence of formoterol compared with TGF-β1 alone. Due to its effects on TMLC reporter cells, formoterol was not used in a co-culture assay as any inhibitory action of formoterol on HASM cells might be counteracted by the stimulatory effect of formoterol on TMLCs.
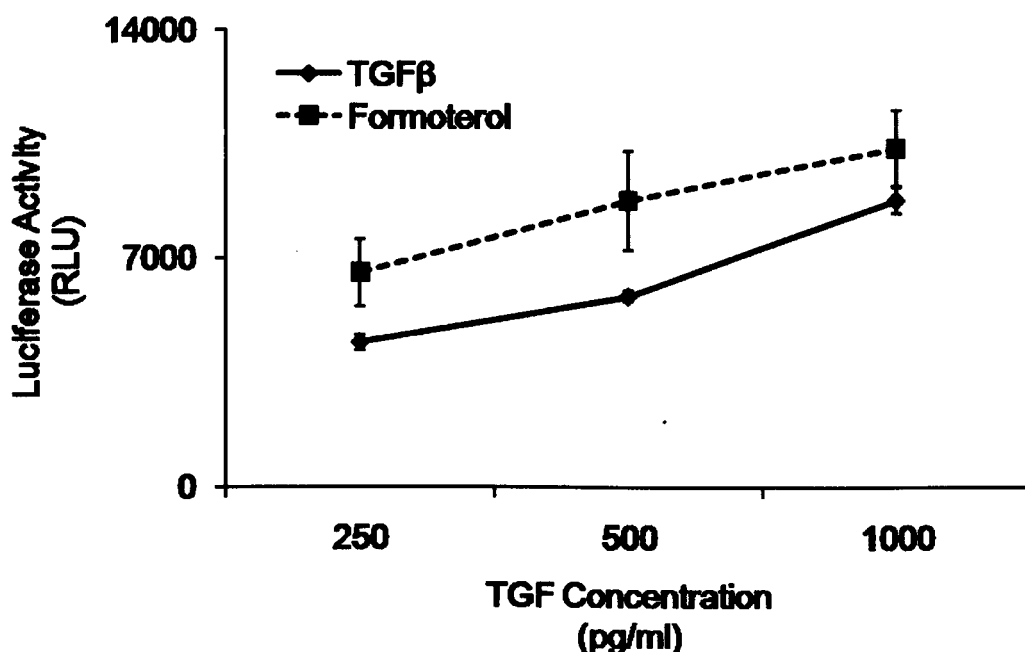
Figure 5.19: Effect of Formoterol on Co-Culture

A co-culture of HASM cells with TMLC's was stimulated with 0, 250, 500 and 1000 pg/ml TGF-β1 with or without 1μM formoterol. Formoterol increased the response of a co-culture of HASM cells and TMLC to a TGF-β1 standard curve. This figure represents 1 independent experiment. Data is expressed as mean RLU ± SEM.

5.3.15 Effect of LPA on TGF-β Activation by Asthmatic HASM Cells

A direct comparison of the response of asthmatic HASM cells to LPA stimulation with non-asthmatic HASM cells was performed using co-culture assay. Due to differences in the proliferation rate of asthmatic and non-asthmatic HASM cells experiments using different HASM cell lines were performed on different days. Differences in HASM cell size between asthmatics

and non-asthmatics have been reported (Ebina *et al.* 1993). In order to exclude the variable of these differences in cell size, data was corrected for cell number. Cell number was determined by CellTiter Glow assay (see Methods section 2.8), which quantifies cell number by determining ATP concentrations following cell lysis. Non-asthmatic and asthmatic HASM cells were co-cultured with TMLCs and stimulated with 20μM LPA with or without 1D11. All data were corrected for cell number. Figure 5.20 shows that asthmatic HASM cells elicit an exaggerated response to LPA stimulation compared with non-asthmatic HASM cells (P<0.05). There was very little difference in basal TGF-β activation, non-asthmatic cells activated 0.8 ± 0.2 pg/ml x10^4 cells whereas asthmatic cells activated none. However, when stimulated with 5μM LPA non-asthmatic cells activated 1.5 ± 1.5 pg/ml x10^4 cells compared with asthmatic cells which activated 11.7 ± 7.6 pg/ml x10^4 cells. When stimulated with 10μM LPA, non-asthmatic cells activated 2.8 ± 1.1 pg/ml x104 cells but the asthmatic cells activated 26.8 ± 10.5 pg/ml x10^4 cells. Following stimulation with 20μM LPA, asthmatic cells activated significantly more TGF-β (p<0.05) at 30.3 ± 9.1 pg/ml x10^4 cells compared with 7.4 ± 0.6 pg/ml x10^4 cells by the non-asthmatic HASM cells. These data show asthmatic HASM cells activate more TGF-β in response to LPA than non-asthmatic HASM cells.
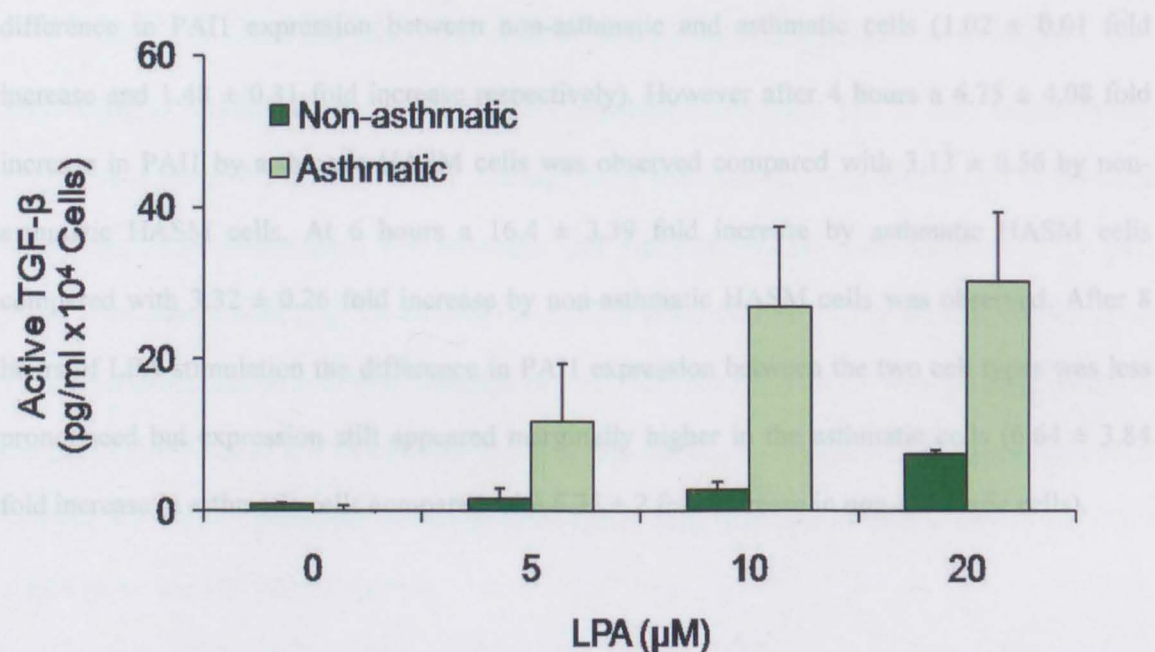
Figure 5.20: Effect of LPA on TGF-β Activation by Asthmatic HASM Cells

Non-asthmatic and asthmatic HASM cells were co-cultured with TMLCs and stimulated with 0, 5, 10 and 20μM LPA. Asthmatic HASM cells activated more TGF-β in response to increasing concentrations of LPA compared with non-asthmatic HASM cells. This figure shows the amalgamated data from 6 separate experiments using cells isolated from 3 non-asthmatic donors and 3 asthmatic patients.

5.3.16 Effect of LPA on PAI1 Expression by Asthmatic HASM Cells

Expression of the TGF-β-inducible gene PAI1 by asthmatic and non-asthmatic HASM cells in response to LPA was assessed using QPCR. Non-asthmatic and asthmatic HASM cells were stimulated in duplicate with 20μM LPA for 0, 4, 6 and 8 hours. Expression of PAI1 and β2-M mRNA was assessed by QPCR. LPA-induced increases in PAI1 have been shown to be mediated by TGF-β (Figure 5.4). Confirming earlier findings LPA induced a time-dependent increase in PAI1 expression by both non-asthmatic and asthmatic HASM cells (see Figure 5.21). Expression of PAI1 by asthmatic HASM cells was greater than expression by non-asthmatic cells at each time point tested following LPA stimulation (P<0.05), although basally there was very little

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difference in PAI1 expression between non-asthmatic and asthmatic cells (1.02 ± 0.01 fold increase and 1.48 ± 0.31 fold increase respectively). However after 4 hours a 6.75 ± 4.08 fold increase in PAI1 by asthmatic HASM cells was observed compared with 3.13 ± 0.56 by non-asthmatic HASM cells. At 6 hours a 16.4 ± 3.39 fold increase by asthmatic HASM cells compared with 3.32 ± 0.26 fold increase by non-asthmatic HASM cells was observed. After 8 hours of LPA stimulation the difference in PAI1 expression between the two cell types was less pronounced but expression still appeared marginally higher in the asthmatic cells (6.64 ± 3.84 fold increase in asthmatic cells compared with 6.35 ± 2 fold increase in non-asthmatic cells).
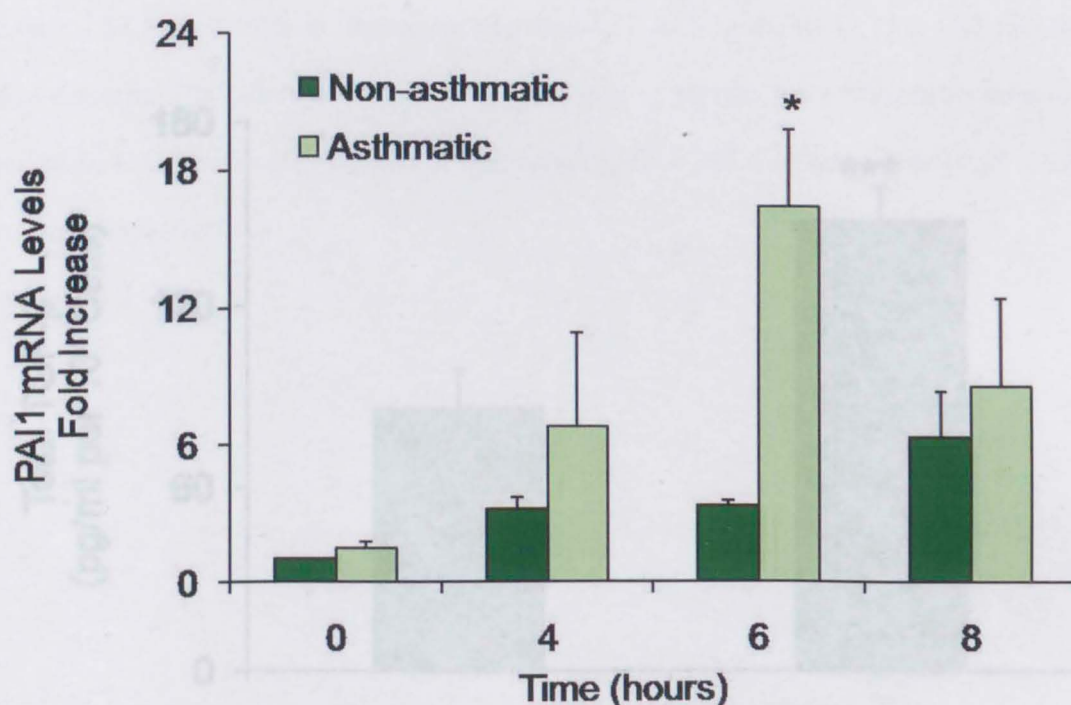
Figure 5.21: Effect of LPA on PAI1 Expression by Asthmatic HASM Cells

Non-asthmatic and asthmatic HASM cells were stimulated in duplicate with 20µM LA and PAI1 gene expression assessed after 0, 4, 6 and 8 hours. LPA induced PAI1 expression in both non-asthmatic and asthmatic HASM cells, however, larger increasing in PAI1 were observed in asthmatic HASM cells. N=3 each group.

5.3.17 Levels of Total TGF-β Released from Non-asthmatic and Asthmatic HASM Cells

To investigate whether asthmatic HASM cells sequester more latent TGF-β than non-asthmatic HASM cells the levels of total TGF-β released from non-asthmatic and asthmatic HASM cells were compared. Unstimulated non-asthmatic and asthmatic HASM cells cultured in 24 well plates were treated with 25µl 1N HCl for 10 minutes to activate all TGF-β present (Brown *et al.* 1990). Following neutralisation by addition of 25µl 1.2N NaOH, TMLCs were plated directly on top of the HASM cells and co-cultured for 16 hours. Asthmatic HASM cells released more total TGF-β (147.5 ± 11.1 pg/ml per 104 cells) than HASM cells from non-asthmatic individuals (85.9 ± 12.4 pg/ml per 104 cells, P<0.005).
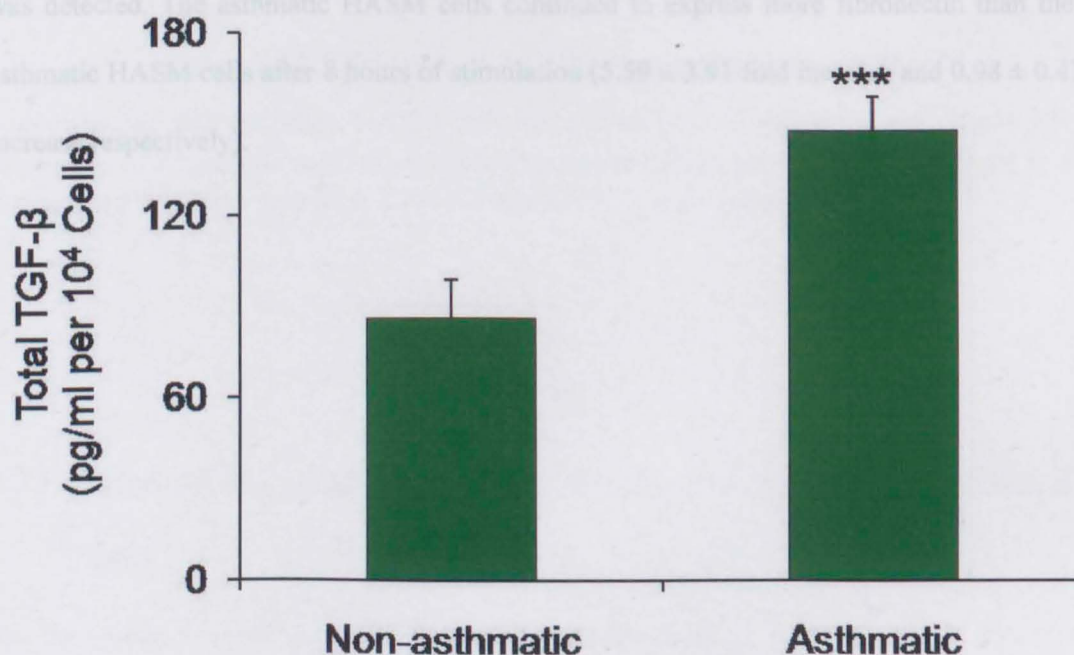
Figure 5.22: Expression of Total TGF-β by Non-asthmatic and Asthmatic HASM Cells

Unstimulated non-asthmatic and asthmatic HASM cells were acid-treated to activate all TGF-β present and levels of TGF-β determined by co-culture assay. Asthmatic HASM cells expressed higher levels of total TGF-β than non-asthmatic HASM cells. N=3 each group.. Data is expressed as mean ± SEM.

5.3.18 Effect of LPA on Fibronectin Expression by HASM Cells

To investigate whether the increased activation of TGF-β in response to LPA by asthmatic cells contributes to airway remodelling, expression of fibronectin was assessed using QPCR. Fibronectin is a component of the extracellular matrix that is up-regulated in asthma (Roche *et al.* 1989; Araujo *et al.* 2008). Non-asthmatic, and asthmatic, HASM cells were stimulated in duplicate with 20μM LPA, mRNA was collected after 0, 4, 6 and 8 hours and expression of fibronectin assessed by QPCR (see section 2.4). LPA had no effect on fibronectin expression in non-asthmatic HASM cells. However, expression of fibronectin by asthmatic HASM cells in response to LPA stimulation was increased at all time points tested and was a statistically significant increase compared with non-asthmatic cells ($P < 0.005$). After 4 hours of stimulation a 5.46 ± 1.53 fold increase in fibronectin was recorded. At 6 hours, an 11.13 ± 1.93 fold increase was detected. The asthmatic HASM cells continued to express more fibronectin than the non-asthmatic HASM cells after 8 hours of stimulation (5.59 ± 3.91 fold increase and 0.98 ± 0.43 fold increase respectively).
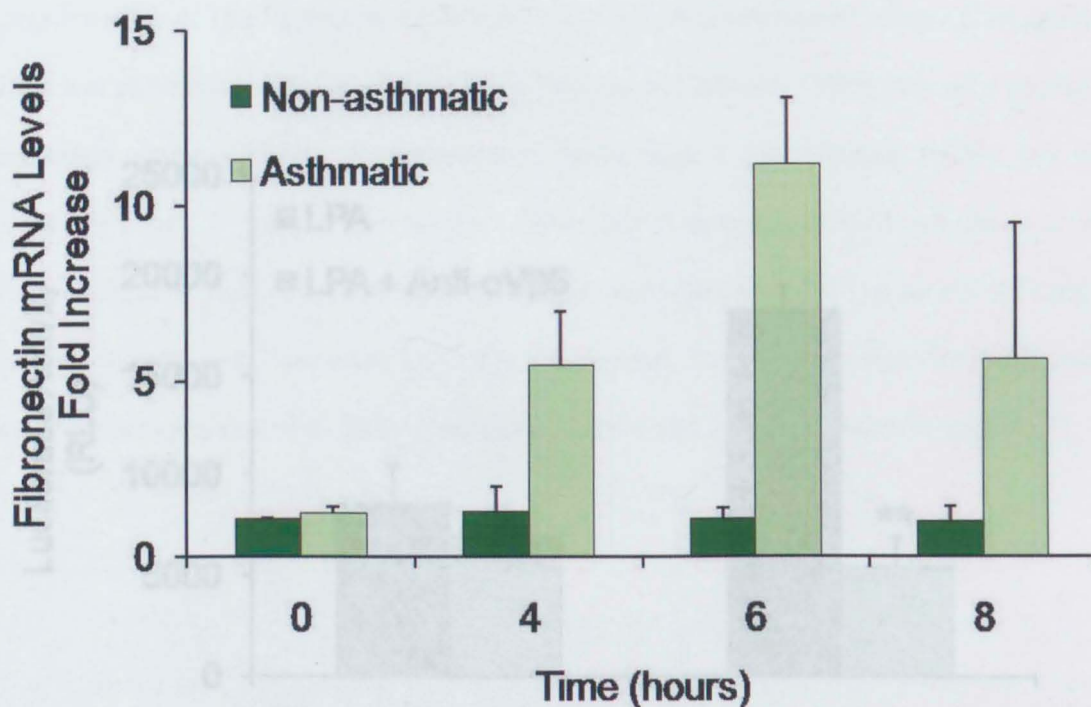
Figure 5.23: Effect of LPA on Fibronectin Expression by HASM

Non-asthmatic and asthmatic HASM cells were stimulated in duplicate with 20µM LPA and expression of fibronectin mRNA assessed. Expression of fibronectin mRNA increased in a time-dependent manner in response to LPA stimulation. N=3 each group.

5.3.19 Effect of Anti-αVβ5 on Increased Activation of TGF-β by Asthmatic HASM

Cells

To determine whether an αVβ5-independent mechanism is responsible for the increased activation of TGF-β in response to LPA stimulation an anti-αVβ5 neutralising antibody was used. A co-culture of asthmatic HASM cells with TMLCs was stimulated in duplicate with 20µM LPA in the presence or absence of 10µg/ml of an αVβ5 neutralising antibody (clone P1f6). Asthmatic HASM cells activated TGF-β in response to LPA stimulation as shown by an increase in RLU from 8583 ± 1946 to 18221 ± 1146. Addition of this antibody completely inhibited the LPA-induced increase in RLU, reducing it to 5392 ± 1511 (P<0.01). Anti-αVβ5 also reduced the basal RLU to 6911 ± 1577.

Figure 5.24: Effect of Anti-αVβ5 on LPA-Induced RLU by Asthmatic HASM

A co-culture of asthmatic HASM cells with TMLCs was stimulated in duplicate with 20µM with or without 10µg/ml anti-αVβ5. Addition of anti-αVβ5 antibody completely inhibited LPA-induced increases in RLU when asthmatic HASM cells were co-cultured with TMLC reporter cells. This is a representative figure from 3 separate experiments using HASM cells from 3 asthmatic donors. Data is expressed as mean fold increase in RLU over basal ± SEM.

** P<0.01

5.3.20 <u>Cell Surface Expression of αVβ5 Integrin on HASM Cells</u>

To determine whether the enhanced activation of TGF-β of asthmatic cells in response to LPA is caused by increased cell surface expression of αVβ5, flow cytometry was performed using an anti-αVβ5 antibody (clone ALULA). Unstimulated non-asthmatic and asthmatic HASM cells were stained with 20µg/ml anti-αVβ5 followed by a phycoerythrin (PE)-labelled anti-mouse secondary antibody (1:200 dilution). Some cells were stained with secondary antibody alone as a

negative control. The fluorescence intensity of the cells was determined using a flow cytometer. Data was expressed as fold increase in mean fluorescence intensity (MFI) over cells stained with secondary antibody alone. A representative figure from a non-asthmatic HASM cell line is shown as Figure 5.25 and a representative figure from a asthmatic HASM cell line is shown as Figure 5.26. The mean fold increase in mean fluorescence intensity (MFI) from HASM cells from 3 non-asthmatic and 3 asthmatic donors was compared. There was no significant difference in cell surface expression of αVβ5 between non-asthmatic and asthmatic donors (Figure 5.27).

Figure 5.25: Expression of αVβ5 Integrin by Non-asthmatic HASM Cells

This is a representative figure from 3 experiments using HASM cells from 3 separate non-asthmatic donors.

Figure 5.26: Expression of αVβ5 Integrin by Asthmatic HASM Cells

This is a representative figure from 3 separate experiments using HASM cells from 3 separate asthmatic donors.
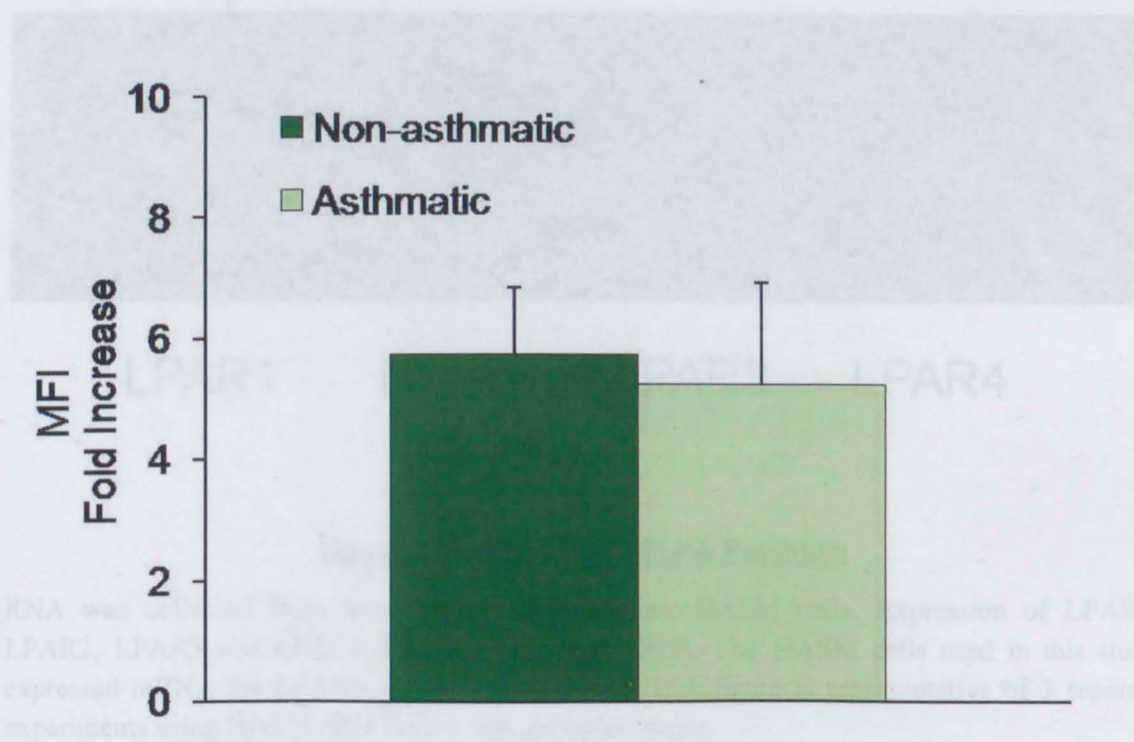
Figure 5.27: Cell Surface Expression on αVβ5 Integrin on HASM Cells

Expression of αVβ5 by both non-asthmatic and asthmatic HASM cells was assessed by flow cytometry. There was no difference in the cell surface expression of αVβ5 between non-asthmatic and asthmatic HASM cells. Figure shows the data from HASM cells isolated from 3 non-asthmatic and 3 asthmatic donors. Data is expressed as mean fold increase in MFI ± SEM.

5.3.21 Expression of LPA Receptor by HASM Cells

At present 4 distinct LPA receptors have been identified termed LPAR1, LPAR2, LPAR3, LPAR4. To establish which receptors are expressed by the HASM cells used in this study the expression of each of the four receptors at the mRNA level was investigated using RT-PCR (see Methods section 2.3). RNA was isolated from unstimulated non-asthmatic HASM cells and reverse transcribed into cDNA. The cDNA was subjected to PCR analysis to determine which of the four LPA receptors are expressed at the mRNA level. The HASM cells used in the current study expressed mRNA for LPAR1, LPAR2 and LPAR4 (see Figure 5.28).

LPAR1   LPAR2   LPAR3   LPAR4
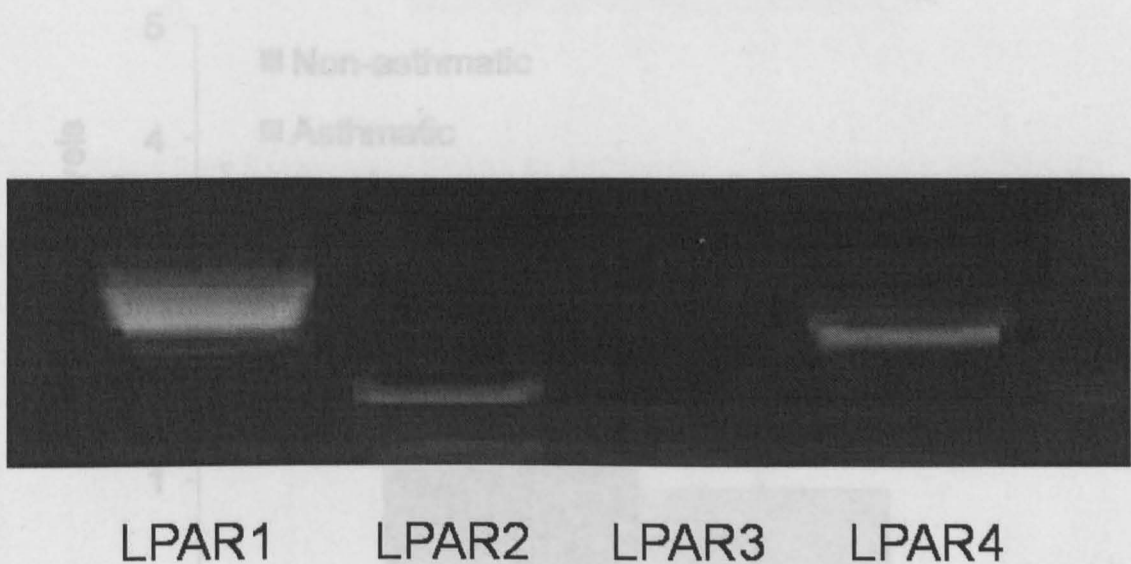
Figure 5.28: Expression of LPA Receptors

RNA was collected from unstimulated non-asthmatic HASM cells. Expression of LPAR1, LPAR2, LPAR3 and LPAR4 was assessed by RT-PCR. The HASM cells used in this study expressed mRNA for LPAR1, LPAR2 and LPAR4. This figure is representative of 3 separate experiments using HASM cells from 1 non-asthmatic donor.

As Figure 5.28 shows, HASM cells express mRNA for LPAR1, LPAR2 and LPAR4. To investigate whether there was any difference in expression of these three LPA receptors between non-asthmatic and asthmatic HASM cells QPCR was utilised (Methods section 2.4). RNA from

unstimulated non-asthmatic and asthmatic HASM cells was collected and reverse transcribed into cDNA (see Methods section 2.3.4). Expression of LPAR1 mRNA by both non-asthmatic and asthmatic HASM cells was assessed by QPCR. The basal expression of both LPAR1 (Figure 5.29) and LPAR2 (Figure 5.30) did not differ between non-asthmatic and asthmatic HASM cells. Unlike LPAR1 and LPAR2, basal expression of LPAR4 was higher in asthmatic HASM cells than in non-asthmatic HASM cells (Figure 5.31). However, this was not statistically significant.
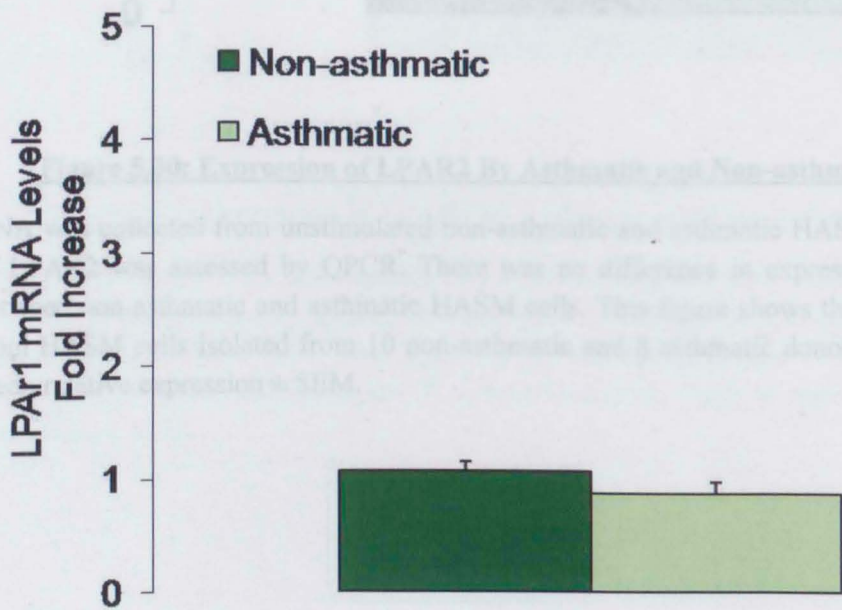
Figure 5.29: Expression of LPAR1 by HASM Cells

RNA was collected from unstimulated non-asthmatic and asthmatic HASM cells and expression of LPAR1 was assessed by QPCR. There was no difference in expression of LPAR1 mRNA between non-asthmatic and asthmatic HASM cells. This figure shows the amalgamation of data from HASM cells isolated from 10 non-asthmatic and 8 asthmatic donors. Data is expressed as mean relative expression ± SEM.
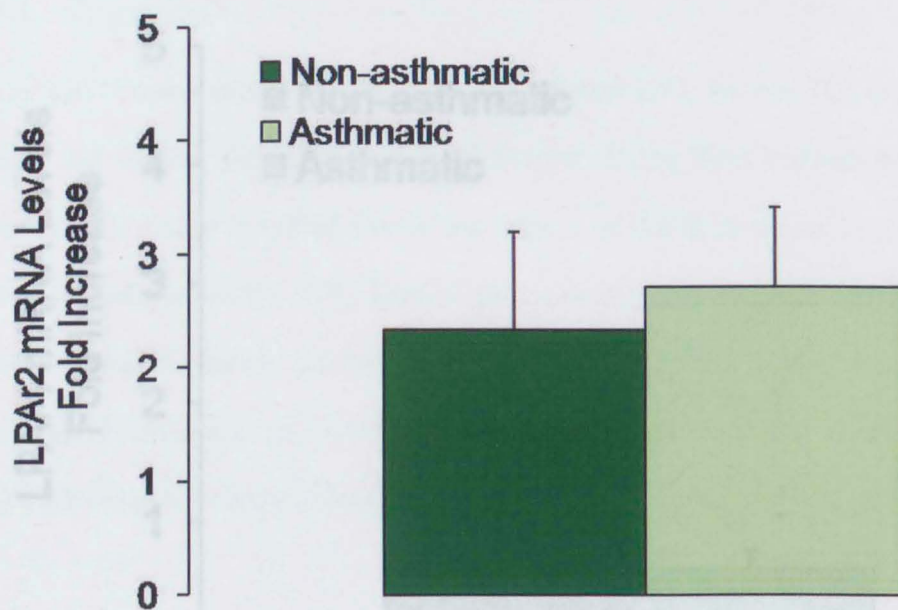
Figure 5.30: Expression of LPAR2 By Asthmatic and Non-asthmatic HASM Cells

RNA was collected from unstimulated non-asthmatic and asthmatic HASM cells and expression of LPAR2 was assessed by QPCR. There was no difference in expression of LPAR1 mRNA between non-asthmatic and asthmatic HASM cells. This figure shows the amalgamation of data from HASM cells isolated from 10 non-asthmatic and 8 asthmatic donors. Data is expressed as mean relative expression ± SEM.

Figure 5.31: Expression of LPAR4 by Asthmatic and Non-asthmatic HASM Cells

RNA was collected from unstimulated non-asthmatic and asthmatic HASM cells and expression of LPAR4 was assessed by QPCR. Non-asthmatic HASM cells expressed more LPAR4 mRNA than asthmatic HASM cells. This figure shows the amalgamation of data from HASM cells isolated from 8 non-asthmatic and 7 asthmatic donors. Data is expressed as mean relative expression ± SEM.

5.4 Discussion

The aim of these studies was to investigate whether LPA induces TGF-β activation in HASM cells and explore the mechanism of activation. Using three methods of measuring TGF-β activation we have identified a novel mechanism of TGF-β activation in HASM cells involving the cytoskeleton and the αVβ5 integrin. Furthermore, these data show that HASM cells isolated from asthmatic patients activate more TGF-β via this pathway than cells isolated from non-asthmatic individuals. This is the first time that a mechanism of TGF-β activation in response to cell activation has been described in asthma.

During this study a previously unpublished method of measuring TGF-β activation has been used. Detection of phosphorylated Smad2 or Smad3 is a commonly used method of assessing TGF-β activity (Jenkins *et al.* 2006; Xu *et al.* 2009). However, phosphorylated Smad2 and Smad3 were very difficult to detect in HASM cells by conventional western blot of whole cell protein extracts. Therefore I isolated nuclear and cytoplasmic fractions of cell protein from HASM cells to detect the translocation of Smad2 and Smad3 from the cytoplasm to the nucleus, as has been shown for other transcription factors (Clarke *et al.* 2008; Clifford *et al.* 2008; John *et al.* 2009). This is the first time that TGF-β activity has been measured in this way.

Previous studies have shown that the αVβ5 integrin can activate TGF-β in myofibroblasts (Wipff *et al.* 2007) and fibroblasts (Asano *et al.* 2005b; Scotton *et al.* 2009). However, this is the first description of αVβ5 integrin-mediated TGF-β activation in airway smooth muscle cells. Smooth muscle cells are contractile cells, and Wipff and colleagues demonstrated that contraction of myofibroblasts causes αVβ5-mediated TGF-β activation via a mechanism involving the cytoskeleton and requires mechanical tension from the ECM (Wipff *et al.* 2007). LPA is known to induce reorganisation of the cytoskeleton and the formation of stress fibers in several cell types (Chrzanowska-Wodnicka *et al.* 1996; Hirakawa *et al.* 2007; Xu *et al.* 2009), suggesting that contraction of smooth muscle cells may promote TGF-β activation. The β2 agonist formoterol, which induces relaxation of ASM cells (Nials *et al.* 1994; Delmotte *et al.* 2009) and is a

commonly used asthma therapy (Price *et al.* 2007), completely inhibited LPA-induced PAI1 expression, which was previously shown to be mediated by TGF-β. Furthermore, inhibiting cytoskeletal reorganisation with cytochalasin D had an inhibitory effect on LPA-induced TGF-β activation in HASM cells supporting this hypothesis. However, inhibition of TGF-β activation by cytochalasin D was not complete, failing to reduce TGF-β activation to basal levels. This may suggest that αVβ5-mediated TGF-β activation is not entirely mediated via the cytoskeleton and other, as yet undiscovered, pathways are involved. It is also possible that cytochalasin D does not completely depolymerise microfilaments and thus they could still function in inducing cytoskeletal changes. Certainly, incomplete inhibition of integrin-mediated TGF-β activation by cytochalasin D has been observed in several other studies (Munger *et al.* 1999; Wipff *et al.* 2007) suggesting that it is not unique to αVβ5-mediated TGF-β activation in HASM cells.

To help corroborate the hypothesis that HASM cell contraction results in TGF-β activation we used the muscarinic receptor agonist methacholine, which induces ASM contraction and is used clinically to induce bronchoconstriction (Boulet *et al.* 1997; Anderson *et al.* 2009), to stimulate HASM cells and assessed TGF-β activation in response. Similar to the effects of LPA, methacholine induced a concentration dependent increase in TGF-β activation and a TGF-β-dependent increase in PAI1 expression by HASM cells. Although there have been no reports of methacholine inducing TGF-β activation in HASM cells, activation of TGF-β in epithelial and subepithelial cells has been observed following allergen challenge in mild asthmatics (Torrego *et al.* 2007). The number of nuclei that stained positively for Smad2, Smad3 and Smad4 increased significantly. These patients elicited both EAR and LAR associated with significant decreases in FEV1 indicating that bronchoconstriction had occurred. Together these data suggest that bronchoconstriction could indeed lead to activation of TGF-β in the airway wall.

Data presented here show that HASM cells from asthmatic patients activate more TGF-β via the αVβ5 integrin in response to LPA stimulation than cells from non-asthmatic individuals. Increased TGF-β activation by the αVβ5 integrin in diseased cells has previously been

demonstrated in scleroderma fibroblasts when compared with control fibroblasts (Asano *et al.* 2006). This enhanced TGF-β activation was also detected in unstimulated cells, which was not seen in asthmatic HASM cells in the present study. Increased activation of TGF-β by scleroderma fibroblasts was attributed to increased cell surface expression of αVβ5 (Asano *et al.* 2006), however, no difference in cell surface expression of αVβ5 was found between asthmatic and non-asthmatic HASM cells in this study. This may reflect a difference in the nature of defect in the αVβ5-mediated TGF-β activation pathway between the two diseases. Increased duration of exposure of scleroderma fibroblasts to active TGF-β due to their enhanced TGF-β activation basally, could induce ITGB5 gene expression (Lai *et al.* 2000), promoting a positive feedback loop that ultimately leads to increased activation of TGF-β. However, this does not seem to be involved in the enhanced activation of TGF-β by asthmatic HASM cells.

Our data show for the first time that HASM cells from asthmatic patients activate more TGF-β than cells from non-asthmatic individuals in response to LPA stimulation. It is possible that the enhanced TGF-β activity is merely due to the enhanced total TGF-β secreted by HASMs from asthmatic patients. However, this possibility is not favoured because the level of total TGF-β in HASMs from both control and asthmatics was in considerable excess of levels of active TGF-β following stimulation with LPA, as we have found in other cell systems (Xu *et al.* 2009). This means that the amount of latent TGF-β available was not a rate limiting factor in LPA-induced TGF-β activation. Furthermore, the enhanced TGF-β activity observed in asthmatic HASM cells was completely inhibited by blocking the function of the αVβ5 integrin. These data show that there was no difference in αVβ5 integrin expression between diseased and control cells. This suggests that changes in the interaction between the αVβ5 integrin and its ligand, latent TGF-β, control the activation status of TGF-β rather than increased expression of either latent TGF-β or the αVβ5 integrin. Such interaction changes have previously been described for TGF-β activation mediated by the epithelially restricted integrin αVβ6 (Munger *et al.* 1999). Further study is required to investigate the role of such interaction changes in αVβ5-mediated TGF-β activation.

Although the data presented in this chapter show that asthmatic HASM cells activate more TGF-β in response to LPA stimulation than non-asthmatic cells, the mechanism of this enhanced response has not been fully elucidated. It has been shown that it is not mediated by increased expression of the αVβ5 integrin, and it is unlikely to be caused by the increased levels of total TGF-β released from asthmatic HASM cells. Furthermore, these data suggest that LPA-induced αVβ5-mediated TGF-β activation is caused by contraction of the HASM cells. Enhanced bronchoconstriction is a significant feature of asthma and is responsible for the variable airway obstruction observed in asthmatic patients. Published data suggests that this may be caused by a fundamental difference in HASM cells, since primary HASM cells from asthmatic patients are more contractile in *in vitro* models (Ma *et al.* 2002; Matsumoto *et al.* 2007). Increased contractility could be caused by many different factors including increased expression of contractile proteins such as MLCK, RhoA and myosin (Ammit *et al.* 2000; Sakai *et al.* 2001; Leguillette *et al.* 2009). The difference in contractility between non-asthmatic and asthmatic HASM cells could account for the enhanced activation of TGF-β in response to LPA stimulation, which is known to cause contraction and stress fibre formation (Chrzanowska-Wodnicka *et al.* 1996; Toews *et al.* 2002; Ainslie *et al.* 2004; Hirakawa *et al.* 2007; Xu *et al.* 2009). Future studies will address this possibility using in vitro, ex vivo and in vivo models.

As has been discussed in Introduction section 1.4, TGF-β plays a fundamental role in orchestrating structural changes in the airways that are associated with airway remodelling. These data show that asthmatic HASM cells activate more TGF-β in response to LPA stimulation than non-asthmatic HASM cells. This lead us to the hypothesis that expression of TGF-β-inducible genes that are involved in airway remodelling would also be increased in asthmatic cells compared with non-asthmatic cells in response to LPA stimulation. Expression of fibronectin was assessed. Fibronectin expression by asthmatic HASM cells increased over time in response to LPA, however, in non-asthmatic cells no increase was observed. Despite evidence that LPA enhances assembly of fibronectin matrix extracellularly (Checovich *et al.* 1993; Olorundare *et al.* 2001) there is currently no evidence to suggest that LPA can increase expression of fibronectin

transcriptionally. It is possible that the increased expression of fibronectin by asthmatic HASM cells in response to LPA stimulation is mediated by activation of TGF-β, which acts in an autocrine fashion to increase fibronectin transcription (Kucich *et al.* 2000). This could be confirmed by repeating the experiment with the addition of a TGF-β neutralising antibody. It is possible that the lack of increase in fibronectin expression observed in non-asthmatic cells is caused by insufficient activation of TGF-β.

This is the first time that enhanced TGF-β activation by asthmatic HASM cells has been described in response to any stimulus, and is the first description of αVβ5-mediated TGF-β in this cell type. Furthermore, we have found that the contraction agonist methacholine, which is used clinically to induce bronchoconstriction (Anderson *et al.* 2009), can also induce TGF-β activation by HASM cells suggesting that contraction of HASM cells results in TGF-β activation. This hypothesis was corroborated by the finding that inhibiting cytoskeletal reorganisation with cytochalasin D and inducing HASM cell relaxation with the β2 agonist formoterol both inhibit LPA-induced TGF-β activation. These findings could have important implications for asthma pathogenesis. AHR and bronchoconstriction are fundamental features of asthma and involve the ASM layer. These data suggest that contraction of the ASM layer during an asthma exacerbation could result in TGF-β activation, which would potentiate the development of structural changes associated with airway remodelling such as subepithelial fibrosis, mucous gland hyperplasia and epithelial cell apoptosis. There is currently little information about how airway remodelling is initiated in the asthmatic airway and so these findings could shed some light on the link between asthma symptoms and pathogenesis, and the development of airway remodelling, which ultimately is detrimental to lung function over time and contributes to worsening disease (Lange *et al.* 1998; Mascia *et al.* 2005).

Role of the β5 Cytoplasmic Domain in αVβ5-Mediated TGF-β Activation

6 Role of the β5 Cytoplasmic Domain in αVβ5-Mediated TGF-β Activation

6.1 Rationale

Integrins are unique in that they are capable of bi-directional signalling. Ligand binding to the extracellular domain initiates intracellular signalling pathways via what is known as outside-in signalling. In addition, the affinity of an integrin for its ligand, or its activation state, can be influenced by intracellular signalling pathways via a process known as inside-out signalling. Inside-out signalling results in a conformational change in the extracellular domain of the integrin, which increases its ligand binding affinity (Zhu *et al.* 2008). The cytoplasmic domains of integrin subunits are fundamental for this purpose (O'Toole *et al.* 1994). Several cytoplasmic proteins have been shown to interact with integrin cytoplasmic domains to initiate inside-out activation of integrins (Liu *et al.* 2000) the most important of which is thought to be talin. Talin promotes inside-out signalling by binding to both actin filaments and the cytoplasmic domain of β integrin subunits (Calderwood *et al.* 1999; Vinogradova *et al.* 2002) creating a link between the actin cytoskeleton and the integrin on the cell surface.

The data described in the previous chapter shows that stimulation of HASM cells with LPA results in αVβ5-mediated TGF-β activation via a mechanism that involves the cytoskeleton similar to that described for αVβ6-mediated TGF-β activation in epithelial cells (Munger *et al.* 1999; Jenkins *et al.* 2006; Xu *et al.* 2009). Structural interactions of the β5 subunit with latent TGF-β and cytoskeletal proteins are therefore likely to be crucial for αVβ5-mediated TGF-β. Munger and colleagues (1999) used truncation mutants of the β6 subunit cytoplasmic domain to show that regions within this domain are essential for the ability of αVβ6 to mediate TGF-β activation. The role of the cytoplasmic domain in αVβ5-mediated TGF-β activation has not been investigated.

A polymorphism in the β5 cytoplasmic domain has been previously described (Kawahara *et al.* 2005) (see Figure 6.1). This polymorphism occurs within an NxxY talin binding motif and results in the loss of 3 amino acids (FNK767-769) from the cytoplasmic tail. Although a talin binding domain is still present in the polymorphic β5 cytoplasmic tail it is possible that the deletion may affect the ability of talin to bind. A role for talin in integrin-mediated TGF-β activation has not been described for any of the integrins known to mediate this process, although it is expected due to evidence that deletion of a talin binding domain from the cytoplasmic domain of β6 results in an αVβ6 integrin that is unable to activate TGF-β (Munger *et al*, 1999).

Full Length β5 Subunit (FNKFNK)
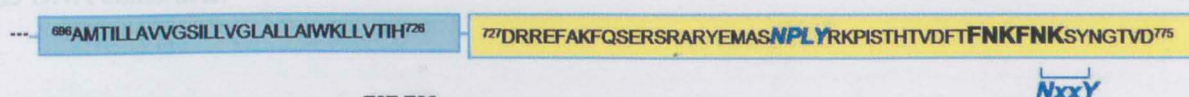
β5 Subunit Lacking FNK$^{767-769}$ (FNK)
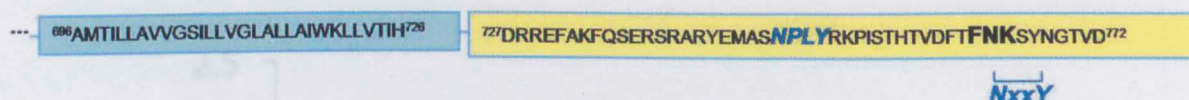
Figure 6.1: Sequence of Cytoplasmic Domains of Full Length and Polymorphic β5

6.2 Aims

The aim of these experiments was to investigate the role of the talin binding domain at amino acids 768-771 of the β5 cytoplasmic domain in αVβ5-mediated TGF-β activation. DNA constructs corresponding to the full length β5 subunit and the polymorphic β5 subunit were over-expressed in CS-1 cells that express no endogenous β5. Activation of TGF-β by these cells was investigated using a co-culture assay. The ability of the two variants of β5 to interact with talin was investigated using co-immunoprecipitation.

6.3   Results

6.3.1   Kill Curve of G418 on Untransfected CS-1 Cells

The two DNA constructs to be used (FNKFNK and FNK) contain a neomycin resistance gene which gives cells expressing the construct resistance against the antibiotic G418. In order to determine the concentration of G418 sufficient to kill cells not expressing the construct $1x10^6$ untransfected CS-1 cells were cultured in serum plus media containing 0, 31.25, 65, 125, 250, 500 and 1000μg/ml of the antibiotic G418. After three days the cells were harvested and counted. The antibiotic G418 killed untransfected CS-1 cells in a concentration-dependent manner. The two highest concentrations tested, 500 and 1000μg/ml, killed all cells after 3 days of culture. G418 at 500μg/ml was therefore used in future experiments to select CS-1 cells that express the β5 DNA constructs.
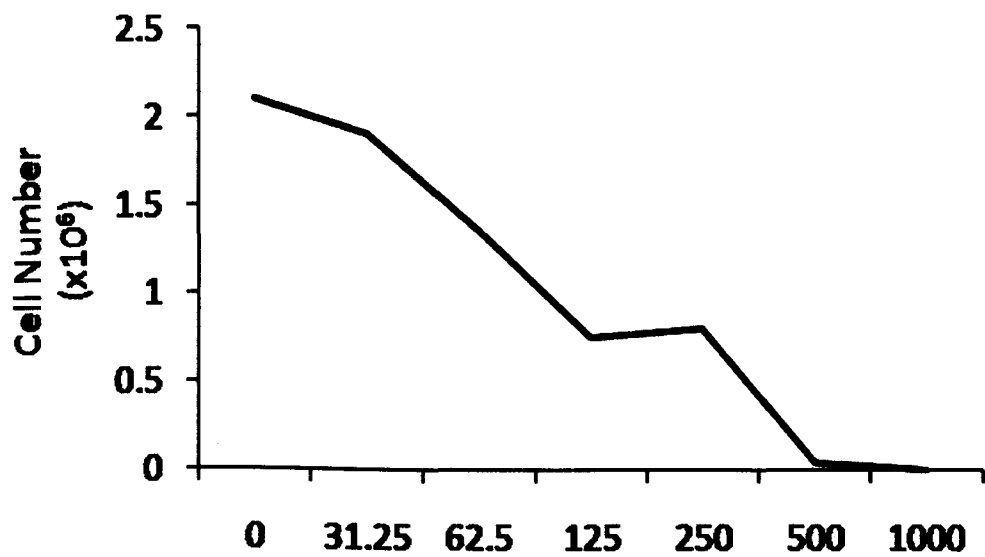
Figure 6.2: Kill Curve of G418 on Untransfected CS-1 Cells

Untransfected CS-1 cells ($1x10^6$ cells) were cultured in the presence of 0, 31.25, 65, 125, 250, 500 and 1000μg/ml of the antibiotic G418. After three days the cells were harvested and counted. G418 killed CS-1 cells in a concentration-dependent manner. This figure represents a single experiment.

6.3.2 Generation of Stable CS-1/FNK and CS-1/FNKFNK Cell Lines

CS-1 cells at 1.5×10^6 were transfected with the two β5 DNA constructs using Transfast transfection reagent (see Methods section 2.12). A variety of DNA concentrations and DNA: Transfast ratios were used, these are outlined in Table 6.1. Expression of the constructs by the CS-1 cells resulted in their adhesion to plastic. Since all ratios and all DNA concentrations resulted in successful transfections and subsequent expression of β5 by the CS-1 cells, the result was several CS-1 cell lines expressing varying levels of β5.

CS-1 Cell Line Created	DNA Concentration Used	DNA: Transfast Ratio
CS-1/FNKFNK-1	1μg	1:2
CS-1/FNKFNK-2	2μg	1:2
CS-1/FNKFNK-3	3μg	1:2
CS-1/FNKFNK-4	1μg	1:3
CS-1/FNKFNK-5	2μg	1:3
CS-1/FNKFNK-6	3μg	1:3
CS-1/FNK-1	1μg	1:2
CS-1/FNK-2	2μg	1:2
CS-1/FNK-3	3μg	1:2
CS-1/FNK-4	1μg	1:3
CS-1/FNK-5	2μg	1:3
CS-1/FNK-6	3μg	1:3

Table 6.1: Generation of CS-1/FNKFNK and CS-1/FNK Cell Lines

This table outlines the DNA concentrations and DNA: Transfast ratios used in the generation of stable CS-1 cell lines that express either the full length β5 construct (FNKFNK) or the polymorphic β5 construct.

6.3.3 Expression of αVβ5 by CS-1/FNKFNK and CS-1/FNK Cell Lines

Following transfection and clonal expansion of CS-1/FNKFNK and CS-1/FNK cells cell surface

expression of αVβ5 was confirmed by flow cytometry (see Methods section 2.14). Cells were

stained with 20μg/ml anti-αVβ5 and a PE-labelled secondary antibody. The fluorescence intensity

of the cells was determined using a flow cytometer. These data show that αVβ5 integrin was

expressed on the surface of both CS-1/FNKFNK and CS-1/FNK (Figure 6.3).
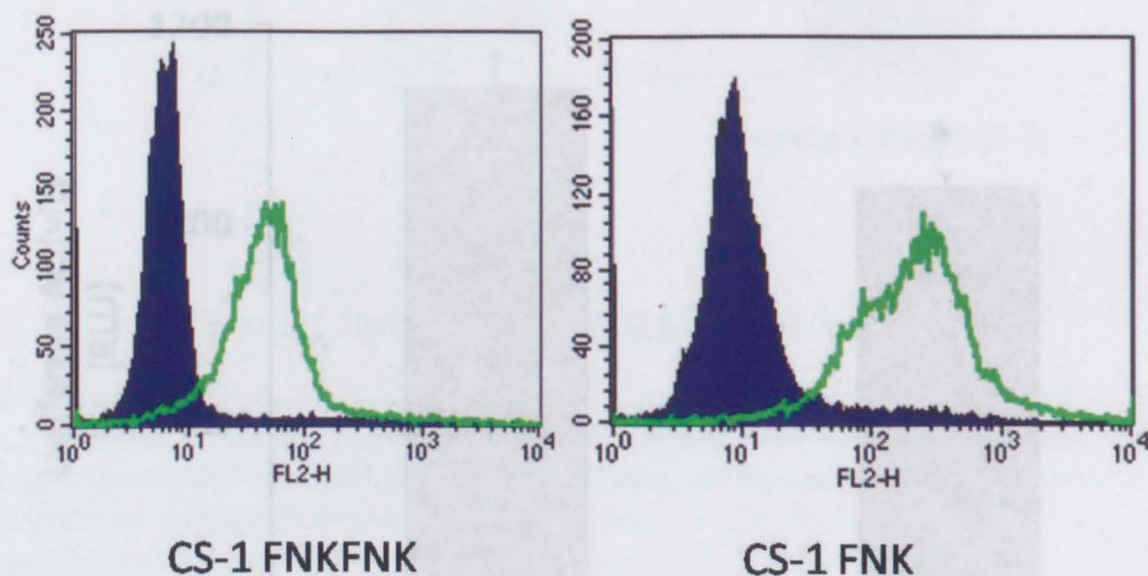
CS-1 FNKFNK   **CS-1 FNK**

Figure 6.3: Expression of αVβ5 by CS-1/FNKFNK and CS-1/FNK Cell Lines

Cell surface expression of αVβ5 by unstimulated CS-1/FNKFNK and CS-1/FNK was assessed by
flow cytometry. Both CS-1 cell lines expressed αVβ5 on their cell surface as shown by flow
cytometry. This figure shows representative data from one CS-1/FNKFNK cell line and one CS-
1/FNK cell line. Data is expressed as fold increase in MFI over negative control (secondary
antibody only).

6.3.4 TGF-β Activation by CS-1/FNKFNK and CS-1/FNK Cells

CS-1/FNKFNK and CS-1/FNK cells were co-cultured with TMLC reporter cells to investigate

both basal and LPA-induced TGF-β activation. The co-cultures were either left unstimulated with

or without 10μg/ml anti-TGF-β, or stimulated with 20μM LPA with or without 10μg/ml anti-

TGF-β. All stimulations were performed in duplicate. CS-1/FNKFNK cells were able to activate TGF-β basally (Figure 6.4) as shown by the decrease in luciferase activity from 1074 ± 65 to 869 ± 27 in the presence of a pan-TGF-β neutralising antibody (P<0.05). However, CS-1/FNK cells could not activate TGF-β basally (Figure 6.5) as there was no decrease in RLU upon addition of the pan-TGF-β neutralising antibody.
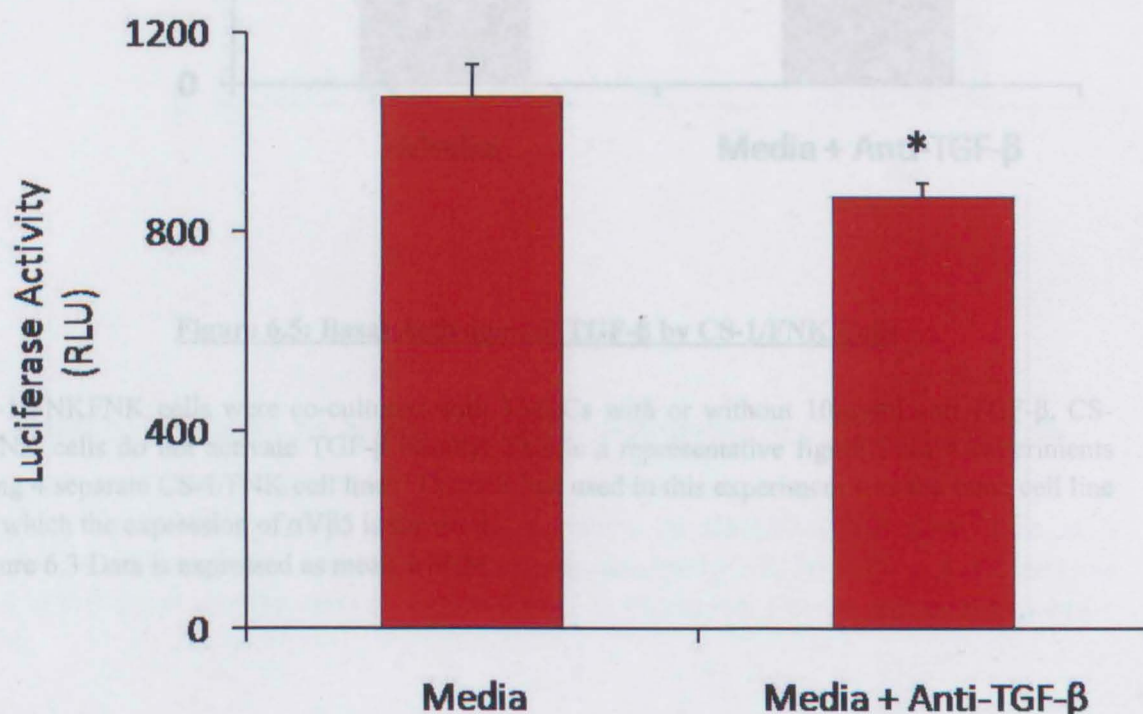
Figure 6.4: Basal Activation of TGF-β by CS-1/FNKFNK Cells

CS-1/FNKFNK cells were co-cultured with TMLCs with or without 10μg/ml anti-TGF-β. CS-1/FNKFNK cells activate TGF-β basally. This is a representative figure from 4 experiments using 4 separate CS-1/FNKFNK cell lines. The cell line used in this experiment was the same cell line for which the expression of αVβ5 is shown in Figure 6.3. Data is expressed as mean ± SEM. P values < 0.05 were accepted as significant.

*   P<0.05
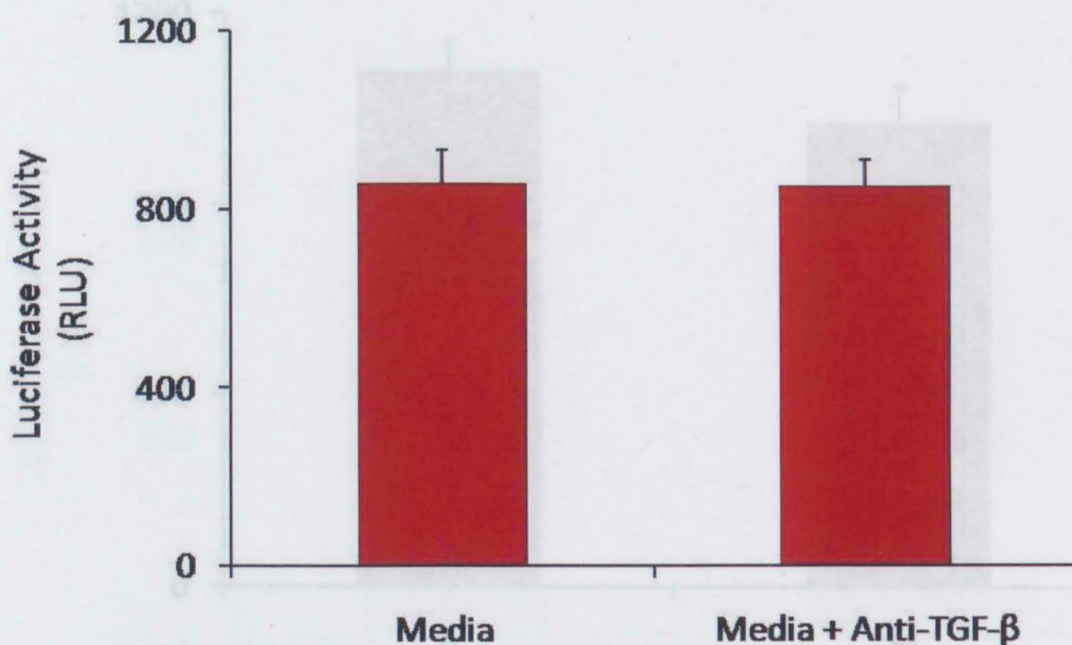
Figure 6.5: Basal Activation of TGF-β by CS-1/FNK Cells

CS-1/FNKFNK cells were co-cultured with TMLCs with or without 10µg/ml anti-TGF-β. CS-1/FNK cells do not activate TGF-β basally. This is a representative figure from 4 experiments using 4 separate CS-1/FNK cell lines. The cell line used in this experiment was the same cell line for which the expression of αVβ5 is shown in Figure 6.3 Data is expressed as mean ± SEM.

The effect of LPA stimulation on TGF-β activation by CS-1/FNKFNK and CS-1/FNK cells was also investigated by stimulating co-cultures of each cell line and TMLCs with 0 and 20µM LPA. Activation of TGF-β in response to LPA stimulation did not occur in either CS-1/FNKFNK (Figure 6.6) or CS-1/FNK cells (Figure 6.7), as shown by no difference in luciferase activity between 0 and 20µM LPA stimulation.
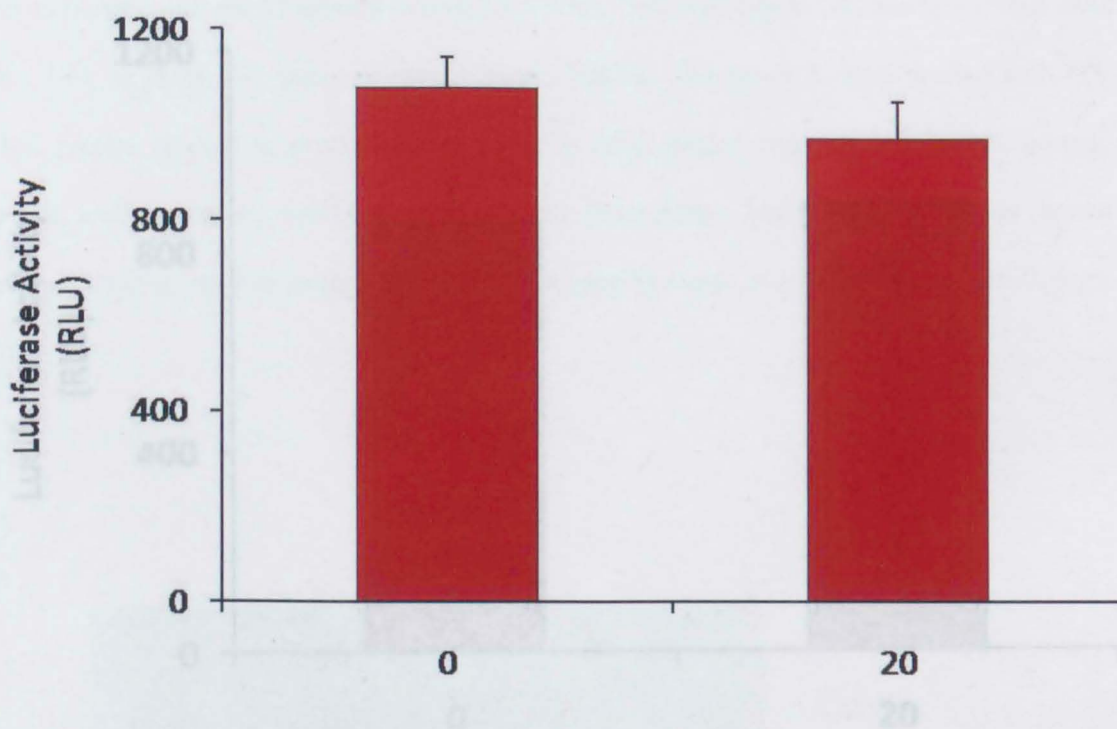
Figure 6.6: Effect of LPA Stimulation on TGF-β Activation by CS-1/FNKFNK Cells

A co-culture of CS-1/FNKFNK cells and TMLCs was stimulated with 0 and 20μM LPA in duplicate. LPA had no effect on TGF-β activation by CS-1/FNKFNK cells. This is a representative figure from 4 experiments using 4 separate CS-1/FNKFNK cell lines. The cell line used in this figure was the same as the one shown in Figure 6.3. Data is expressed as mean ± SEM.

Figure 6.7: Effect of LPA Stimulation on TGF-β Activation by CS-1/FNK Cells

A co-culture of CS-1/FNK cells and TMLCs was stimulated with 0 and 20µM LPA in duplicate. LPA had no effect on TGF-β activation by CS-1/FNK cells. This is a representative figure from 4 experiments using 4 separate CS-1/FNK cell lines. The cell line used in this figure was the same as the one shown in Figure 6.3. Data is expressed as mean ± SEM.

6.3.5   Co-immunoprecipitation (Co-Ip) of Talin with β5

To assess whether the deletion of FNK[767-769] affects the ability of talin to interact with the β5 cytoplasmic domain, unstimulated protein from CS-1/FNKFNK and CS-1/FNK cells was subjected to a co-immunoprecipitation assay. Three CS-1/FNKFNK and three CS-1/FNK cells lines were cultured and unstimulated cell protein collected according to the manufacturer's protocol (Active Motif, see chapter 2.13). Talin was immunoprecipitated from 500µg of total cell protein using magnetic protein G beads and 5µg of antibody directed against both talin1 and talin2. The immunoprecipitated protein was separated by SDS-PAGE and immunoblotted for β5.

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Talin associates with the β5 subunit in both CS-1/FNKFNK cells (lanes 1-3) and CS-1/FNK cells (lanes 4-6), as shown by bands at approximately 95kDa. The bands in lanes loaded with CS-1/FNK protein are not as pronounced as those in lanes loaded with CS-1/FNKFNK protein, however, loading was not equal between lanes (see Input protein bands). β5 protein was present in protein samples prior to immunoprecipitation as seen by bands in all 6 lanes loaded with input protein.

Figure 6.8: Co-immunoprecipitation of Talin and β5 in CS-1/FNKFNK and CS-1/FNK Cells

Co-immunoprecipitation assay was used to determine whether talin and β5 are associated with one another intracellularly by immunoprecipitating talin and western blotting the resulting protein for the β5 subunit. Lanes 1-3 were loaded with protein from unsitmulated CS-1/FNKFNK cell lines and lanes 4-6 were loaded with protein from unstimulated CS-1/FNK cell lines. 10μg of total cell protein prior to immunoprecipitation was loaded and used as an loading control. An anti-talin antibody was used to immunoprecipitate talin from the total protein mixture. The immunoprecipitated protein was then subjected to western blot analysis for the β5 integrin subunit. Talin associates with β5 in both CS-1/FNKFNK cells and CS-1/FNK cells. This is a representative figure from 1 experiment using N=3 CS-1/FNKFNK cell lines and N=3 CS-1/FNK cell lines.

To confirm that talin associates with β5 in both CS-1/FNKFNK and CS-1/FNK cells, the co-immunoprecipitation was repeated in reverse i.e. cell protein was immunoprecipitated with an anti-β5 antibody and then immunoblotted for talin. The reverse Co-Ip confirmed that talin and β5 associate with one another in both CS-1/FNKFNK and CS-1/FNK cells. Bands at the expected molecular weight for talin (225kDa) were observed in lanes loaded with both CS-1/FNKFNK and CS-1/FNK immunoprecipitated protein (Figure 6.9).
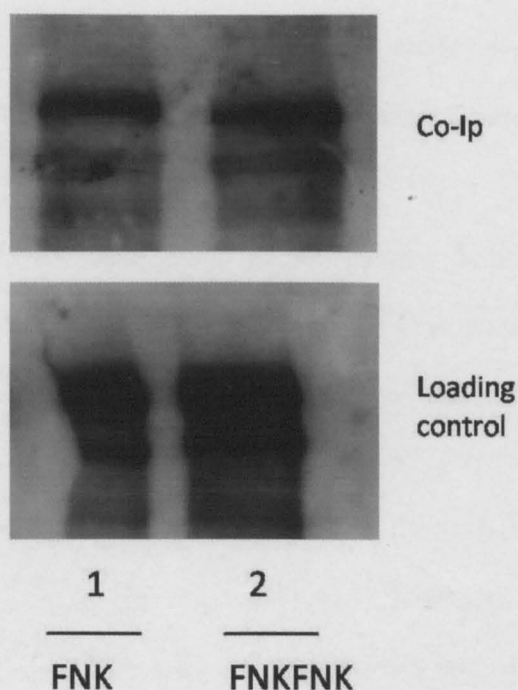
Figure 6.9: Reverse Co-Ip of β5 and Talin in CS-1/FNKFNK and CS-1/FNK Cells

Co-immunoprecipitation assay was used to confirm that talin and β5 are associated with one another by immunoprecipitating the β5 subunit and western blotting the resulting protein for talin. Lane 1 was loaded with protein from an unsitmulated CS-1/FNK cell line and lane 2 was loaded with protein from an unstimulated CS-1/FNKFNK cell line. 10µg of total cell protein prior to immunoprecipitation was loaded and used as an loading control. An anti-β5 antibody was used to immunoprecipitate β5 from the total protein mixture. The immunoprecipitated protein was then subjected to western blot analysis for talin. Talin associates with β5 in both CS-1/FNKFNK cells and CS-1/FNK cells. This is a representative figure from 1 experiment using N=1 CS-1/FNKFNK cell line and N=1 CS-1/FNK cell line.

6.4   Discussion

The results presented in Results Chapter 5 show that HASM cells are capable of activating TGF-β

via the integrin αVβ5. Structural interactions of the β5 subunit with both latent TGF-β and

cytoskeletal proteins are likely to be crucial for αVβ5-mediated TGF-β as has been shown for the

αVβ6 integrin (Munger *et al.* 1999). A polymorphism in the β5 cytoplasmic domain involving a

9 base pair deletion has been previously described (Kawahara *et al.* 2005). This polymorphism

occurs within an NxxY talin binding region and results in the loss of three amino acids (FNK767-

769) from the cytoplasmic tail. Here, I show that this polymorphism results in a β5 subunit that is

unable to mediate TGF-β activation. Cells expressing this polymorphic β5 are unable to activate

TGF-β, however, cells that expressed the full length β5 subunit activated TGF-β. This is the first

description of a biologically relevant integrin mutant that can't activate TGFβ.

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Previous work studying the role of the β6 cytoplasmic domain in αVβ6-mediated TGF-β

activation has highlighted the importance of the β subunit cytoplasmic domain in this process. By

studying β6 mutants that have truncated cytoplasmic domains it was shown that distinct regions

of integrin β subunits are responsible for TGF-β activation (Munger *et al.* 1999). These data

suggest that this is also true of αVβ5-mediated TGF-β activation since a polymorphism that

results in the deletion of just three amino acids from the cytoplasmic domain of β5 results in an

inability to activate TGF-β. However, this polymorphism doesn't abrogate all biological functions

of β5 as adhesiveness of αVβ5 to extracellular ligands is not affected (Kawahara *et al.* 2005).

The frequency of individuals heterozygous for this polymorphism in β5 was determined to be

11% (Kawahara *et al.* 2005). The results described here suggest that individuals that express this

polymorphism in ITGB5 would not be able to activate TGF-β via this integrin. The relevance of

this polymorphism in disease is not yet clear. It would be predicted that this mutation would

protect against fibrotic conditions, although it is possible that compensation through the αvβ3

integrin may occur (Asano *et al.* 2005a; Wipff *et al.* 2007). It may also be relevant in diseases

such as cancer where a failure to activate TGFβ may mitigate against TGFβ's known effect

inhibiting epithelial cell proliferation (Guasch *et al.* 2007). Although it is unlikely that this specific mutation has any relationship with fibrotic disease, there are 720 currently described polymorphisms in the β5 gene, 3 of which lead to coding changes in the latent TGFβ binding domain and it is quite possible that a mutation in this region could lead to enhanced TGFβ activation and fibrogenesis. However, this hypothesis remains to be tested.

CS-1 cells were utilised in these studies because they express endogenous αV integrin subunits but no β subunits (Thomas *et al.* 1993), making them an excellent model for investigating structural differences in the β5 cytoplasmic domain. Cells that over-expressed the full length β5 subunit (CS-1/FNKFNK) were capable of activating TGF-β basally but cells over-expressing the β5 subunit that lacks amino acids 767-769 (CS-1/FNK) were not, despite these cells actually expressing more αVβ5 on their cell surface. Unfortunately, these cells did not respond to stimulation with LPA and so the effect of the two different forms of β5 on LPA-induced αVβ5-mediated TGF-β activation could not be investigated. There are several possible reasons for this. Expression of LPA receptors by CS-1 cells was not assessed and it may be that they are unable to respond to LPA stimulation due to a lack of LPA receptors. It is also possible that for some reason these cells are unable to contract, and if our hypothesis that LPA induces TGF-β activation via induction of cell contraction is correct this would result in no increased TGF-β activation in response to LPA.

These data show that the deletion of amino acids FNK[767-769] from the β5 cytoplasmic tail did not completely abrogate the interaction of β5 with the cytoskeletal protein talin, despite it abrogating TGF-β activation. Although the deletion occurs within an NxxY talin binding domain, two talin binding domains are still present within the polymorphic β5 cytoplasmic domain (see Figure 6.1). This suggests that the inability of the polymorphic β5 subunit to activate TGF-β is not the result of an inability of talin to interact with the β5 cytoplasmic tail. αVβ6-mediated TGF-β activation is associated with the formation of stress fibers and reorganisation of the αVβ6 integrin into focal adhesions (Munger *et al.* 1999; Xu *et al.* 2009). However, it is not known at present whether the

polymorphic β5 subunit can still organise into focal adhesions. Further investigation is required to establish how the deletion of FNK$^{767-769}$ affects the ability of the integrin to activate TGF-β.

The data presented in this chapter show that a deletion of just three amino acids from the cytoplasmic domain of the β5 subunit results in an αVβ5 integrin that is unable to activate TGF-β. This is the first description of a biologically relevant integrin that is unable to activate TGF-β. The data suggests that talin is still is still able to interact with the polymorphic β5 subunit, however, it is not currently clear whether this interaction is impaired by the deletion. Further investigation is therefore required to elucidate the mechanism that results in the inability of the polymorphic β5 to activate TGF-β.

Conclusions and Future

Directions

7   Conclusions and Future Directions

Asthma is an extremely common inflammatory disease of the airways. Airway remodelling is the term given to the structural changes that occur within the airways of asthmatic patients, including subepithelial fibrosis and increased ASM mass, and is more common in individuals suffering from severe forms of the disease. The causes responsible for the development of airway remodelling are currently unknown, however, the pro-fibrotic cytokine TGF-β has been implicated. TGF-β is released from cells as a latent complex that requires activation for functionality. Activation of TGF-β by airway epithelial cells has been well documented. However, activation of TGF-β by ASM cells has never been investigated, despite these cells being implicated in asthma pathogenesis.

The results presented in this thesis highlight two biologically-relevant mechanisms of TGF-β activation employed by HASM cells in vitro. The first is proteolytic activation by the serine protease mast cell tryptase. Mast cell infiltration of the ASM layer is a defining characteristic of asthma compared with other inflammatory airway diseases (Brightling *et al.* 2002). This means that ASM cells in vivo are influenced by mast cell mediators following mast cell degranulation in response to inhaled asthmatic stimuli. Tryptase is the most abundant protease secreted by mast cells and these data suggest that release of tryptase could cause TGF-β activation in vivo. This would provide a direct link between inflammation of the airways and the development of airway remodelling. It has been shown that an inhaled tryptase inhibitor, APC366, can inhibit tryptase-induced airway hyper-responsiveness and bronchoconstriction (Molinari *et al.* 1996). Clinical trials using this inhibitor have been promising. Administration of APC366 abrogates decreases in FEV$_1$ associated with the late asthmatic response (LAR) following allergen challenge (Krishna *et al.* 2001). The data presented in this thesis suggest that using this treatment might help reduce airway remodelling in asthma.

The second mechanism of TGF-β activation described here was mediated by the integrin αVβ5 in response to LPA stimulation via the cytoskeleton. LPA is present in the serum and levels of LPA in BALF are increased following allergen challenge (Eichholtz *et al.* 1993; Georas *et al.* 2007). It has multiple effects on ASM cells but this is the first description of LPA-induced TGF-β activation by HASM cells. During an asthma exacerbation serum is exudated into the airways (Belda *et al.* 2005) and levels of LPA in the airways are likely to increase. Furthermore, data presented in this thesis using a wounding model suggest that HASM cells might be able to activate TGF-β via αVβ5 independently of LPA. These data suggest that HASM cells may activate TGF-β locally in asthmatic airways, which could lead to airway remodelling. This is the first description of αVβ5-mediated TGF-β activation in this cell type and these findings provide evidence that αVβ5 is a potential target for treatment or prevention of airway remodelling in asthma.

Angiogenesis is a key feature of asthmatic airway remodelling (see Introduction section 1.2.4), as well as many other pathologies including cancer and ischemic diseases. The integrin αVβ5, in addition to several other integrins including αVβ3 and αVβ8, is thought to be involved in angiogenesis. αVβ5 does not appear to affect developmental angiogenesis since mice null for either β5 or αV exhibit extensive angiogenesis during development (Bader *et al.* 1998; Huang *et al.* 2000). However, published data indicates a role for αVβ5 in pathological angiogenesis. A dual inhibitor of αVβ5 and the closely related integrin αVβ3 inhibits angiogenesis *in vitro* and in a murine xenograft tumour model (Kumar *et al.* 2001), and an αVβ5 neutralising antibody inhibits angiogenesis *in vitro* (Friedlander *et al.* 1995). However, the precise role of αVβ5 in mediating angiogenesis is still very unclear as some studies have shown that inhibition of αVβ5 can promote angiogenesis and tumourigenesis. Mice null for both β5 and β3 exhibit enhanced tumour-associated angiogenesis *in vivo* (Reynolds *et al.* 2002). Moreover, RGD-mimetic inhibitors of αVβ5 and αVβ3 enhance angiogenesis within tumours when used at low concentrations (Reynolds *et al.* 2009). Further work is needed to fully elucidate how αVβ5 affects pathological angiogenesis in asthma. These studies and the data presented in this thesis raise the possibility

that αVβ5 might promote angiogenesis in asthmatic airway remodelling in two separate ways. The first involves direct stimulation of angiogenesis via an as yet unclear, direct αVβ5-mediated mechanism, and the second by activating the pro-angiogenic mediator TGF-β. The data presented here suggest that distinct regions of the β5 cytoplasmic domain are responsible for mediating TGF-β activation, as has been shown for αVβ6-mediated TGF-β activation (Munger *et al.* 1999). It is possible that distinct regions of β5 are responsible for promoting angiogenesis in disease. Such regions might prove to be a more effective target for combating pathological angiogenesis than completely abrogating all αVβ5 functions.

The finding that LPA causes TGF-β activation by HASM cells may have wider implications for asthma pathogenesis. It is well known that LPA induces cytoskeletal changes via activation of RhoA and Rho kinase (Hashimoto *et al.* 2003; Xu *et al.* 2009). The observation that the contraction agonist methacholine can also induce TGF-β activation and the bronchodilator formoterol can inhibit LPA-induced TGF-β activation has led us to hypothesise that contraction of ASM cells causes TGF-β activation (see Figure 7.1). If correct, this could be fundamental to our understanding of the development of airway remodelling in asthma. Furthermore, we have found that asthmatic cells activate more TGF-β in response to LPA stimulation than non-asthmatic cells. There have been several descriptions of asthmatic HASM cells being more contractile than non-asthmatic HASM cells. Our hypothesis suggests that this may be a reason that asthmatic cells activate more TGF-β and develop airway remodelling. However, further work is needed to confirm this. Ultimately, it is hoped that these data and future work will identify a therapeutic target for the development of novel treatments for airway remodelling and asthma.

Following the discovery that HASM cells can activate TGF-β via the integrin αVβ5 we investigated the role of the β5 cytoplasmic domain in mediating TGF-β activation. We found that a melanoma cell line over-expressing a polymorphic β5 subunit that lacks three amino acids (767-769) in its cytoplasmic domain, were unable to activate TGF-β compared with cells over-

expressing full length β5, which were able to activate TGF-β. This suggests an essential role for specific regions of the β5 cytoplasmic domain αVβ5-mediated TGF-β activation and is also the first description of a biologically-relevant integrin-variant that is unable to activate TGF-β.
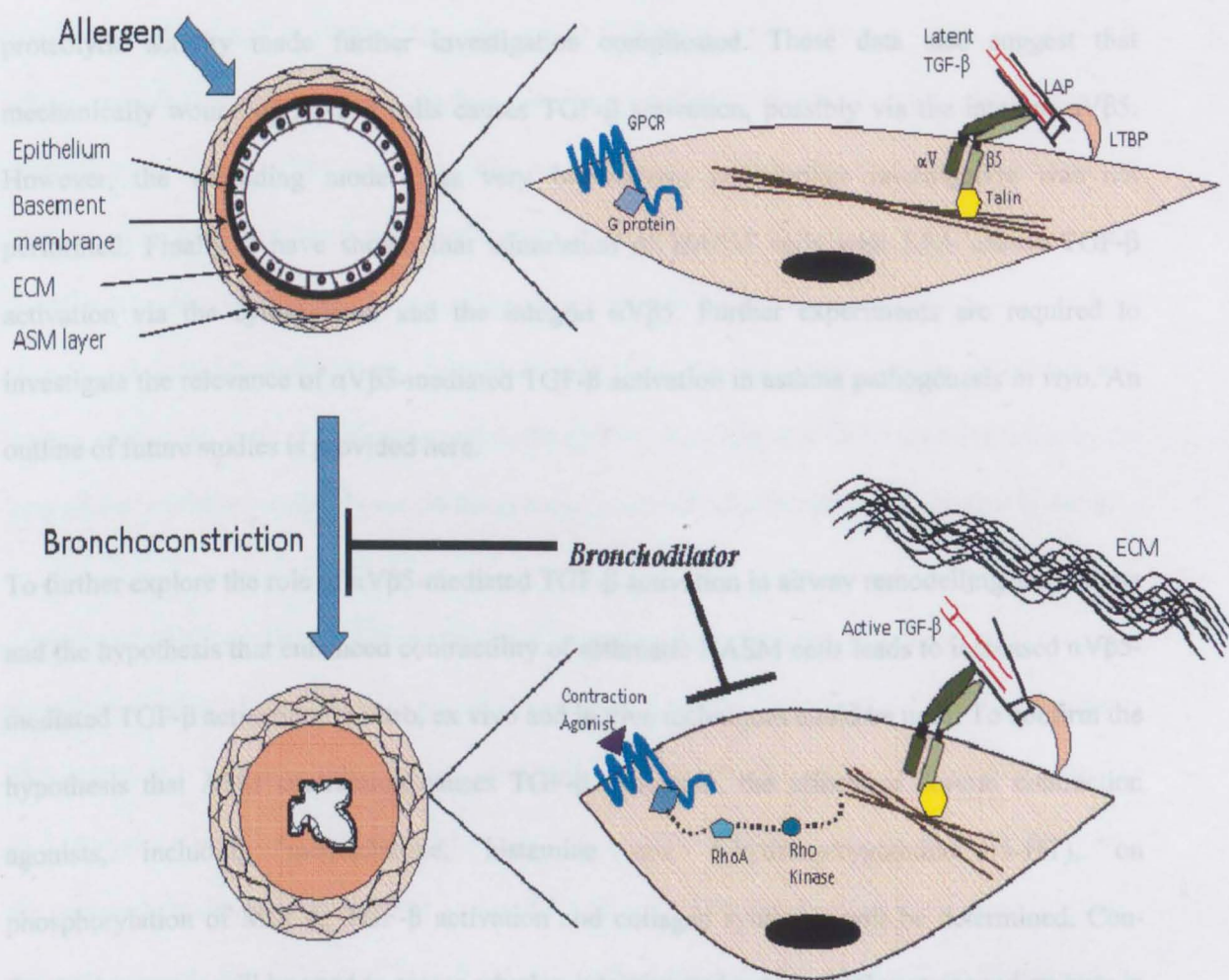
Figure 7.1: Overview of Proposed Mechanism of Contraction-Induced αVβ5-Mediated TGF-β Activation by HASM Cells

The results presented in this thesis have led us to hypothesise that ASM contraction during an asthma exacerbation results in TGF-β activation via the cytoskeletal changes and the integrin αVβ5. This schematic shows an overview of the proposed hypothesis. Allergen-induced bronchoconstriction would cause TGF-β activation but treatment with a bronchodilator such as a β2-agonist would inhibit this activation by inhibiting ASM contraction.

7.1   Future Directions

This thesis has highlighted two biological relevant mechanisms of TGF-β activation employed by HASM cells *in vitro*. I have found that mast cell tryptase proteolytically activates TGF-β independently of its receptor PAR2. However, difficulties obtaining tryptase with consistent proteolytic activity made further investigation complicated. These data also suggest that mechanically wounding HASM cells causes TGF-β activation, possibly via the integrin αVβ5. However, the wounding model was very inconsistent and further investigation was not performed. Finally, I have shown that stimulation of HASM cells with LPA causes TGF-β activation via the cytoskeleton and the integrin αVβ5. Further experiments are required to investigate the relevance of αVβ5-mediated TGF-β activation in asthma pathogenesis *in vivo*. An outline of future studies is provided here.

To further explore the role of αVβ5-mediated TGF-β activation in airway remodelling and asthma and the hypothesis that enhanced contractility of asthmatic HASM cells leads to increased αVβ5-mediated TGF-β activation, in vitro, ex vivo and in vivo techniques could be used. To confirm the hypothesis that ASM contraction causes TGF-β activation, the effects of several contraction agonists, including methacholine, histamine and 5-hydroxytryptamine (5-HT), on phosphorylation of MLCK, TGF-β activation and collagen synthesis will be determined. Confocal microscopy will be used to assess whether integrins and cytoskeletal proteins redistribute in response to these agonists as previously described (Xu *et al.* 2009). Furthermore, the effects of a variety of relaxation agents, including the β2-agonists formoterol, the anti-muscarinic atropine and the potassium-channel opener chromokalin, will be investigated. Together, the results of these experiments should confirm the role of HASM cell contraction in TGF-β activation and airway remodelling.

The results presented in this thesis have shown that asthmatic HASM cells activate more TGF-β via αVβ5 in response to LPA stimulation than non-asthmatic HASM cells and this was not the result of increased expression of αVβ5 by the asthmatic cells. However, the mechanism of this

enhanced response was not investigated. Future studies will therefore focus on investigating the mechanism responsible for this observation. Several studies have suggested that asthmatic ASM cells are more contractile than non-asthmatic cells (Ma *et al.* 2002; Matsumoto *et al.* 2007), which may explain the enhanced response of asthmatic cells. To investigate this, levels of MLCK between asthmatic and non-asthmatic cells will be compared. Furthermore, the inhibitor H-1152, which inhibits Rho Kinase and therefore the Rho signalling pathway and Rho dependent cell contraction, will be used to determine whether inhibition of this pathway reduces activation of TGF-β and ECM protein synthesis by asthmatic cells. In addition to these in vitro studies, the role of cell contraction on TGF-β activation by ASM cells will be investigated using an ex vivo lung slice model. Intact bronchial lung slices from ovalbumin sensitised mice will be stimulated with the contraction agonists previously mentioned and TGF-β activity and ECM gene synthesis by the lung slices will be determined over 24 hours using immunohistochemistry and western blotting.

The role of αVβ5-mediated TGF-β activation in airway remodelling and asthma *in vivo* will be investigated using αVβ5-null mice and an *Aspergillus fumigates (Asp.f)* model of allergic asthma. This model will be used over the more commonly used ovalbumin model of asthma because the background strain of the αVβ5-null mice is not compatible with the ovalbumin model. Following the repeated challenges of wild-type and αVβ5-null mice with the Asp.f antigen, lung histology, collagen content and markers of TGF-β activity such as phosphorylated Smad2 or Smad3 will be compared between the two types of mice.

7.2 Concluding Remarks

Airway remodelling is associated with severe asthma and leads to a progressive decline in lung function over time. TGF-β has been implicated in the development of airway remodelling as it is both mitogenic and pro-fibrotic. However, activation of extracellular stores of latent TGF-β is the rate limiting step in TGF-β bioavailability. The findings outlined in this thesis have provided evidence that HASM cells are capable of activating TGF-β in vitro, which could contribute to the development of airway remodelling in vivo. Two mechanisms of TGF-β activation employed by HASM cells have been identified, both of which may be relevant for asthma pathogenesis and airway remodelling in vivo. Future studies will concentrate on assessing the role of these mechanisms of TGF-β activation in vivo. Data concerning $\alpha V\beta 5$-mediated TGF-β activation in HASM cells has led us to hypothesise that contraction of HASM cells leads to TGF-β activation in vivo. If correct this could be vital to our understanding of how airway remodelling is initiated in asthma and could lead to the development of new treatments aimed at inhibiting contraction-induced TGF-β activation. This would hopefully improve symptoms in asthmatics and help to inhibit the decline in lung function over time that is commonly observed.

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Appendix

9 Appendix

9.1 Materials

Hyperfilm ECL™..Amersham Biosciences

Pure nitrocellulose membrane ....Gelman Sciences

9.2 Reagents

Ammonium persulphate ....Sigma-Aldrich
Dissolved in distilled water

Amphotericin B ....Sigma-Aldrich

Bisacrylamide ....Sigma-Aldrich

β-mercaptoethanol ....Sigma-Aldrich

Bradford protein reagent ....BioRad

Bovine serum albumin (BSA) ....Sigma-Aldrich

Bromophenol blue ....Sigma-Aldrich

Bronchial epithelial cell medium and supplements ....Cambrex BioSciences

Cycloheximide...Sigma-Aldrich
Dissolved in water

Cytochalasin..Sigma-Aldrich
Dissolved in dimethyl sulphoxide

DEPC treated water (RNase free) ....Sigma-Aldrich

Dulbecco's modified Eagle's medium (DMEM)..................................Sigma-Aldrich

Dimethyl sulphoxide (DMSO) ....Sigma-Aldrich

Deoxynucleosides (dNTPs)...Promega

Dithiothreitol (DTT)...Sigma-Aldrich

ECL™ detection reagents ....Amersham Biosciences

Ethylenediamine tetraacetic acid (EDTA) ....Sigma-Aldrich

Ethanol ....BDH

Ethidium bromide ....Sigma-Aldrich

Foetal calf serum (FCS) ....Harlan UK

Goat serum ....Sigma-Aldrich

Glycerol ....Sigma-Aldrich

B-glycerophosphate..Sigma-Aldrich

Glycine ....Sigma-Aldrich

GoTaq® DNA polymerase ....Promega

GoTaq® 5x green buffer ....Promega

G418 antibiotic..Sigma-Aldrich

Leupeptin ....Sigma-Aldrich

L-glutamine ....Sigma-Aldrich

Lysophosphatidic acid (LPA)...Sigma-Aldrich
Dissolved in 0.1% BSA in PBS

Methanol ....BDH

MgCl2 ....Promega

Moloney murine leukaemia virus (M-MLV) ....Promega
reverse transcriptase

N,N,N,N-Tetramethylethylenediamine (Temed) ....Sigma-Aldrich

Nafamostat mesilate (FUT175) ....Biomol International LP

Non-fat dry milk ....Santa Cruz Technology

Oligo(DT) primer ....Promega

PAR2 activating peptide (PAR2-AP) ....Bachem
SLIGKV-NH2

PCR DNA ladders ....Sigma-Aldrich

Phosphate buffered saline (PBS) tablets ....Sigma-Aldrich

Penicillin/streptomycin ....Sigma-Aldrich

Phenylmethanesulphonylfluoride (PMSF) ....Sigma-Aldrich

Ponceau S solution ....Sigma-Aldrich

Protein inhibitor cocktail ....Sigma-Aldrich

Rainbow™ molecular weight marker ....Amersham Biosciences

RPMI ....Sigma-Aldrich

RNase free DNase ....Qiagen

RNasin RNase inhibitor ....Promega

RT buffer (5 x) ....Promega

Sodium azide   (NaN3) ....Sigma-Aldrich

Sodium chloride (NaCl) ....Sigma-Aldrich

Sodium dodecyl sulphate (SDS) ....Sigma-Aldrich

Sodium orthovanadate (Na3VO4) ....Sigma-Aldrich

Sulphuric acid (H2SO4) ....BDH

Takara SYBR Premix Ex Taq..Lonza

Transfast transfection reagent ....Promega

Tryptase, purified human lung ....Europa Bioproducts
Supplied in 50mM NaOAc, 1M NaCl, 0.05mM heparin.
Specific activity 63.3 U/mg using n-benzyl-Phe-Arg-pNA as substrate.

Tris buffered saline (TBS) ....Sigma-Aldrich

Trizma® base   (Tris-base) ....Sigma-Aldrich

Trizma® hydrochloride (Tris-HCl) ....Sigma-Aldrich

Triton X 100 ....Sigma-Aldrich

Trypsin/EDTA ....Sigma-Aldrich

Tween-20 ....Sigma-Aldrich

9.3   Antibodies

Integrin αVβ5 antibody (clone P1F6)...Tebu-bio
Raised in mouse

Integrin αVβ5 antibody (ALULA) ....Professor Dean Sheppard, UCSF
Raised in mouse

Integrin β5 polyclonal antibody ....Abcam
Raised in rabbit.

GAPDH monoclonal antibody ....ABD Serotec
Raised in mouse

Pan TGF-β monoclonal antibody (clone 1D11) ....R & D Systems
Raised in mouse.

TGF-β1 monoclonal antibody ....R & D Systems
Raised in mouse

TGF-β2 monoclonal antibody ....R & D Systems
Raised in mouse

PAR2 monoclonal antibody (clone SAM11) ....Santa Cruz Biotechnology
Raised in mouse.

Talin 1 and 2 monoclonal antibody (clone 8D4) ....Abcam
Raised in mouse

Lamin AC monoclonal antibody (clone) .... Santa Cruz Biotechnology
Raised in mouse

Anti-mouse polyclonal antibody ....Dakocytomation
Raised in rabbit

Anti-rabbit polyclonal antibody ....Dakocytomation
Raised in goat

9.4   Kits

QIAEX II Gel purification kit .... Qiagen

Luciferase reporter assay kit ....Promega

NucleoSpin RNA II RNA Extraction kit..Macherey Nagel

Universal CoIp Kit ....Active Motif

NXtract CellLytic nuclear extraction kit ....Sigma-Aldrich

Cell Titer Glow kit ....Promega

BCA Protein Assay ....Pierce

9.5 Buffer Recipes

9.5.1 RT-PCR Buffers

0.5xTris-borate-EDTA Buffer   89mM Tris borate, 2mM EDTA, pH 8.3

9.5.2 Western Blotting Buffer Recipes

Lysis Buffer: 50mM Tris-HCl pH 6.8, 150mM NaCl, 1% TritonX 100, 0.1% SDS, 0.5%

deoxycholic acid and 0.01M EDTA

Prior to use, the buffer was diluted 1:2 and the following protease inhibitors

added:

1µl/ml leupeptin, 20µl/ml PMSF, 40µl/ml protease inhibitor cocktail and 10µl/ml

NaVO4

4x Lamellae Buffer:

62.5mM Tris-HCl pH 6.8, 10% v/v glycerol, 1% w/v SDS and 0.01%

bromophenol blue.

Just before use 1% v/v β-mercaptoethanol was added.

10x Transfer Buffer:

50mM Tris base pH 8.3, 192mM glycine and 20% v/v methanol

Buffer 1:   1.5M Tris-HCl, 0.4% SDS, pH 8.8

Buffer 2:   0.5M Tris-HCl, 0.4% SDS, pH 6.8

Tris Buffered Saline plus Tween 20 (TBST):

24.2g Tris base, 87.6g NaCl, 1L dH20, pH 7.4-7.6, 10mls Tween 20 in 1L

10% Resolving Gel:

6.66ml 30% Bis/acrylamide, 5.2ml buffer 1, 7,92ml distilled water

Then add 200µl 10% ammonium persulphate and 20µl tetramethylethylenediamine (temed) just prior to use

Stacking Gel: 1.3ml 30% bis/acrylamide, 2.5ml buffer 2, 6.1ml distilled water,

Then add 50µl 10% ammonium persulphate and 10µl temed just prior to use

9.6   Media Recipes

Serum plus DMEM: DMEM plus 10% FCS, 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotercin B

Serum free DMEM: DMEM plus 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotercin B

Serum plus TMLC media:

DMEM plus 10% FCS, 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml amphotercin B and 25µg/ml G418.

Serum free TMLC media:

DMEM plus 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml amphotercin B and 25µg/ml G418

Serum plus RPMI: RPMI plus 10% FCS, 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml amphotercin B

Serum free RPMI: RPMI plus 4mM L-glutamine, 100U/ml penicillin, 100µg/ml

streptomycin and 2.5µg/ml amphotercin B

Transfection media: RPMI plus 10% FCS and 4mM L-glutamine.

CS-1 media: RPMI plus 4mM L-glutamine, 100U/ml penicillin, 100µg/ml

streptomycin, 2.5µg/ml amphotercin B and 500µg/ml G418.

9.7   PCR Primers and Cycling Conditions

Gene	Primer Sequences	QPCR Cycling Conditions	RT-PCR Cycling Conditions
PAI1	TCTGCAGACCTGGTTCCCAC AGCCCCGTAGTTCCATCCTG	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	N/A
β2-microglobulin	AATCCAAATGCG GCATCT GAGTATGCCTGCCGTGTG	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	N/A
Protease Activated Receptor-1 (PAR1)	TGTGAACTGATCATGTTTATG TTCGTAAGATAAGAGATATGT	N/A	95°C for 7min 35 cycles: 94°C for 1min, 60°C for 1min, 72°C for 1min, 72°C for 5 min
Protease Activated Receptor-2 (PAR2)	CCTTTGTATGTCGTGAAGCAGAC TTCCTGGAGTGTTTCTTTGAGGTG	N/A	95°C for 2min 35 cycles: 94°C for 30s, 55°C for 30s, 68°C for 1min, 68°C for 5 min

Protease Activated Receptor-3 (PAR3)	CTGATACCTGCCATCTACCTCC AGAAAACTGTTGCCCACACC	N/A	95°C for 2min 35 cycles: 94°C for 30s, 55°C for 30s, 68°C for 1min, 68°C for 5 min
Protease Activated Receptor-4 (PAR4)	ATTACTCGGACCCGAGCC TGTAAGGCCCACCCTTCTC	N/A	95°C for 2min 35 cycles: 94°C for 30s, 55°C for 30s, 68°C for 1min, 68°C for 5 min
LPA receptor 1 (LPAR1)	GGTGATTGGGACTTGGAAT AAACCGTAATGTGCCTCT	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	95°C for 2min 35 cycles: 95°C for 1min, 58°C for 1min, 72°C for 1min, 72°C for 5 min
LPA receptor 2 (LPAR2)	CCCAACCAACAGGACTGACT GAAGAGCCAGATTCCTG	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	95°C for 2min 35 cycles: 95°C for 1min, 60°C for 1min, 72°C for 1min, 72°C for 5 min
LPA receptor 3 (LPAR3)	CCGCAGGTACACCACAAC TGCTTCCCTCACCAACTT	N/A	95°C for 2min 35 cycles: 95°C for 1min, 58°C for 1min, 72°C for 1min, 72°C for 5 min
LPA receptor 4 (LPAR4)	TGCGCTCCCAAGCTATTACA GGCTTTGTGGTCAAAGGT	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	95°C for 2min 35 cycles: 95°C for 1min, 58°C for 1min, 72°C for 1min, 72°C for 5 min
Integrin αV	ACTCAAGCAAAAGGGACGAA TGCAAGCCTGTTGTATCAGC	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	N/A
Integrin β3	GCAAAGGGCTCTGGAGACAG CTTGGTACCCAGGTTCTGGA	95°C for 30s 40 cycles: 95°C for 5s, 62°C for 30s, 72°C for 15s,	95°C for 1min 30 cycles: 95°C 1min, 62°C 1min, 72°C 1min 72°C for 5 min
Integrin β5	GGAACGTCACCAGCAACTTC CTGTCTGTGAGAGGCAGCAG	40 cycles: 95°C for 5s, 60°C for 30s, 72°C for	95°C for 1min 30 cycles: 95°C for 1min, 60°C for

		15s.	1min, 72°C for 1min 72°C for 5 min
Integrin β6	GCAAGCTGCTGTGTGTAAGGA CTTGGGTTACAGCGAAGATCA	N/A	95°C for 1min 30 cycles: 95°C for 1min, 60°C for 1min, 72°C for 1min 72°C for 5 min
Integrin β8	CATTAAGTGTTTAAAAATCTTTTT TTCATCATTTTCATAGTTACATTC	N/A	95°C for 1min 30 cycles: 95°C for 1min, 60°C for 1min, 72°C for 1min 72°C for 5 min
Fibronectin	CAGGATCACTTACGGAGAAACAG GCCAGTGACAGCATACACAGTG	95°C for 30s 40 cycles: 95°C for 5s, 60°C for 30s, 72°C for 15s.	N/A
Vascular endothelial growth factor (VEGF)	ATCTGCATGGTGATGTTGGA GGGCAGAATCATCACGAAGT	95°C for 30s 40 cycles: 95°C for 5s, 59°C for 30s, 72°C for 15s.	N/A

Table 9.1: PCR Primers and Cycling Conditions

This table shows the primer sequences and thermal PCR profile for all of the primers used throughout this thesis.