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Actions of Mast Cell Tryptase on Myofibroblasts: Implications for Inflammation and Tissue Remodelling in the Human Lung

by

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ABSTRACT

FACULTY OF MEDICINE HEALTH AND BIOLOGICAL SCIENCES

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Mast cell activation is a prominent feature in conditions associated with inflammation and tissue remodelling. The myofibroblast, a cell present with fibroblasts between the basement membrane of the epithelium and the smooth muscle layer has been implicated in remodelling of the asthmatic airways. The potential for interaction between mast cells and myofibroblasts has been little investigated. Tryptase, the major secretory product of the mast cell is emerging as a key mediator that can modulate cell function through the activation of protease activated receptor (PAR)-2, a G protein coupled receptor. The aim of these studies has been to investigate the ability of tryptase and other agonists of PAR-2 to alter myofibroblast and fibroblast function and to act as stimuli for inflammation and tissue remodelling in the lung.

Tryptase was isolated from a recombinant expression system and from human lung tissue using butyl agarose, benzamidine agarose and S-200 gel filtration chromatography, and the purified material characterised by western blotting and enzyme assays. Cells of the myofibroblast phenotype were induced from primary cultures of human lung fibroblasts using TGF-β. Expression of mRNA for PAR-2 was detected in both fibroblasts and myofibroblasts by PCR, and the presence of this receptor on the cell membrane and within the cytoplasm was indicated by immunocytochemistry with specific monoclonal antibody P2A. Functional PAR-2 on these cells was demonstrated by the ability of a peptide agonist to induce calcium flux, though surprisingly this effect was not induced by tryptase. Mitogenic responses were stimulated with tryptase in fibroblasts and myofibroblasts, but cell proliferation was not observed under the conditions used. Pre-incubation of tryptase with protease inhibitors, or heat inactivation of the enzyme did not consistently inhibit the actions on cells, but responses similar to those with tryptase were observed following stimulation with peptide agonists of PAR-2.

The synthesis and release of IL-6, IL-8 and GM-CSF by fibroblasts and myofibroblasts was indicated by quantitative Taqman PCR and ELISA techniques, though there was considerable heterogeneity between cell preparations in patterns of cytokine generation. Tryptase induced increased IL-8 release from myofibroblasts (but not from fibroblasts), and there was no overall alteration in IL-6 release, or in expression of mRNA for any of the three cytokines examined. The PAR-2 agonist peptide induced increased release of IL-6 from myofibroblasts, but otherwise was found to have little effect on cytokine generation by either cell type.

Incubation of fibroblasts and myofibroblasts with tryptase provoked increased release of collagens as determined by measurement of [^{3}H]-proline. PCR studies suggested that collagen type III rather than type I was generated by these cells. Matrix metalloproteinase (MMP)-2 release in both the pro and active forms was stimulated by tryptase. A similar pattern of MMP-2 release in response to tryptase was seen with myofibroblasts. The peptide agonist of PAR-2 failed to induce collagen synthesis in fibroblasts and myofibroblasts, and although it induced increased release of the pro-form of MMP-2 from fibroblasts, induction of the active form was not observed.

Tryptase being present in substantial quantities in the asthmatic airways could modulate myofibroblast and fibroblast behaviour, and represent an important stimulus for inflammation and tissue remodelling. However, these studies indicate that there is no simple relationship between tryptase-induced alterations in cell function and the activation of PAR-2. It seems likely that tryptase may stimulate non-PAR-2 mediated processes as well as those in which this receptor is involved.

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Abbreviations

APRT	adenine phosphoribosyltransferase
Ab	antibody
APC 1390	N-(1-hydroxy-nathothoyl)-L-arginyl-proliamide
APS	ammonium persulphate
αSMA	α-smooth muscle actin
BAL	bronchoalveolar lavage
BAPNA	N-α-benzoyl-$_{DL}$-arginine-*p*-nitroanilide hydrochloride
BSA	bovine serum albium
cDNA	complementary deoxyribonucleic acid
$[Ca^{2+}]_i$	intracellular calcium concentration
COX-2	cyclo-oxygenase-2
CPM	count per minute
DAB	diaminobenzadine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked-immuno-sorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulation factor
GPCR	G-protein coupled receptor

G-protein	GTP-binding protein
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
Ig	immunoglobulin
IL	interleukin
ITS	insulin-transferrin-selenium suppliment
JNK	c-Jun-NH_2-terminal kinase
kDa	kilo Dalton
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MC	mast cell
MEM	minimum essential medium
MES	2-[*N*-morpholino]ethanesulphonic acid
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTS	(3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PG	prostaglandin
PKC	protein kinase C
PNGase	peptide N-glycanase
PSG	penicillin-streptomycin-glutamine mixture
RANTES	regulated on activation, normal T-cell expressed and secreted
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SCF	stem cell factor

SE	standard error
tc-	*trans*-cinnamoyl
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteases
TMB	tetramethyl benzidine
TNF	tumour necrosis factor
Tris-HCl	tris[hydroxymethyl]aminomethane hydrochloride
Tween 20	polyoxyethylane-sorbitan monoaurate

Chapter 1

Introduction

1 Introduction

Mast cell activation is a prominent feature of asthma and other allergic conditions. This cell can release a range of mediators including serine proteases, histamine, arachidonic acid metabolites, proteoglycans and cytokines. The most abundant protein product of the mast cell is the tetrameric serine protease tryptase, and substantial quantities are secreted following mast cell activation. A number of mediator actions for tryptase have been proposed and the finding that it may activate protease activated receptor-2 (PAR-2) has provided a possible explanation for observations that this protease can alter cell function. The roles of tryptase in allergic disease, however, remain poorly defined.

The inflammatory nature of asthma is well established and it is becoming clear that processes of airway remodelling can be of central importance to the development of asthma. Increased numbers of fibroblasts and myofibroblasts, and an increased deposition of extracellular matrix (ECM) proteins has frequently been observed in the airways of asthmatic subjects. Tissue remodelling may occur as a wound healing response to damage induced during inflammation, and this may be critical for disease chronicity. Mast cells have been associated with inflammation and remodelling and they may make key contributions to the process.

The aim of these studies has been to investigate the actions of the major mast cell product tryptase on human fibroblasts and myofibroblasts, as these cells are of particular relevance to airway inflammation and remodelling. A focus has been to investigate the potential role of PAR-2.

1.1 Allergy

The immune system is necessary for protection from invading microorganisms and parasites, but under certain circumstances it may be directed inappropriately, leading to allergic responses being triggered against harmless antigens (allergens). Hypersensitivity reactions have been classified into four types by Coombs and Gell (Table 1.1.1). Type I acute reactions or anaphylactic reactions are triggered mainly by mast cell activation following cross-linking of cell-bound IgE, resulting in the release of mediators that can lead to infiltration of inflammatory cells. Type II hypersensitivity involves cytotoxic actions of complement against cell surface antigens detected by antibodies. Type III reactions are mediated by activation of the complement pathway, triggered by the formation of antigen/antibody complexes. Type IV or delayed hypersensitivity reactions are mediated by effector cells that are drawn to the site of inflammation by cytokines from T cells presented with antigens.

Type I hypersensitivity reactions, when they occur in the airways, can be a key step in the development of an asthmatic reaction. The association of mast cells with type I hypersensitivity has prompted investigation of how this cell type can participate in mechanisms of asthma. The classification of hypersensitivity reactions into four types was an important advance in the understanding of allergy, but the scheme represents a simplification. Allergic reactions are now seen as a result of an imbalance between multiple processes, and the contribution of mast cells seems likely to extend beyond the rapid release of mediators in type I hypersensitivity reactions.

Table 1.1.1

Classification of hypersensitivity by Coombs and Gell.

Type	Description
I	IgE mediated (acute)
II	Antibody mediated (cytotoxic)
III	Immune complex mediated
IV	Cell mediated (delayed)

1.2 Asthma

The prevalence of asthma has increased in recent decades (Nadel & Busse 1998). This condition has considerable impact on society and despite the availability of a number of treatments, there is no cure. The consequences of asthma can be serious and result in sudden death by airflow obstruction, with blockage of airways with mucus (Kay 1996). The incidence of allergic asthma is highest in childhood, although this disease can start at any age (Dodge & Burrows 1980). Asthma diagnosed in early life can remit with increasing age, but it remains a strong risk factor for the development of asthma in adult life (Jenkins *et al.* 1994, Horak *et al.* 2003). Developed countries tend to have a higher prevalence of diagnosed allergic asthma than developing countries (Jason *et al.* 1997).

Variability in lung function is a characteristic of patients with asthma. Asthmatic reactions can be provoked by allergens, upper respiratory tract infection, exercise and by changes in humidity and temperature. The term asthma is relatively non-specific, and is used to describe conditions with different patterns of airflow obstruction, and different degrees of reversibility, and there are no standard criteria for clinical diagnosis. There are three main clinical aspects of asthma, (1) airway inflammation associated with activation of mast cells and infiltration of eosinophils and other inflammatory cells into the airways, (2) airway obstruction that is reversible either spontaneously or with treatment and (3) increased airway responsiveness to a variety of stimuli. Environmental factors are important in the development of asthma, and family studies have shown genetic association with susceptibility to asthma (Weiss & Raby 2004). Genetic variability has also been linked with different responses to drug treatment (Fenech & Hall 2002).

Epithelial cell shedding, thickening of the collagen layer under the basement membrane, infiltration of inflammatory cells such as mast cells, eosinophils, and CD4+ T lymphocytes, and smooth muscle hypertrophy and hyperplasia are pathogenic features of asthma (Hogg 1993). The presence of mast cells in the smooth muscle layer of bronchial biopsy tissue has been suggested to be a distinctive characteristic of asthmatic airways (Brightling *et al.* 2002). Infiltration of dendritic cells into the bronchial mucosa of asthmatic individuals after local allergen challenge, suggests that antigen presentation by these cells could be important (Jahnsen *et al.* 2001). Class switching and the production of IgE by B lymphocytes is stimulated by Th2 cells. Infiltration of eosinophils and their activation have been thought to be major contributing factors in the pathogenesis of asthma (Lee *et al.* 2001). However, suppression of eosinophilia by reducing IL-5 levels with a blocking antibody, or by priming Th1 cells with IL-12, have not been found to alter airflow and airway hyperresponsiveness (Leckie *et al.* 2000, Bryan *et al.* 2000).

Inflammation represents a response of vascularized tissue to injury, and an attempt to restore tissue function to normal. Epithelial injury can induce processes of healing and structural changes that can lead either to complete restoration or to chronic inflammation. The walls of the conducting airways in asthma are thickened and there is luminal narrowing caused by the presence of excessive mucus and an inflammatory exudate.

In recent years, asthma has come to be seen as a condition in which tissue remodelling as well as inflammation is important (Bousquet *et al.* 2000). A key process in asthma is likely to involve not only inflammation and epithelial injury but tissue remodelling arising from close interactions between epithelial cells, fibroblasts and other cell types (Holgate *et al.* 2000, Knight 2001). A number of cytokines have been suggested to provide links between inflammation and remodelling. TNF and

GM-CSF can induce predominantly inflammatory responses in rat lung (Xing *et al.* 1996, Sime *et al.* 1998). TGF-β acts downstream of both TNF and GM-CSF, and it has been shown that adenovirus vector mediated transfer of the TGF-β gene in rat lung can induce a dramatic fibrotic response, with increased extracellular matrix deposition observed histologically (Sime *et al.* 1997), a type of remodelling seen in asthma. However, the precise relationship between remodelling and inflammation is unclear.

The principal features of airway remodelling in asthma are subepithelial fibrosis, myofibroblast hyperplasia, airway smooth muscle hypertrophy and hyperplasia, mucous gland and goblet cell hyperplasia and disruption of the epithelium (Elias *et al.* 2003). The excess matrix deposition below the basemant membrane that is such a prominent feature of asthma, has been found to be composed of collagen I, collagen III, fibronectin and tenascin in bronchial biopsy tissues (Roche *et al.* 1989, Laitinen *et al.* 1997).

Mild or moderate asthma can often be controlled with corticosteroids and β-agonists, and these may be supplemented with new treatments such as those that target leukotrienes (leukotriene receptor antagonists or lipoxygenase inhibitors) (Leru 2003) or IgE, using the anti-IgE monoclonal antibody omalizumab (Holgate *et al.* 2004). However, medications currently available rarely provide complete relief from symptoms and substantial numbers of patients have poorly controlled disease. There is a pressing need for new therapeutic approaches.

1.3 The Mast Cell

1.3.1 Origin of mast cells

Mast cells have been ascribed important roles in allergy, parasite infection, inflammation, angiogenesis and tissue remodelling (Church & Levi-Schaffer 1997). Human mast cells are derived from CD34 positive progenitor cells in the bone marrow (Bressler *et al.* 1990, Kirshenbaum *et al.* 1991). Cell growth and maturation are under the control of stem cell factor (SCF) (Irani *et al.* 1992, Valent *et al.* 1992, Kirshenbaum *et al.* 1992, Austen & Boyce 2001) which acts via c-kit (CD117) the SCF receptor (Geissler *et al.* 1988, Chabot *et al.* 1988, Boyce 2003). Other cytokines including IL-3, IL-4 and IL-10 also stimulate mast cell growth and maturation in cell culture systems (Huang *et al.* 1990, Rennick *et al.* 1985, Thompson-Snipes *et al.* 1991). Mast cell chemotactic agents have been found to include SCF, TGF-β, eotaxin and RANTES, when this has been investigated with cells of a human mast cell line *in vitro* (Frangogiannis *et al.* 1998, Quackenbush *et al.* 1998, Berger *et al.* 2003, de Paulis *et al.* 2001).

1.3.2 Activation of mast cell

Cross-linking of IgE bound to the high affinity IgE receptor (FcεRI) on mast cells can lead to degranulation with release of pre-formed products, and the synthesis and secretion of cytokines and eicosanoids (Reischl *et al.* 1999). Mast cell degranulation can be induced also by non-IgE dependent processes. Thus mediator release may be stimulated by neuropeptides such as substance P from sensory neurons (Foreman *et al.* 1982, Foreman *et al.* 1983, Fewtrell *et al.* 1982), by complement components C3a and C5a (Hartman & Glovsky 1981, Swerlick *et al.* 1986), and by eosinophil granule

proteins (Henderson *et al.* 1980), though mast cells from different tissues can respond to the different stimuli to different extents.

1.3.3 Mast cell heterogeneity

Human mast cells may be categorised into two distinct sub-populations on the basis of protease content. Mast cells with tryptase and chymase have been termed MC_{TC} and those with tryptase but not chymase, MC_T cells (Irani *et al.* 1986). The MC_{TC} phenotype is predominant in skin and connective tissue, whereas the MC_T phenotype predominates in normal mucosal tissues of the lung and intestine (Irani *et al.* 1989).

There is evidence for functional heterogeneity of mast cells based on differences in responsiveness to pharmacological or biological stimuli. The extent of IgE dependent or independent release of histamine, and also the speed of release, has been found to differ between mast cells of different tissue origins. Thus, for instance, substance P can be a potent stimulus for histamine release from mast cells dispersed from human skin tissue, but not from lung, tonsil or adenoid tissue (Lowman *et al.* 1988). Mast cell heterogeneity assessed by such functional criteria, is not related directly to heterogeneity as determined by protease content.

1.3.4 Mediators in mast cells (Table 1.3.1)

1.3.4.1 Neutral proteases

Neutral proteases of mast cells include tryptase (Schwartz *et al.* 1981, Smith *et al.* 1984), chymase (Schechter *et al.* 1986), and carboxypeptidase (Goldstein *et al.* 1987,

1989, Schechter *et al.* 1983). A cathepsin G-like protease has been found, but this has not yet been isolated from mast cells (Shechter *et al.* 1990). Tryptase, a major focus of the present studies, will be described in detail in a separate section that follows.

Chymase is a monomeric serine protease of 30 kDa stored in an active form in mast cell granules. It has been purified from human skin (Schechter *et al.* 1986), heart (Urata *et al.* 1990), and tonsil tissue (Sukenaga *et al.* 1993). A single chymase is present in human mast cells, unlike the situation in rodents and some other animals in which multiple types have been found. Chymase can cleave angiotensin I very effectively to generate angiotensin II (Urata *et al.* 1990, Kinoshita *et al.* 1991, Reilly *et al.* 1982, Wintroub *et al.* 1984), may possibly degrade neurotensin (Kinoshita *et al.* 1991), and can stimulate mucus secretion from glandular cells (Sommerhoff *et al.* 1989). Possible contributions of chymase in processes of inflammation, could be to convert IL-1β into an active form (Mizutani *et al.* 1991), and to degrade IL-4 (Tunon de Lara *et al.* 1994). Moreover, a massive influx of neutrophils and eosinophils can be induced when rodents are injected with human chymase (He & Walls 1998). Procollagen I, collagen IV and interstitial collagenase are substrates for chymase (Lees *et al.* 1994), findings that are consistent with chymase having important roles in tissue remodelling.

Carboxypeptidase is a Zn^{2+} dependent metalloprotease exopeptidase with a molecular weight of approximately 35 kDa. This enzyme has been purified from human mast cells (Goldstein *et al.* 1987, Goldstein *et al.*1989) and also from rodent mast cells (Serafin *et al.* 1987, Everitt & Neurath 1980, Cole *et al.* 1991). Carboxypeptidase is bound to the same proteoglycan complex as chymase and these proteins are co-released on mast cell activation (Irani *et al.* 1991, Goldstein *et al.* 1992).

1.3.4.2 Histamine

Histamine is stored mainly in mast cells (Riley & West 1956). Concentrations of histamine are elevated in BAL fluid from mild asthmatic subjects (Liu *et al.* 1990), and levels can be further increased following allergen challenge (Liu *et al.* 1991). Four histamine receptors have been identified, and all are G-protein coupled receptors (GPCR) (Wass 1997, Gether 2000). The H_1 receptor may mediate most of the allergic symptoms induced by histamine. Activation of H_1 receptors induces bronchial smooth muscle contraction and mucus production in the lung. Processes induced by H_2 receptors have been described mostly in the gastrointestinal tract and in neurological tissue where expression is localised. H_2 receptor activation results in down-regulation of histamine release from basophils (Kazimierczak *et al.* 1981). The H_3 receptor is expressed on nerves and can act as an autoreceptor (Arrang *et al.* 1983, Schwartz *et al.* 1991). Activation of H_3 receptors may serve to modulate amine neurotransmitters such as noradrenaline (Schlicker *et al.* 1994). The H_4 receptor has been suggested to be important in the regulation of immune function in allergy and asthma, and is associated with immune cells such as eosinophils (Liu *et al.* 2001, Zhu *et al.* 2001, Nguyen *et al.* 2001).

1.3.4.3 Proteoglycans

Proteoglycans are single chain proteins with a glycosaminoglycan side chain (Stevens *et al.* 1988). The major proteoglycans of human mast cells are heparin and chondroitin sulphate E (Stevens *et al.* 1988, Thompson *et al.* 1988). Heparin can bind to other preformed mediators, and facilitate their packaging in granules for

storage. The anticoagulant activity of heparin is the best known property (Damus *et al.* 1973) but this proteoglycan possesses a wide range of functions, binding to enzymes, cytokines and numerous other factors following its release from cells. Of interest for the present studies is the observation that heparin can help to stabilise the active tetrameric form of tryptase (Schwartz & Bradford 1986).

1.3.4.4 Lipid mediators

Prostaglandins (PG) and leukotrienes (LT) are metabolised from arachidonic acid and are products of the cyclooxygenase and the lipoxygenase pathways, respectively. PGD_2 is generated after the activation of mast cells, and inhalation of PGD_2 can induce bronchoconstriction in both non-asthmatic and asthmatic subjects (Hardy *et al.* 1984). Levels of PGD_2 are increased in BAL fluid collected from allergen challenged asthmatic subjects (Wenzel *et al.* 1988, Wenzel *et al.* 1989, Wenzel *et al.* 1990). LTC_4, LTD_4 and LTE_4 are derived from human lung mast cells by IgE-dependent stimulation (Peters *et al.* 1984).

Levels of leukotrienes are increased in BAL fluid from allergen- challenged asthmatic subjects (Wenzel *et al.* 1988, Wenzel *et al.* 1990, Wenzel *et al.* 1991). LTs are potent bronchoconstrictors when administrated to human subjects by inhalation (Dahlen *et al.* 1980, Weiss & Mullick 1983, Barnes *et al.* 1984). In vitro, they have been found to act as selective chemotactic agents for human eosinophils (Spada *et al.* 1994). LTs may also induce mucus hypersecretion from the bronchial mucosa (Coles *et al.* 1983). Platelet activating factor (PAF), a low molecular weight phospholipid (Demopoulos *et al.* 1979), may also be released from mast cells, but with a rapid rate of re-uptake, its mediator actions are not well established.

1.3.4.5 Cytokines

Mast cells are an important source of range of cytokines. Human lung mast cells express mRNA for TNF-α, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and GM-CSF following stimulation with SCF and anti-IgE (Okayama *et al.* 1995), and mRNA for IL-8 is expressed in HMC-1 cells, a human mast cell leukaemia line, following activation (Selvan *et al.* 1994). A more extensive list of cytokines has been reported to be generated and released from rodent mast cells. Cytokines released from mast cells are likely to play roles in host defence as well as to be involved in initiating, controlling and maintaining allergic reactions. The mast cell also releases the growth factors, TGF-β and bFGF, which have been suggested to make key contributions to extracellular matrix deposition in asthma (Reed *et al.* 1995, Kendall *et al.* 1997).

1.3.5 The roles of mast cells in asthma

In patients with asthma, the degree of airway hyperresponsiveness is associated with the numbers of mast cells in the BAL fluid (as estimated by tryptase concentration in cells recovered) (Ferguson *et al.* 1992). A higher than normal degree of mast cell degranulation can be observed histologically in bronchial biopsy tissues from asthmatic subjects (Pesci *et al.* 1993). Moreover, increased levels of the preformed granule constituents, tryptase and histamine have been measured in BAL fluid, even in the absence of clinical symptoms, suggesting that there may be continuous mast cell degranulation in the airways of asthmatic subjects (Broide *et al.* 1991). Exposure of the airways to allergen induces increased numbers of mast cells at the epithelial surface (Laitinen *et al.* 1993) and in the smooth muscle layer of human

bronchial tissue (Ammit *et al.* 1997, Robinson 2004), but this has not been observed in the submucosa.

Mast cell products could play important roles in triggering and maintaining the inflammatory changes and the remodelling in asthma. Histamine could contribute to the bronchoconstriction, oedema and mucus secretion that is characteristic of asthma (White 1990). Leukotrienes and prostanoids may act as more potent bronchoconstrictors and vasodilators than histamine, and could increase vascular permeability, and stimulate eosinophil accumulation and activation. Moreover, various cytokines and chemokines from mast cells could play key roles in the initiation and modulation of processes of inflammation and remodelling in asthma (Hart 2001). The potential actions of tryptase in asthma will be described separately (section 1.4).

Studies with mast cell deficient mice have shown that the presence of mast cells is required at least in part for the induction of airway hyperreactivity in a model of asthma that involves administration of ovalbumin as allergen (Kobayashi *et al.* 2000). The importance of mast cells in acute airway responses has been shown in an allergen challenged mast cell-deficient mouse model of chronic asthma (Williams & Galli 2000). Studies with c-kit and c-kit ligand knockout mice have indicated that mast cells may play a crucial role in inducing fibrosis in mouse models of allergen-induced asthma (Masuda *et al.* 2003).

Both mast cells and myofibroblasts have been recognised to play major roles in wound healing (Hebda *et al.* 1993). However, there have been few investigations of the interaction of these cells. Histological studies have indicated that mast cells in tissue from cases of idiopathic pulmonary fibrosis contain bFGF (Inoue *et al.* 2002), and the presence of bFGF receptors on myofibroblasts as well as on airway smooth

muscle cells has been taken as evidence that mast cells may provide a stimulus for the proliferation of these other cell types (Inoue *et al.* 2002). Interestingly, lysates of the mast cell line HMC-1 can induce the transformation of human foreskin fibroblasts to cells of the myofibroblast phenotype, that express α-smooth muscle (Gailit *et al.* 2001). It will be important to investigate in more detail the potential interactions between mast cells and myofibroblasts in the airways in asthma and other conditions associated with inflammation and tissue remodelling.

Table 1.3.1

Mediators produced by in human mast cells.

Class	Mediator	Category
Neutral proteases	tryptase	preformed
	chymase	
	carboxypeptidase	
	cathepsin G	
Biogenic amines	histamine	preformed
Proteoglycans	heparin	preformed
Arachidonic acid metabolites	PGD_2	newly generated
	LTC_4	
	PAF	
Cytokines	TNF-α	preformed and newly generated
	IL-3	
	IL-4	
	IL-5	
	IL-6	
	IL-10	
	IL-13	
	GM-CSF	
	TGF-β	
	bFGF	

1.4 Tryptase

1.4.1 Biochemical characteristics

Tryptase is the most abundant protein in human mast cells, and makes up some 20% of the total protein present (Schwartz 1990). This enzyme is largely unique to the granules of mast cells with much smaller quantities in basophils (Craig & Schwartz 1990, Walls *et al.* 1990), and it has thus been widely used as a marker for mast cells (Walls *et al.* 1990). Tryptase is a tetrameric serine protease with a molecular weight of 134 kDa with subunits of approximately 35 kDa (Schwartz *et al.* 1981, Smith *et al.* 1984, Cromlish *et al.* 1987). The tetrameric structure may be stabilized by heparin while present in the mast cell granules and immediately following secretion (Schwartz & Bradford 1986, Fukuoka & Schwartz 2004).

Though often thought of as a single enzyme, there are different types of human mast cell tryptase with differences in sequence and in post translational modification. There are a cluster of genes for tryptase located at chromosome 16p13.3 (Pallaoro *et al.* 1999). The first sequence to be elucidated was for a form of tryptase that was subsequently termed α-tryptase (Miller *et al.* 1989). There have been conflicting reports over the extent to which this form may possess enzymatic activity, and it has been suggested that it is released constitutively from mast cells and basophils in an inactive form (Schwartz *et al.* 1995). It was once thought α-tryptase represents the main form of tryptase in the circulation of normal subjects, but the antibody employed in the studies that led to this conclusion has since been re-categorised as specific for pro-tryptase rather than α-tryptase (Schwartz *et al.* 2003). Moreover substantial proportions of the human population have been found to lack α-tryptase. Beta-tryptase is stored in secretory granules in the active form (Schwartz & Bradford 1986) and is released upon mast cell degranulation. It appears to be the major form

of tryptase isolated from lung and skin (Xia *et al.* 1995), and is the most well studied. There are several isoforms of β-tryptase termed I, II and III which have 98-99% homology in amino acid sequence (Vanderslice *et al.* 1990). There is 91% homology in amino acid sequence between α and β tryptases.

Several other members of the tryptase family have been identified on chromosome 16p13.3. These include γ-tryptase, a trans-membrane form of tryptase which may be bound to the cell membrane of mast cells (Caughey *et al.* 2000), δ-tryptase which was first characterised as a pseudogene but which may in fact be translated as an enzymatically active form of tryptase (Wang *et al.* 2002), and ε-tryptase, a prostasin –like enzyme from airway epithelial cells (Wong *et al.* 2001). These, the later variants of tryptase to be described, remain relatively poorly characterised and their enzymatic and potential biological actions have been little investigated. They are structurally quite different from β-tryptase, and despite the similar nomenclature, they should be regarded as distinct enzymes.

Beta tryptase is the form that would appear to represent the major form in human mast cells, and it is secreted in substantial quantities in the human lung in asthma. The present studies focus on β-tryptase, and the term 'tryptase' will be used to describe this form.

1.4.1.1 Tryptase isolation

Tryptase was originally purified from human mast cells (Schwartz *et al.* 1981), and afterwards was purified from human lung (Smith *et al.* 1984) and skin tissue (Harvima *et al.* 1988). Subsequently recombinant human β-tryptase has been expressed in the methylotrophic yeast, *Pichia pastoris* (Niles *et al.* 1998). The

purified enzyme has been reported to have enzymatic properties similar to those of tryptase purified from lung tissue in its ability to cleave fibrinogen, and its sensitivity to inhibitors (Chan *et al.* 1999). The advent of recombinant tryptase has opened the way for larger quantities of well characterised enzyme to be available for studies of tryptase function.

1.4.1.2 Enzymatic activities of tryptase

Tryptase cleaves peptide and ester bonds preferentially at lysine or arginine residues at the P1 position, and in this respect is similar to pancreatic trypsin (Tanaka *et al.* 1983). Proteins which are components of the ECM, such as type VI collagen, gelatinase MMP-2 and fibronectin may be cleaved by human mast cell tryptase to some extent (Lohi *et al.* 1992, Kielty *et al.* 1993). Mast cell tryptase can also activate MMP-3 (Gruber *et al.* 1989), but not MMP-2 nor MMP-9 (Lees *et al.* 1994). Degradation of MMP-2 by this protease can result in a product of smaller size than active MMP-2 (Suzuki *et al.* 1995). Human mast cell β-tryptase has been reported to be a gelatinase which can degrade denatured collagen type 1 (Fajardo & Pejler 2003). Such findings implicate tryptase in processes of remodelling and repair.

Tryptase can efficiently degrade certain neuropeptides, such as vasoactive intestinal peptide and peptide histidine methionine (Tam & Caughey 1990), and calcitonin-gene related peptide (Walls *et al.* 1992), and could thus participate in processes of neurogenic inflammation. The ability to inactivate fibrinogen as a clottable substrate for thrombin, suggests that tryptase release could limit the clotting reaction at sites of mast cell activation (Schwartz *et al.* 1985). Of particular interest for an understanding of the pro-inflammatory actions of tryptase could be the report that tryptase can cleave and activate protease activated receptor-2 (PAR-2) (Molino *et al.*

1997). The potential consequences of PAR-2 activation by tryptase will be described in detail in a separate section.

1.4.1.3 Actions of tryptase on cells

Increased levels of tryptase are present in BAL fluid from asthmatic subjects (Wenzel *et al.* 1988), suggesting that cells of the human airways may be exposed constantly to this protease. Though tryptase has relatively few protein substrates, it can have profound actions on the behaviour of a number of cell types. This property was first identified when it was fond that tryptase can stimulate the proliferation of rodent fibroblasts (Hartmann *et al.* 1992, Ruoss *et al.* 1991). Subsequently mast cell tryptase has been reported to be able to stimulate mitogenic and proliferative responses in the MRC-5 human fibroblast line (Cairns & Walls 1997), in primary cultures of human lung fibroblasts (Aker *et al.* 2000) and human dermal fibroblasts (Gruber *et al.* 1997, Abe *et al.* 1998). Tryptase has been found to be a stimulus for the release of type I collagen from the MRC-5 human lung fibroblast cell line (Cairns & Walls 1997) and also from human dermal fibroblasts (Abe *et al.* 1998, Abe *et al.* 2000). However, it has been reported that tryptase does not alter expression of mRNA for MMP-1, MMP-2, MMP-3 & MMP-9 and TIMP-1, or their release from human dermal fibroblasts (Zhang *et al.* 1999a).

A potential role for tryptase as a growth factor has been reported for a number of cells other than fibroblasts. These include airway smooth muscle cells (Berger *et al.* 2001a), epithelial cells (Cairns & Walls 1996) and dermal microvascular endothelial cells (Blair *et al.* 1997), but not vascular smooth muscle cells (Hartmann *et al.* 1992). The effect of tryptase on the proliferation of fibroblasts and various other cell types, calls attention to a role for this protease in having key roles in tissue remodelling.

However, the extent to which tryptase may stimulate proliferation of myofibroblasts, a cell type important in remodelling of asthmatic airways, has yet to be investigated.

Tryptase has been found to stimulate the release of cytokines and other factors from several cell types. This protease can stimulate the release of IL-8 from epithelial cells (Cairns & Walls 1996), and endothelial cells (Compton *et al.* 1998) as well as being able to upregulate expression of mRNA for IL-1β and IL-8 from endothelial cells (Compton *et al.* 1998). Moreover, IL-6 and IL-8 release has been reported from human peripheral blood eosinophils (Temkin *et al.* 2002), and TGF-β from human airway smooth muscle cells (Berger *et al.* 2003). Cytokine release from fibroblasts or myofibroblasts in response to tryptase has not been investigated.

The precise mechanisms involved in the diverse pro-inflammatory and pro-fibrotic actions of tryptase on cells are not clear. The report by Molino *et al.* (1997) that tryptase can activate protease activated receptor-2 has raised the possibility that this could be a key mechanism. This is an area that needs further investigation in seeking to understand the actions of tryptase on cells.

1.5 Protease Activated Receptor 2 (PAR-2)

Four protease activated receptors (PAR) have been described; PAR-1, PAR-2, PAR-3 and PAR-4. They belong to the G-protein-coupled receptor (GPCR) superfamily (Dery *et al.* 1998), and are characterized by seven trans-membrane domains with an extracellular N terminus, and an intracellular C terminus (Fig. 1.5.1).

1.5.1 Mechanism of PAR activation

PARs are activated by a 'tethered ligand', whose peptide sequence is exposed by the proteolytic cleavage of the extracellular NH_2-terminus of the receptor (Lerner et al. 1996, Ossovskaya & Bunnett 2004). Molecular recognition of the agonist by the receptor is at the second extracellular loop (Schmidt *et al.* 1996, Lerner *et al.* 1996) (Fig. 1.5.2). PAR-2 may be internalised rapidily and incorporated into endosomes and lysosomes within 30-60 minutes of receptor activation (Hoxie *et al.* 1993, Derian *et al.* 1997). Re-sensitisation has been found to be dependent on a pool of PAR-2 in the Golgi apparatus (Bohm *et al.* 1996a).

PAR-2 is likely to be linked with Gαq or αo, G proteins which activate phospholipase C (PLC) β to produce inositol triphosphate (IP_3) and diacylglycerol (DAG), which, in turn increase cytoplasmic Ca^{2+} concentrations and activate protein kinase C (PKC) (Dery *et al.* 1998) (Fig. 1.5.3).

1.5.2 Agonists for PAR activation (Table 1.5.1)

PAR-1, PAR-3 and PAR-4 are activated by thrombin but not by tryptase (Hollenberg 1999). PAR-2 is activated by the tryptic enzymes trypsin (Bohm *et al.* 1996a) and mast cell tryptase (Molino *et al.* 1997, Mirza *et al.* 1997) but not by thrombin. PAR-2 activation can be mimicked by the synthetic peptides SLIGKV-NH_2 and SLIGRL-NH_2, whose sequences correspond to those of the human and mouse tethered ligands, respectively (Bohm *et al.* 1996b), by trans-cinnamoyl (tc) LIGRO-NH_2 (Al Ani *et al.* 1999) and by 2-furoyl-LIGKV (ASKH95) (Maryanoff *et al.* 2001). Agonist peptides have deficiencies as investigational tools. Thus the PAR-1 agonist peptide, SFLLRNP-NH_2 can cross-activate PAR-2 (Blackhart *et al.* 1996), and the peptide, tc-LIGRO-NH_2 described as a PAR-2 agonist has been reported to activate mast cells by non-PAR-2 specific activation (Stenton *et al.* 2002). Nevertheless, synthetic peptides can be useful as a tool to study PAR-2 activation. Unfortunately, no antagonist is yet available for PAR-2.

Table 1.5.1

Peptide and protease agonists of human PARs (adapted from Steinhoff *et al.* 2005).

PAR	enzyme	Ligand sequence	Agonist peptide
PAR-1	Thrombin	SFLLRN	TFLLR
PAR-2	Trypsin	SLIGKV	SLIGRL
	Tryptase		SLIGKV
	Coagulation factor VIIa, Xa		
PAR-3	Thrombin	TFRGAP	Not available
PAR-4	Thrombin	GYPGQV	GYPGQV
			AYPGKV

1.5.3 Molecular Biology of PARs

PAR-2 is a protein of 397 amino acids (Nystedt *et al.* 1994), encoded by a gene located at chromosome 5q13 (Nystedt *et al.* 1995). The genes for PAR-1 (Vu *et al.* 1991) and PAR-3 (Ishihara *et al.* 1997) are also located nearby in the same region (Schmidt & Schachner 1998) and with the same exon structure (Kahn *et al.* 1998). However, the gene for PAR-4 is located on a separate chromosome (19p12) (Xu *et al.* 1998). A polymorphism in PAR-2 has been described with a change in an amino acid residue in the second extracellular loop domain, and this difference can alter the sensitivity to peptide agonists (Compton *et al.* 2000).

1.5.4 Expression of PAR-2

PAR-2 is expressed in a wide variety of human tissues, including those of the pancreas, kidney, colon, liver and small intestine (Bohm *et al.* 1996b). This receptor has now been reported to be expressed on a variety of cells including lung fibroblasts (Akers *et al.* 2000), bronchial smooth muscle cells (Cocks *et al.* 1999, Schmidlin *et al.* 2001), airway epithelial cells (D'Andrea *et al.* 1998), vascular smooth muscle cells (Molino *et al.* 1998), vascular endothelial cells (D'Andrea *et al.* 1998, Hamilton *et al.* 2001), colonic myocytes (Corvera *et al.* 1997), skeletal muscle cells (Chinni *et al.* 2000), cardiomyocytes (Sabri *et al.* 2000, Lan *et al.* 2001), keratinocytes (Mascia *et al.* 2002), neurons and astrocytes (D'Andrea *et al.* 1998, Steinhoff *et al.* 2000) and neutrophils (Howells *et al.* 1997).

The patterns of cellular expression of PAR-2 are consistent with the idea that PAR-2 could play roles in inflammation. In mice injected perivenularly with PAR-2 agonists, a delayed onset of inflammation in response to surgical trauma was

observed, which was associated with a delay in leukocyte infiltration (Lindner *et al.* 2000). PAR-2 expression in endothelial cells has been reported to be increased by incubation in vitro with inflammatory mediators such as TNF-α and IL-1α (Nystedt *et al.* 1996, Hamilton *et al.* 2001). There appears to be a strong link between PAR-2 and inflammation, and upregulation of PAR-2 has been reported in the respiratory epithelium of human airway from patients with asthma (Knight *et al.* 2001). However, the potential roles of PAR-2 in allergic disease remain unclear.

1.5.5 Functions of PAR-2

The proliferation of airway smooth muscle cells (Berger *et al.* 2001b) and mouse skeletal myoblasts (Chinni *et al.* 2000) can be induced by PAR-2 agonist peptides. Activation of PAR-2 can also inhibit ion transport in human epithelial cells (Danahay *et al.* 2001). A key role for PAR-2 in mediating chronic inflammation was suggested by the finding that intra-articular injection of PAR-2 agonists could have potent pro-inflammatory actions, and could prolong swelling and synovial hyperaemia in mice (Ferrell *et al.* 2003).

PAR-2 activation has been reported to induce release of the pro-inflammatory cytokines, IL-6 and IL-8 from the A549, BEAS-2B (Asokananthan *et al.* 2002) and 16HBE lung epithelial cells lines (Page *et al.* 2003). In addition, PAR-2 stimulation can induce release of GM-CSF from primary cultures of epithelial cells from the small airways, but not from the human airway epithelial cell line A549 (Vliagoftis *et al.* 2001). Thus, PAR-2 could be involved in modulation of processes of inflammation in the lung. Consistent with a proinflammatory role, has been the finding that IL-6 can be released from an oral epithelial cell line in response to PAR-2 stimulation (Lourbakos *et al.* 2001). Moreover, increased expression of mRNA for

IL-6 and GM-CSF, and the release of IL-8 from human keratinocytes has been reported (Wakita *et al.* 1997, Hou *et al.* 1998).

The effects of PAR-2 activation on tissue remodelling have been little investigated. However, PAR-2 activation can induce the deposition of ECM components and the release of MMP-9 from airway epithelial cells in response to trypsin and a peptide agonist (Vliagoftis *et al.* 2000). In contrast, intranasal administration of a peptide agonist of PAR-2 in mice has been shown to inhibit LPS-induced MMP-2 release into BAL fluid (Moffatt *et al.* 2002).

In the guinea pig, activation of PAR-2 by intravenous injection of a PAR-2 agonist peptide has been reported to induce bronchoconstriction (Ricciardolo *et al.* 2000). No PGE_2 release was found to be induced following PAR-2 activation in an airway smooth muscle cell line, but PAR-2 activation can increase cytoplasmic Ca^{2+} concentrations (Chambers *et al.* 2001), and to result in the contraction of airway smooth muscle cells (Schmidlin *et al.* 2001). On the other hand, it has been reported that activation of PAR-2 in lung parenchymal strips from guinea pigs fails to alter constriction responses in vitro (Saifeddine *et al.* 2001).

In contrast to the observations that PAR-2 activation can induce bronchoconstriction, a bronchoprotective effect has been reported in a mouse model (Lan *et al.* 2001). This has been attributed to PGE_2 dependent relaxation of tracheal smooth muscle. PAR-2 activation has been found to induce inositol triphosphate (IP_3) production, PGE_2 and $PGF_1\alpha$ generation and arachidonic acid release from PAR-2 transfected epithelial and enterocyte rat cell lines (Kong *et al.* 1997). PAR-2 activation can also induce release of PGE_2 and COX-2 from airway smooth muscle cells (Chambers *et al.* 2003). PGE_2 can inhibit local immune responses and increase blood flow, and this could facilitate faster resolution of local inflammation (Cocks *et al.* 1999).

However, there is a report suggesting the possibility of a bronchoprotective effect for PAR-2 activation that does not involve prostaglandin generation. Intravenous administration of a peptide agonist of PAR-2 can inhibit histamine-induced increases in lung resistance and protein leakage in a guinea-pig model, and it was found that this bronchoprotection was not mediated by COX (Cicala *et al.* 2001). In contrast, PAR-2 activation can result in COX-2-dependent proliferation of human foreskin fibroblasts, associated with expression of mRNA for COX-2 (Frungieri *et al.* 2002). The possibility of COX-2, or PGE_2 dependent protective roles for PAR-2 have been suggested, but, there have been major differences in findings with different species and some of these may be related to the experimental system. There remains a pressing need for the investigation of the roles of PAR-2 on cells of the human airways.

1.6 Myofibroblasts

Myofibroblasts were first described in the context of wound repair (Gabbiani *et al.* 1971), and these cells have emerged as key participants in wound healing. Myofibroblasts form a layer in the *lamina reticularis* between the muscle and fibroblast layers in the lung(Gabbiani 1981), and they are important contractile cells (Hinz & Gabbiani 2003). Myofibroblasts express vimentin and α-smooth muscle actin, but not smooth muscle myosin (Zhang *et al.* 1994, Low 1999). TGF-β can promote the morphological and functional differentiation of human dermal fibroblasts into myofibroblasts (Vaughan *et al.* 2000). Increased α-smooth muscle actin expression in rat subcutaneous fibroblasts treated with TGF-β is associated with increased activity in rat fibroblasts (Hinz *et al.* 2001). Myofibroblasts can respond to proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 to release increased amounts of extracellular matrix proteins such as collagen I and III, and also can release growth factors into the local area (Powell *et al.* 1999).

In the asthmatic lung, the numbers of myofibroblasts have been reported to be increased, and the layer beneath the basement membrane has been found to be thickened in endobronchial biopsy tissues (Roche *et al.* 1989). The thickening is associated with increased deposition of sub-epithelial collagen (Brewster *et al.* 1990). The thickness of the area under the basement membrane is correlated closely with the numbers of fibroblasts and TGF-β expressing cells in the asthmatic airways (Vignola *et al.* 1997). Myofibroblast numbers have also been found to be increased in the allergen induced late response in mild atopic asthma (Gizycki *et al.* 1997).

Numbers of myofibroblast progressively decline at sites of wound healing (Rudolph *et al.* 1977), and these cells have been suggested to undergo apoptosis (Desmouliere *et al.* 1995). IL-1β can increase expression of PDGF-Rα leading to myofibroblast

hyperplasia in the rat lung (Lindoors *et al.* 1998). The rate of myofibroblast proliferation can be affected by levels of PGE_2, which can downregulate IL-1β expression, and hence PDGF-α receptor expression in rat lung myofibroblasts (Boyle *et al.* 1999). IL-1β can inhibit the differentiation of rabbit myofibroblasts by inducing apoptosis via inducible nitric oxide synthase (iNOS), and TGF-β can inhibit myofibroblast apoptosis by inhibiting IL-1β-induced iNOS expression in these cells (Zhang & Phan 1999).

1.6.1 Actions of TGF-β on myofibroblasts

Modulation of the myofibroblast phenotype can be influenced by cytokines, such as TGF-β (Desmouliere *et al.* 1993), PDGF (Bostrom *et al.* 1996), TNF-α (Bachem *et al.* 1993), and GM-CSF (Vyalov *et al.* 1993, Serini & Gabbiani 1999) which may also act as mitogenic factors for fibroblasts. As well as these cytokines, heparin has been reported to induce an increase in α-smooth muscle actin expression (Desmouliere *et al.* 1992a). It has been suggested that IFN-γ can downregulate α-smooth muscle actin expression (Desmouliere *et al.* 1992b).

TGF-β can play an important role in regulating α-smooth muscle actin expression in fibroblasts. Desmouliere *et al.* (1993) reported that induction of α-smooth muscle actin expression was increased in dermal fibroblasts by TGF-β but not by TNF-α and platelet-derived growth factors. TGF-β can also upregulate α-smooth muscle actin expression in liver fibroblasts and induce the proliferation of liver myofibroblasts (Desmouliere *et al.* 1995). A member of the TGF-β supergene family, activin A, can also stimulate the proliferation of a human foetal lung fibroblast cell line and also their differentiation into myofibroblasts (Ohga *et al.* 1996). The extent to which α-

smooth muscle actin expression may be increased in response to TGF-β_1 is dependent on tension present between cells and collagen (Arora *et al.* 1999).

TGF-β can convert human lung fibroblasts to the myofibroblast phenotype in a dose dependent manner which is regulated by c-Jun-NH_2-terminal kinase (JNK) (Hashimoto *et al.* 2001). However, regulation of the α-smooth muscle actin promoter by TGF-β in the differentiation of myofibroblasts from fibroblasts is different from that for smooth muscle cells by transfection of the known α-smooth muscle actin promoter (Roy *et al.* 2001).

Levels of TGF-β are increased in BAL fluid collected from asthmatic subjects (Redington *et al.* 1997). The epithelium can release TGF-β_2 during processes of damage and repair, and this is the most likely source of TGF-β in the asthmatic lung and could be responsible for the transformation fibroblasts into myofibroblasts at this site (Vignola *et al.* 1997). Increases in TGF-β_2 levels have been reported from mechanically scraped cultures of an epithelial cell line (Puddicombe *et al.* 2000). TGF-β in a sonicated preparation of eosinophils has been shown to induce increased proliferation, collagen release and lattice contraction in human lung and dermal fibroblast cell lines (Levi-Schaffer *et al.* 1999). Similarly there have been reports of increased α-smooth muscle actin expression in primary cultures of human dermal fibroblasts cultured with eosinophils, which has been attributed to TGF-β released from eosinophils (Phipps *et al.* 2002). As myofibroblasts can themselves release TGF-β, it has been suggested that an autocrine source of TGF-β_2 could lead to the generation of intestinal (McKaig *et al.* 2002) and liver myofibroblasts (Wells *et al.* 2004).

As well as inducing the myofibroblast phenotype, TGF-β has been found to act directly on rat lung fibroblasts to induce the release of the ECM proteins, collagen, fibronectin and elastin (Sime *et al.* 1997). Moreover, TGF-β_1, can act in concert with endothelin-1 and PDGF-BB to activate the proliferation of primary cultures of bronchial fibroblasts from normal and asthmatic subjects, and to induce collagen synthesis in these cells (Dube *et al.* 2000). In contrast, in another study it has been suggested that TGF-β can decrease collagen Iα1 mRNA expression in human lung fibroblasts through the generation of PGE_2 that can act on EP2 receptors (Choung *et al.* 1998).

1.6.2 Cytokine production by myofibroblasts

The immune system is under the control of a diverse array of cytokines which can induce cell proliferation, and apoptosis, regulate gene expression and have other actions on various cell types. Overlapping functions can be explained in part by sharing of receptor subunits within cytokine receptor families. Cytokines and chemokines can play important roles in bronchial hyperresponsiveness and airway inflammation in murine models of respiratory disease (Vasquez & Spina 2000, Lloyd 2002), as well as in the regulation of mediators involved in tissue fibrosis (Atamas 2002).

Myofibroblasts have been reported to release numerous cytokines. Bronchial subepithelial myofibroblasts from human bronchial biopsies are capable of releasing GM-CSF, IL-6, IL-8 and SCF constitutively (Zhang *et al.* 1996a). Expression of mRNA for IL-1, IL-6, TNF-α, IL-10, TGF-β, GM-CSF, IL-8, and RANTES, has been reported in colonic myofibroblasts of both the mouse (Powell *et al.* 1999) and the human (Rogler *et al.* 2001).

The potential actions of tryptase and PAR-2 activation in altering cytokine production in fibroblasts and myofibroblasts have not been investigated. Tryptase has been shown to stimulate IL-8 release from epithelial cells (Cairns & Walls 1996) and endothelial cells (Compton *et al.* 1998), IL-6 and IL-8 release from human peripheral blood eosinophils (Temkin *et al.* 2002) and TGF-β from human airway smooth muscle (Berger *et al.* 2003). PAR-2 stimulation has been found to induce GM-CSF release from airway epithelial cells (Vliagoftis *et al.* 2001), IL-6 and IL-8 release from human dermal endothelial cells (Shpacovitch *et al.* 2002, Uehara *et al.* 2003).

It will be important to extend such studies to the fibroblast and myofibroblast. In the present studies, IL-6, IL-8 and GM-CSF were selected for particular investigation on account of their potential importance as fibroblast and myofibroblast-derived cytokines that are released and synthesised in response to various stimuli, and because it has been suggested that these cytokines have actions on fibroblasts consistent with roles in remodelling. Moreover, IL-6, IL-8 and GM-CSF can have regulatory actions on mast cells (Valent 1995, Welker *et al.* 2001, Conti *et al.* 2002), providing a further reason to study the effects of tryptase and PAR-2 stimulation on the production of these cytokines.

1.6.2.1 IL-6

IL-6 is a pleiotropic cytokine with a molecular weight of 26 kDa. A number of human cell types produce IL-6 in addition to fibroblasts, including T- cells, B-cells monocytes, epithelial cells keratinocytes, endothelial cells, mesangial cells and some tumour cells. IL-6 can regulate the growth and differentiation of various cells with

major activities on the immune system and inflammation (Kelley 1990, Kishimoto 1989). The multiple actions of IL-6 are integrated in a complex cytokine network in which IL-6 can have synergistic or antagonistic activities with other cytokines, which include IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF and IFN-γ. This is consistent with IL-6 being involved in inflammatory processes. IL-6 can induce the final maturation of B cells into antibody-producing cells, stimulate T-cell growth and differentiation of cytotoxic T-cells, and it is a potent growth factor for myeloma and plasmacytoma cells. This cytokine is an endogenous pyrogen with IL-1 and TNF-α, and can provoke acute phase reactions in response to inflammation (Heinrich *et al.* 1990). IL-6 is a cytokine that is crucial for mast cell maturation. In vitro, the differentiation of blood CD34+ cells into mast cells induced by stem cell factor (SCF), can lead in the presence of IL-6 to an increase in the proportion of chymase positive cells and increased intracellular histamine levels, when compared with cells treated with SCF alone (Conti *et al.* 2002). IL-6 can prevent mast cell apoptosis (Kambe *et al.* 2001) and IL-6 and SCF from a lung epithelial cell line have been reported to induce tryptase production by the HMC-1 mast cell line (Pompen *et al.* 2000). There has been some controversy over the actions of IL-6 on fibroblasts. IL-6 can have a suppressive effect on the proliferation of alveolar fibroblasts (Shahar *et al.* 1996), but oncostatin M, a member of the IL-6 family has been reported to induce procollagen synthesis, inhibit spontaneous apoptosis, and also to suppress mitogenic activity in lung fibroblasts (Scaffidi *et al.* 2002).

1.6.2.2 IL-8

IL-8 is a polypeptide isolated originally from LPS stimulated monocytes (Schroder *et al.* 1987). It was first described as a neutrophil chemotactic agent but subsequently found to be chemotactic also for T cells and eosinophils (Shute 1994, Erger & Casale

1995). Antibodies against IL-8 can block neutrophilic inflammation in various models (Kunkel *et al.* 1991, Alam 1997). IL-8 has a molecular weight of approximately 8 kDa and is produced not only by fibroblasts but also by a variety of other cell types including monocytes, neutrophils, endothelial cells, epithelial cells and lymphocytes (Standifoid *et al.* 1990, Strieter *et al.* 1989, Alam 1997). Established stimuli for the release of IL-8 from these cells are IL-1, TNF-α, IFN-γ and LPS. IL-8 levels have been found to be increased in bronchial tissue and in serum in severe asthma (Shute *et al.* 1997) and cystic fibrosis (Koller *et al.* 1997).

1.6.2.3 GM-CSF

Human granulocyte-macrophage colony-stimulation factor (GM-CSF) is produced in response to IL-1, TNF-α and LPS by macrophages, endothelial cells, epithelial cells, smooth muscle cells and activated T cells, as well as by fibroblasts (Hamilton 2002). Increased levels of GM-CSF have been found in BAL fluid from the asthmatic airways (Mattoli *et al.* 1991). GM-CSF can enhance the expression of adhesion molecules on epithelial cells and endothelial cells, prime granulocytes to release substantial amounts of mediators on stimulation, and prolong the survival of inflammatory cells (Lopez *et al.* 1986, Soloperto *et al.* 1991, Wislez *et al.* 2001). This cytokine could promote the accumulation and activation of eosinophils and other inflammatory cells (Soloperto *et al.* 1991). The actions of GM-CSF on mast cells could be important. GM-CSF has been shown to downregulate β-tryptase expression in HMC-1 cells (Finotto *et al.* 1996) and in normal cord blood-derived mast cells (Welker *et al.* 2001).

1.7 Extracellular Matrix (ECM)

The ECM is a network of macromolecules of protein and proteoglycans, and includes collagens, fibronectin and elastin, that are produced from cells adjacent to the scaffolding (Hay 1981, Moseley *et al.* 2004). Complex interactions between cells and the ECM regulate processes of remodelling, cytokine availability, inflammation and aging. In airway inflammation, the normal balance of the ECM components is altered, and in asthma the increased deposition of ECM components can contribute to narrowing of the airways and prolonged cell responses (Johnson 2001).

There are twenty distinct collagens that have been identified on the basis of gene sequence analysis in vertebrates (Brown &Timpl 1995). Collagen has a triple helical conformation, with single strands of a Gly-X-Y triplet repeat, where X is proline and Y is hydroxyproline (Prockop *et al.* 1979). Collagens I, III and IV are the main constitutents of the ECM.

Matrix metalloproteases (MMP) are capable of degrading collagens and other ECM components (Birkedal-Hansen *et al.* 1993). MMP has a catalytic domain that contains a Zn^{2+} active site, and so far approximately 30 different MMP have been described (Parks & Shapiro 2001). MMP are secreted as proenzymes (the latent form) and require activation (Nagase & Woessner 1999). Increased levels of MMP-2 and MMP-9 have been reported in supernatants of lung epithelial cell monolayers following LPS administration (taken as a model of acute lung injury) (Yao *et al.* 1996) and also in BAL fluid of subjects following cardiopulmonary bypass surgery (Eichler *et al.* 2003). MMP-2 has a molecular weight of 72 kDa (Okada *et al.* 1990), and MMP-9 a molecular weight of 92 kDa in the latent form. Both are known as gelatinases and they are capable of degrading collagens I, IV and V (Welgus *et al.* 1990).

The ratio between MMPs and their endogenous inhibitors, tissue inhibitors of metalloprotease (TIMP), controls the degree of ECM deposition. There are four TIMPs to have been described TIMP-1 TIMP-2, TIMP-3 and TIMP-4. These have molecular weights of 22 kDa to 30 kDa (Gomez *et al.* 1997). TIMPs bind to the catalytic site of MMP in a 1:1 molar ratio, and TIMP-1 can form a complex with pro MMP-9 (Ogata *et al.* 1995) and TIMP-2 with proMMP-2 (Itoh *et al.* 1995). The MMP/TIMP ratio has been found to be decreased in BAL fluid from asthmatic airways. The ratio is lower in severe disease, and is associated with extracellular matrix deposition (Mautino *et al.* 1997). MMP-9/TIMP-1 ratios in sputum have been reported to be correlated with airflow obstruction in asthma (Vignola *et al.* 1998). Similarly, a higher expression of TIMP has been observed in idiopathic pulmonary fibrosis (Selman *et al.* 2000).

TIMP-1, TIMP-2, TIMP-3, TIMP-4, MMP-2 and MMP-9 have been detected by immunohistochemistry in human lung fibroblasts and myofibroblasts (Selman *et al.* 2000). Expression of mRNA for these proteases and inhibitors has also been found in these cells, but greater amounts have been found in myofibroblasts, and in the case of MMP-9 in myofibroblasts and not fibroblasts (Ramos *et al.* 2001). The extent to which tryptase and agonists of PAR-2 may modulate levels of MMP, TIMP and other components of the ECM from fibroblasts and myofibroblasts needs investigation.

1.8 Aim

The aim of this study has been to investigate the actions of mast cell tryptase on fibroblasts and myofibroblasts. In particular the objective has been to systematically examine the effects of this major mast cell product on:

1. Processes of mitogenesis and cell proliferation
2. The generation and release of cytokines
3. The ability to stimulate the production and release of MMP and components of the extracellular matrix.

In seeking to assess the roles of tryptase as a stimulus for altered fibroblast and myofibroblast function, the potential contribution of protease activated receptor 2 was investigated.

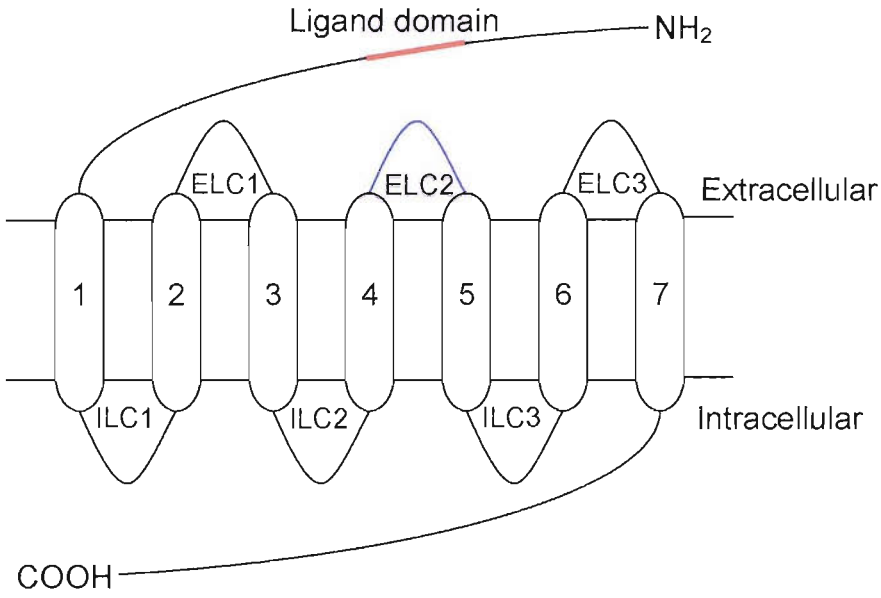


Fig. 1.5.1

Structure of the protease activated receptor, diagramatic presentation of 7 trans-membrane domains. Intracellular loop (ILC) and extracellular loop (ECL) are shown (adapted from Dery *et al.* 1998).

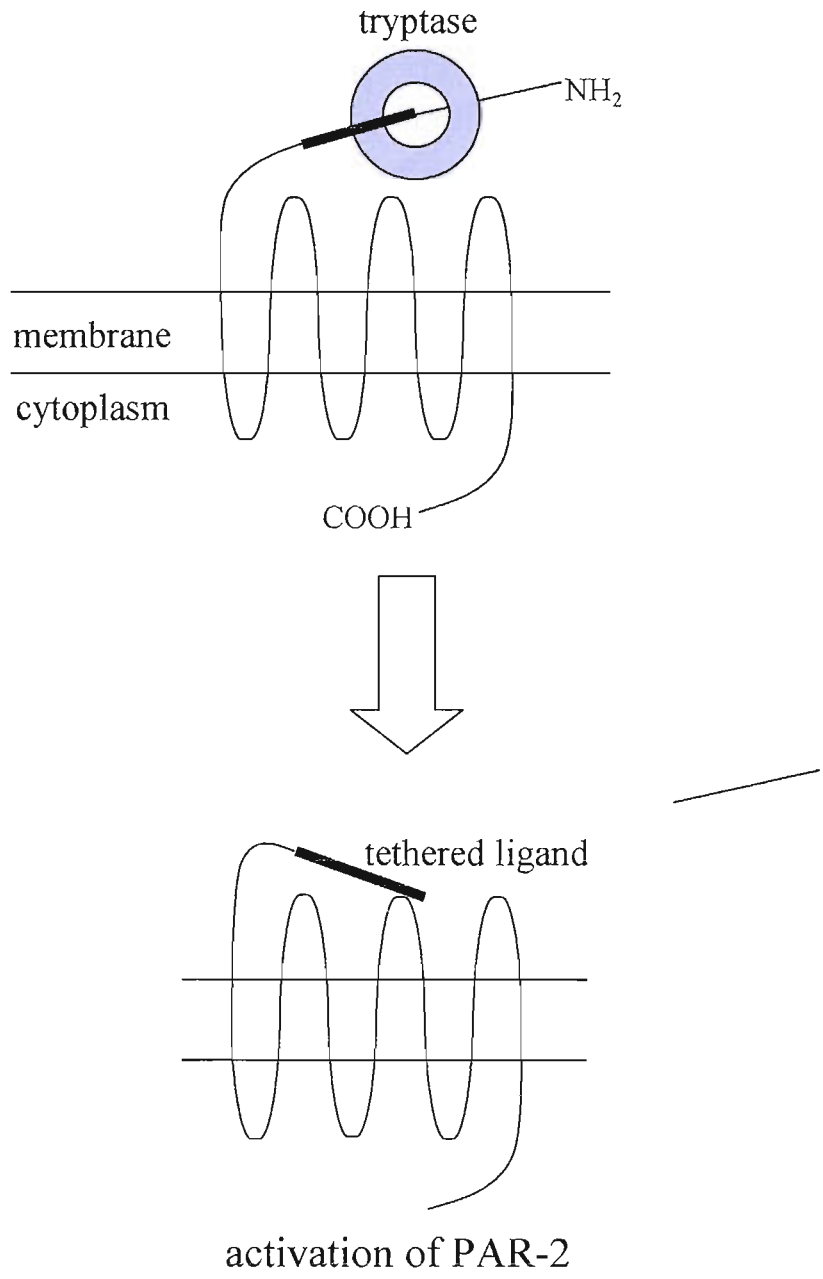


Fig. 1.5.2

Diagram to illustrate mechanism of PAR-2 (GPCR) activation by tryptase (adapted from Dery *et al.* 1998).

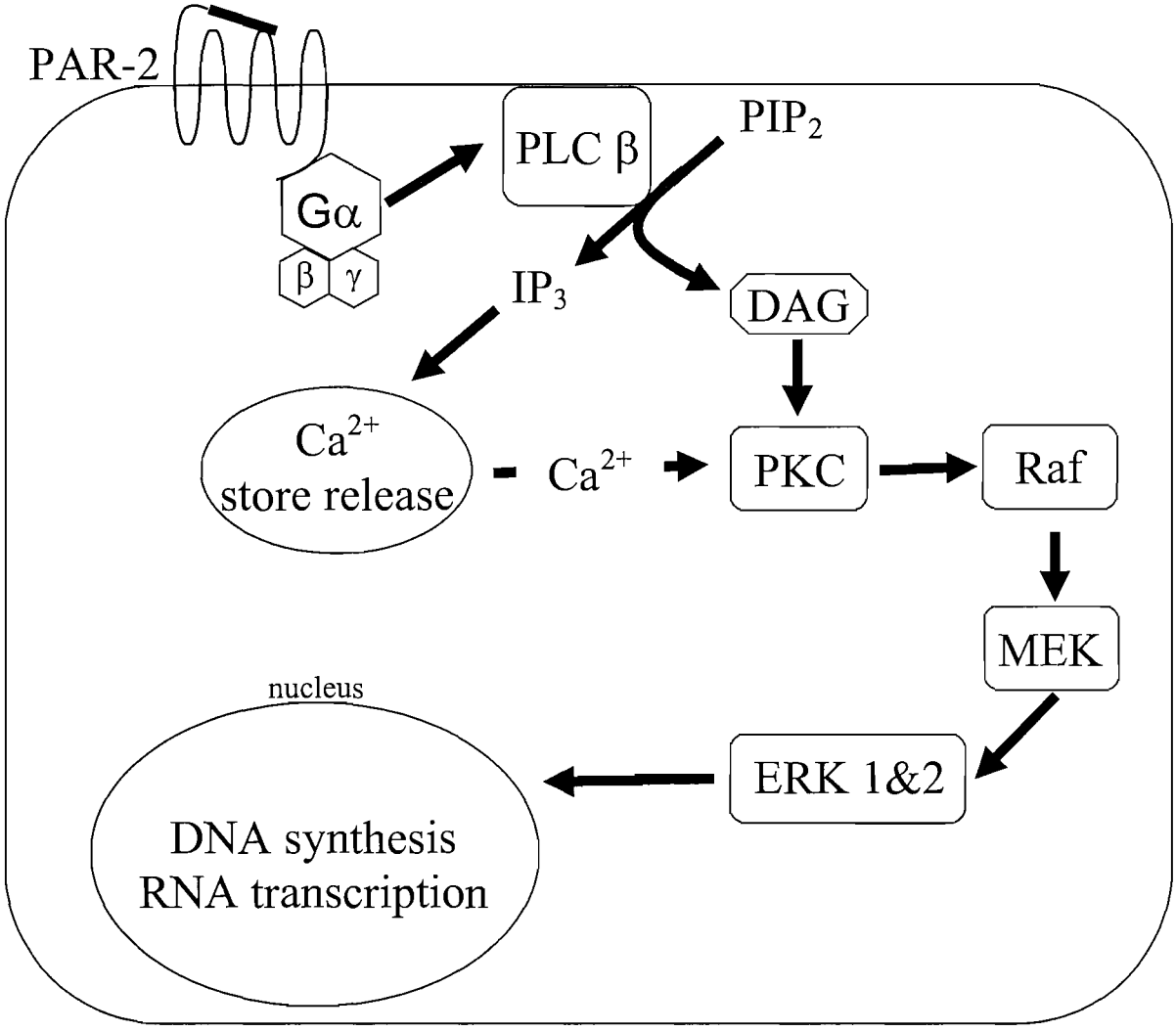


Fig. 1.5.3

DAG - diacylglycerol

ERK - extracellular signal-regulated kinase

IP_3 - inositol triphosphate

MEK - mitogen activated protease kinase kinase

PIP_2 - phosphatidylinositol 4, 5,-bisphosphate

PKC - protein kinase C

PLC - phospholipase C

Diagram to illustrate signal transduction by GPCR (PAR-2) activation, Gαq model (adapted from Dery *et al.* 1998).

Chapter 2

Materials and Methods

2 Materials and methods

2.1 Materials

FITC conjugated mouse monoclonal anti-human α-smooth muscle actin (clone 1A4), mouse monoclonal anti-human vimentin (clone V9) and mouse monoclonal anti-human myosin (smooth) (clone hSM-V), *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA), benzamidine, butyl agarose, cellulose membrane dialysis tubing, glycerol, PCR primers, Sephacryl S-300 gel filtration chromatography, transforming growth factor (TGF)-β_2, tumour necrosis factor (TNF)-α, saponin, Tween 20, NaCl, $(NH_4)_2SO_4$, 7-amino-actinomycin-D (7-AAD), leupeptin and trypsin (human pancreas) were from Sigma-Aldrich, (Poole, Dorset). EDTA, MES and Tris were from BDH (Poole). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS), Hank's balanced salts solution (HBSS), insulin-transferrin-selenium supplement (ITS), minimum essential medium (MEM), penicillin-streptomycin-glutamine mixture (PSG), Trizol, typsin-EDTA were from GIBCO BRL (Paisley, Scotland). Acrylamide, ammonium persulphate, nitrocellurose membrane, protein molecular marker, silver stain reagents, sodium dodecyl sulphate (SDS) and TEMED were from Bio-Rad (Hemel, Hempstead, Hertfordshire). Horseradish proxidase (HRP) conjugated rabbit anti-mouse immunoglobulin antibody and HRP conjugated rabbit anti-sheep immunoglobulin antibody were from Dako Cytomation (Cambridge). Sheep monoclonal anti-human matrix metalloproteinase (MMP) 2 and MMP-9 were from The Binding Site (Birmingham). The chemiluminescent substrate for western blotting was from Pierce (Chester, Cheshire). Centricon C-30 concentrators, Cytofluor fluorescent plates and 0.45 μm filter papers were from Millipore (Bedford MA, USA). Heparin agarose was from Kem-En-Tec (Copenhagen, Denmark). Benzamidine agarose was from Affinity Chromatography

(Ballasalla, Isle of Man). Filter paper (1, 50, GF/F and GF/A) was from Whatman (Maidstone, Kent). The enzyme linked-immunosorbant assay (ELISA) kits, CytoSet TM (IL-6, IL-8 and GM-CSF) were from Biosource International (Nivelles, Belgium). Cell culture flasks, cell culture plates and 8-chamber slides were from NUNC (Naperville, IL, USA). Cell Titre® AQ_{ueous} Solution, lactate dehydrogenase (LDH) assay kits, PCR master mix, DNAse and the Reverse Transcription System were from Promega (Southampton, UK). The GAPDH probe conjugated with VIC fluorophore was from Applied Biosystems (Warrington, Cheshire). FAM (6-carboxyfluorescein) fluorophore Taqman PCR probes, Taqman PCR primers and Taqman PCR core kit were from Eurogenic (Seraing, Belgium). [^{3}H]-thymidine, [^{3}H]-proline and ECL film were from Amersham (Little Chalfont, Bucks). The scintillation solution, Micro Scint 40 was from Perkin Elmer (Beaconsfield, Bucks). The PAR agonist and control peptides were prepared by the University of Calgary peptide synthesis facility (Calgary, Alberta, Canada). The tryptase specific mouse monoclonal antibody AA5 (Walls *et al.* 1990) and PAR-2 specific monoclonal antibody (P2A) (Aslam *et al.* 2002) were prepared in the Immunopharmacology Group, University of Southampton. The tryptase inhibitors, APC366 and APC1390 were kindly provided by Celera Genomics (South San Francisco, CA, USA). A MMP-2 and MMP-9 positive cell supernatant prepared as described (Pender *et al.* 1997) was kindly provided by Dr S. Pender (Infection, Inflammation and Respiration Division, University of Southampton).

Equipment and Software

The ABI PRISM ® 7700 Sequence Detection System, GeneAmp PCR system 9600 and Primer Express software were from Applied Biosystems. The Top Count NXT^{TM} microplate scintillation and luminescence counter was from Packard

BioScience (Bucks, UK). The ThermoMAX platereader was from Molecular Devices (Sunnyvale, CA, USA). The fluorescence spectrophotometer was from Photon Technologies (Oxford). The band quantifing software, GeneGenius was from SynGene (Cambridge).

2.2 Methods

2.2.1 Purification and characterisation of tryptase

2.2.1.1 Purification of recombinant tryptase

Recombinant tryptase was obtained from a *Pichia pastoris* expression system. A crude extract of the cells in 2 M ammonium sulphate buffer (kindly provided by Dr. Peter Kilby, Roche, Welwyn Garden City) was passed through a 0.45 μm filter. The extract was loaded on to a butyl agarose chromatography column of 50 cm^3 (diameter = 2 cm) equilibrated with buffer A (2.0 M $(NH_4)_2SO_4$, 0.5 M NaCl, 10% glycerol, 20 mM MES, pH 6.1), and tryptase was eluted with a linear gradient with buffer B (2 M NaCl, 10 mM MES pH 6.1) at a flow rate of 1 ml/min. The tryptase enzymatic activity (section 2.2.1.3) and protein content (section 2.2.1.4) of each fraction was measured, and fractions with high tryptase activity were pooled.

The pooled sample was applied to a benzamidine agarose chromatography column of 5 cm^3 (diameter = 1 cm) equilibrated with buffer 2.0 M NaCl and 10 mM MES, pH 6.1. Bound tryptase was eluted with 0.15 M benzamidine, 2.0 M NaCl and 10 mM MES, pH 6.1 at a flow rate of 0.5 ml/min and collected into 2 ml fractions. The protein content was assessed by measuring the absorbance at 280 nm for each fraction and those with the highest absorbance were pooled. The volume was reduced to approximately 1 ml using Centricon C-30 centrifugal concentrators and the concentration of benzamidine in the sample reduced by diafiltration.

Sephacryl S-300 gel filtration chromatography was applied to the partially purified preparation and the tryptase was eluted with a buffer consisting of 2.0 M NaCl and 10 mM MES, pH 6.1. The presence of tryptase in the fractions collected was

detected enzymatically and the protein content assessed, and fractions with high tryptase activity were pooled, concentrated as described above and stored in 50 µl aliquots at –70°C. All purification steps were carried out at 4°C.

2.2.1.2 Purification of human lung tryptase

Tryptase was purified from approximately 500 g human lung tissue obtained *post mortem* as previously described (Smith *et al.* 1984). Frozen lung tissue was defrosted and excess blood was decanted. Lung tissue was blended with low salt buffer (0.1 M NaCl, 1 mM EDTA, 0.05 M MES, pH 6.1), centrifuged at 40,000 × *g* for 30 min, and the process repeated until the supernatant became clear (free of haemoglobin), and tryptase was extracted using a high salt buffer (2.0 M NaCl, 1 mM EDTA, 0.05 M MES, pH 6.1). Both low salt buffer and high salt buffer extracts were tested for tryptase activity. The high salt buffer extract was filtered progressively through 1, 50, GF/F, GF/A (Whatman) and 0.45 µm filters before dialysis in cellulose membrane dialysis tubing against 0.4 M NaCl and 10 mM MES (pH6.1). The dialysed extract was again passed through GF/F, GF/A and 0.45 µm filters.

The extract was loaded on to a heparin agarose column of 135 cm^3 (diameter = 3.6 cm) equilibrated with low salt buffer. Tryptase was eluted with an increasing concentration gradient of NaCl from 0.4 M to 2.0 M in 10 mM MES, pH 6.1 at a flow rate of 1.8 ml/min, and collected into 5 ml fractions. The tryptase activity and protein content of each fraction were measured, and fractions with high tryptase activity were pooled.

The pooled sample was purified further by sequential application of benzamidine agarose and S-300 gel chromatography, as described for the purification of the recombinant tryptase. Potential contamination of preparations with chymase or elastase was investigated by the enzyme assay.

2.2.1.3 Enzyme assay

The enzymatic activity of tryptase was measured using the peptide substrate *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) (McEuen *et al.* 1996). To each well of the microtitre plate, 90 µl of the assay mixture (1mM BAPNA in standard tryptase buffer 1M glycerol, 0.1 M Tris, pH 8.0) was added to 10 µl of enzyme. The change in absorbance was measured at 410 nm for 10 minutes in a ThermoMAX platereader in the kinetic mode. The OD value was converted into milliunits (mU) per ml according to the equation mOD/min × 3.888 (mU/ml)/(mOD/min) (Appendix 1), where 1 U represents the enzymatic activity required to hydrolyse 1 µmol of substrate per minute at room temperature. Chymase and elastase were measured in a similar manner using the substrates, 0.7 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and 0.5 mM *N*-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, respectively, in 1.5 M NaCl, 0.3 M Tris, pH 8.0.

2.2.1.4 Protein assay

The protein present was measured spectophotometrically at 280 nm. The concentration of purified tryptase (w/v) was calculated using the extinction coefficient for purified tryptase $E_{280nm}^{1mg/ml} = 2.81$ (Smith *et al.* 1984).

2.2.1.5 Tryptase characterisation

The purity of extracted tryptase was assessed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Tryptase was boiled 5 minutes with a loading buffer (1% SDS, 20% β2-mercaptoethanol, 0.001% bromophenol blue and 40% glycerol) and run on a 10% gel (375 mM Tris, 2.5 μM TEMED, 10% acrylamide, 0.1% SDS, 0.05% ammonium persulphate, pH8.9-9.1) followed by silver staining. The SDS-PAGE gel was fixed in 40% methanol and 10% acetic acid (v/v). The gel was then incubated sequentially with fixative, oxidiser, silver stain reagent and developer reagent according to the manufacturer's protocol (Bio-Rad).

The protein was confirmed as tryptase by western blotting using the tryptase specific monoclonal antibody AA5. Tryptase was transferred from SDS-PAGE gel to a nitrocellurose membrane at 30 V at 4°C in transfer buffer with 48 mM Tris (pH8.3), 3.9 mM glycine, 0.0375% SDS and 20% methanol over 90 min. The membrane was blocked with 1% BSA in PBST (PBS with 0.2% Tween - 20) and incubated with AA5 diluted 1: 2000. HRP-conjugated rabbit antibody against mouse immunoglobulins antibody was applied as the secondary antibody and the blot was exposed on ECL film using chemiluminescent substrate.

Active site titration was performed using 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) (Jameson *et al.* 1973). MUGB (10 μl of 20 μM) was added to 90 μl of enzyme. Fluorescence was measured at 360 nm excitation and 460 nm emission with four scans at one minute intervals using a fluorescent plate reader, (Cytofluor).

2.2.2 Cell culture

Cultures of primary human lung fibroblasts derived from lung biopsy tissue from normal and asthmatic volunteers were prepared as described by Richter *et al.* (2001) kindly supplied for these studies by Dr Audrey Richter and Dr Mark Buckley (Table 2.2.1). Local Research Ethics Committee approval was granted for this work. Primary cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM), with 10% foetal calf serum (FCS) and a penicillin-streptomycin-glutamine mixture (PSG). Cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere. Cells used in experiments were at passage 4 to 8, except mitogenesis and proliferation studies that used up to passage 12. The MRC-5 fibroblast cell line (ECACC, Wilts) was maintained in Minimum Essential Medium (MEM) with 10% FCS and PSG. Trypsin-EDTA (20 mM trypsin and 540 μM EDTA) was used to detach cells from the container prior to passage. Trypan blue exclusion was used to determine cell viability, and cells counted using an Improved Neubauer haemacytometer. Myofibroblasts were prepared from fibroblasts which had been transformed by addition of transforming growth factor TGF-β_2 (1 ng/ml) for 72 hours. It was estimated that 73% of fibroblasts were transformed into the myofibroblast phenotype as assessed by the expression of α-smooth muscle actin (detected by immunocytochemistry).

2.2.3 Immunocytochemistry

Eight chamber slides were seeded with 1×10^5 cells/ml (300 μl/chamber) in the presence or absence of TGF-β_2 (1 ng/ml) for a minimum of 72 hours. After air drying overnight, slides were washed with PBS, and fixed with 1% paraformaldehyde. Cells were permeabilised with methanol and 0.5% saponin and

0.1% Tween 20. Slides were blocked with goat serum, and fluoroscein isothiocyanate (FITC)-conjugated monoclonal antibodies were added specific for α-smooth muscle actin (antibody clone 1A4), vimentin (V9), myosin (hSM-V), and PAR-2 (P2A). The nuclei were stained with 7-amino-actinomycin-D (7-AAD) to facilitate cell counts. A computerised image analysis system (Zeiss, Oberkochen, Germany) was employed to quantify the staining.

2.2.4 Cell proliferation

2.2.4.1 MTS assay

Fibroblasts were cultured to confluency as described above. Cells were detached by using trypsin-EDTA solution and counted following addition of trypan blue dye to prepare a 5×10^4 cell/ml stock in cell culture medium. Cells were seeded into a 96 well cell culture plate at 100 µl/well (5,000 cells/well) with or without addition of TGF-β_2 (1ng/ml). A standard curve was prepared by seeding cells at 0 (medium only), 2500, 5000, 7500, and 10,000 cells/well in triplicate (Appendix 2). At 72 h following seeding, cells were washed twice with Hank's balanced salts solution (HBSS) without Ca^{2+} and Mg^{2+} and the medium was changed to serum free media (DMEM with insulin-transferrin-selenium supplement (ITS) and PSG). After maintaining cells for 24 h in serum free medium, the stimulus (prepared in serum free medium) was added. Medium alone was added as the negative control, and 10% FCS and 5 ng/ml epidermal growth factor (EGF) as the positive controls. The concentration range of tryptase employed in the cell proliferation studies was 3, 10, 30 and 100 mU/ml. Tryptase (100 mU/ml) was heat inactivated at 60°C for 30 minutes using a thermocycler. After 48 h, Cell Titre® AQ_{ueous} Solution was added and incubated for 1 h before reading at 490 nm. Cell number was determined from

OD using standard curves (Appendix 2). Cell proliferation was expressed as a percentage of the cell number without the stimulus.

2.2.4.2 [^{3}H]-thymidine incorporation

Fibroblasts were cultured to confluency as described above. Cells were detached by trypsin-EDTA and counted with trypan blue to prepare a 5×10^4 cell/ml stock in cell culture medium. Cells were seeded into a 96 well cell culture plate at 100 µl/well with or without TGF-β_2 (1 ng/ml). At 72 h following seeding, cells were washed twice with HBSS and the medium was changed to serum free media (DMEM with ITS and PSG). After maintaining cells for 24 h in serum free media, the stimulus (prepared in serum free media) was added. In cell stimulation experiments, medium alone was added as the negative control, and 10% FCS and 5 ng/ml EGF as the positive controls. Human lung tryptase was applied at 1 and 10 mU/ml, and recombinant tryptase at 3, 10, 30 and 100 mU/ml in both cases with heparin added (1:1 wt/wt) to help stabilise activity. Tryptase activity was inhibited by treatment with 50 µM leupeptin, 100 µM APC366 or 10 µM APC1390 or by heat inactivation. SLIGKV-NH_2, the human PAR-2 agonist peptides were tested at 1, 10 and 100 µM and LSIGKV-NH_2, at non-PAR-2 activating peptide included as a control, at 100 µM. At 48 h after stimulation, 0.5 µCi/well of [^{3}H]-thymidine was added and incubated for 8 h in a cell culture incubator. Supernatants were removed, and cells were fixed in 5% trichloroacetic acid (TCA) at 4°C for 10 min. Cells were washed twice with TCA and methanol, and further disrupted by addition of 40 µl/well of 0.2 M NaOH. The scintillation solution Micro Scint 40 was added at 150 µl/well for counting by the Top Count NXT^{TM} microplate scintillation and luminescence counter. Cell mitogenesis was expressed as a percentage of the control with medium alone.

2.2.4.3 Cytotoxicity assays

Cytotoxicity was assessed by measurement of lactate dehydrogenase (LDH) which is a stable cytosolic enzyme that is released upon cell lysis. The colorimetric LDH activity assay kit was used to determine LDH in cell supernatants. Results were expressed as a percentage of the LDH measured in cell lysates.

2.2.5 Cytokines and mediator synthesis and release

Fibroblasts were cultured to confluency as described above. Cells were detached by trypsin-EDTA and a 2×10^5 cell/ml stock prepared. The preparation was added to a 6 well culture plate with or without addition of TGF-β_2 (1 ng/ml). At 72 h, cells were washed twice with HBSS and serum free medium added (DMEM with ITS and PSG). After 24 h in serum free medium, the stimulus (prepared in serum free medium) was added to cells. In cell stimulation experiments, medium alone was added as the negative control, and 10% FCS and 1 ng/ml TNF-α as positive controls. Tryptase, PAR-2 activating peptides and appropriate controls were added at concentrations similar to these used in cell proliferation studues. For other experiments, cell supernatants were collected 24 h after the stimulation.

2.2.5.1 Extraction and sampling of mRNA

After removing supernatant, cells were washed twice with HBSS and total RNA was extracted from cultured cells using Trizol according to the manufacturer's instructions (GIBCO BRL). The preparation of Trizol lysed cells was mixed with

chloroform, and RNA was extracted in the aqueous phase after centrifugation at 12,000 × *g* for 15 min. The RNA was precipitated with isopropanol, incubating at -20°C for 30 min, and then centrifuged at 12,000g for 15 min. The RNA pellet was washed with 80% cold ethanol (v/v) twice and air dried. The RNA pellet was resuspended in H_2O and incubated with DNase for 1h at 37°C. DNase activity was inhibited by the addition of "stop solution" and DNase inactivated by incubating at 65°C for 10 min. The concentration of RNA was assessed by measuring the absorption at 260 nm, and the purity was assessed by measuring OD_{260}/OD_{280}. RNA was reverse transcribed into cDNA using the Reverse Transcription System according to the manufacturer's instructions (Promega). One μg of RNA was reverse transcribed using reverse transcriptase AMV, and the oligo d(T) primer incubated with dNTP and RNase inhibitor at 42°C for 60 min. All enzymes were inactivated by incubation at 94°C for 3 min.

2.2.5.2 Quantitative PCR for cytokine mRNA expression (TaqMan PCR)

The expression of cytokine mRNA in response to different stimuli was assessed by measuring the release of fluorescence in quantitative PCR (Fig. 2.2.1). TaqMan PCR was performed using an ABI PRISM ® 7700 Sequence Detection System with thermal cycle conditions of 50°C for 2 min, 95°C for 10 min and 50 cycles of 95°C for 15 sec / 60°C for 1 min.

The primers and probe set for GAPDH were from the published TaqMan PCR protocols (Applied Biosystems). Other primers and probes were designed by Primer Express software, with restrictions of product size (≈ 70 bp), annealing temperature of primers (≈ 60°C) and probe (≈ 70°C), % of GC in the range of 20-80%, and no runs of more than three consecutive G (Table 2.2.2). The GAPDH probe was

conjugated with VIC fluorophore, and other probes were conjugated with FAM (6-carboxyfluorescein) fluorophore. The cDNA sample was added to PCR master mix with 900 nM primers and 250 nM probe. The efficiency and the limit of detection of each set of primers and probes was investigated by performing serial dilutions of the template. The efficiency was confirmed to be similar with gradients of curves from -3.15 to -3.31, and an efficiency of 100% to 108%. The specificity of each set of primers was confirmed by the presence of a product of the predicted size on the agarose gel.

Expression of mRNA was standardised with GAPDH and expressed relative to the medium control at each time point calculated according to the equation below, where Ct represents cycle number at threshold (based on amplification plot of fluorescence production during the PCR cycle), x and y represent different treatments of sample and z represents the target gene.

$$\Delta Ctx\ (z) = Ctx\ (z) - Ctx\ (GAPDH)$$

$$\Delta\Delta Cty\ (z) = \Delta Cty\ (z) - \Delta Ctx\ (z)$$

$$\text{relative value} = 2^{-\Delta\Delta Cty}$$

$$\%\ \text{of control} = \text{relative value} \times 100$$

For control

$$\Delta Cty\ (z) = \Delta Ctx\ (z)$$

$$\Delta\Delta Cty\ (z) = 0$$

$$\text{relative value} = 2^{-\Delta\Delta Cty}$$

$$= 2^{-0}$$

$$= 1$$

2.2.5.3 ELISA measurement of cytokine release

Cytokine release was measured in cell supernatants using specific enzyme linked-immunosorbant assays (ELISA) according to the manufacturer's instructions (Biosource). Polystyrene microtitre plates were coated with the monoclonal antibody specific for the cytokine of interest and sample added in a defined volume. The cytokine was detected by addition of biotinylated secondary antibody and streptavidin conjugated horseradish peroxidase added. Colour development using 25 mM TMB (tetramethyl benzidine) as the substrate was measured reading at 450 nm after the reaction was stopped by the addition of 2 M H_2SO_4. Cytokine concentrations were determined from a standard curve with the cytokine serially diluted in two fold series from a top concentration of 1 μg/ml. The limits of detection of the ELISA cytokines were in each case to be 10 pg/ml (the lowest standard giving an absorbance greater than that of the zero standard plus two standard deviations).

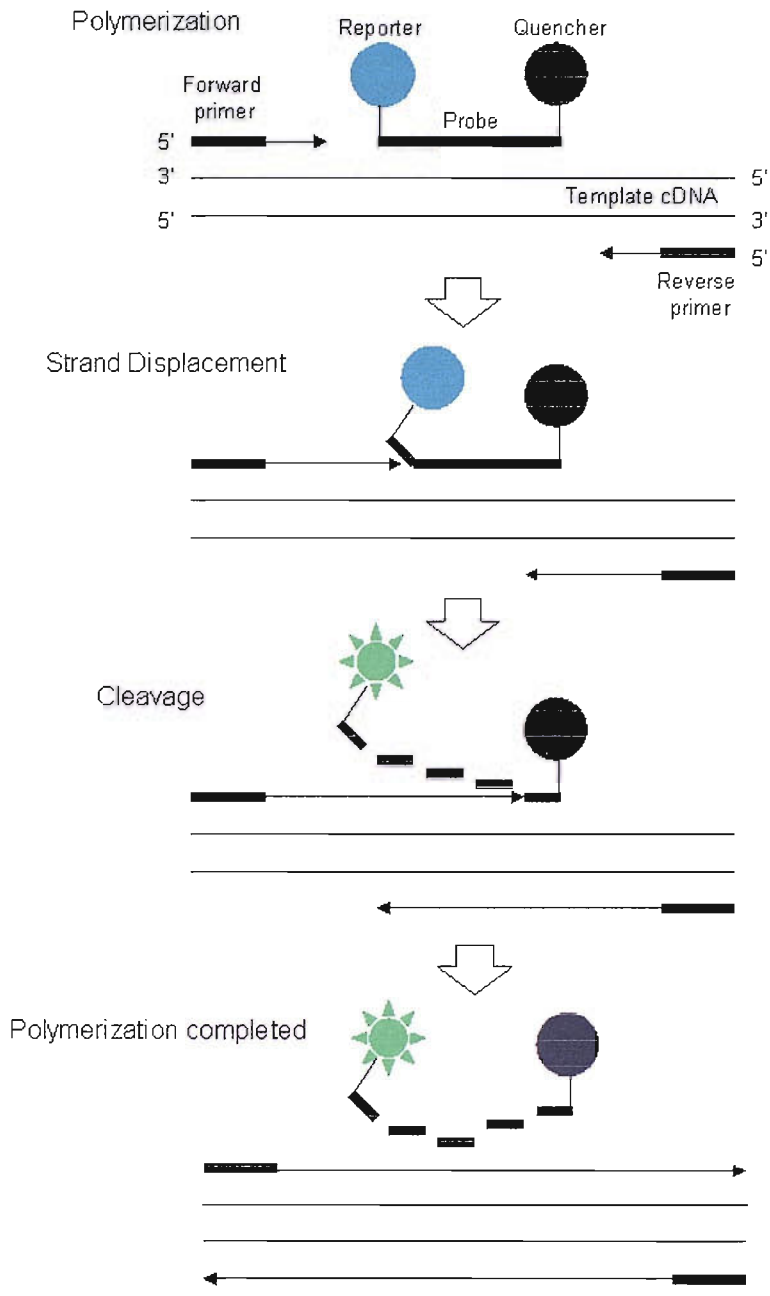


Fig. 2.2.1

Diagramatic illustration of the mechanism of fluorescent (reporter) release in quantitative real time PCR by 5'-3' nuclease activity of AmpliTaq Gold™ during one extension phase of PCR (adapted from Lyamichev *et al.* 1993)

2.2.6 Extracellular Matrix Protein Production and Release

2.2.6.1 Collagen assay

Fibroblasts were cultured to confluency as described above. Cells were detached by trypsin-EDTA and counted with trypan blue to prepare a 1×10^5 cell/ml stock in cell culture medium. Cells were seeded into a 24 well cell culture plate at 500 µl/well with or without TGF-β_2 (1 ng/ml). At 72 h following seeding, cells were washed twice with HBSS and the medium was changed to serum free media (DMEM with ITS and PSG). After maintaining for 24 h in serum free media, the stimulus (prepared in serum free media) was added to cells. In cell stimulation experiments, medium alone was added as the negative control, and 10% FCS and 5 ng/ml TNF-α as the positive controls. Tryptase and PAR-2 activating peptides and their controls were added as described for the cell proliferation studies. Following stimulation, 1 µCi/well of [^{3}H]-proline was added and cells incubated for 24 h. Cells were fixed in 5% trichloroacetic acid (TCA) at 4°C for 10 min and washed twice with TCA. Cell supernatants were treated with and without collagenase. Protein in supernatants was precipitated by addition of 20% TCA using gelatine as a carrier, and tubes were centrifuged at 12,000 × *g* for 15 minutes at 4°C. The pellet obtained was washed twice with 5% TCA. Cells and protein pellet were dissolved with 0.2 M NaOH and scintillation solution, Micro Scint 40, was added for counting using a Top Count NXT™ microplate scintillation and luminescence counter. Collagen production was expressed as a percentage of the medium control.

2.2.6.2 Zymography

A 10% SDS-polyacrylamide gel was prepared with 1 mg/ml gelatine. Cell supernatants were loaded as described above for SDS-PAGE without 2-mercaptoethanol and boiling. Trypsin was used as a positive control. The gel was washed twice with 2.5% Triton-X100 and incubated in the zymography buffer (50 mM Tris, 10 mM $CaCl_2$ and 1 μM $ZnCl_2$, pH adjusted to 7.5 with HCl) at 37°C overnight. Staining was with Coomassie Blue staining solution (0.25% Coomassie Brilliant Blue R250, 10% acetic acid, 45% methanol) and destained with 10% acetic acid and 25% methanol. Band density was quantified using GeneGenius software.

2.2.6.3 Western blotting

The protein was confirmed as MMP by western blotting using the MMP-2 and MMP-9 specific monoclonal antibodies as described earlier in tryptase characterisation. Protein was transferred from SDS-PAGE gel to a nitrocellurose membrane. The membrane was blocked with 1% BSA in PBST (PBS with 0.2% Tween-20) and incubated with anti-MMP-2 and anti-MMP-9 antibodies in 1:750. HRP-conjugated rabbit antibody against sheep immunoglobulins antibody was applied as the secondary antibody and the blot was exposed on ECL film using chemiluminescent substrate.

2.2.7 Polymerase chain reaction (PCR)

PCR was performed by GeneAmp PCR system 9600 on cDNA from purified total RNA of challenged cells using primer listed in Table. 2.2.2 and *Taq* DNA polymerase. For collagens αI and αIII, TIMP-1 and TIMP-2, a set of primers were used, which have been described earlier (Nusgens *et al.* 2001). Amplification conditions were as follows:

APRT primers

94°C for 2 min
30 cycles { 94°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec }
72°C for 5 min

PAR-2 primers

92°C for 2 min
30 cycles { 94°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec }
72°C for 5 min

All other primers

94°C for 2 min
30 cycles { 94°C for 15 sec; 66°C for 20 sec; 78°C for 10 sec }
72°C for 5 min

The amplified products and the base pair ladder marker were separated by electrophoresis on a 1.5% agarose TAE buffer gel with loading buffer. The bands were visualised by ethidium bromide under a UV illuminator and analysed using GeneGenius software. The result was standardised with the APRT signal.

2.2.8 Measuring release of intracellular calcium

Cells were detached from the culture vessel using a Ca^{2+} and Mg^{2+} free HBSS based non-enzymatic cell dissociation buffer. The cells were conditioned and loaded with dye, Fluo-3 acetoxymethyl ester (Fluo-3 AM) in challenge buffer (HBSS with Ca^{2+} and Mg^{2+} supplemented with 20 mM HEPES pH7.2, 0.1% BSA and 250 μM sulphinyrazone) in the dark. The acetoxymethyl side chain allows the dye to diffuse into the cells, and its cleavage by the endogenous de-esterified enzyme present in the cytoplasm stops the diffusion of dye from out of the cells. Sulphinyrazone was added to inhibit the transporter which actively pumped the dye out of the cells. Cells were suspended in a challenge buffer and aliquoted (1×10^6 cells in each cuvette) for challenge. The contents of cuvettes were mixed continuously using a magnetic stirrer. Fluorescence was measured at an excitation wavelength of 488 nm, and an emission wavelength of 530 nm using a fluorescence spectrophotometer. Fluorescence was monitored and the stimulus added to cells once a steady baseline was established. Calcium ionophore A23187 was added to cells to confirm the calcium releasability of cells, and 0.1% triton X-100 was added in order to lyse the cells to obtain the maximum calcium content.

Calcium concentration can be calculated from fluorescence (F) by the equation

$$[Ca^{2+}] = K_D \times \left[\frac{F_x - F_{min}}{F_{max} - F_x} \right]$$

K_D = calcium dissociation constant

= 390 nM for Fluo-3 AM (Nair *et al.* 2002)

F_{min} = 100 mM EDTA

F_{max} = 0.1% Triton X-100

2.2.9 Statistical analysis

Statistical analysis was performed using PRIZM software (GraphPad Software, CA, USA). When data were not normally distributed, the Kruskal-Wallis test for multiple comparisons was performed first, and if significant differences were detected Dunnett's test was applied to pre-planned comparisons of interest. Student's t test was used where sample were normally distributed. Spearman's coefficient (r_s) of rank correlation was applied to assess associations between treatments. Values of $P<0.05$ were considered significant.

Table 2.2.1

Clinical details of donors of fibroblasts

Non-asthmatics

Prep.	Age at Bronchoscopy	Gender	PC_{20} (mg/ml)	FEV_1 (%predicted)	Inhaled Steroid	β_2 agonist	Duration
1	21	M	>32	104	-	-	-
2	20	M	>32	118	-	-	-
3	28	F	>32	108	-	-	-
4	21	F	>32	97	-	-	-
mean	22.5		>32	106.8			

Asthmatics

Prep.	Age at Bronchoscopy	Gender	PC_{20} (mg/ml)	FEV_1 (%predicted)	Inhaled Steroid	β_2 agonist	Duration
1	20	F	5.70	83	N	Y	10
2	20	F	1.49	75	N	Y	6
3	46	M	0.04	67	Y	Y	nd
4	23	F	8.00	nd	nd	nd	nd
5	32	M	0.29	83	N	Y	8
6	40	F	1.10	110	Y	Y	33
7	29	F	7.97	97	Y	Y	nd
mean	30		3.51	74.2			

nd – no data

Table 2.2.2

TaqMan PCR primer probes

IL-6	
5' primer	179 AGCCGCCCCACACAGA
3' primer	250 CCGTCGAGGATGTACCGAAT
probe	203 CACCTCTTCAGAACGAATTGACAAACA

IL-8	
5' primer	48 GAAGGAACCATCTCACTGTGTGTAA
3' primer	118 ATCAGGAAGGCTGCCAAGAG
probe	74 CATGACTTCCAAGCTGGCCGT

GM-CSF	
5' primer	394 CTTTCTGCTTGTCATCCCCTTT
3' primer	469 GGCTTGGCCAGCCTCAT
probe	430 TGGCCGGTCTCACTCCTGGACT

PCR primers

APRT	242 bp
5' primer	GCTGCGTGCTCATCCGAAAG
3' primer	CCTTAAGCGAGGTCAGCTCC

PAR-2	493 bp
5' primer	TGGATGAGTTTTCTGCATCTGTCC
3' primer	CGTGATGTTCAGGGCAGGAATG

collagen I	214 bp
5' primer	CCCACCAATCACCTGCGTACAGA
3' primer	TTCTTGGTCGGTTGGGTGACTCTGA

collagen III	207 bp
5' primer	GAGATGTCTGGAAGCCAGAACCAT
3' primer	GATCTCCCTTGGGGCCTTGAGGT

TIMP-1	170 bp
5' primer	CATCCTGTTGTTGCTGTGGCTTGAT
3' primer	GTCATCTTGATCTCATAACGCTGG

TIMP-2	155 bp
5' primer	CTCGCTGGACGTTGGAGGAAAGAA
3' primer	AGCCCATCTGGTACCTGTGGTTCA

Chapter 3

Results

3 Results

3.1 Preparation of tryptase

There was difficulty in obtaining human lung as a source of tryptase, and recombinant tryptase was more readily available. For this reason the effects of recombinant tryptase rather than human lung tryptase were investigated in these studies. Recombinant tryptase also has the advantage of being less likely to be contaminated with endogenous human stimuli of cell function.

3.1.1 Purification and characterization of recombinant tryptase

Two preparations of recombinant tryptase were purified from high salt extracts of a *Pichia pastoris* expression system using butyl agarose and S-300 gel chromatography (Fig. 3.1.1). There were double bands on the silver stained SDS-PAGE gel for both preparations at approximately 35 kDa and 32 kDa for the first preparation and 33 kDa and 30 kDa for the second (Fig. 3.1.2). The two bands from both preparations were confirmed as tryptase by western blotting with tryptase specific antibody AA5 (Fig. 3.1.2). The enzymatic activity was calculated according to the equation:

$$\%\ \text{of activation} = \frac{\text{molar concentration of active tryptase } (\mu M) \times 10^{-6}\ \text{mol}/\mu\text{mol} \times 100\%}{\left(\frac{\text{protein concentration (mg/ml)}}{\text{molecular mass of tryptase (g/mol)}}\right)}$$

An activity as 133% was found for the first preparation, and 106% for the second preparation on the basis of active site titration measured using a known concentration of 4-methylumbelliferone as a substrate and the molecular mass calculated from a silver stained SDS-PAGE gel. The specific activities of the two tryptase preparations were 1.4 U/mg and 2.2 U/mg with BAPNA as substrate.

Bands of low molecular weight were observed on SDS-PAGE for the second preparation of recombinant tryptase purified, which were not detected by the tryptase-specific antibody AA5, on western blotting. For this reason, the tryptase was subjected to further purification on a heparin agarose column. Tryptase failed to elute with a NaCl concentration gradient of 0.4 M to 2.0 M and the NaCl concentration was increased stepwise to 3.0 M and then 4.0 M when the tryptase eluted from the heparin agarose. However, this additional step still failed to remove the low molecular weight material from the preparation (data not shown).

The activity of the first preparation of purified recombinant tryptase decreased from 112 U/ml to 16 U/ml after a single freeze and thaw cycle, possibly due to precipitation of protein with an extremely high concentration of the tryptase. The second preparation of recombinant tryptase was concentrated down to 3.4 U/ml (45 μM) and did not lose its activity after a cycle of freezing and thawing.

3.1.2 Purification and characterization of human lung tryptase

Tryptase was isolated to high purity from high salt extracts of human lung tissue. Little tryptic activity was detected in the low salt buffer extracts. Maximal tryptic activity was eluted from the heparin agarose column at approximately 1.0 M NaCl, and it was associated with the peak of protein content. On SDS-PAGE, there was a

clear single band on SDS-PAGE with silver staining corresponding to a molecular mass of approximately 34 kDa (Fig. 3.1.2). The identity of the band was confirmed as tryptase by western blotting with tryptase-specific antibody AA5 (Fig. 3.1.2). The percentage of enzymatic activity calculated for the purified preparation was 110% on the basis of active site titration. The specific activity of the tryptase was 2.1 U/mg with BAPNA as substrate. There was no significant enzymatic activity detected for chymase and elastase using the substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide respectively.

3.1.3 Heat inactivation of tryptase

In studies designed to investigate the dependence of tryptase's actions on an intact catalytic site, tryptase was heat inactivated at 94°C for 5 minutes and at 60°C for 30 minutes (Table 3.1.1). The activity of recombinant tryptase and human lung tryptase against the BAPNA was reduced by heat inactivation in all experiments. The recombinant tryptase was more resistant to a high temperature treatment for a short period than the purified human lung tryptase.

Summary

The recombinant β-tryptase isolated from a *Pichia pastoris* expression system was characterised in detail and was demonstrated to have similar enzymatic and physicochemical properties to tryptase purified from human lung tissue.

Table. 3.1.1

Elution conditions, molecular weight, and effect of heat treatment on preparations of recombinant and human lung tryptase.

	Recombinant Tryptase β	Human Lung Tryptase
[NaCl] required for elution from heparin	4.0 M	1.0 M
Molecular mass	35 / 33 kDa (multiple bands)	34 kDa (single band)
Activity lost by heat inactivation		
at 94°C for 5 minutes	65%	99%
at 60°C for 30 minutes	97%	99%

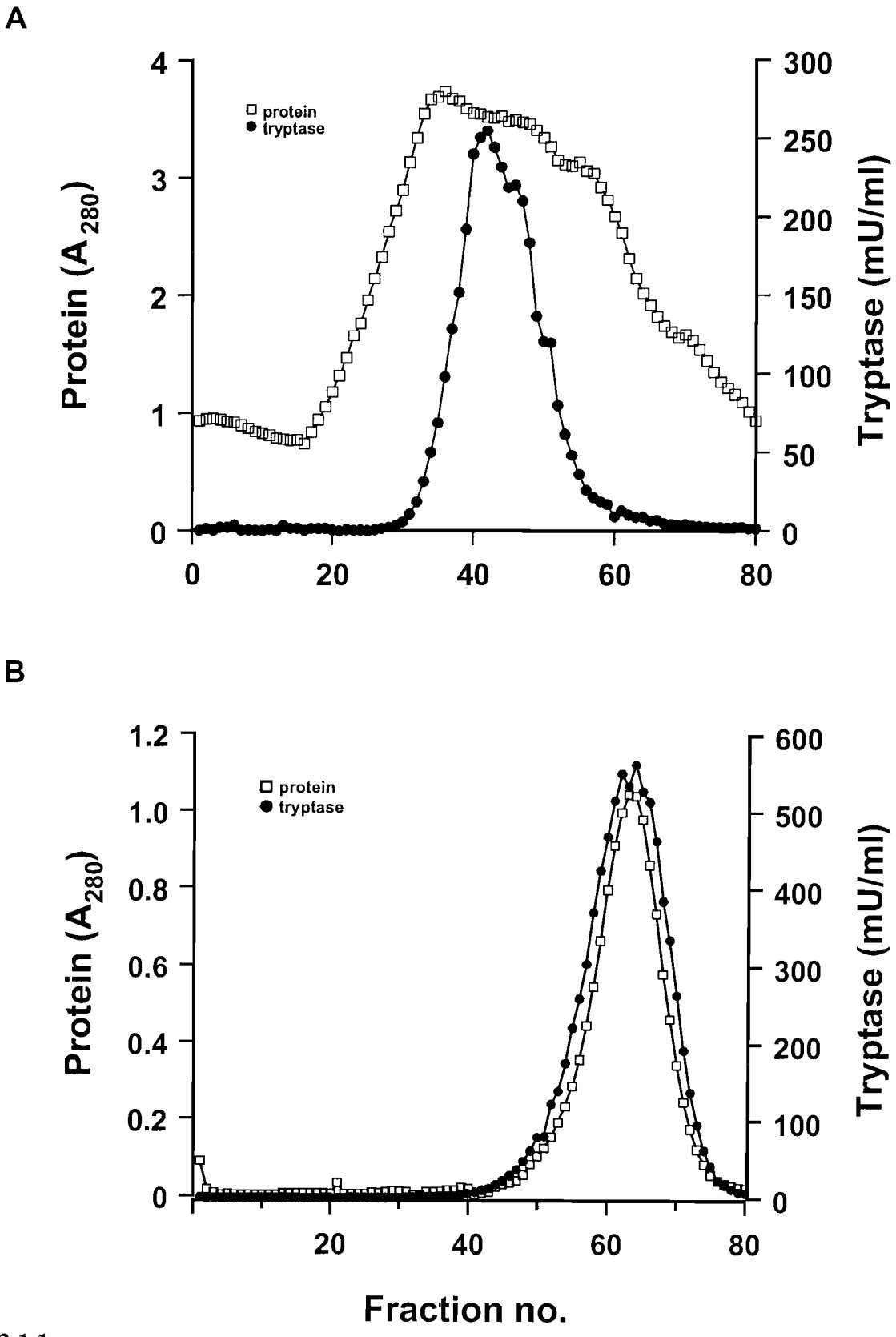


Fig. 3.1.1

The tryptic activity and absorbance of fractions of recombinant tryptase from (A) butyl agarose affinity chromatography and (B) S-300 gel filtration.

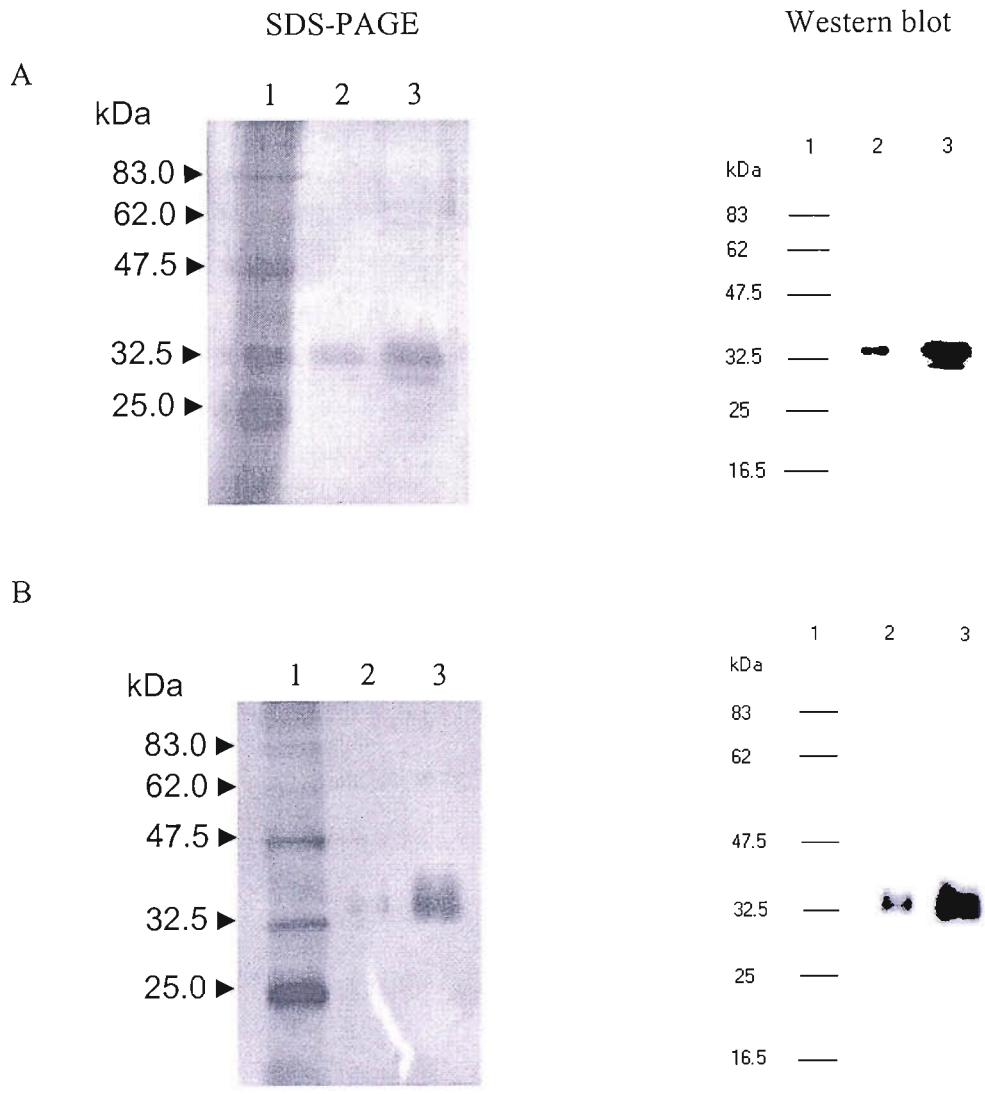


Fig. 3.1.2

(A) Purified recombinant tryptase and (B) human lung tryptase on silver stained 10% SDS polyacrylamide gels and on a matching western blot with tryptase-specific antibody, AA5. Lane 1: molecular weight standards, lane 2: tryptase (1ng/ml protein), lane 3: tryptase (5 ng/ml protein).

3.2 Fibroblast and myofibroblast cultures

No staining for α-smooth muscle actin was detected in fibroblasts cultured from bronchial biopsy tissue, but treatment with TGF-β_2 stimulated expression of actin in a high proportion of cells (Fig. 3.2.1). Incubation with serum free medium for 48 h (without TGF-β_2) after the TGF-β treatment did not alter the degree of α-smooth muscle actin expression in myofibroblasts. Fibroblasts either with or without TGF-β_2 stimulation stained positively for the fibroblast marker vimentin, but were negative for the smooth muscle cell marker, myosin, after 120 h incubation (Fig. 3.2.2). There was no staining observed with the isotype-matched control antibodies applied.

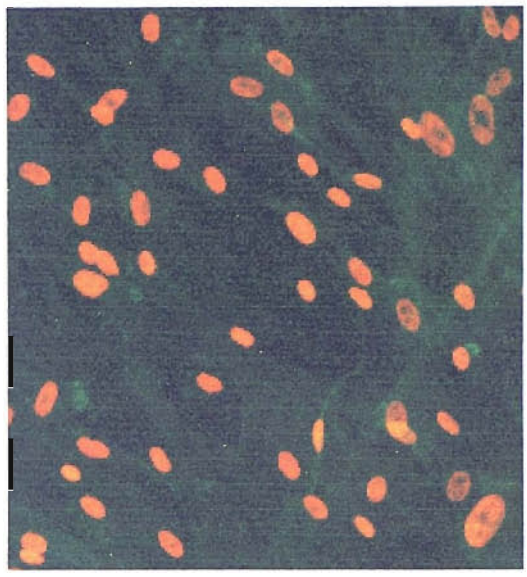
Efficiency of the transformation from a fibroblast to a myofibroblast phenotype was investigated. It was estimated that 73% ± 17% (mean ± SD) (n=10) of fibroblasts were transformed into the myofibroblast phenotype following incubation with TGF-β for 72 h as assessed by expression of α-smooth muscle actin.

The potential for a high concentration of TGF-β to exert an inhibitory effect on cell growth was suggested by Zhao & Young (1996). Therefore, the possible inhibitory effect of TGF-β on proliferation was examined in MRC-5 fibroblast line. At the concentration of TGF-β_2 used in these studies for transformation of fibroblasts into the myofibroblast phenotype (1 ng/ml), there was no inhibitory effect on cell proliferation (Fig. 3.2.3).

Summary

Human lung fibroblasts were successfully transformed into cells of a myofibroblast phenotype after incubation with TGF-β_2 for 72 h, and without inhibiting cell proliferation. The TGF-β_2-induced myofibroblasts clearly possessed a myofibroblast phenotype, and expressed α-smooth muscle actin and vimentin, but not smooth muscle myosin.

A



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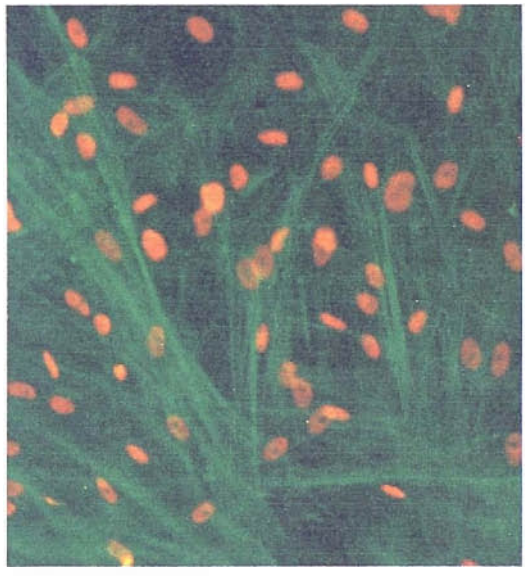
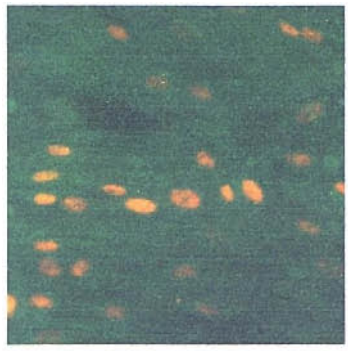


Fig. 3.2.1

Expression of α-smooth muscle actin in fibroblasts cultured from bronchial tissue (A) without and (B) with 1 ng/ml TGF-β_2 for 72 h followed by 48 h in serum free medium. Cells were immunostained with FITC conjugated α-smooth muscle actin specific monoclonal antibody, and counterstained with nuclear stain 7AAD.

A



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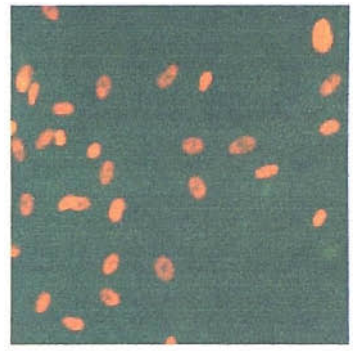


C

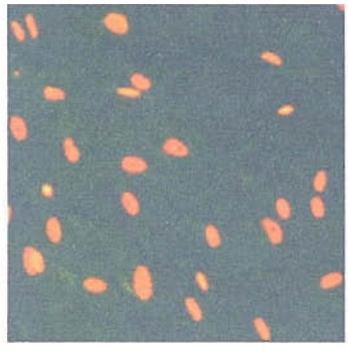


Fig. 3.2.2

Expression of the myofibroblast phenotype in fibroblasts cultured from bronchial tissue following incubation for 120 h with 1 ng/ml TGF-β_2. Cells were stained for (A) vimentin (B) myosin heavy chain and (C) isotype control using FITC conjugated monoclonal antibodies and counterstained with nuclear stain 7AAD.

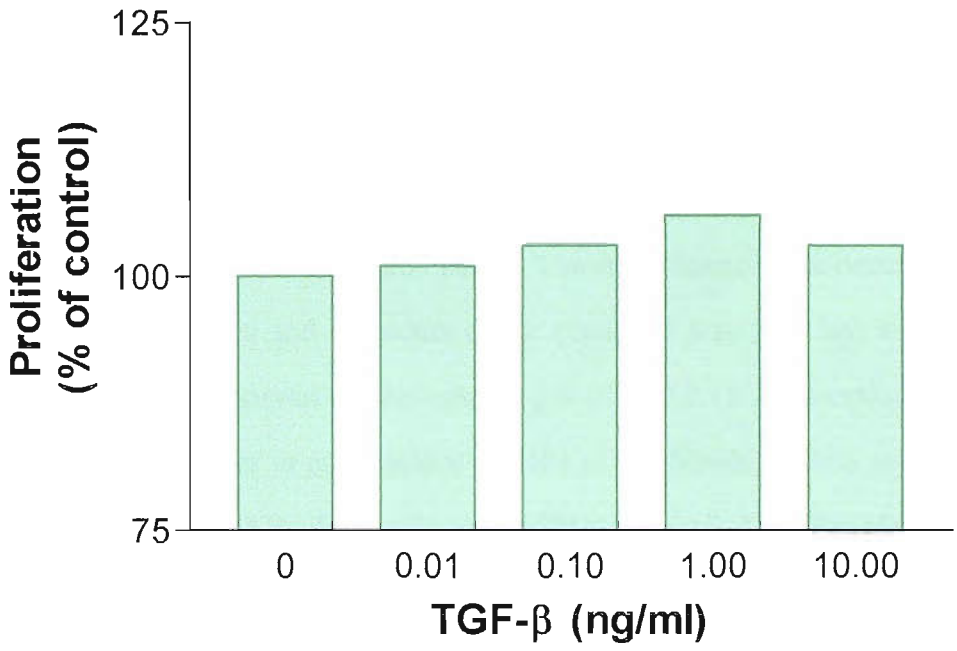


Fig. 3.2.3

Effect of TGF-β on proliferation of cells of the MRC-5 fibroblast line as assessed by the MTS assay. Relative numbers of cells are shown, 48 h after stimulation with TGF-β_2. Results represent the mean of a single experiment performed in triplicate.

3.3 PAR-2 expression in fibroblasts and myofibroblasts

3.3.1 mRNA expression of PAR-2

Total RNA was purified from stimulated primary cultured fibroblasts and myofibroblasts, and transcribed into cDNA. The amplification was performed using PAR-2 specific primers and a product of the predicted size (493 bp) was obtained from the PCR and observed on the agarose gel (Fig. 3.3.1). Expression of PAR-2 was significantly higher in preparations of cells of the fibroblast than myofibroblast phenotype (Fig. 3.3.3(A)). Expression of mRNA for PAR-2 in fibroblasts was not correlated with that in the corresponding myofibroblast preparations (r_s=0.460: p=0.077: n=11).

3.3.2 Detection of PAR-2 by immunocytochemistry

Expression of PAR-2 was observed on fibroblasts and myofibroblasts that had been cultured in chamber slides, by immunocytochemistry with the PAR-2 specific monoclonal antibody P2A (Fig. 3.3.2). Expression of PAR-2 was evenly distributed over the surface of cells. Permeablisation of cells indicated the presence of PAR-2 within the cytoplasm. No immunostaining was observed with an isotype-matched control antibody (IgM) or by P2A antibody pre-absorbed with its epitope peptide, TNRSSKGRSLIGKV. There was no difference between fibroblasts and myofibroblasts in the degree of PAR-2 staining observed (n=9) when the staining intensity (adjusted to take account of cell numbers) was compared using a computerised analysis system (Fig. 3.3.3 (B)). Expression of PAR-2 detected in permeablised was correlated with that in non-permeablised cells in both fibroblasts (r_s=0.583: $p<0.05$: n=9) and myofibroblasts (r_s=0.6167: $p<0.05$: n=9).

3.3.3 Induction of calcium flux by PAR-2 agonists

Expression of functional PAR-2 was examined by measuring the calcium influx following addition of peptide agonists of PARs. The PAR-2 agonist peptide SLIGRL-NH_2 at 100 μM induced a calcium influx of 244 nM in primary cultures of myofibroblasts (Fig. 3.3.4 (A)). The response was absent following a second addition of the peptide. A calcium influx was induced also in response to the PAR-1 agonist peptide TFLLR-NH_2, but not to the PAR-4 agonist peptide ATPGKF-NH_2. The non PAR-2 activating peptide LSIGRL-NH_2 did not induce a calcium influx (Fig. 3.3.4 (B)). Surprisingly, there was no response to recombinant tryptase at 10 mU/ml and 30 mU/ml (Fig. 3.3.4 (C)) or to tryptase purified from human lung at 30 mU/ml. Stimulation of cells with calcium ionophore A23187 was associated with a clear increase in calcium flux (data not shown).

Summary

The preparations of lung fibroblasts and myofibroblasts both expressed PAR-2 mRNA and protein. The receptors are functional on myofibroblasts but not activated by tryptase.

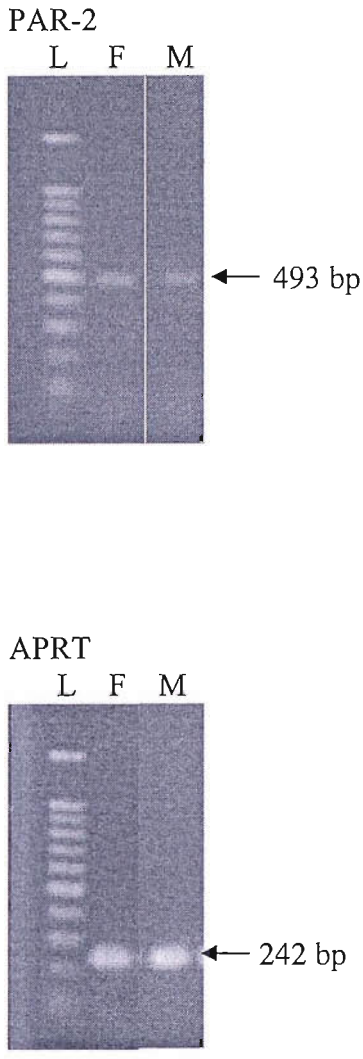


Fig. 3.3.1

PCR products for mRNA for PAR-2 in primary cultures of fibroblasts (F) and myofibroblasts (M). Lane L is 100 bp ladder marker.

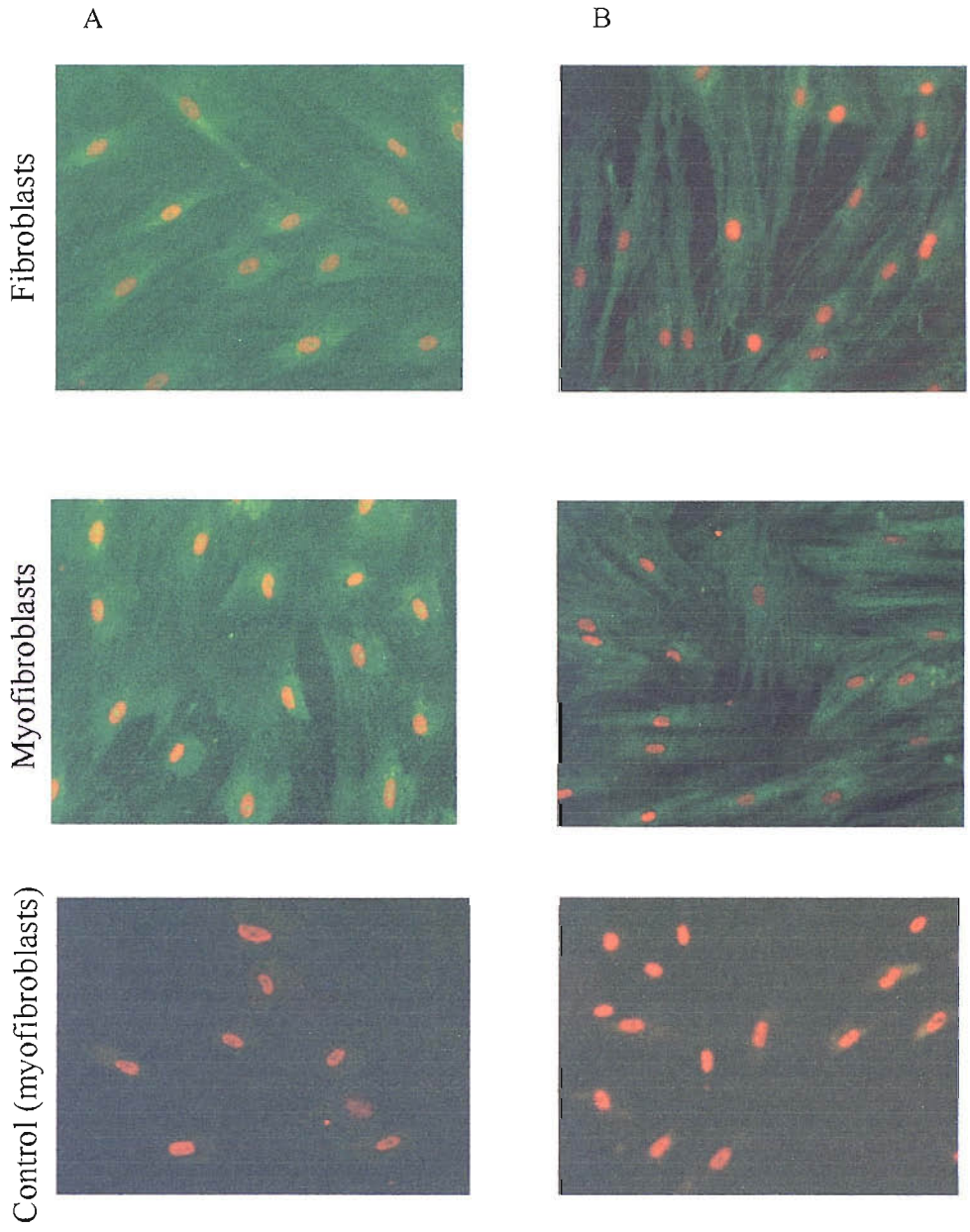


Fig. 3.3.2

Immunostaining of PAR-2 with specific antibody P2A by FITC in (A) permeablised and (B) non-permeablised primary cultures of human bronchial fibroblasts and myofibroblasts. Nuclei were stained with 7AAD. In the control slides, P2A antibody was pre-absorbed with the peptide, TNRSSKGRSLIGKV whose sequence corresponds to that of the rejoin of PAR-2 to which the antibody is directed.

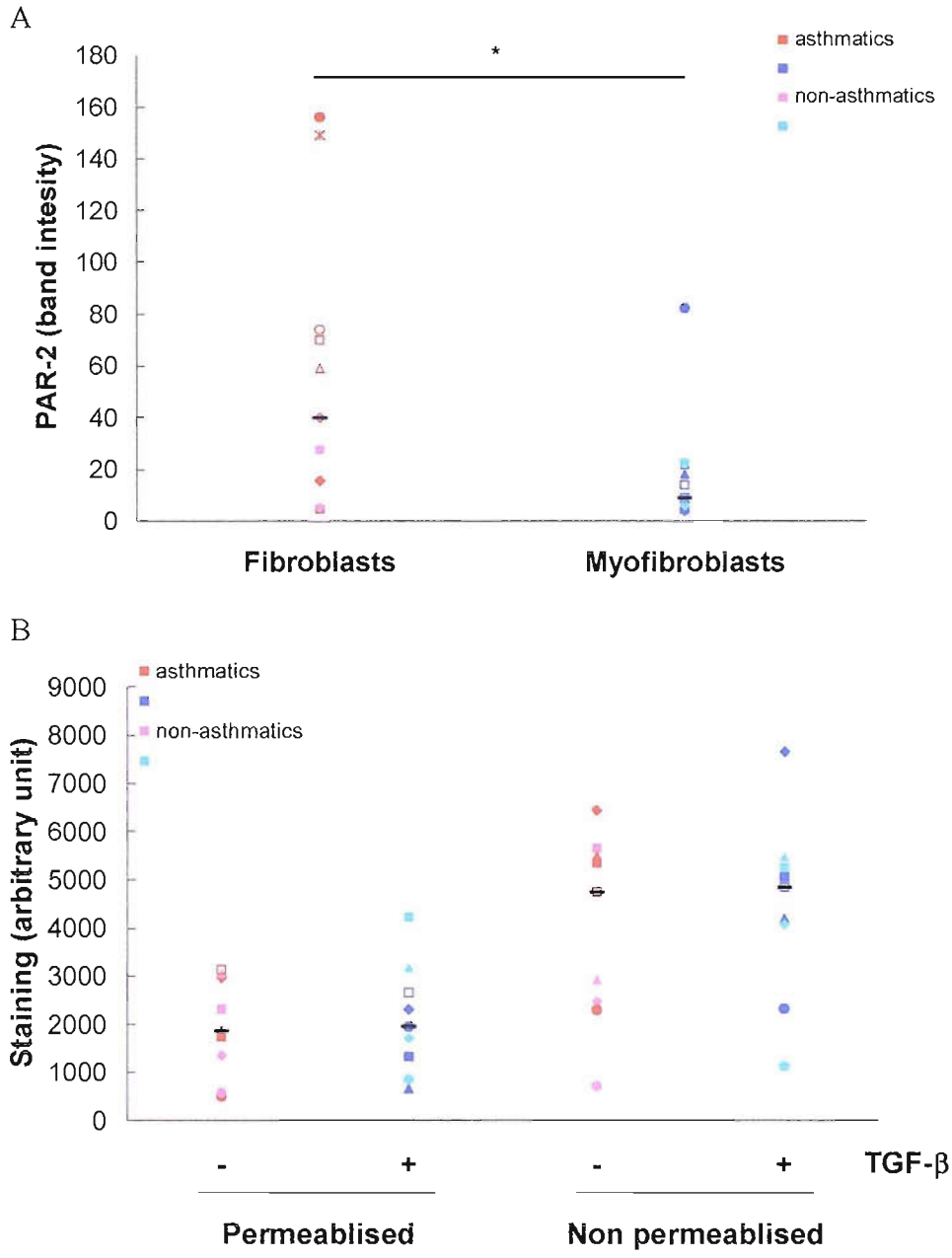


Fig. 3.3.3

Expression of PAR-2 in primary cultures of fibroblasts and myofibroblasts. (A) mRNA expression of mRNA assessed by RT-PCR (normalised to APRT), and (B) the degree of receptor expression detected by immuocytochemistry with the PAR-2 specific monoclonal antibody P2A (adjusting for cell number). Median values are shown for 9 to 11 experiments. *P<0.05 by student's t test.

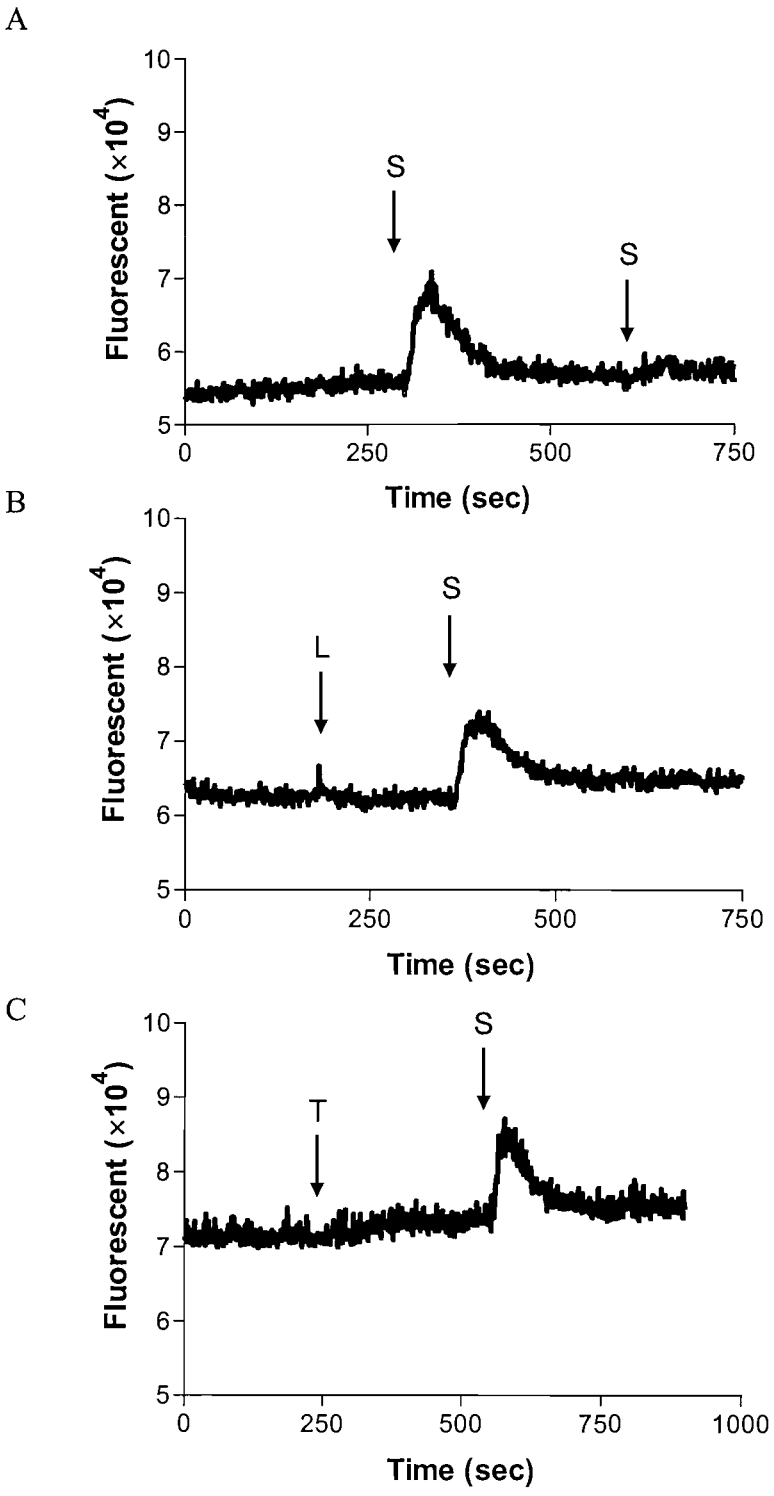


Fig. 3.3.4

Intracellular calcium flux fluorescence measurements in primary cultures of myofibroblasts. Cells were stimulated with 100 μM PAR-2 agonist peptide, SLIGRL-NH_2 (S), 100 μM control non-PAR-2 activating peptide, LSIGRL-NH_2 (L), and 30 mU/ml recombinant tryptase (T).

3.4 Fibroblast and myofibroblast mitogenesis and proliferation

The rate of mitogenesis in cells was assessed by measurement of [^{3}H]-thymidine incorporation, and cell proliferation by measurement of MTS bioreduction to formazan by the dehydrogenase enzymes found in metabolically active cells. In seeking to optimize the methods for use in these studies, various experimental conditions were employed, and different approaches adopted to evaluate tryptase as a stimulus for cell proliferation.

Cultures of the MRC-5 foetal lung fibroblast cell line were employed in initial experiments for reasons of consistency and because they were more easy to obtain than primary cultures of fibroblasts. Particular attention was directed to reducing the degree of variation between replicates. Avoiding wells at the edge of the microplate proved effective in reducing variability, and in further experiments the outer 36 wells were excluded. Seeding cell density was also altered. In early experiments, cells were seeded at 1×10^5 cells/ml, as this density has been used widely in previous fibroblast cell proliferation studies (Cairns & Walls 1997, Akers *et al.* 2000). However, at this cell density some evidence was found for contact inhibition reducing the rate of cell proliferation. For this reason, cell density was reduced to 5×10^4 cells /ml in all wells. The effects of recombinant tryptase on the rate of mitogenesis and cell proliferation were similar to that of native lung tryptase added at the same concentration to fibroblasts or myofibroblasts (Fig. 3.4.1). However, human lung tryptase stimulated proliferation of fibroblasts, where as recombinant tryptase did not. There was a trend for an increase in mitogenesis but this did not reach significance with either preparation of tryptase. The rate of proliferation of MRC-5 cells without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts) was not significantly increased by addition to cells of tryptase or the peptide agonist of PAR-2 (Fig. 3.4.2).

3.4.1 Mitogenic responses of fibroblasts and myofibroblasts

Mitogenesis in primary cultures of fibroblasts and TGF-β_2-induced myofibroblasts were observed by [^{3}H]-thymidine incorporation. The optimum time to measure mitogenesis was determined as 48 h after stimulation with FCS and EGF (Fig. 3.4.3). These were employed as positive controls as they are well established as strong stimuli for fibroblast and myofibroblast mitogenesis.

Fibroblast mitogenesis was induced by tryptase at concentrations ranging from 3 mU/ml to 30 mU/ml (Fig. 3.4.4 (A)). However there was no significant stimulation of mitogenesis with 100 mU/ml tryptase. Similarly mitogenesis was induced in myofibroblasts, with a bell-shaped response with a peak at 30 mU/ml tryptase (Fig. 3.4.4 (B)).

Tryptase activity towards the chromogenic substrate BAPNA was inhibited by heat inactivation or by preincubation of the enzyme with the protease inhibitors leupeptin or APC1390. The mitogenic activity of tryptase was significantly inhibited by APC1390 but not by the less potent inhibitor leupeptin (Fig. 3.4.5). When tryptase was heated at 60°C for 30 minutes using a thermocycler, the enzymatic activity was reduced to less than 3% of that of the non-heated control. Surprisingly the heat treatment did not significantly reduce the mitogenic activity of tryptase. The mitogenic response to active tryptase was correlated with that to the heat-inactivated tryptase (r_s=0.683: $p<0.05$: n=9) and also with that of APC 1390 inhibited tryptase (r_s=0.738: $p<0.05$: n=9) in fibroblasts.

Myofibroblasts exhibited a similar trend to that of the fibroblasts in these studies of inhibition, though the reduction in mitogenic activity with APC1390 did not reach significance. The mitogenic response to tryptase was not associated with that with

tryptase inactivated by heating or by addition of inhibitors, though responses to APC1390 and leupeptin inhibited tryptase were correlated (r_s=0.829: $p<0.05$: n=6). At the concentrations employed, addition of leupeptin and APC1390 had no effect on cell mitogenesis, either when these inhibitors were added alone or when added with FCS as the stimulus (data not shown).

The PAR-2 agonist peptide, SLIGKV-NH_2 stimulated significant mitogenesis in fibroblasts at the highest concentration employed (100 μM) (Fig. 3.4.6). This effect did not reach significance with myofibroblasts though there was considerable variation between cell preparations in the responses seen. The control peptide, LSIGKV-NH_2 did not stimulate mitogenesis in fibroblasts or myofibroblasts.

3.4.2 Cell proliferation responses

Cell proliferation of fibroblasts and myofibroblasts in response to tryptase and PAR-2 agonist were assessed by the MTS assay. Cell proliferation was observed from 24 h following the addition of FCS and EGF and the greatest difference between stimulated and non-stimulated cells in the cell proliferation response was observed at 48 h (Fig. 3.4.7). These positive control stimuli both induced fibroblast and myofibroblast proliferation, although EGF failed to stimulate a significant myofibroblast proliferative response. The rates of proliferation of fibroblasts and myofibroblasts were not significantly increased by addition to cells of tryptase or the peptide agonist of PAR-2 (Fig. 3.4.8).

The reproducibility of the effect of different stimuli on primary cultures of fibroblasts and myofibroblasts were assessed using the cells of passage 6 and passage 7. The pattern of cell proliferation observed with these two cell preparations was

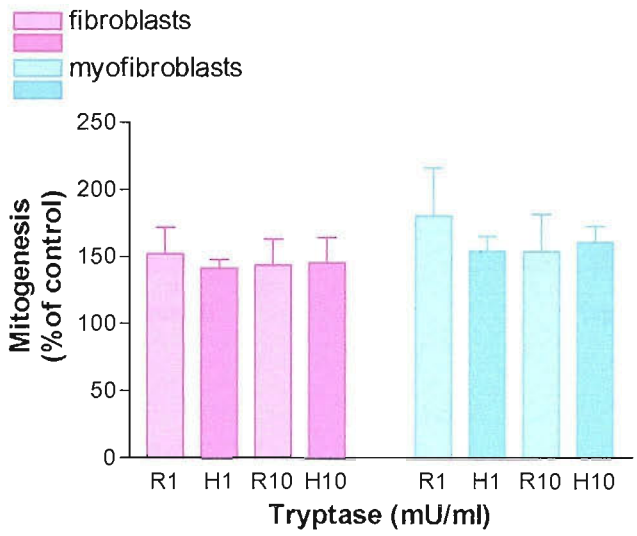
very similar, and the results were correlated. Difference in response cells from the different passages were of a minor nature (Fig. 3.4.9).

The potential for cytotoxic effects were investigated by measuring the levels of lactate dehydrogenase (LDH) in supernatants, and expressing them relative to those in the whole cell lysate. The various stimuli employed in the study did not lead to increased levels of LDH compared with those in controls at 48 h, and levels were consistently below 5% (n=3).

Summary

There were no major differences between human lung and recombinant tryptase as stimuli for mitogenesis and proliferation. Tryptase and the PAR-2 agonist peptide were without effect on mitogenesis and proliferation of cells of MRC-5 line. Tryptase was able to induce dose-dependent alterations in [^{3}H]-thymidine incorporation in both fibroblast and myofibroblast cultures, but this was not observed with the PAR-2 agonist peptide. Although there was significant mitogenesis, tryptase did not appear to be particularly potent in eliciting this effect. Neither tryptase nor the PAR-2 agonist peptide were able to stimulate cell proliferation.

A



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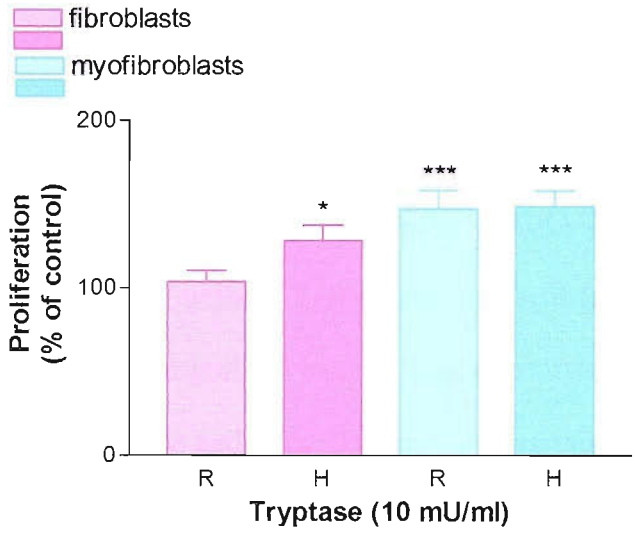


Fig. 3.4.1

Effect of human lung (H) and recombinant (R) tryptase at the concentration indicated on (A) mitogenesis, and (B) proliferation in cells of the MRC-5 cell line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). Mean ± SEM are shown for three separate experiments performed in triplicate. *P<0.05 and *** P<0.001compared with the response with no stimulus (taken as 100%) by Student's t test.

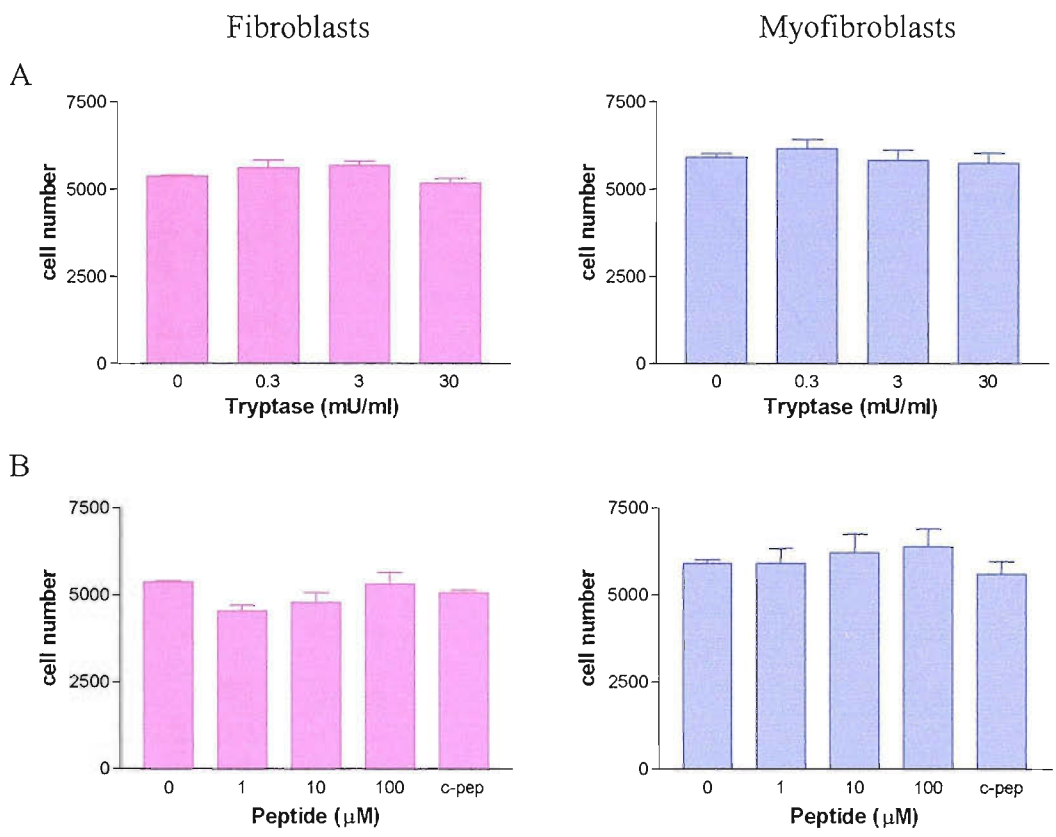


Fig. 3.4.2

Effect of tryptase and the PAR-2 agonist peptide SLIGKV-NH_2 on the proliferation of cells of the MRC-5 line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). Numbers of cells are shown, 48 h after stimulation with (A) tryptase and with (B) the PAR-2 agonist peptide SLIGKV-NH_2. The non-PAR-2 activating peptide, LSIGKV-NH_2 (c-pep) was included at 100 μM. Mean ± SEM are shown for three separate experiments performed in triplicate.

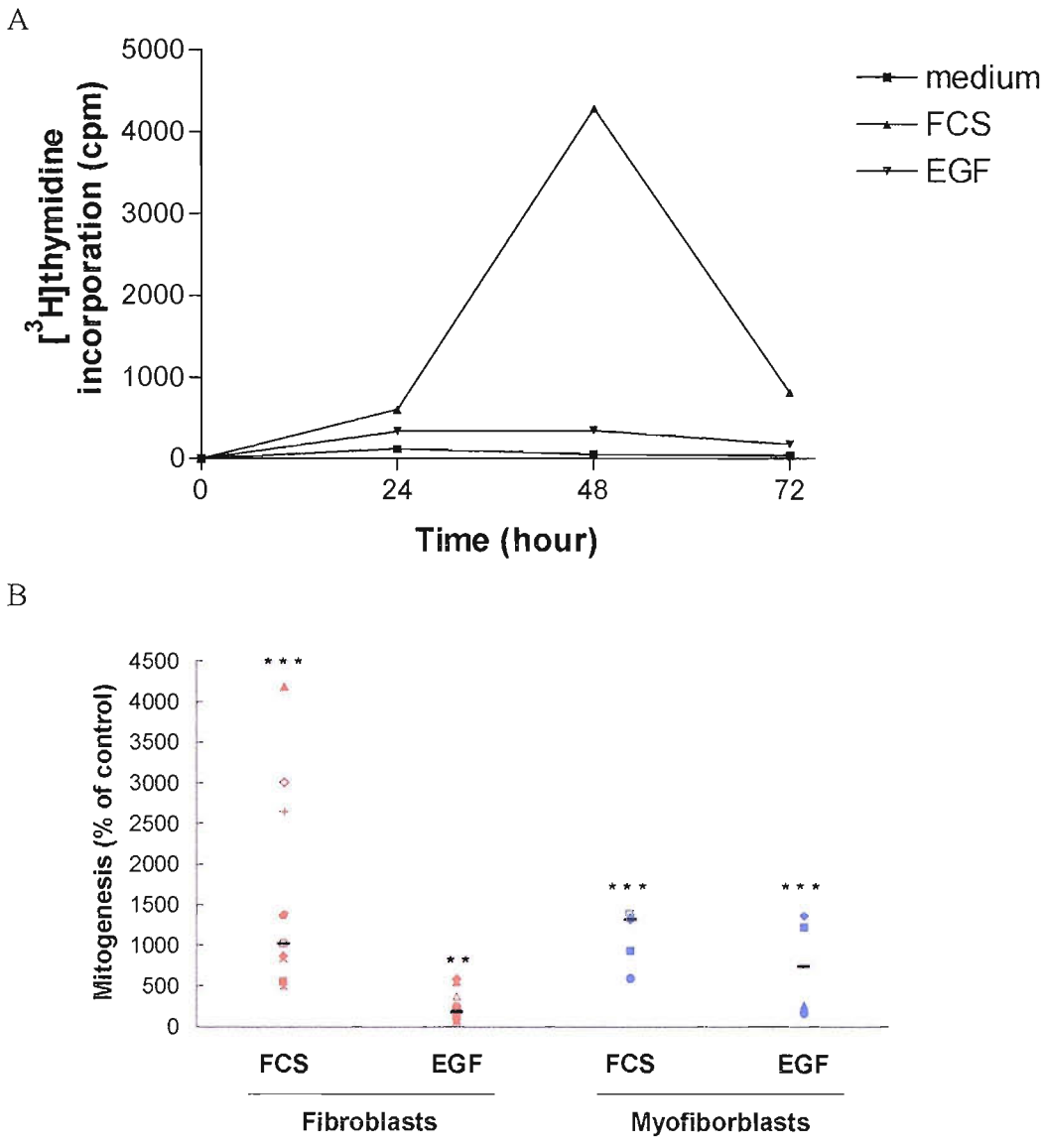
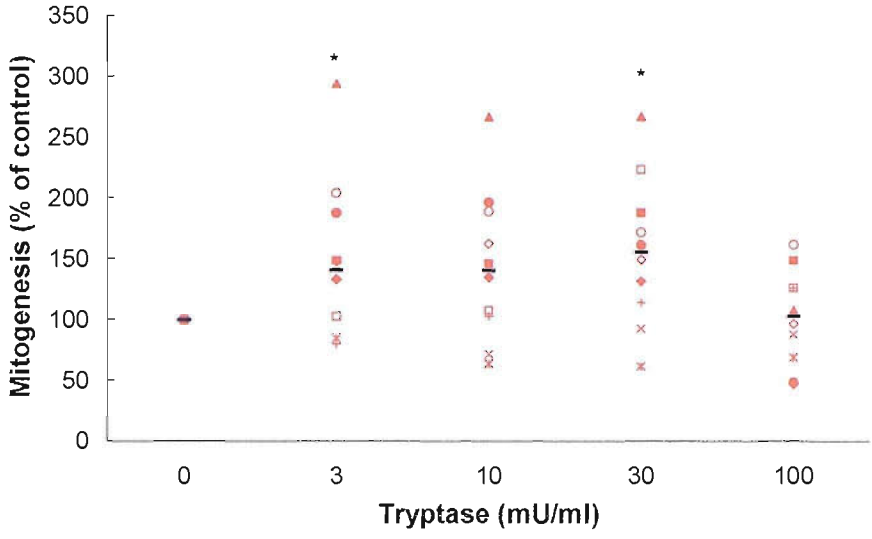


Fig. 3.4.3

(A) Time course of [^{3}H]-thymidine incorporation in primary cultures of fibroblasts following stimulation with 10% FCS or 5 ng/ml EGF represent triplicate determination of a single experiment. (B) Effect of FCS and EGF on fibroblast and myofibroblast mitogenesis 48 h after addition of 10% FCS or EGF 5ng/ml. Median values are shown for 4 to 10 experiments in triplicate. **P<0.01 and ***P<0.001 compared with no stimulus (taken as 100%) by Dunnett's test. The median [^{3}H]-thymidine incorporation in unstimulated fibroblasts was 138 (78-537) CPM and in the unstimulated myofibroblasts was 330 (129-344) CPM.

A



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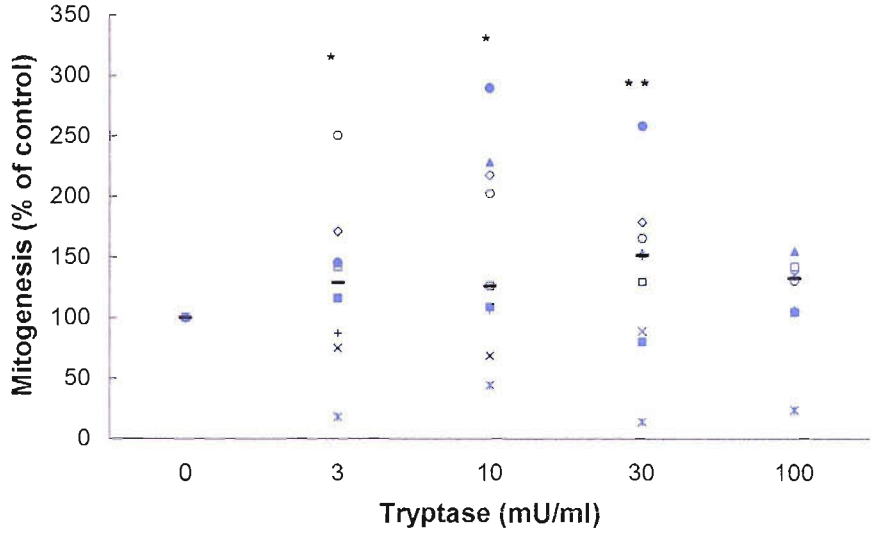


Fig. 3.4.4

Effect of tryptase on mitogenesis of (A) fibroblast preparations (n=9) and (B) myofibroblast preparations (n=6 to 8), 48 h after stimulation with tryptase. Median values are indicated. * $P<0.05$ and ** $P<0.01$ compared with response with no stimulus by Dunnett's test. The median [^{3}H]-thymidine incorporation in unstimulated fibroblasts was 138 (78-537) CPM and in the unstimulated myofibroblasts was 330 (129-344) CPM.

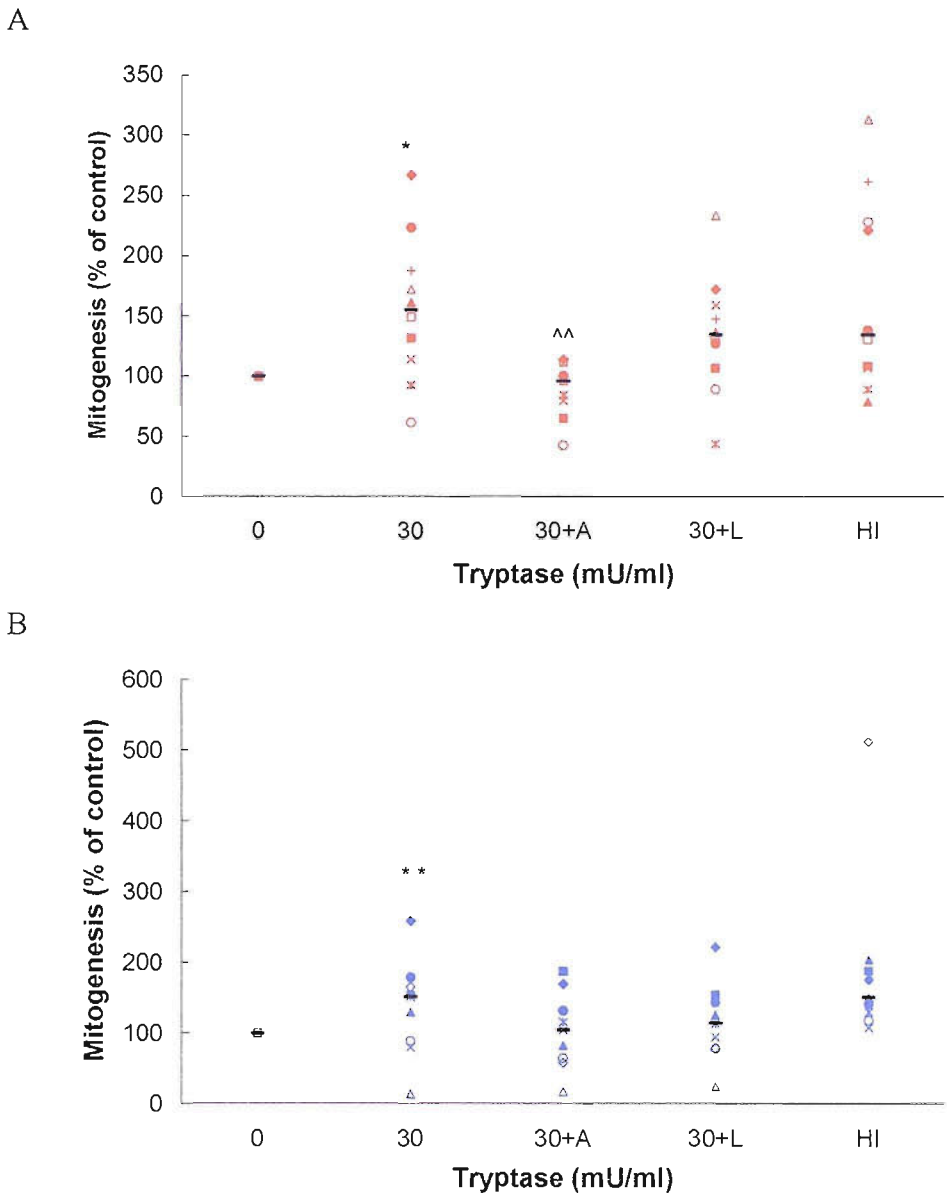
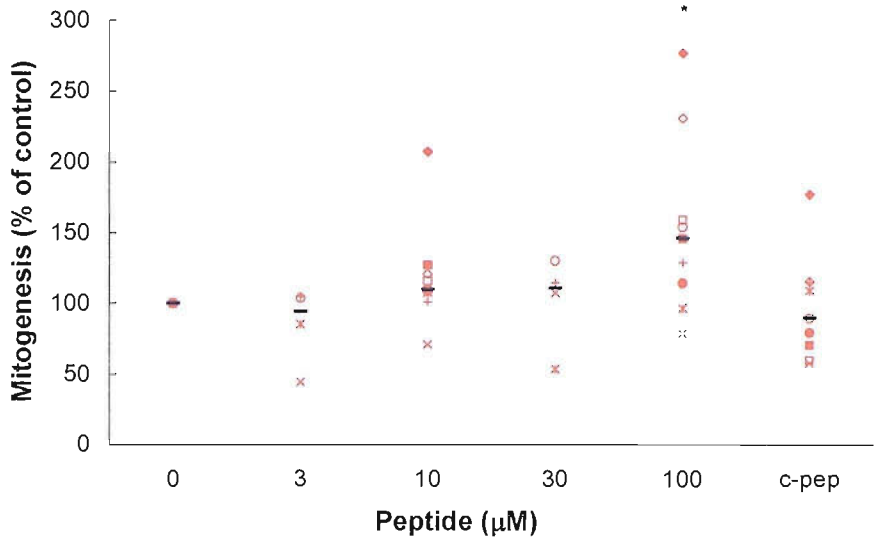


Fig. 3.4.5

Catalytic site dependency of the mitogenic actions of tryptase on (A) fibroblast preparations (n=8 or 9) and (B) myofibroblast preparations (n=6 or 7). The tryptase at 30 mU/ml was inhibited by preincubation for 30 minutes with 100 μM APC1390 (+A) or 50 μM leupeptin (+L), or heat inactivated (HI). Median values are shown. * $P<0.05$ and ** $P<0.01$ compared with the response with no stimulus, and ^^ $P<0.01$ compared with 30 mU/ml active tryptase by Dunnett's test. Refer to Fig. 3.4.4 for amount of radioactivity in the unstimulated controls.

A



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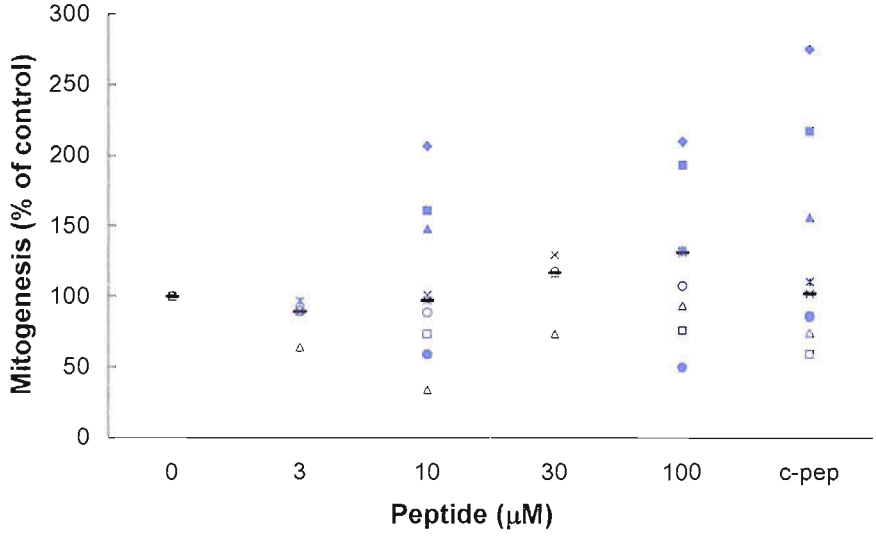
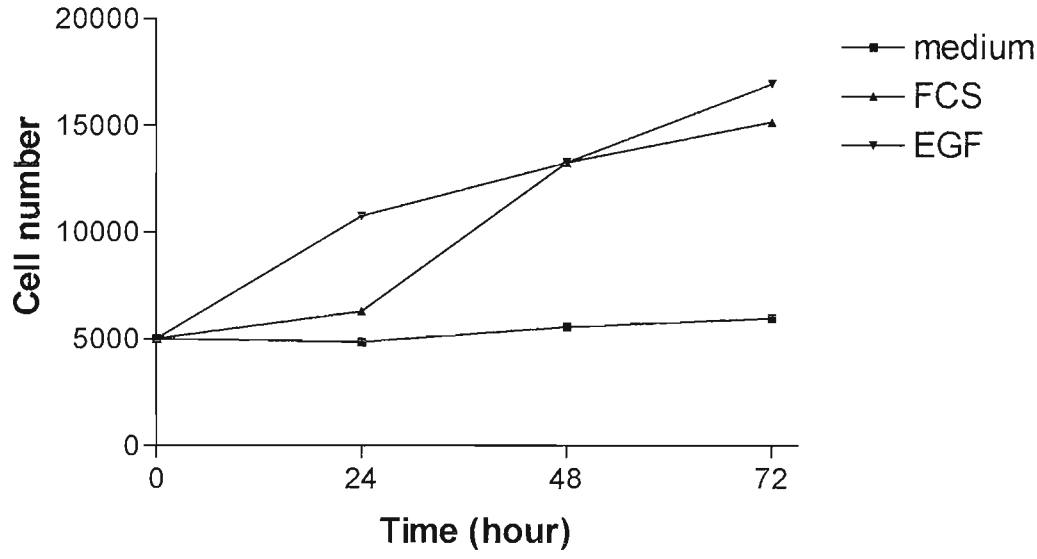


Fig. 3.4.6

Effect of the PAR-2 agonist peptide SLIGKV-NH_2 on (A) fibroblast and (B) myofibroblast mitogenesis at 48 h (n=4 to 9). The non-PAR-2 activating peptide LSIGKV-NH_2 (c-pep) was included at 100 μM. Median values are shown. * $P<0.05$ compared with response with no stimulus by Dunnett's test. Refer to Fig. 3.4.4 for amount of radioactivity in the unstimulated controls.

A



B

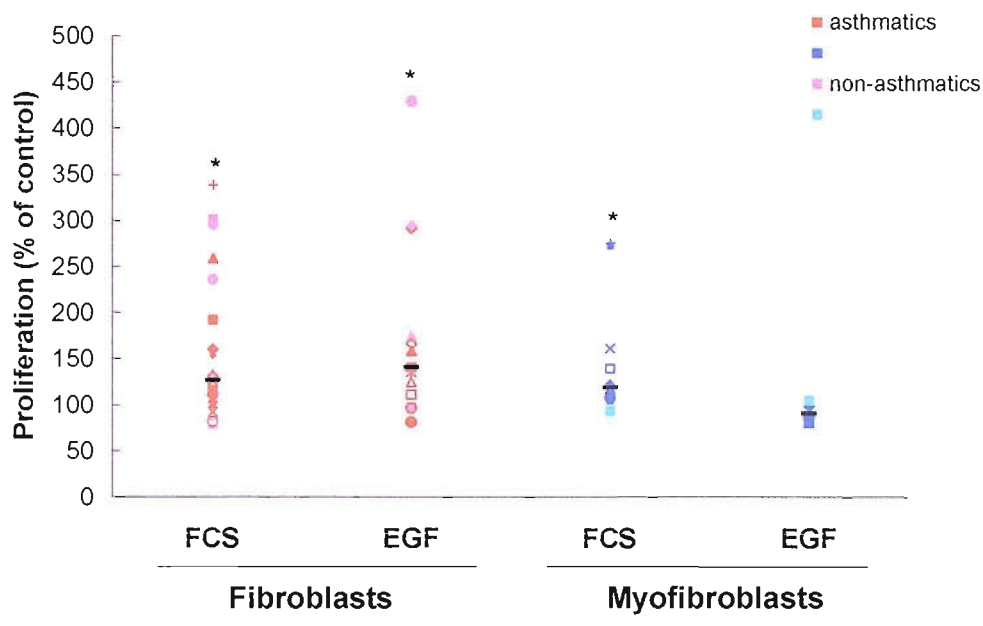


Fig. 3.4.7

(A)Time course for changes in cell number in primary cultures of fibroblasts following stimulation with 10% FCS and EGF at 5 ng/ml. (B) Effect of FCS and EGF on fibroblast (n=13 to 20) and myofibroblast (n=5 to 10) proliferation 48 h after stimulation with 10% FCS and EGF at 5 ng/ml. Median values are shown. * $P<0.05$ compared with response with no stimulus (taken as 100%) by Dunnett's test. The median cell number in unstimulated fibroblasts was 5940 (4982-8335) and in the unstimulated myofibroblasts was 6015 (4774-8384),

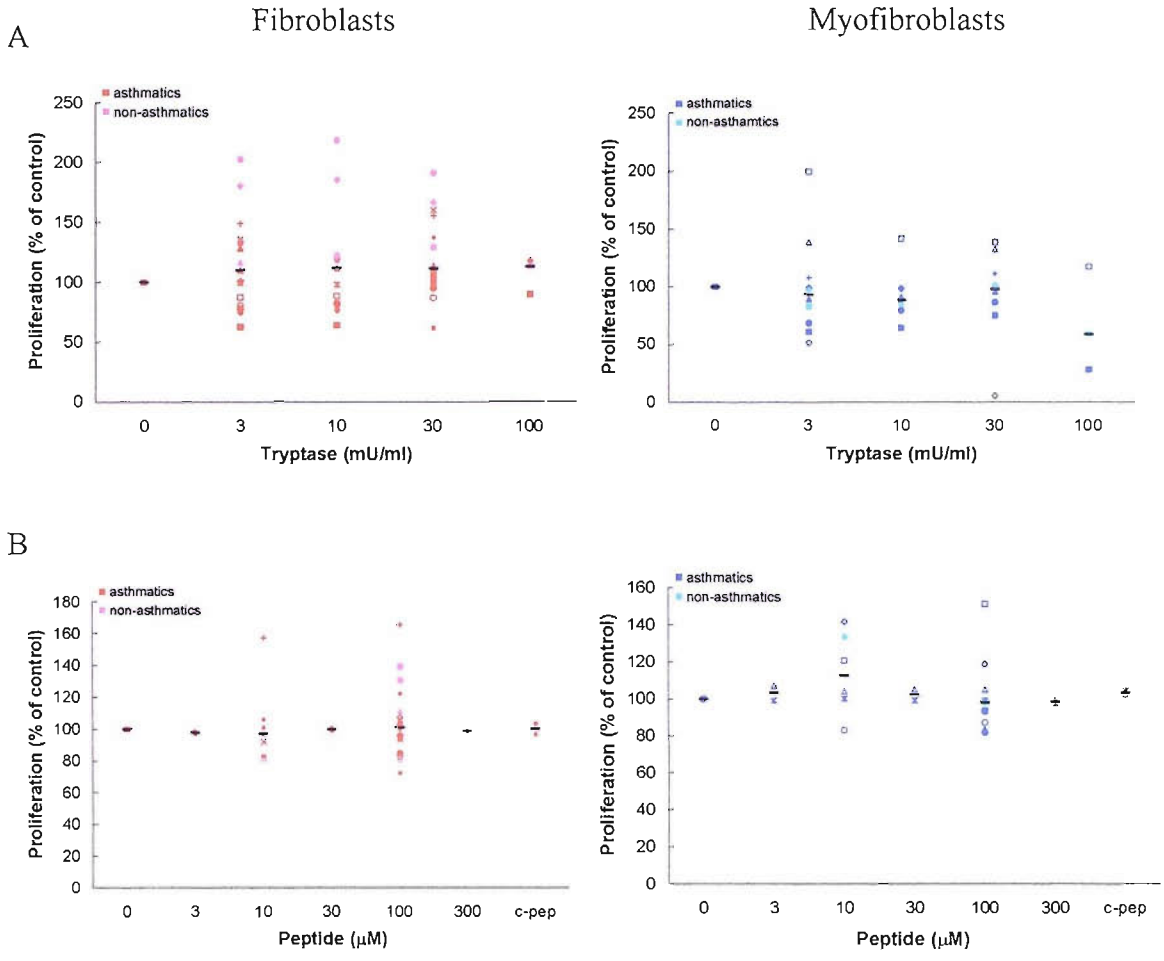
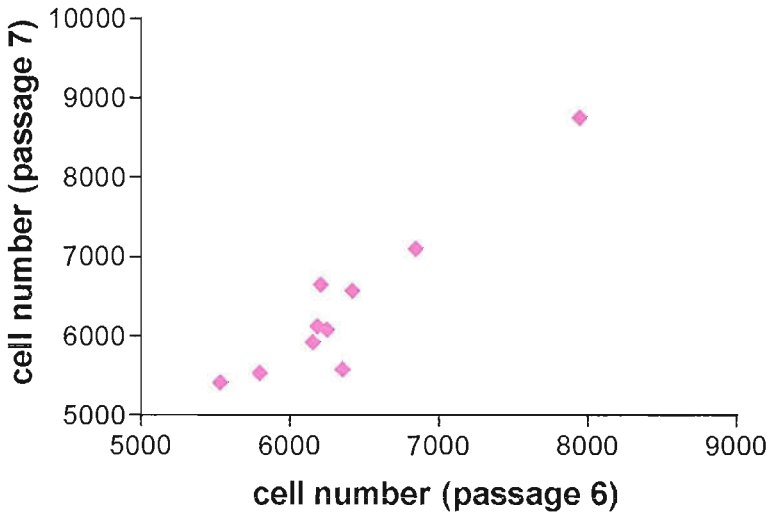


Fig. 3.4.8

Effect of tryptase and the PAR-2 agonist peptide SLIGKV-NH_2 on fibroblast and myofibroblast proliferation. Relative numbers of cells are shown, 48 h after stimulation with (A) tryptase and with (B) the PAR-2 agonist peptide SLIGKV-NH_2. The non-PAR-2 activating peptide, LSIGKV-NH_2 (c-pep) was included at 300 μM. Median values are shown for 2 to 18 experiments. Refer to Fig. 3.4.7 for the cell number in the unstimulated controls.

A



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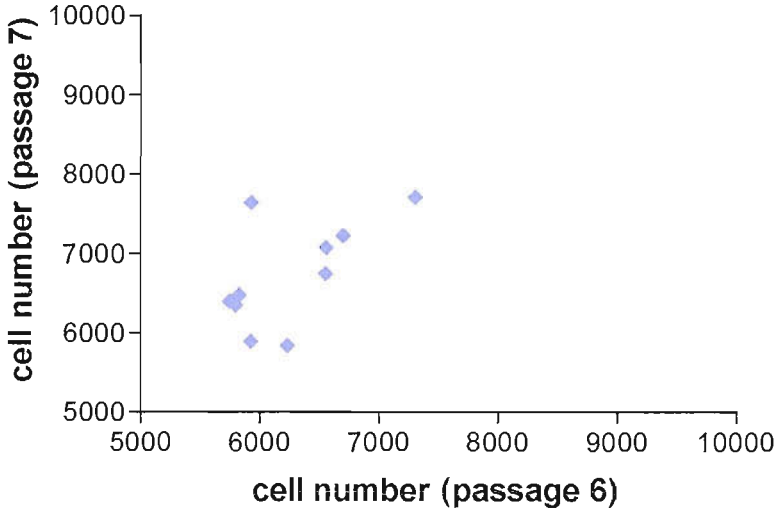


Fig. 3.4.9

Reproducibility of effect of stimuli, 5 ng/ml of EGF, 10% FCS, tryptase at 3, 10, and 30 mU/ml and the PAR-2 agonist peptide SLIGKV-NH_2 at 50, 100, 200 μM, in cell number in primary cultures of (A) fibroblasts (r_s=0.806: $p<0.01$: n=10), and (B) myofibroblasts from non asthmatics (r_s=0.626: $p<0.05$: n=10), 48 h after stimulation.

3.5 Proinflammatory actions of tryptase and PAR-2 agonists on fibroblasts and myofibroblasts

3.5.1 Cytokine release from cells

3.5.1.1 Response to FCS and TNF-α

The proinflammatory cytokines, IL-6 and IL-8 were found to be released from primary cultures of fibroblasts and myofibroblasts as determined by measuring the concentrations of these cytokines in cell supernatants by ELISA. IL-6 release could be detected within 12 h of addition of FCS, added as a positive control stimulus (Fig. 3.5.1 (A)). The level of IL-6 detected increased greatly between 12 h to 24 h when it appeared to reach a plateau at least until 48 h. The pattern of release of IL-8 was similar though an increase could be detected as early as 3 h after stimulation (Fig. 3.5.1 (B)). Concentrations of GM-CSF in cell supernatants were below the detection limit of the ELISA employed.

Significant release of IL-6 and IL-8 from fibroblasts and myofibroblasts was detected 24 h after stimulation with 1 ng/ml TNF-α (Fig. 3.5.2). There was no significant difference between the responses of cell preparations of the fibroblast and myofibroblast phenotype in the quantities of these cytokines released per cell. Comparison of cytokine release from cells from non-asthmatic and asthmatic subjects did not reveal significant differences in response to TNF-α stimulation (Fig. 3.5.3). Although the responses of cells from non-asthmatic and asthmatic subjects were compared, the relative small numbers of cells from non-asthmatic subjects, prevents detailed statistical analysis. The response of fibroblasts to TNF-α was

significantly correlated with that of myofibroblasts in the release of IL-6 (r_s=0.733: $p<0.05$: n=9) and IL-8 (r_s=0.917: $p<0.001$: n=9).

3.5.1.2 Actions of tryptase

The ability of tryptase to stimulate cytokine release from fibroblasts and myofibroblasts was tested. Initially the effect of recombinant tryptase on the release of cytokines was compared with that of native lung tryptase added at the same concentration (Fig. 3.5.4). There was no significant difference in response of fibroblasts and myofibroblasts to the different preparations of tryptase. The effect of using a range of tryptase concentrations was investigated on cells of the MRC-5 fibroblast cell line, with and without TGF-β_2. There was a trend for the dose dependent release of IL-6 (Fig. 3.5.5) and IL-8 (Fig. 3.5.6) in response to tryptase, though the increase did not reach significance in the case of IL-6. Concentrations of GM-CSF in cell supernatants of tryptase stimulated cells were below the detection limit of the ELISA.

As numbers of cells in primary cultures of fibroblasts and myofibroblasts were more limited, just two different concentrations of tryptase (3 and 30 mU/ml) were employed. At these concentrations significant release of IL-6 or IL-8 from either fibroblasts or myofibroblasts was not observed for the group as a whole (Fig. 3.5.7). However, for certain preparations there appeared to be substantial release.

There was a considerable variation between preparations of cells in the responses observed. This would appear to reflect fibroblast heterogeneity rather than random assay variation, as an apparent response for a cell preparation at one concentration was in some cases associated with one at the other concentration. Thus, release of

IL-6 from fibroblasts in response to 3 mU/ml tryptase was significantly correlated with that from tryptase at 30 mU/ml (r_s=0.789: $p<0.001$: n=14), and there was a similar association in the case of IL-8 release from myofibroblasts (r_s=0.903: $p<0.001$: n=10). IL-6 release was not associated with IL-8 release in fibroblast preparations. The pattern of cytokine release in myofibroblasts was similar to that in fibroblasts with apparent heterogeneity in responsiveness. However, responses in myofibroblasts were not correlated with those in fibroblasts, despite these preparations containing cells of the fibroblast phenotype (section 3.2).

Responses with the heat-inactivated tryptase were not significantly different from those with the corresponding concentration of active enzyme in either fibroblasts or myofibroblasts. This would argue against a role for the catalytic site. Moreover, the amount of IL-6 release from fibroblasts in the presence of heat inactivated tryptase was correlated with that released in the presence of active tryptase (r_s=0.876: $p<0.0001$: n=14), though this did not reach significance for IL-8 release in these cells. This degree of correlation between heat inactivated and active tryptase, serves to validate the cytokine measurements and suggests that the variation reflected heterogeneity in fibroblast and myofibroblast populations from different subjects. These differences were not associated with the cell donors being asthmatic (n=5) or non-asthmatic (n=4). Release of IL-6 or IL-8 from fibroblasts and myofibroblasts in response to tryptase had no association with that stimulated in response to FCS or TNF-α.

3.5.1.3 Actions of PAR-2 agonists

Despite the relatively weak response to tryptase, when trypsin, another PAR-2 activating enzyme was added to fibroblasts and myofibroblasts, there was

appreciable cytokine release. In response to trypsin, fibroblasts released IL-6 and IL-8, and myofibroblasts released IL-6 (Fig. 3.5.8). The IL-8 release from trypsin-treated myofibroblasts was similar to that of fibroblasts, but the increase in levels did not reach significance. The release of IL-6 from fibroblasts was not associated with IL-6 release from the myofibroblasts, though with IL-8 release there was a correlation between the responses of fibroblasts and myofibroblasts (r_s=0.738: $p<0.05$: n=8). The release of IL-6 from myofibroblasts was correlated with IL-8 release from these cells (r_s=0.786: $p<0.05$: n=8), though such a relationship was not found with the fibroblast preparations.

When fibroblasts and myofibroblasts were incubated with a range of concentrations of the peptide agonist of PAR-2 SLIGKV-NH_2, IL-6 release was induced from myofibroblasts in a dose dependent manner (Fig. 3.5.9). In contrast, IL-8 release was not induced from either fibroblasts or myofibroblasts in response to SLIGKV-NH_2 (Fig. 3.5.10). The non-PAR-2 activating control peptide LSIGKV-NH_2 did not provoke significant release of either cytokine when added at a concentration that corresponded to the maximal concentration of SLIGKV-NH_2. IL-6 release from fibroblasts was correlated with IL-8 release from these cells (r_s=0.562: $p<0.0001$: n=32), but this association was not seen with myofibroblasts. There was no correlation between fibroblasts and myofibroblasts in either IL-6 or IL-8 release. IL-8 release from myofibroblasts in the presence of active tryptase was correlated with IL-8 release from these cells in the presence of SLIGKV-NH_2 at 10 μM (r_s=0.867: $p<0.001$: n=10), at 30 μM (r_s=1.000: $p<0.0001$: n=5), at 100 μM (r_s=0.903: $p<0.001$: n=10), and at 300 μM (r_s=1.000: $p<0.0001$: n=5), though such a relationship was not found with the fibroblast preparations or with IL-6 release.

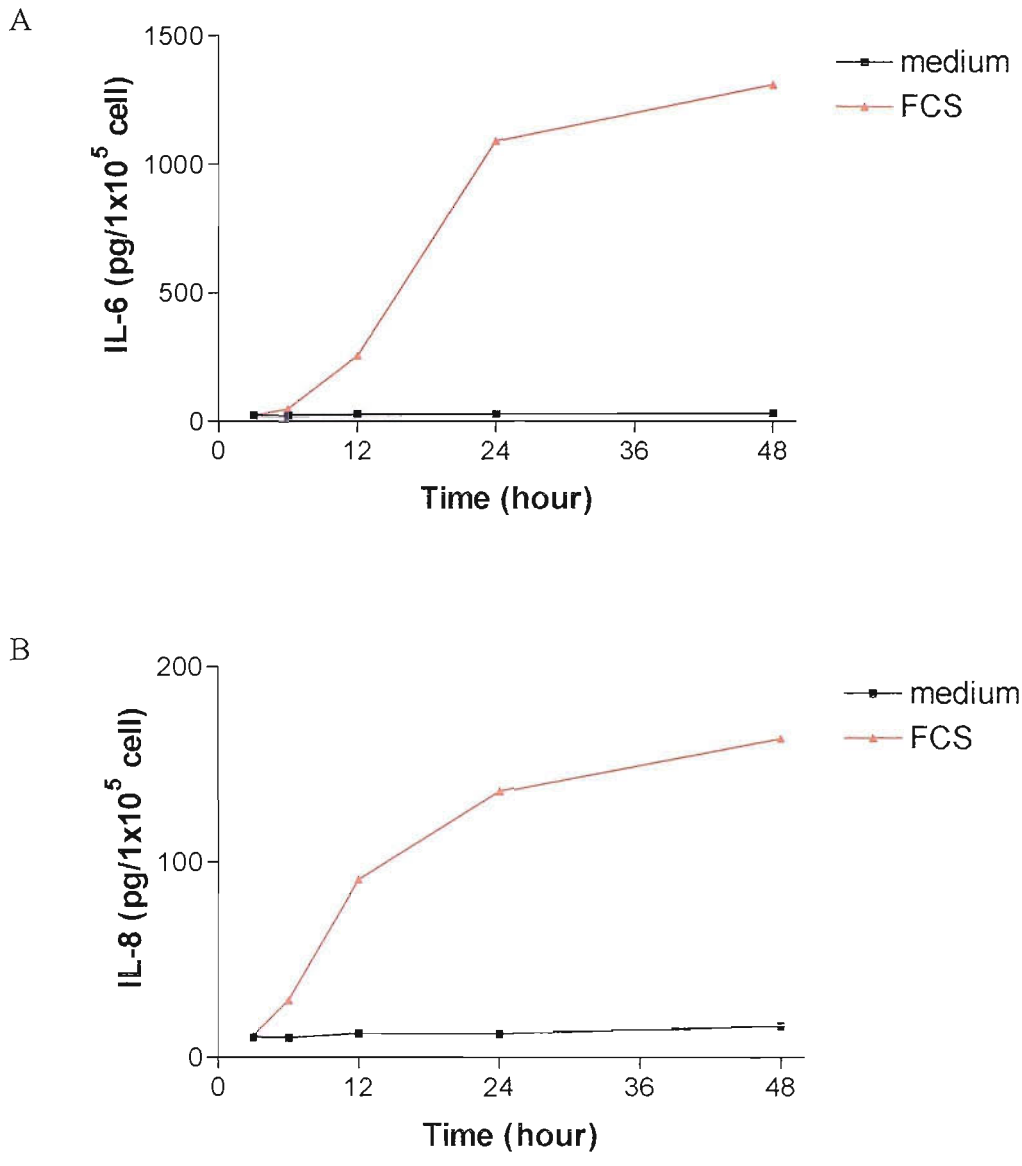
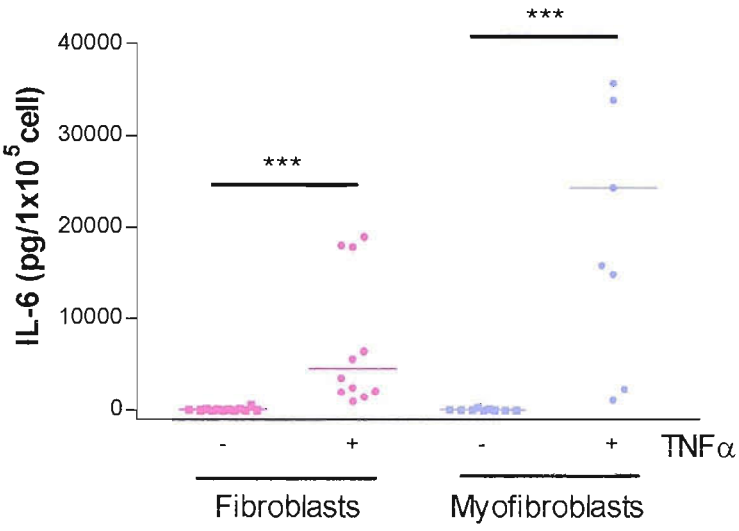


Fig. 3.5.1

Time course of the release of (A) IL-6 and (B) IL-8 from primary cultures of fibroblasts following addition of 10% FCS or of medium alone. Results represent the mean of duplicate determinations of a single experiment (representative of 3 separate experiments performed in duplicate).

A



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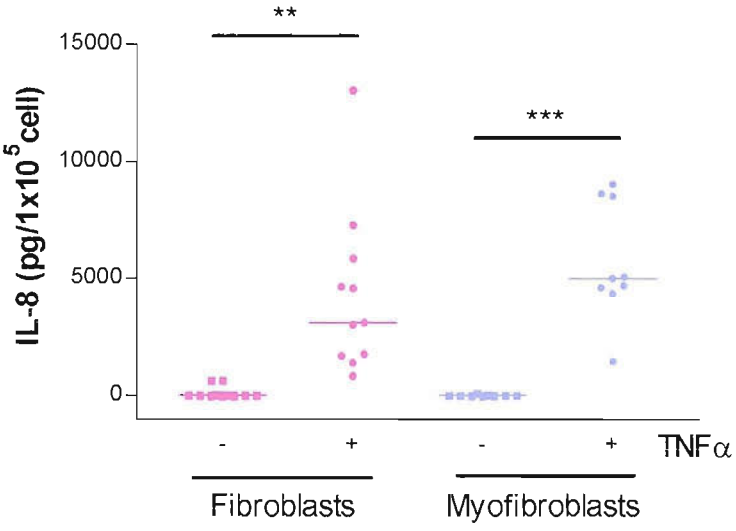


Fig. 3.5.2

Release of (A) IL-6 and (B) IL-8 from primary cultures of fibroblasts (n=11) and myofibroblasts (n=9) stimulated with TNF-α (1 ng/ml) for 24 h. Median values are shown. **P<0.01 and ***P<0.001 compared with the response with no stimulus by Dunnett's test.

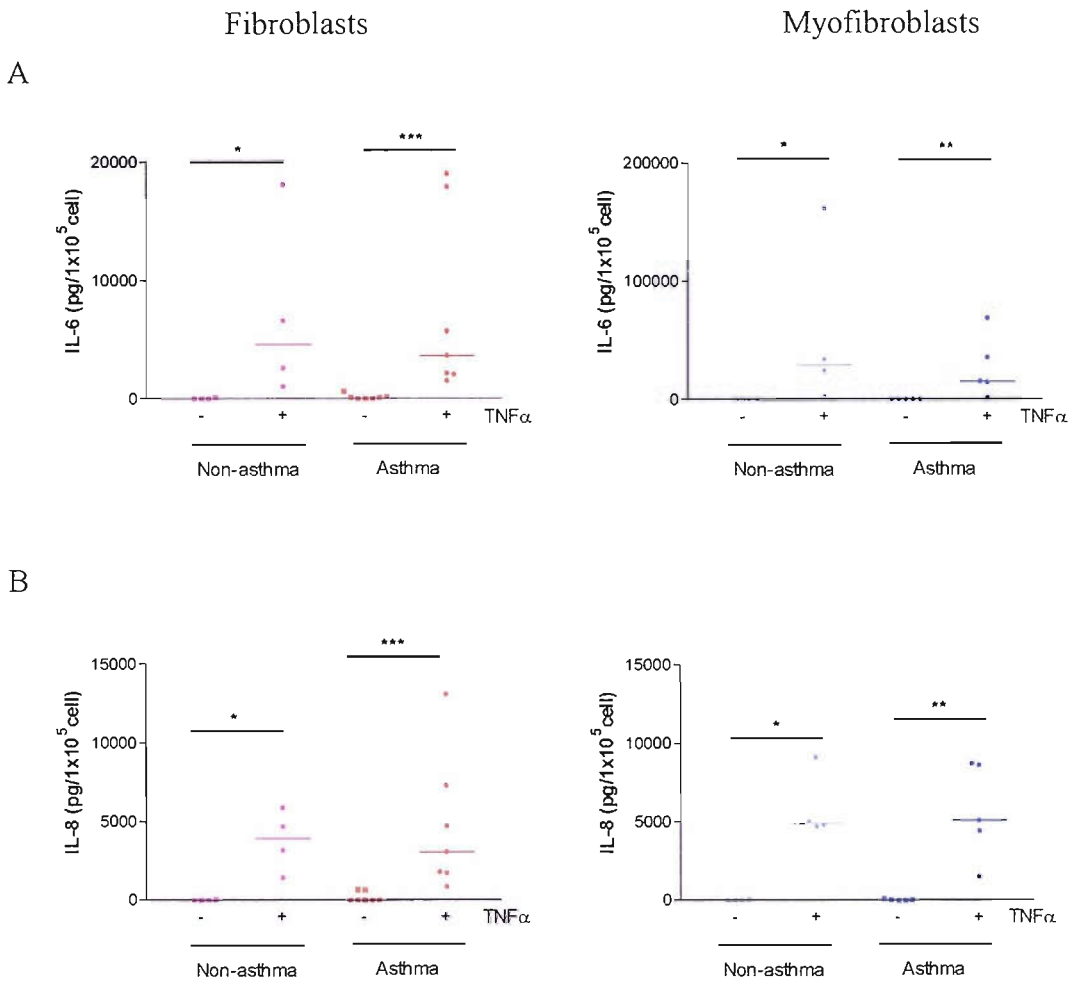


Fig. 3.5.3

Release of (A) IL-6 and (B) IL-8 in response to TNF-α (1 ng/ml) from primary cultures of fibroblasts and myofibroblasts from non-asthmatic and asthmatic subjects. Median values are shown. *P<0.05, **P<0.01 and ***P<0.001 compared with the response with no stimulus by Dunnett's test.

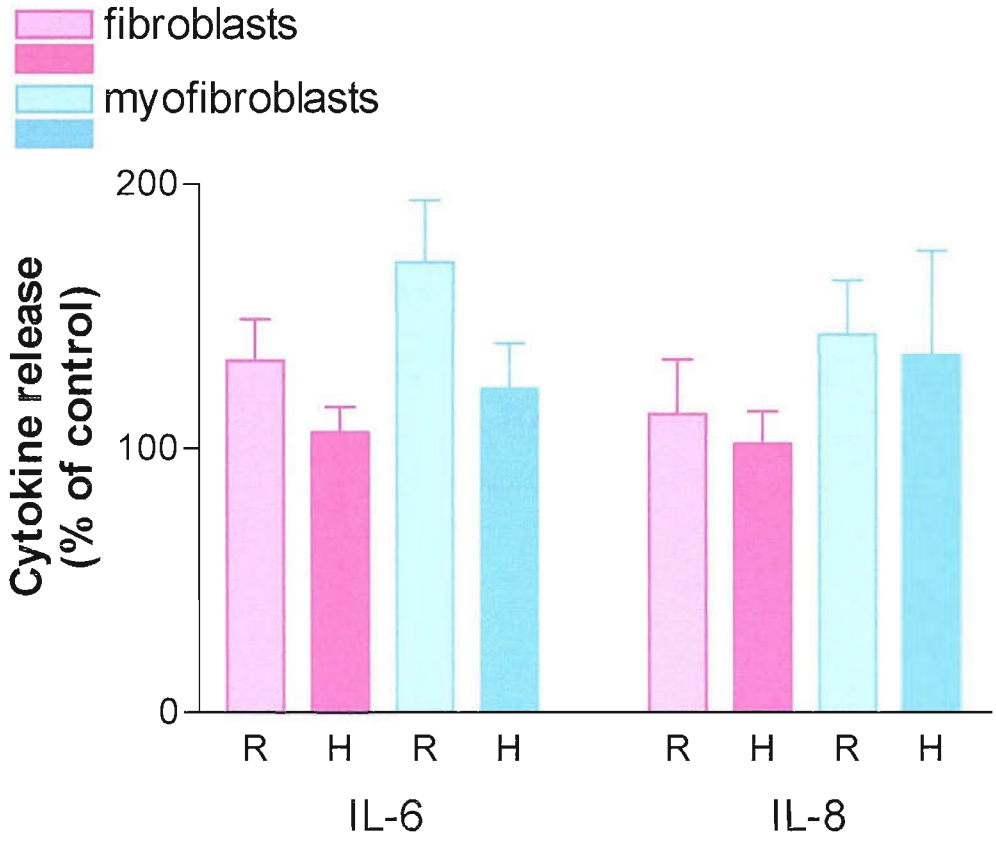
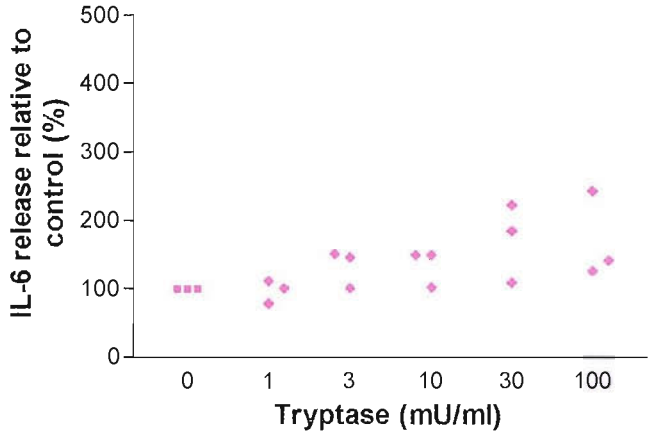


Fig. 3.5.4

Effect of 10 mU/ml human lung (H) and recombinant (R) tryptase on release of IL-6 and IL-8 in cells of the MRC-5 line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). Mean ± SEM are shown for three separate experiments performed in duplicate.

A



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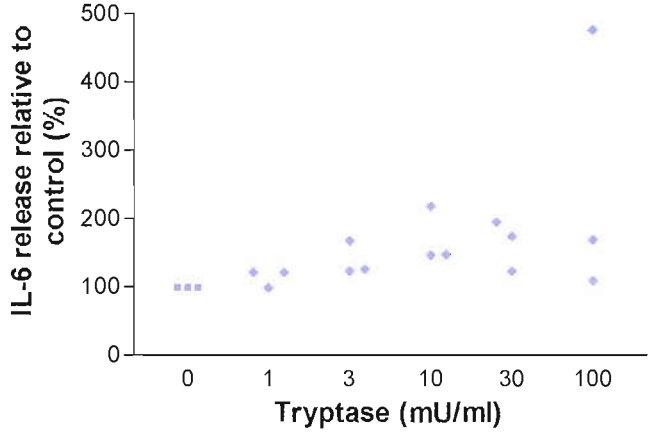
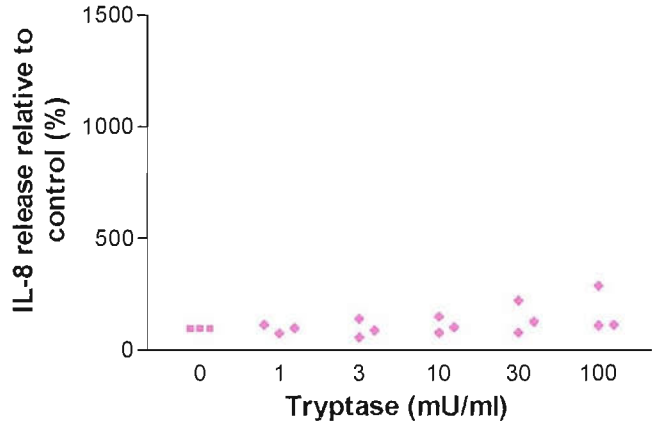


Fig. 3.5.5

Release of IL-6 in response to tryptase in cells of the MRC-5 line (A) without TGF-β pre-treatment or (B) following pre-treatment with TGF-β (for 24 h) for three separate experiments performed in duplicate. The median release of IL-6 per 1×10^5 cells in unstimulated fibroblasts was 242 (177-422) pg and in the unstimulated myofibroblasts was 145 (116-413) pg.

A



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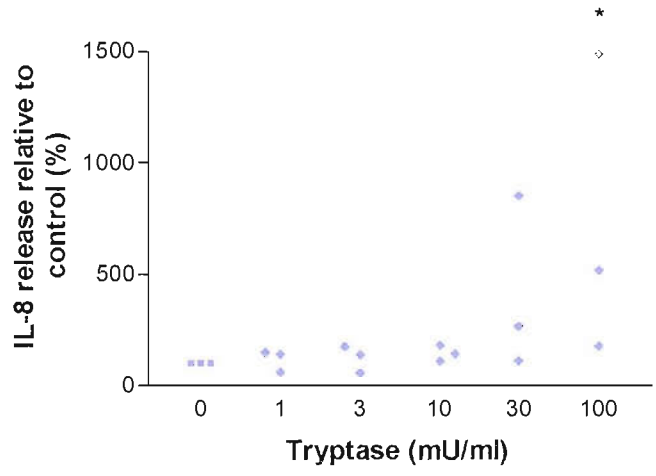


Fig. 3.5.6

Release of IL-8 in response to tryptase in cells of the MRC-5 line (A) without TGF-β pre-treatment or (B) following pre-treatment with TGF-β (for 24 h) for three separate experiments performed in duplicate. The median release of IL-8 per 1×10^5 cells in unstimulated fibroblasts was 360 (289-1015) pg and in the unstimulated myofibroblasts was 77 (75-267) pg. * $P<0.05$ compared with the response with no stimulus by student's t test.

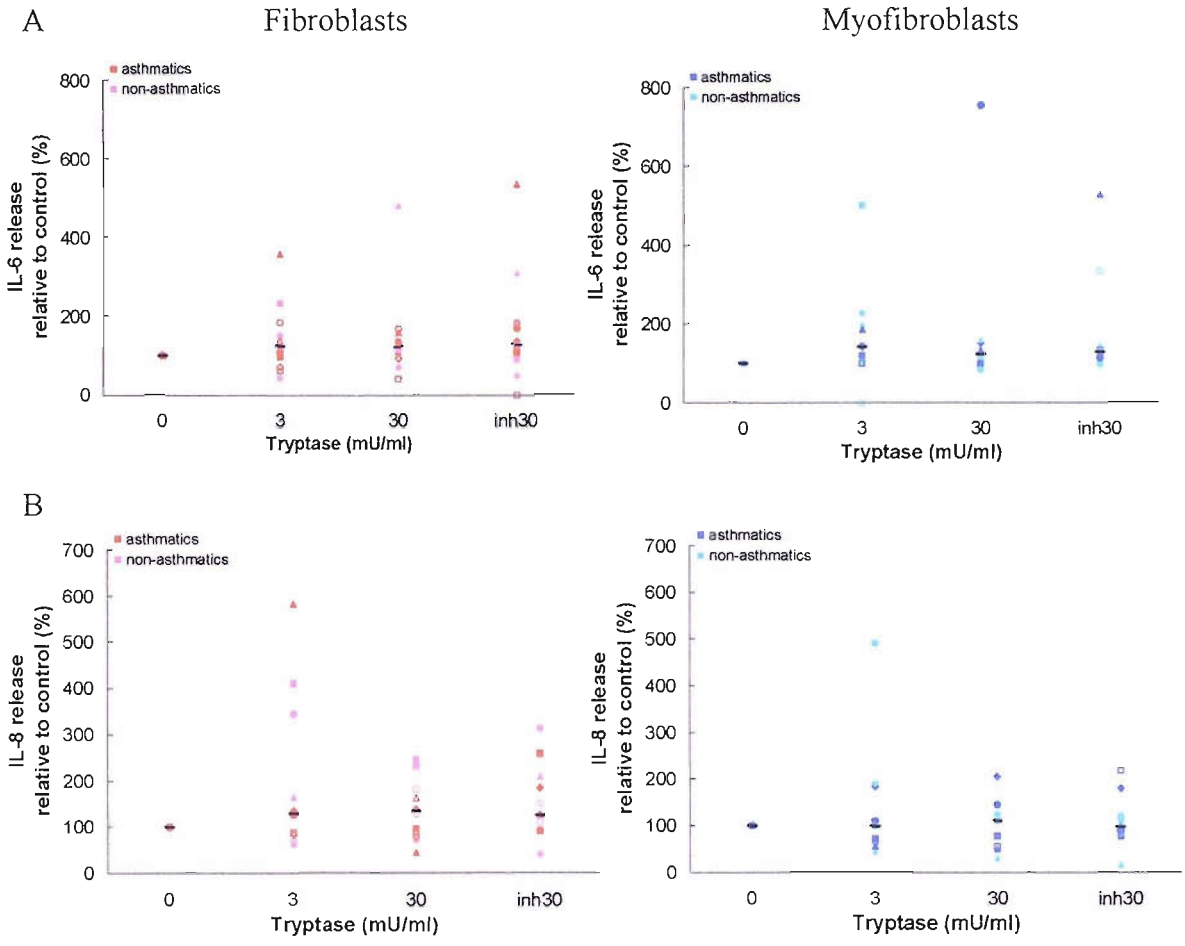


Fig. 3.5.7

Release of (A) IL-6 and (B) IL-8 from primary cultures of fibroblasts (n=13) and myofibroblasts (n=9) stimulated with recombinant tryptase or with a heat inactivated preparation of tryptase original activity corresponded to 30 mU/ml (inh-) for 24 h. Median values are shown for 13 separate experiments performed in duplicate with fibroblasts, and 9 with myofibroblasts. The median release of IL-6 per 1×10^5 cells in unstimulated fibroblasts was 110 (11-2373) pg and in the unstimulated myofibroblasts was 80 (17-2040) pg, and of IL-8 in unstimulated fibroblasts was 22 (10-2311) pg and in the unstimulated myofibroblasts was 21 (10-2069) pg.

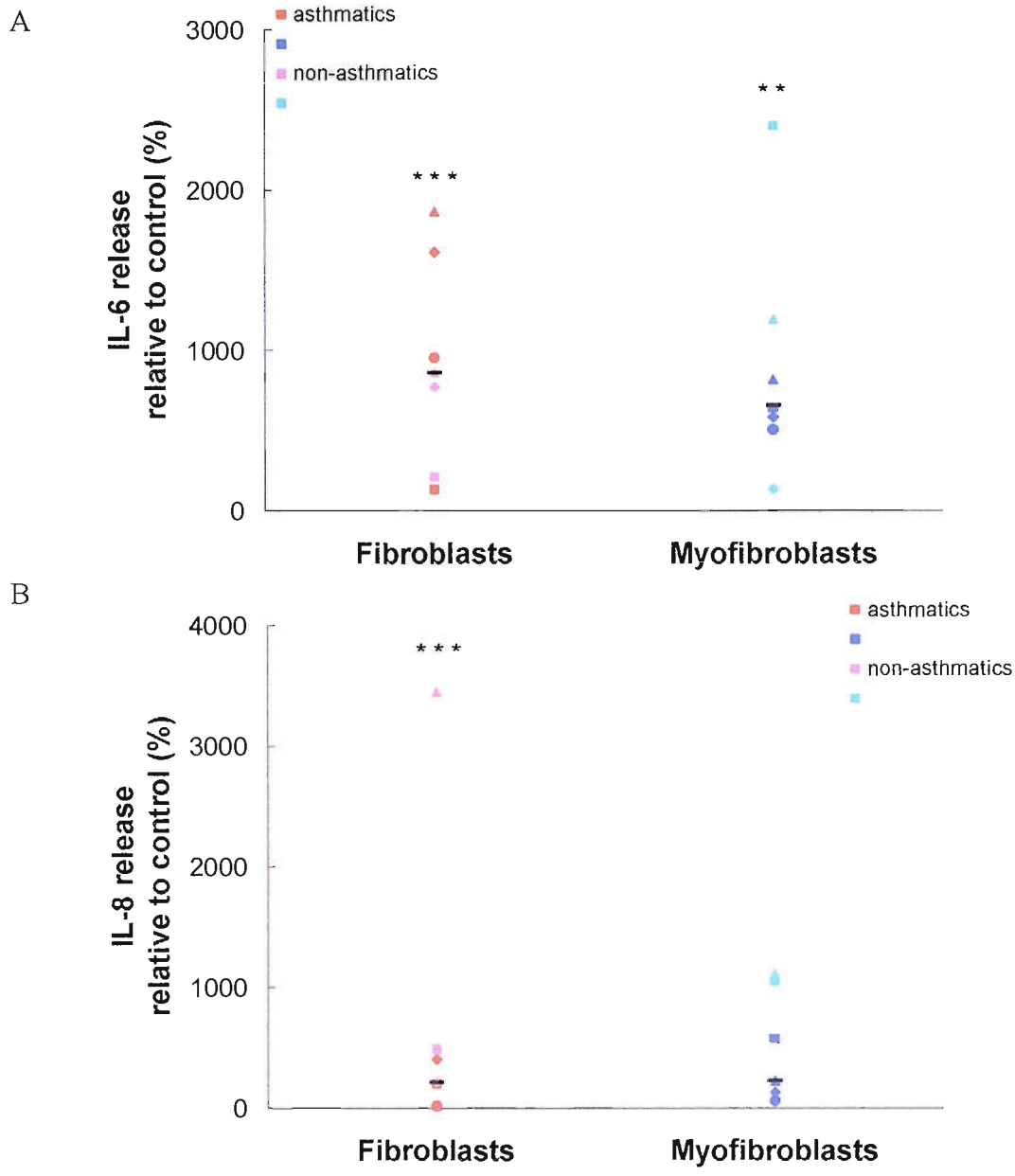


Fig. 3.5.8

Release of (A) IL-6 and (B) IL-8 from primary cultures of fibroblasts (n=7) and myofibroblasts (n=7) stimulated with trypsin at 1 μg/ml for 24 h. Median values are shown. **P<0.01 and ***P<0.001 compared with response with no stimulus (taken as 100%) by Dunnett's test. The median release of IL-6 per 1×10^5 cells in unstimulated fibroblasts was 129 (11-2373) pg and in the unstimulated myofibroblasts was 101 (20-2040) pg, and of IL-8 in unstimulated fibroblasts was 31 (10-2311) pg and in the unstimulated myofibroblasts was 24 (10-2069) pg.

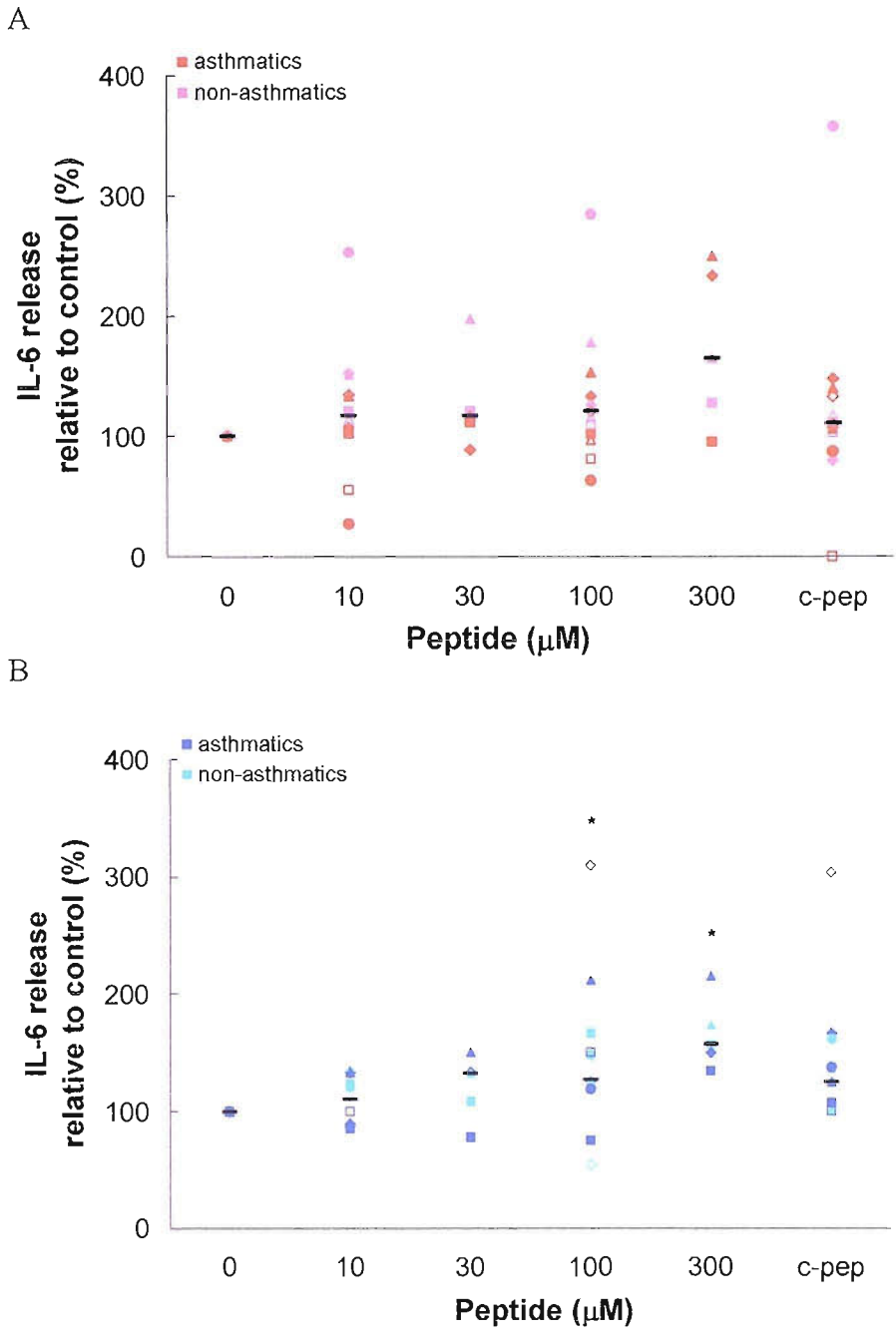


Fig. 3.5.9

Effect of the PAR-2 agonist peptide SLIGKV-NH_2 on release of IL-6 at 24 h for primary cultures of (A) fibroblasts and (B) myofibroblasts. The control peptide, LSIGKV-NH_2 (c-pep) was included at 300 μM. Median values are shown for 5 to 13 separate experiments performed in duplicate. * $P<0.05$ compared with the response with no stimulus by Dunnett's test. Refer to Fig. 3.5.7 for the cytokines (pg) in the unstimulated controls.

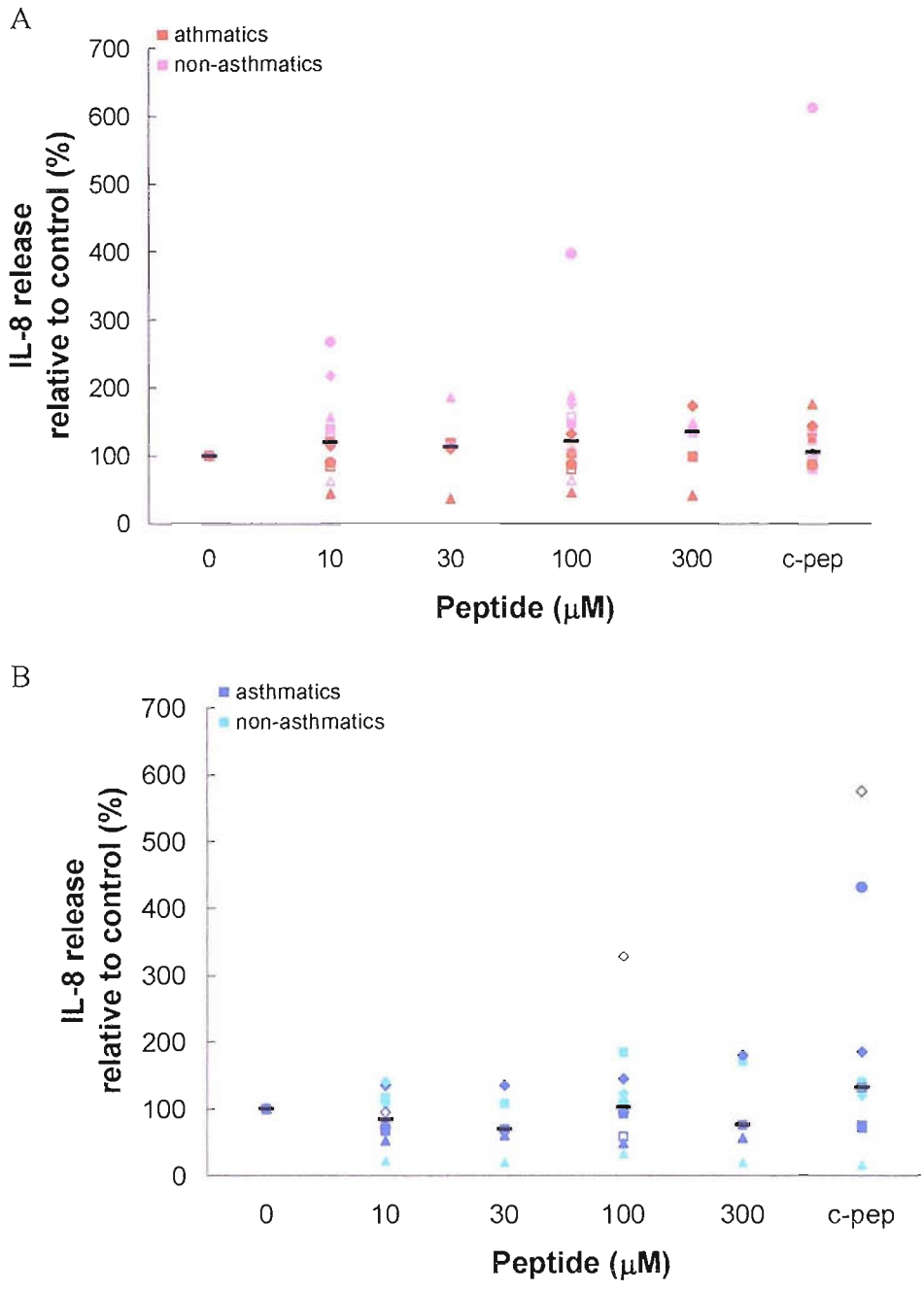


Fig. 3.5.10

Effect of the PAR-2 agonist peptide SLIGKV-NH_2 on release of IL-8 at 24 h for primary cultures of (A) fibroblasts and (B) myofibroblasts. The control peptide, LSIGKV-NH_2 (c-pep) was included at 300 μM. Median values are shown for 5 to 13 separate experiments performed in duplicate. Refer to Fig. 3.5.7 for the cytokines (pg) in the unstimulated controls.

3.5.2 Expression of mRNA for cytokines

3.5.2.1 Response to FCS and TNF-α

Levels of mRNA for IL-6, IL-8 and GM-CSF in primary cultures of fibroblasts and myofibroblasts were detected by measuring cDNA reverse transcribed from purified RNA using quantitative TaqMan PCR. When levels were investigated at 3, 6, 12, 24 and 48 h following stimulation with FCS, it was found that there were quite different time courses for the appearance of mRNA for each of these cytokines (Fig. 3.5.11). There were different patterns observed also for different preparations of cells (data not shown). In subsequent experiments the 6 h time point was selected for each of the three cytokines.

IL-6 mRNA expression was induced by TNF-α in both fibroblasts and myofibroblasts and also by addition of FCS in myofibroblasts (Fig. 3.5.12). IL-8 mRNA expression on the other hand was stimulated by TNF-α in fibroblasts and myofibroblasts, but not by FCS. GM-CSF mRNA expression was significantly increased in response to FCS and TNF-α stimulation in fibroblasts, but not in myofibroblasts under the conditions tested. Expression of mRNA for IL-8 in fibroblasts in response to FCS was significantly correlated with that in response to TNF-α (r_s=1.000: $p<0.001$: n=7), as was GM-CSF (r_s=1.000: $p<0.05$: n=5), raising the possibility that IL-8 generation by these cells may be triggered in the same way by each of these stimuli. However, this association was not seen in the case of IL-6 in fibroblasts or with any of the cytokines examined in myofibroblasts.

Expression of mRNA for IL-8 in fibroblasts treated with TNF-α was correlated with that from myofibroblasts treated with TNF-α (r_s=1.000: $p<0.05$: n=5). Similar

associations were not found with mRNA expression for the other cytokines stimulated by TNF-α, or with any cytokine stimulated by FCS.

3.5.2.2 Actions of tryptase

It proved difficult to prepare intact cDNA from cells stimulated with tryptase and no amplification of products was obtained in a number of experiments. Curiously, this problem was restricted to stimulation with enzymatically active tryptase, and this difficulty did not present itself when amplification was carried out with cDNA from cells incubated with heat-inactivated tryptase, or with tryptase in the presence of the inhibitors, leupeptin and APC1390. There was no difference in the quality (OD_{260} / OD_{280}) of purified RNA in tryptase-stimulated cells. The cause of the problem was not identified, but after repeating the procedure several times, data were obtained from tryptase-treated cells with a procedure little different from that which had been employed unsuccessfully.

Tryptase was found to stimulate expression of mRNA for cytokines in MRC-5 fibroblasts and myofibroblasts over a range of concentrations (Fig. 3.5.13). Tryptase concentrations of 3 and 30 mU/ml were selected for subsequent experiments with primary cultures of cells, so as to be consistent with investigations of cytokine release (section 3.5.1). There was considerable variation between cell preparations in the responses observed. There was no significant increase of mRNA for IL-6, IL-8 or GM-CSF induced by these concentrations of tryptase (Fig. 3.5.14), though the maximal responses of fibroblasts and myofibroblasts to tryptase were similar to those of the FCS and TNF-α controls. Expression of mRNA for any of the cytokines examined was not significantly affected by heat inactivating the tryptase. Expression of mRNA for IL-6 in fibroblasts was correlated with that for IL-8 (r_s=0.637: $p<0.05$:

n=15) and GM-CSF (r_s=0.539: p<0.05: n=15) in tryptase treated cells suggesting that similar processes could be involved in stimulating these responses. Similarly in myofibroblasts, expression of mRNA for IL-6 was correlated with that for IL-8 (r_s=0.510: p<0.05: n=15), though not with GM-CSF. Expression of mRNA for IL-8 in fibroblasts was not correlated with that for GM-CSF, but such a correlation was found in myofibroblasts (r_s=0.615: p<0.05: n=15). No association was found between expression of mRNA for any cytokine in tryptase treated fibroblasts and expression of mRNA for the corresponding cytokine in myofibroblasts. Expression of mRNA for IL-6 from myofibroblasts in response to tryptase was correlated with that in response to FCS (r_s=0.886: p<0.05: n=6), though such a relationship was not found with the fibroblast preparations or with IL-8 and GM-CSF release in response to FCS or TNF-α.

3.5.2.3 Actions of PAR-2 agonist

There was a trend for a dose dependent increase in mRNA expression for IL-6 following addition to cells of the PAR-2 agonist peptide SLIGKV-NH_2, with a peak response at 100 μM in myofibroblasts (Fig. 3.5.15). Such a trend was not seen in fibroblasts. Apart from one exceptionally high value for IL-6 mRNA expression observed in response to the PAR-2 agonist peptide (10 μM) in fibroblasts, the response of these cells to the PAR-2 agonist peptide was uniformly weak. Similarly there was a trend for expression of mRNA for IL-8 to exhibit a dose dependent increase, with a peak at 100 μM for myofibroblasts. A consistent trend for a concentration dependent increase in GM-CSF expression was not observed. The control peptide used for the PAR-2 agonist, LSIGKV-NH_2 did not alter expression of mRNA for any of the cytokines tested.

Expression of mRNA for IL-6 in fibroblasts was correlated with that for GM-CSF (r_s=0.900: $p<0.05$: n=5) in SLIGKV-NH_2 treated cells. Expression of mRNA for IL-8 in myofibroblasts was correlated with that for GM-CSF (r_s=1.00: $p<0.05$: n=4). However, there was no association between expression of mRNA for any of the cytokines in SLIGKV-NH_2 treated fibroblasts and expression of mRNA for the corresponding cytokine in myofibroblasts. Expression of mRNA for any of the cytokines tested from fibroblasts or myofibroblasts in the presence of SLIGKV-NH_2 was not associated with that in response to tryptase. Expression of mRNA for IL-6 from fibroblasts in response to SLIGKV-NH_2 was correlated with that in response to FCS (r_s=1.000: $p<0.05$: n=5), though such a relationship was not found with the myofibroblast preparations, or with IL-8 and GM-CSF release in response to FCS or TNF-α.

Summary

Release of IL-8 from MRC-5 cells treated with TGF-β was increased in response to tryptase at 100mU/ml but tryptase was without effect in release of IL-6 and IL-8 from primary cultures of cells. A peptide agonist of PAR-2 stimulated IL-6 release from myofibroblasts by nearly 2 fold but IL-8 release was not affected. Trypsin (1ng/ml) stimulated an increase in the release of IL-6 from fibroblasts of 9 fold and from myofibroblasts by 7 fold. IL-8 release from fibroblasts was increased by more than 4 fold in response to trypsin and from myofibroblasts by 2 fold. There was no significant change in expression of mRNA for cytokines in primary cultures of fibroblasts and myofibroblasts following addition of by tryptase or the PAR-2 agonist

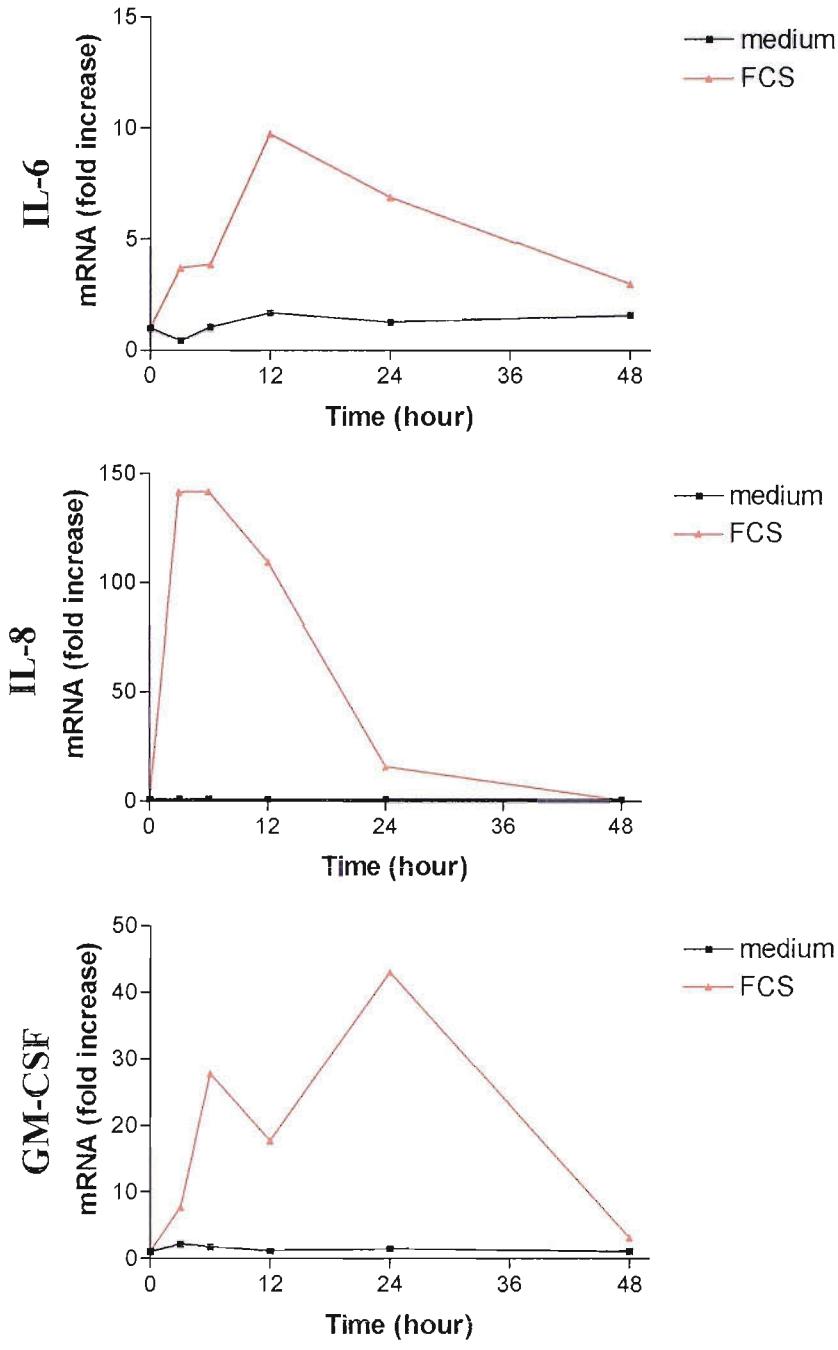


Fig. 3.5.11

Expression of mRNA for cytokines in primary cultures of fibroblasts incubated with 10% FCS for a period of 48h. Results represent the mean of duplicate determinations of a single experiment (that was performed on three separate occasions)

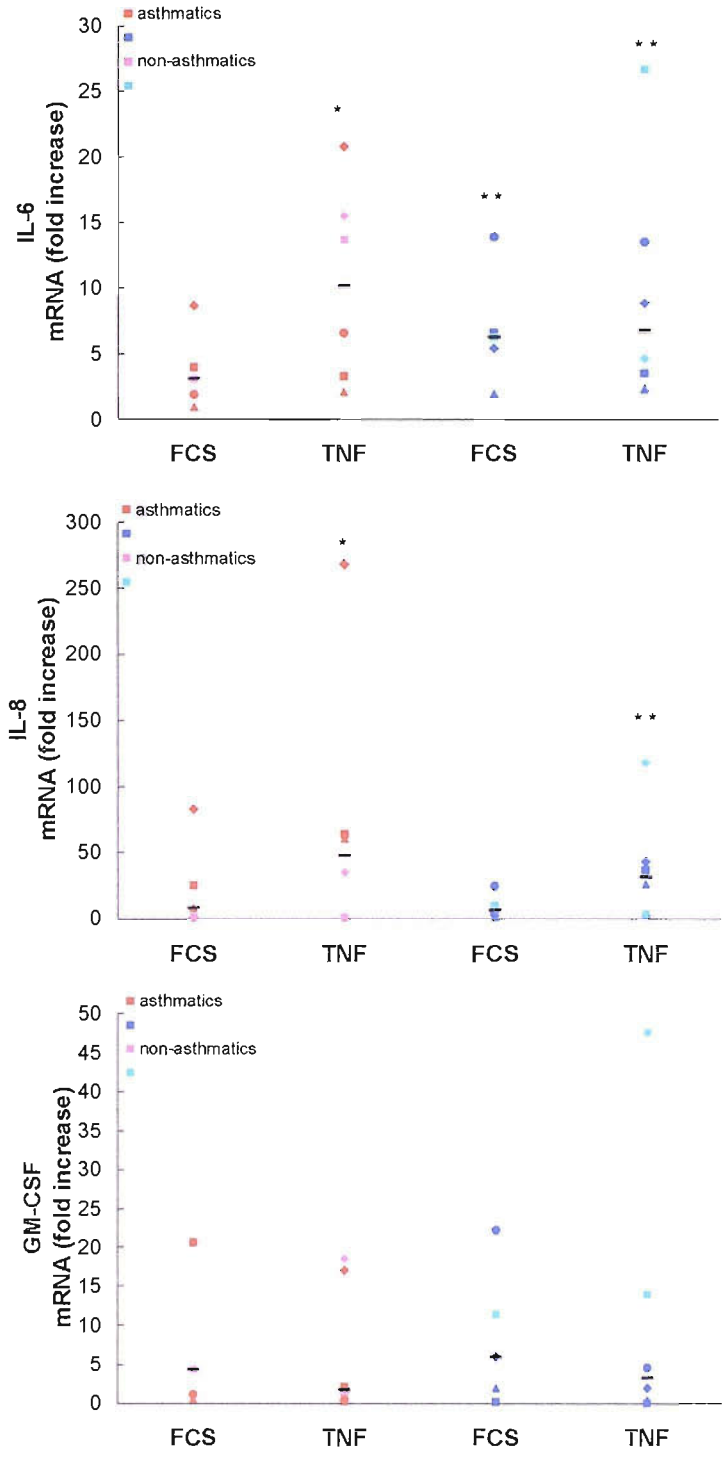


Fig. 3.5.12

Effect of TNF-α on expression of mRNA for cytokines in fibroblasts and myofibroblasts 6 h after stimulation. Median values are shown for 5 to 6 separate experiments. *P<0.05 and **P<0.01 compared with the response with no stimulus (arbitrarily taken as 1.0) by Dunnett's test.

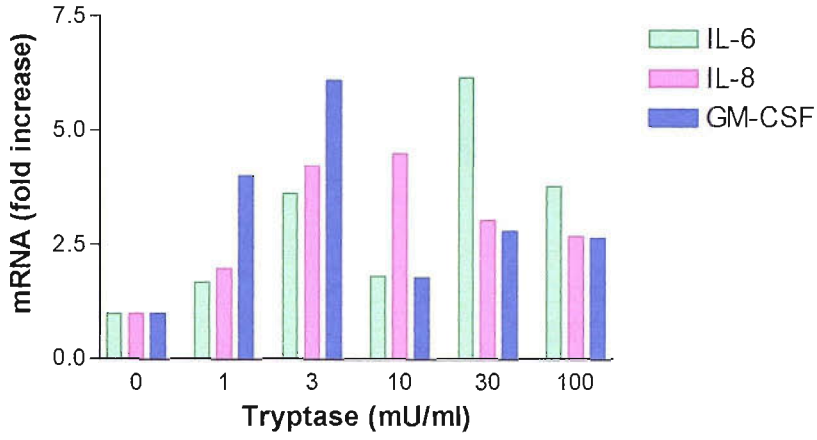


Fig. 3.5.13

Effect of tryptase on expression of mRNA for IL-6, IL-8 and GM-CSF in fibroblasts of the MRC-5 cell line, 6h after stimulation with tryptase. Mean values are shown for 2 separate experiments.

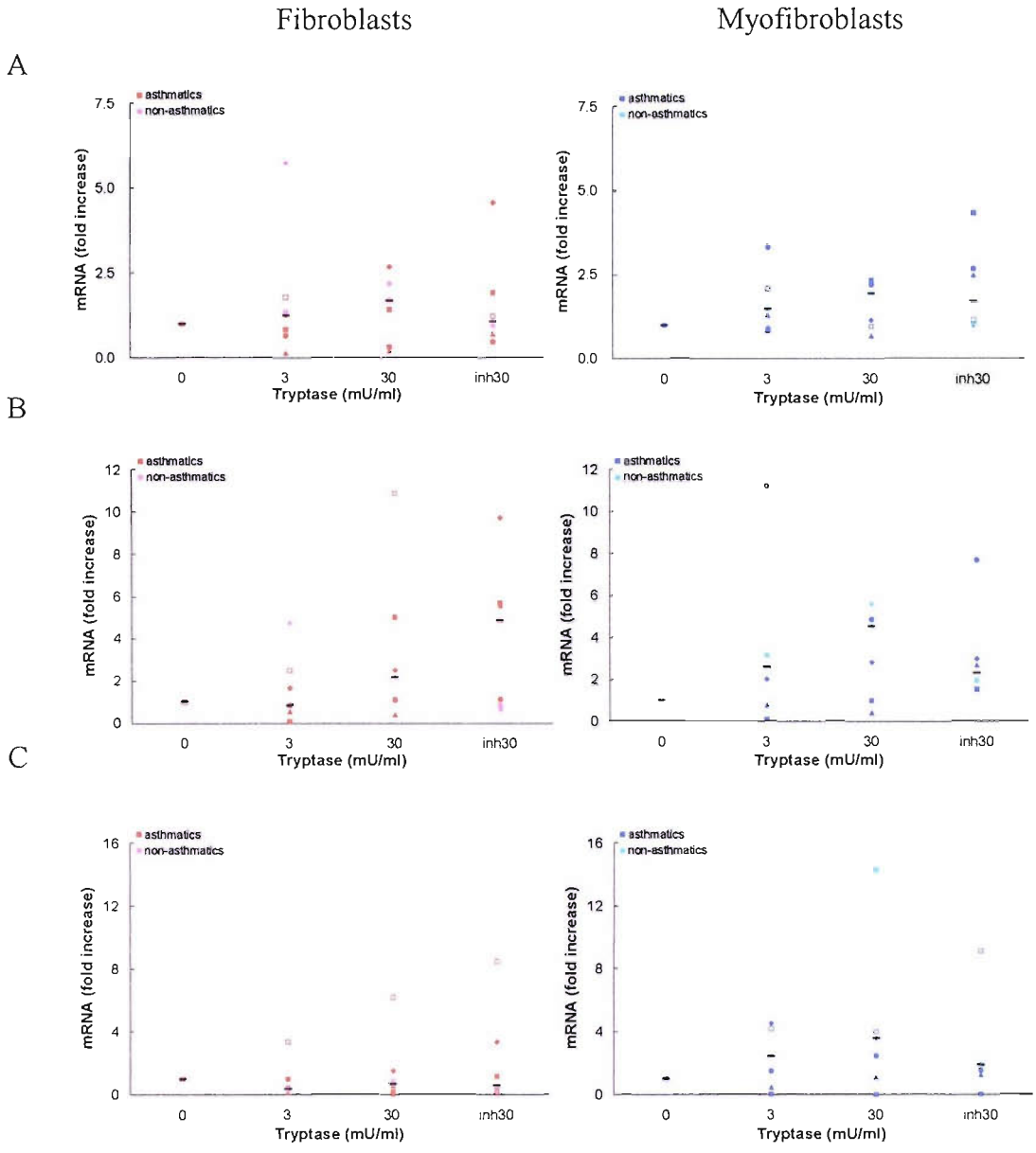


Fig. 3.5.14

Expression of mRNA for (A) IL-6, (B) IL-8 and (C) GM-CSF in primary cultures of fibroblasts and myofibroblasts following incubation with tryptase or with a heat inactivated preparation corresponding to 30 mU/ml tryptase (inh-) 6 h after stimulation. Median values are shown for 8 separate experiments.

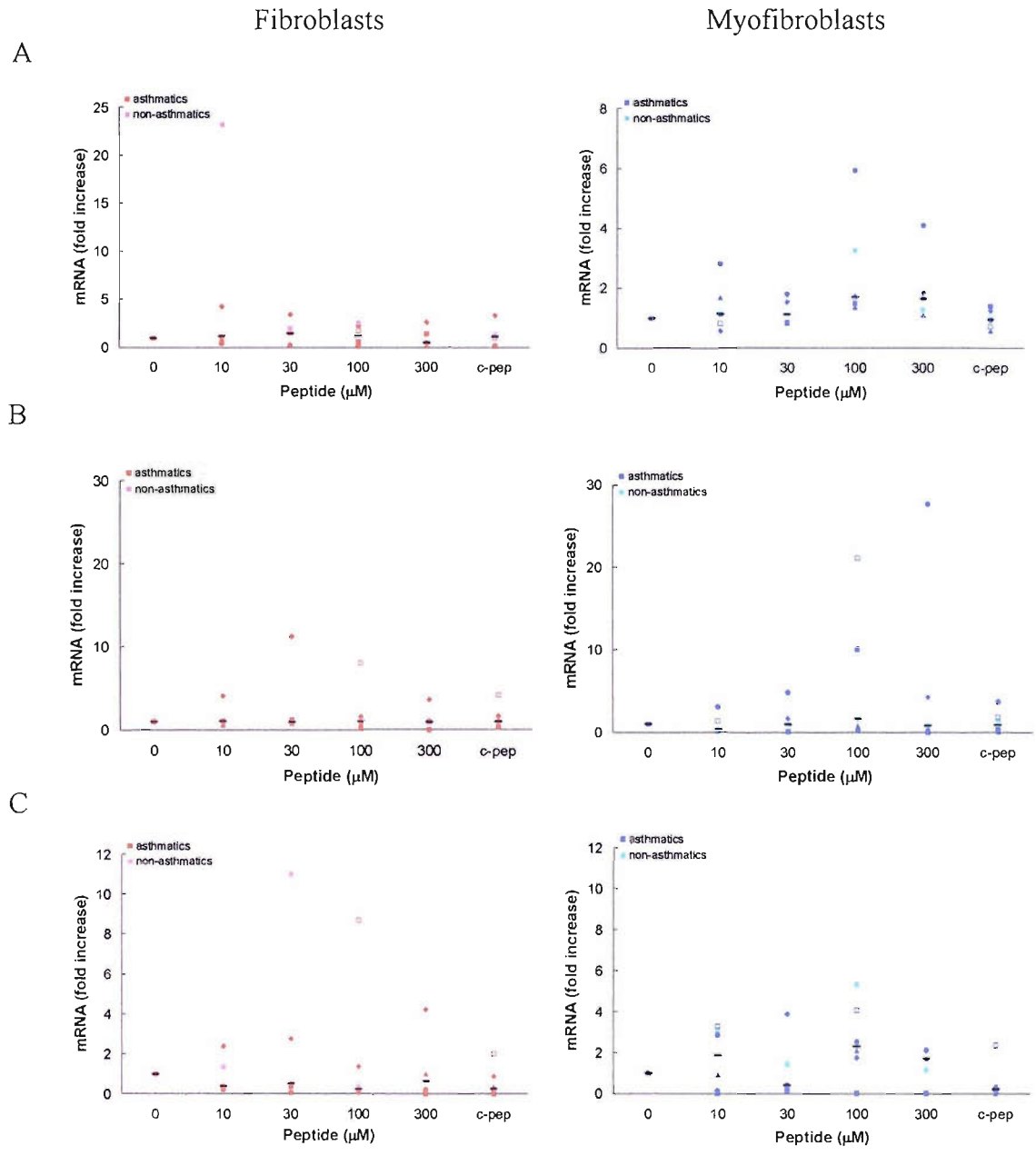


Fig. 3.5.15

Expression of mRNA for (A) IL-6, (B) IL-8 and (C) GM-CSF in primary cultures of fibroblasts and myofibroblasts in response to PAR-2 agonist peptide SLIGKV-NH_2 6 h after stimulation. The control peptide LSIGKV-NH_2 (c-pep) was included at 300 μM. Median values are shown for 5 to 8 separate experiments.

3.6 Effects of tryptase and PAR-2 agonist on expression and release of collagens and collagenase.

3.6.1 Collagen synthesis

Increased synthesis of collagen was induced in primary cultures of fibroblasts and myofibroblasts, 24 h after addition of tryptase, as assessed by measurement of [^{3}H]-proline incorporation. When protein samples were treated with collagenase to differentiate the non-collagen protein synthesised from that of collagen, it was found that the newly synthesised non-collagen protein accounted for 8.3% (5.0-15.4%, n=16) of the total [^{3}H]-proline incorporation in fibroblasts following addition of stimulus. In myofibroblasts the corresponding figures were 10.1% (6.3-16.1%, n=16). Total [^{3}H]-proline incorporation was thus taken as a measure of collagen synthesis in subsequent experiments.

3.6.1.1 Actions of tryptase

The ability of tryptase to stimulate the generation and release of collagen from fibroblasts and myofibroblasts was tested. The effect of recombinant tryptase on the synthesis of collagen was compared with that of native lung tryptase added cells at the same concentration (Fig. 3.6.1). There was no significant difference in response to the different preparations of tryptase. However, human lung tryptase stimulated the synthesis of collagen from fibroblasts, where as recombinant tryptase did not. The effect of using a range of tryptase concentrations was investigated on cells of the MRC-5 fibroblast cell line, with and without TGF-β_2 treatment. Fibroblast [^{3}H]-proline incorporation in cells was increased in response to tryptase at a concentration of 30 mU/ml (Fig. 3.6.2). However, there was no significant induction of [^{3}H]-

proline incorporation with 100 mU/ml tryptase. Significant release of proteins incorporating [^{3}H]-proline was not observed in cell supernatants from fibroblasts. The degree of [^{3}H]-proline incorporation in proteins of cells or of cell supernatants was not affected by addition of tryptase to myofibroblasts.

The degree of [^{3}H]-proline incorporation in fibroblasts in response to tryptase was significantly inhibited by heat inactivation of the enzyme or by preincubation with APC1390, but not with the less potent inhibitor leupeptin (Fig. 3.6.3). Curiously when these protease inhibitors were added alone, there was an increase in [^{3}H]-proline incorporation in myofibroblasts.

In primary cultures of both fibroblasts and myofibroblasts, tryptase at 30 mU/ml stimulated an increase in [^{3}H]-proline incorporation in cell associated collagen. The effects of tryptase on [^{3}H]-proline incorporation were not inhibited when the enzyme was heat inactivated (Fig. 3.6.4). There was a strong correlation between the degree of [^{3}H]-proline incorporation in response to 30mU/ml tryptase and that to heat inactivated tryptase in both fibroblasts (r_s=0.758: $p<0.05$: n=10) and myofibroblasts (r_s=0.650: $p<0.05$: n=9). This strongly suggests that the effect was not dependent on an intact catalytic site.

Tryptase at 30 mU/ml stimulated an increase in [^{3}H]-proline incorporation in cell supernatants of fibroblasts, but not in those of myofibroblasts (Fig. 3.6.5). Once again the effects of tryptase on [^{3}H]-proline incorporation were not inhibited when the enzyme was heat inactivated. The degree of [^{3}H]-proline incorporation in response to tryptase was correlated with that in response to the heat inactivated tryptase in myofibroblasts (r_s=0.905: $p<0.05$: n=8), though this association was not found with fibroblasts.

3.6.1.2 Actions of PAR-2 agonist

The PAR-2 agonist peptide SLIGKV-NH_2 over a range of concentrations failed to induce significant alterations in the degree of [^{3}H]-proline incorporation in the proteins in either cells or cell supernatants (Fig. 3.6.6). The non-PAR-2 activating control peptide, LSIGKV-NH_2 also had little effect on [^{3}H]-proline incorporation and levels were not different from those in the presence of the agonist peptide. There was, however, considerable variation in responsiveness to the peptides. This appears to reflect fibroblast heterogeneity rather than random assay variation as the relative amount of collagen production, as measured by [^{3}H]-proline incorporation at one concentration of agonist, was frequently associated with that at another concentration. Thus, production of collagen from fibroblasts in response to 100 μM SLIGKV-NH_2 was significantly correlated with that from SLIGKV-NH_2 at 30 μM (r_s=0.829: $p<0.05$: n=6) and with that at 300 μM (r_s=0.943: $p<0.05$: n=6). Surprisingly production of collagen from myofibroblasts in response to 100 μM SLIGKV-NH_2 was significantly correlated with that in response to the control peptide LSIGKV-NH_2 at 100 μM (r_s=0.617: $p<0.05$: n=9). Production of collagen from fibroblasts in response to 100 μM SLIGKV-NH_2 was correlated with that from myofibroblasts at the same concentration of agonist (r_s=0.617: $p<0.05$: n=9).

Release of collagen from fibroblasts in response to 100 μM SLIGKV-NH_2 was correlated with that from SLIGKV-NH_2 at 10 μM (r_s=0.817: $p<0.05$: n=9), and also with that from 100 μM LSIGKV-NH_2 (r_s=0.783: $p<0.05$: n=9). Release of collagen from myofibroblasts in response to 100 μM SLIGKV-NH_2 was significantly correlated with that from SLIGKV-NH_2 at 10 μM (r_s=0.810: $p<0.05$: n=8), with that at 300 μM (r_s=0.943: $p<0.05$: n=6), and with that from LSIGKV-NH_2 at 100 μM (r_s=0.738: $p<0.05$: n=8).

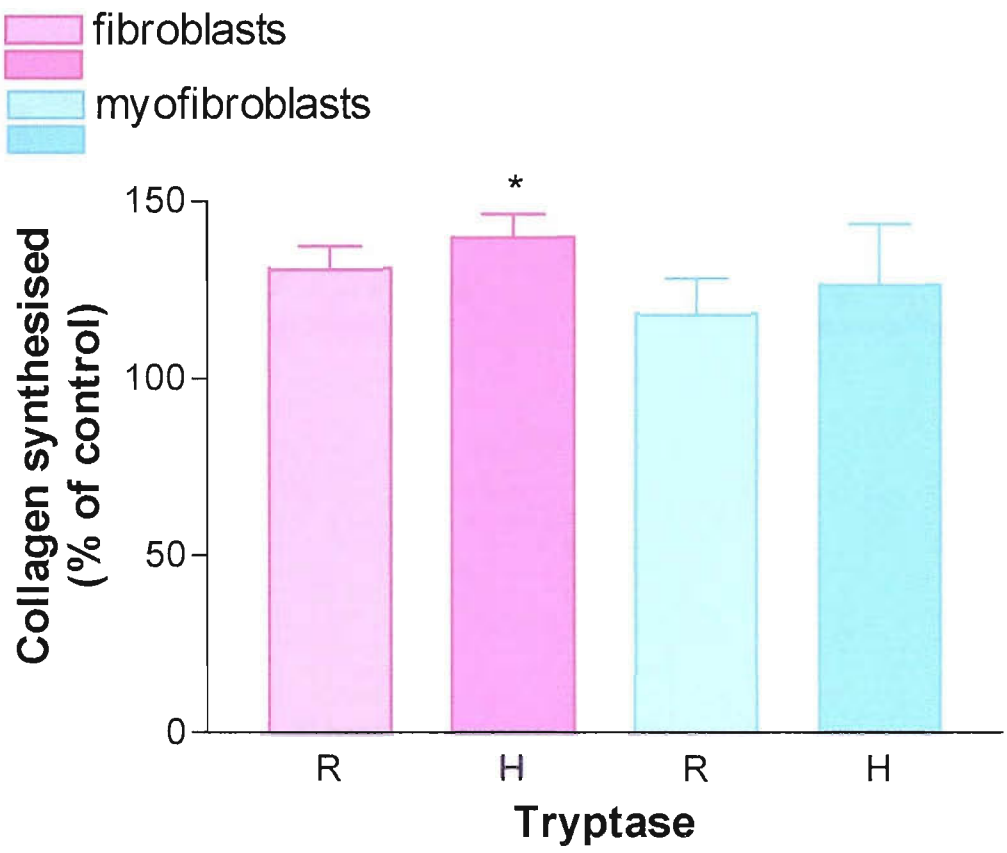


Fig. 3.6.1

Effect of 10 mU/ml human lung (H) and recombinant (R) tryptase on collagen production in cells of the MRC-5 line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). Mean ± SEM are shown for three separate experiments performed in duplicate. *P<0.05 compared with the response with no stimulus (taken as 100%) by Student's t test.

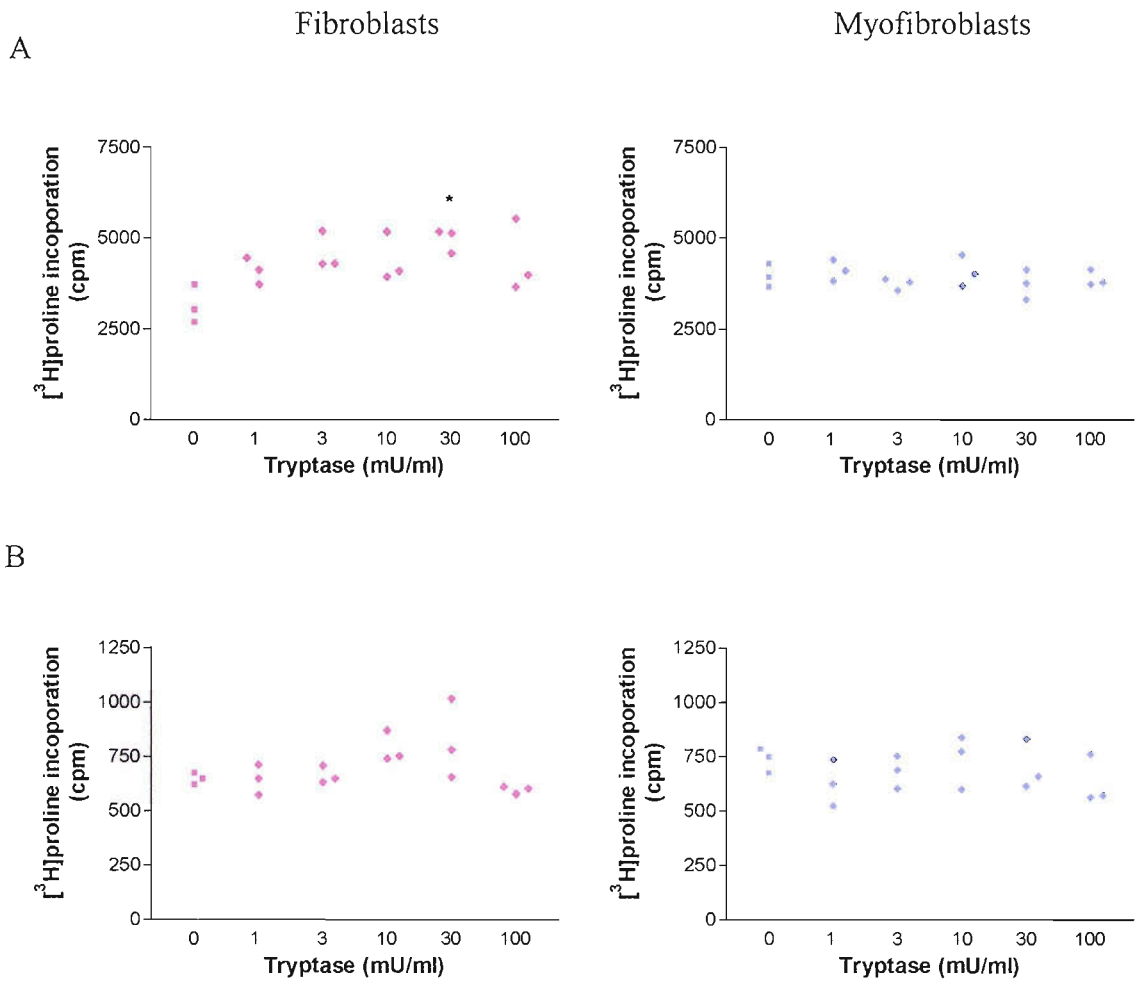


Fig. 3.6.2

Effect of tryptase on collagen production and release from cells of the MRC-5 line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). [^{3}H]-proline incorporation is shown for (A) protein in the cells or (B) in cell supernatants, 24 h after addition of tryptase. *P<0.05 compared with the response with no stimulus by Dunnett's test.

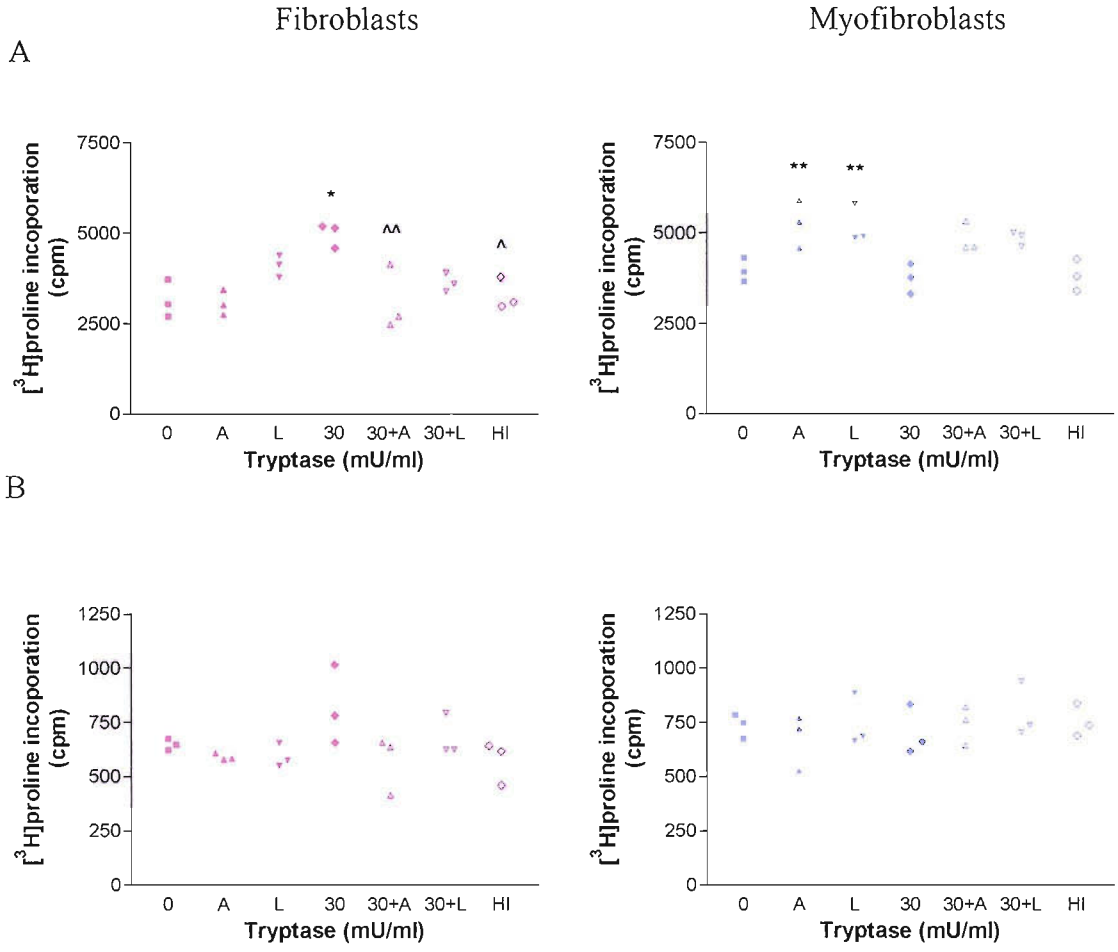
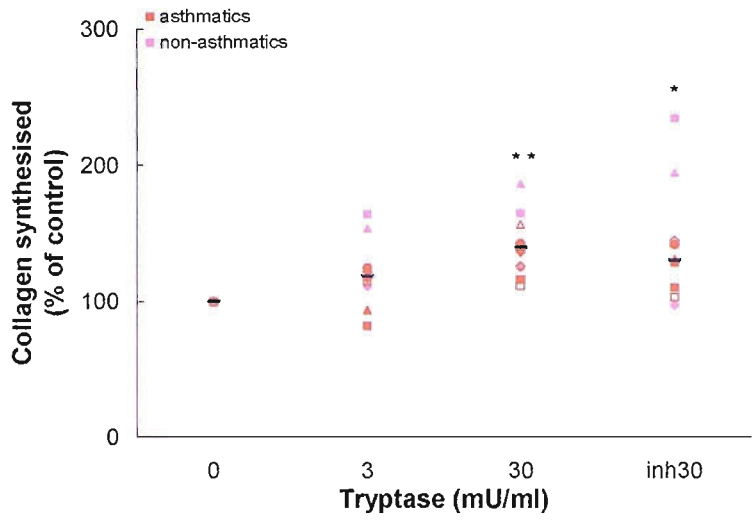


Fig. 3.6.3

Catalytic site dependency of the actions tryptase on (A) the production and (B) the release from fibroblast and myofibroblast-like cells of the MRC-5 line. Tryptase at 30mU/ml was preincubated for 30 minutes with 100 μM APC1390 (+A) or 50 μM leupeptin (+L), or was heat inactivated (HI) prior to addition to cells. [^{3}H] proline incorporation is shown in response to tryptase with and without the inhibitors, and also with the inhibitors with alone. * $P<0.05$ and ** $P<0.01$ compared with the response with no stimulus, and ^ $P<0.05$ and ^^ $P<0.01$ compared with 30mU/ml active tryptase by Dunnett's test.

A



B

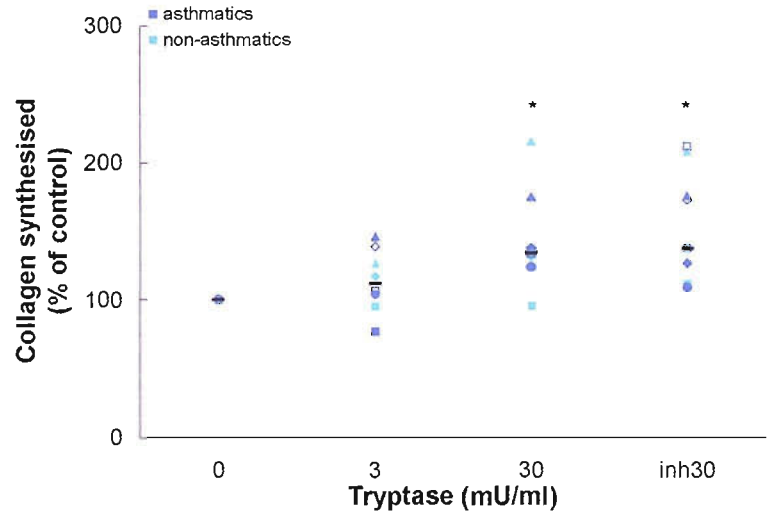
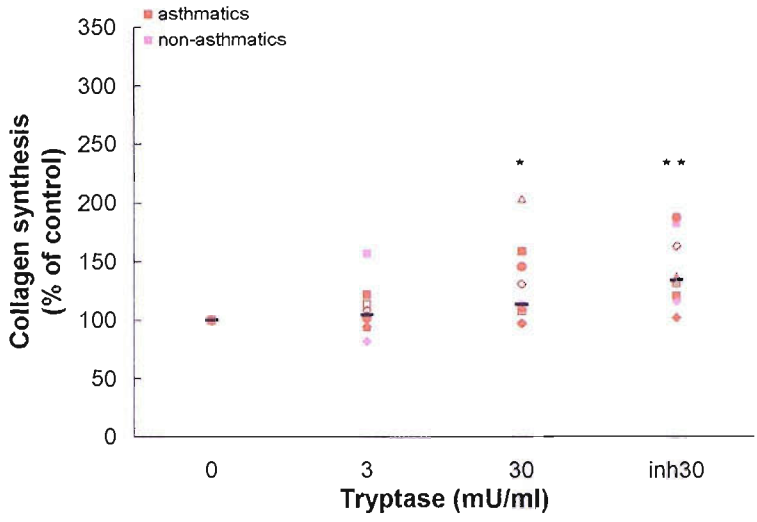


Fig. 3.6.4

Effect of tryptase on production of collagen by primary cultures of (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. Collagen synthesis by cells was assessed by measuring [^{3}H]-proline incorporation in cells following addition of tryptase or heat inactivated tryptase (inh-). Median values are shown for 10 separate experiments, each performed in duplicate. *P<0.05 and**P<0.01 compared with the response with no stimulus by Dunnett's test. The median [^{3}H]-proline incorporation in unstimulated fibroblasts was 3033 (660-8144) CPM and in the unstimulated myofibroblasts was 1634 (829-6319) CPM.

A



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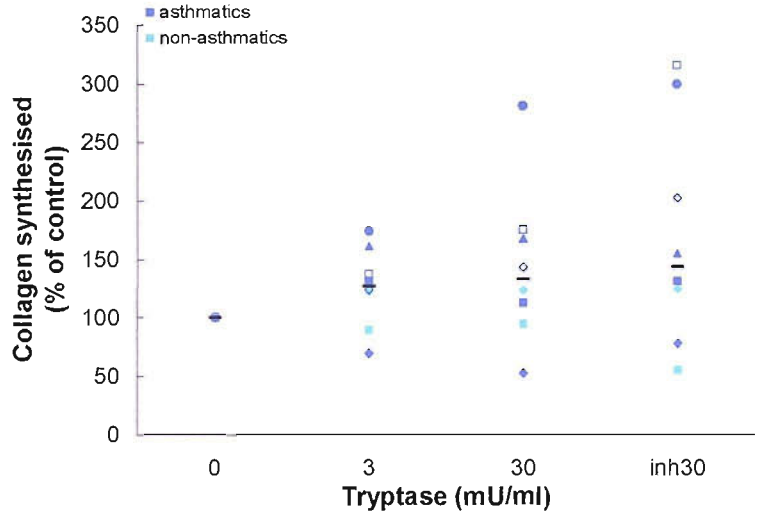


Fig. 3.6.5

Effect of tryptase on release of collagen by (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. Collagen synthesis by cells was assessed by measuring [^{3}H]-proline incorporation in cell supernatants following addition of tryptase or heat inactivated tryptase (inh-). Median values are shown for 8 separate experiments, each performed in duplicate. *P<0.05 and**P<0.01 compared with the response with no stimulus by Dunnett's test. The median [^{3}H]-proline incorporation in unstimulated fibroblasts was 656 (293-1105) CPM and in the unstimulated myofibroblasts was 552 (188-941) CPM

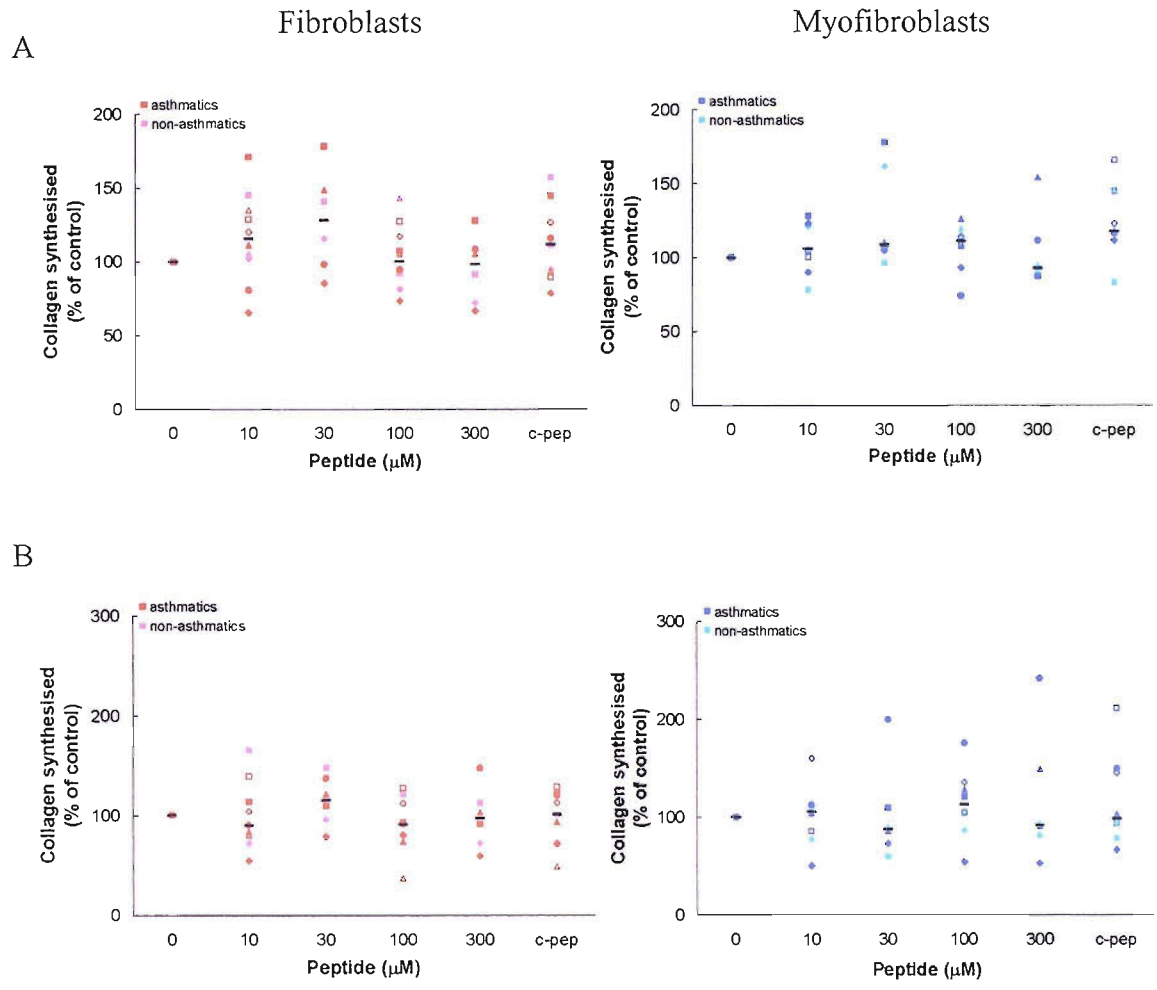


Fig. 3.6.6

Effect of the PAR-2 agonist peptide, SLIGKV-NH_2 on collagen production and release from fibroblasts and myofibroblasts. [^{3}H]-proline incorporation is shown for (A) proteins in the cells or (B) in cell supernatants 24 h after addition of SLIGKV-NH_2 or the control peptide LSIGKV-NH_2 (c-pep; at 100 μM). Median values are shown for 6 to 8 separate experiments performed in duplicate. Refer to Fig. 3.6.4 and 3.6.5 for the amount of radio activity in the unstimulated controls.

3.6.2 MMP production and activation

Gelatinase activity was detected in cell supernatants by zymography on 10% SDS polyacrylamide gel containing gelatine (1 mg/ml), 24 h after stimulation. The presence of gelatinase was investigated by determining the presence of clear bands in Coomassie blue-stained gels, and western blotting allowed identification of the protein (Fig. 3.6.7 (A)). There was one very strong band at approximately 72 kDa and two to four minor bands in each sample. The strong band at 72kDa and a weak band at 67kDa observed in zymography were confirmed to be MMP-2 by western blotting (Fig. 3.6.7 (B)). No MMP-9 was detected by western blotting. The intensity of the band for proMMP-2 in non-stimulated cells was assigned an arbitrary value of 1.0, when quantifying using a computerised image analysis system.

3.6.2.1 Actions of tryptase

The effect of recombinant tryptase on the synthesis of MMP-2 was compared with that of native lung tryptase added at the same concentration to cells (Fig. 3.6.8). There was no significant difference in response to the different preparations of tryptase by fibroblasts and myofibroblasts. Tryptase was found to stimulate increased quantities of proMMP-2 in fibroblasts and this trend was seen also for myofibroblasts (Fig. 3.6.9). ProMMP-2 release in response to 3 mU/ml tryptase was correlated with that to 30 mU/ml tryptase in both fibroblasts (r_s=0.964: $p<0.0001$: n=11) and myofibroblasts (r_s=0.933: $p<0.0001$: n=9). Heat inactivation of tryptase did not reduce the effect significantly. ProMMP-2 release in response to 30 mU/ml tryptase was correlated with that to heat inactivated tryptase of the same original concentration in both fibroblasts (r_s=0.900: $p<0.0001$: n=11) and myofibroblasts (r_s=0.879: $p<0.001$: n=9).

The levels of active MMP-2 were also increased in a dose dependent manner in fibroblasts and myofibroblasts (Fig. 3.6.10). Active MMP-2 was expressed as a proportion of the intensity for the proMMP-2 band in unstimulated cells of the same preparation. Where no active MMP-2 was detected, the value was taken as 0. Heat inactivation of tryptase clearly reduced the effect of tryptase in fibroblasts. A similar trend was observed in myofibroblasts, though it did not reach significance in these studies. There was no correlation between the amount of active MMP-2 released from fibroblasts and that from myofibroblasts in response to tryptase. Active MMP-2 release in response to tryptase from fibroblasts was correlated with that from myofibroblasts (r_s=0.5343: $p<0.05$: n=10). Active MMP-2 release in response to 30 mU/ml tryptase was correlated with that of pro MMP-2 release in myofibroblasts (r_s=0.857: $p<0.05$: n=8) but not in fibroblasts.

3.6.2.2 Actions of PAR-2 agonists

Addition of trypsin to fibroblasts or myofibroblasts was not associated with the release of proMMP-2 into cell supernatants, but active MMP-2 was detected in supernatants from preparations of myofibroblasts ($p<0.05$ compared with the response with no stimulus: n=4) (Fig. 3.6.11).

The PAR-2 agonist peptide, SLIGKV-NH_2 stimulated an increase in levels of proMMP-2 in supernatants from fibroblasts in a concentration dependent manner (Fig. 3.6.12 (A)). However, this pattern was not observed with myofibroblasts (Fig. 3.6.12(B)). Activated MMP-2 was observed in some but not all of the cell preparations investigated (Fig. 3.6.13). A correlation between the relative quantities

of proMMP-2 and active MMP-2 released from myofibroblasts was found (r_s=0.457: $p<0.05$: n=37), but this association was not observed with the fibroblasts.

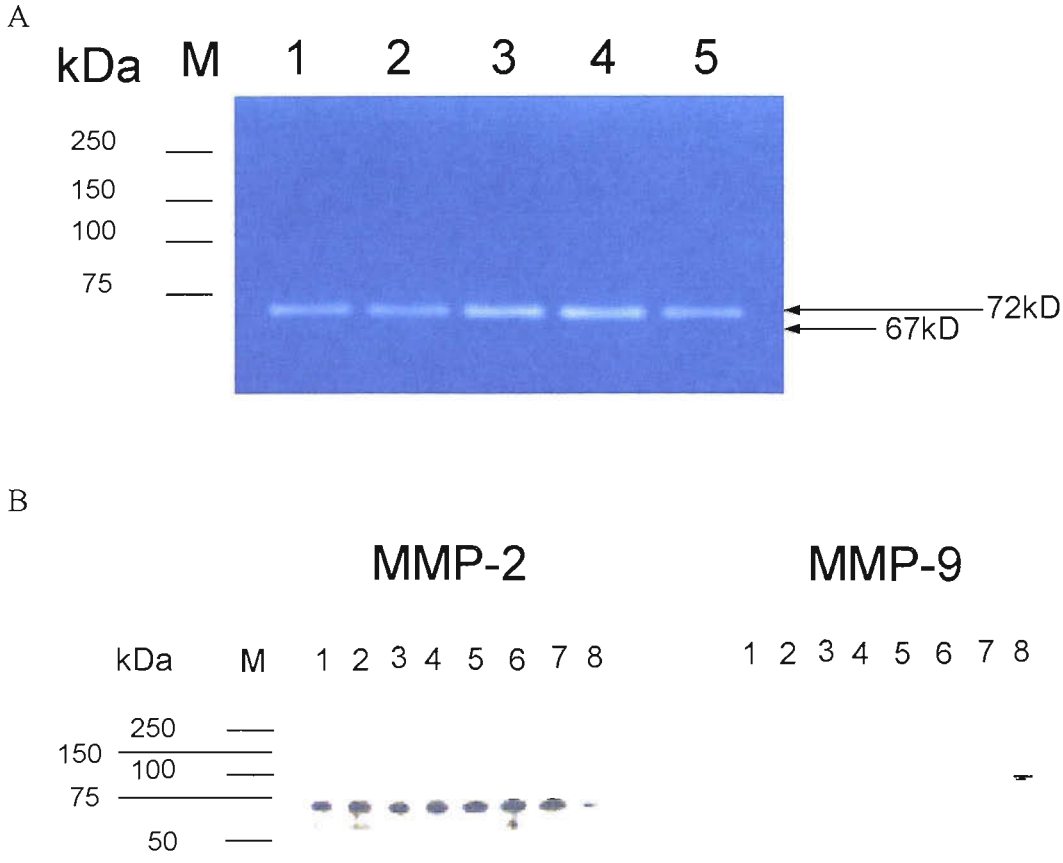


Fig. 3.6.7

(A) Coomassie blue stained gelatin zymography gel showing bands of MMP-2 48 h after addition of (lane 1) medium only, (2) 5 ng/ml of TNF-α, (3) 3mU/ml tryptase, (4) 30mU/ml tryptase and (5) heat inactivated tryptase of 30mU/ml. (B) Western blot for MMP-2 and MMP-9 48 h after stimulation with, (lane 1) medium only, (2) 5 ng/ml of TNF-α, (3) 10 μM PAR-2 activating peptide, SLIGKV-NH_2, (4) 100 μM SLIGKV-NH_2, (5) 100 μM non-PAR-2 activating control peptide, LSIGKV-NH_2, (6) 30mU/ml tryptase, (7) heat inactivated tryptase of 30mU/ml and (8) MMP positive control.

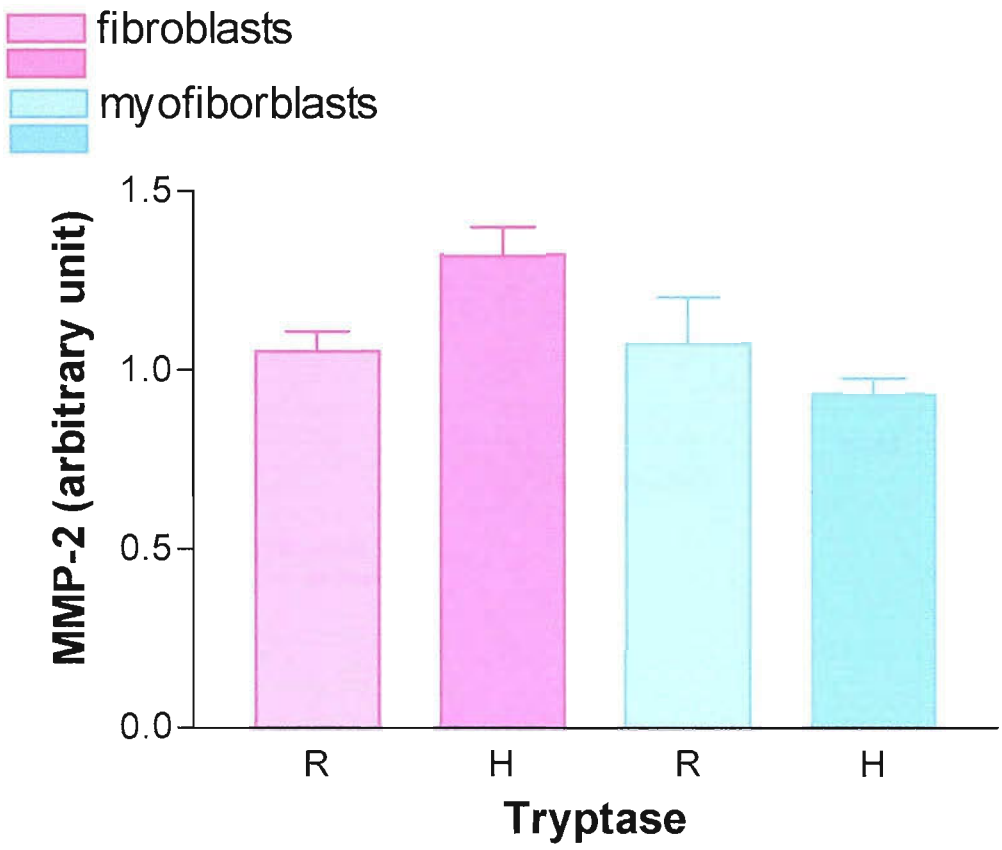


Fig. 3.6.8

Effect of 10 mU/ml human lung (H) and recombinant (R) tryptase on production of proMMP-2 in cells of the MRC-5 line without TGF-β pre-treatment (fibroblasts) or following pre-treatment with TGF-β (myofibroblasts). Mean ± SEM are shown for three separate experiments performed in duplicate.

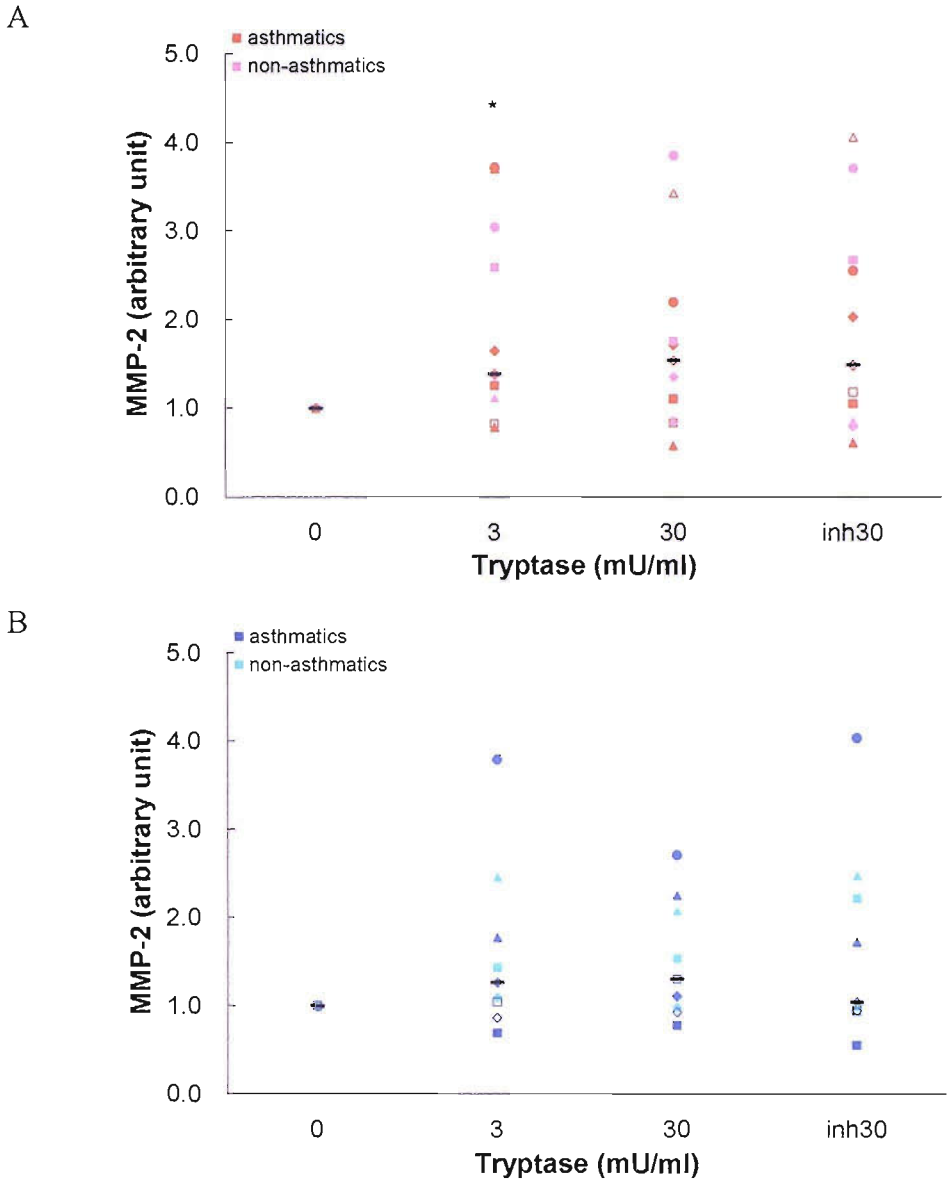


Fig. 3.6.9

Effect of tryptase and heat inactivated tryptase (inh-) on release of proMMP-2 by (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. The relative amount of proMMP-2 production was measured in cell supernatants by zymography. Median values are shown for 9 separate experiments. *P<0.05 compared with the response with no stimulus by Dunnett's test.

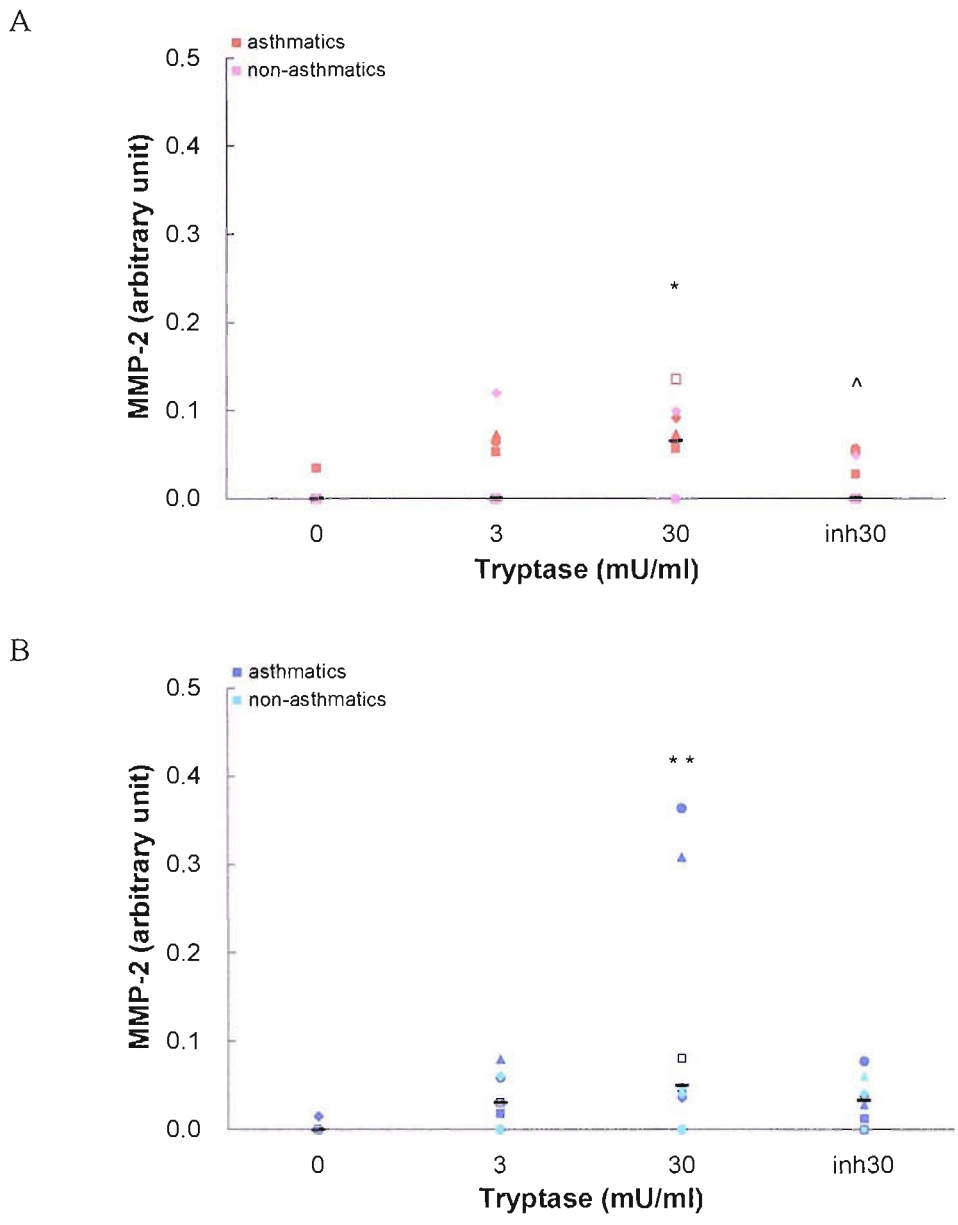
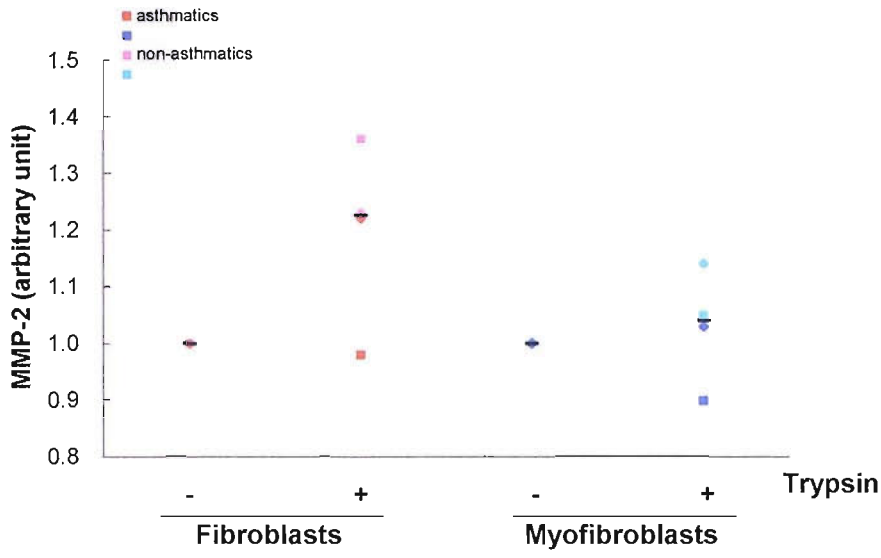


Fig. 3.6.10

Effect of tryptase and heat inactivated tryptase (inh-) on production of activated MMP-2 by (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. The relative amount of activated MMP-2 production was measured in cell supernatants by zymography. Median values are shown for 7 to 8 separate experiments. *P<0.05 and**P<0.01 compared with the response with no stimulus by Dunnett's test. ^P<0.05 compared with 30 mU/ml tryptase.

A



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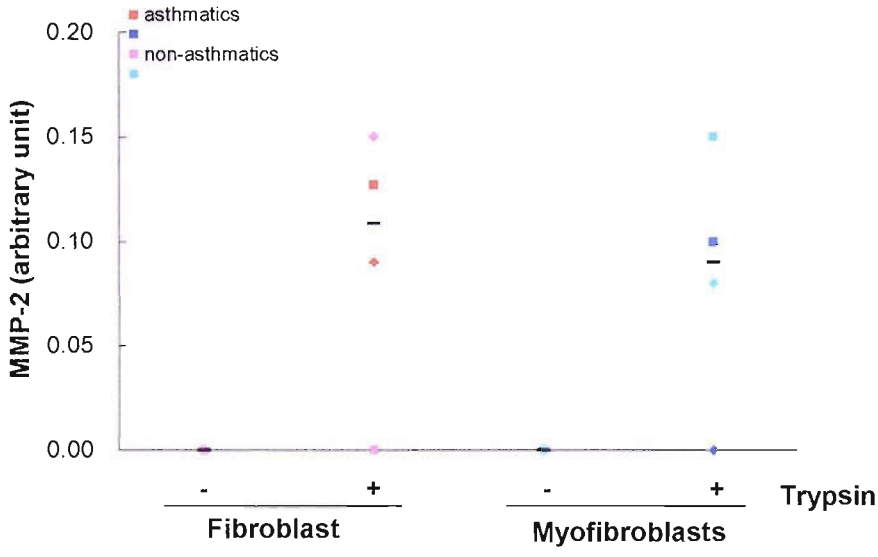
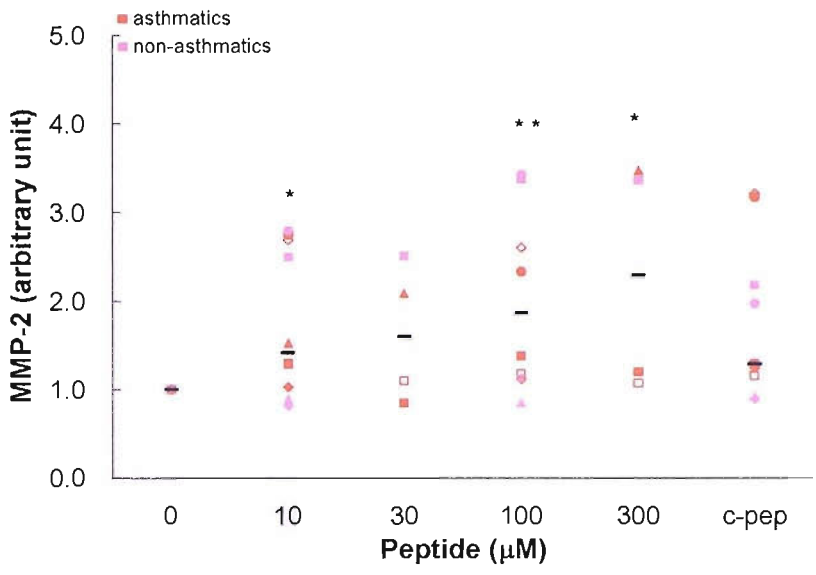


Fig. 3.6.11

Production of (A) pro and (B) active MMP-2 from primary cultures of fibroblasts (n=4) and myofibroblasts (n=4) stimulated with trypsin at 1 μg/ml for 24 h. Median values are shown.

A



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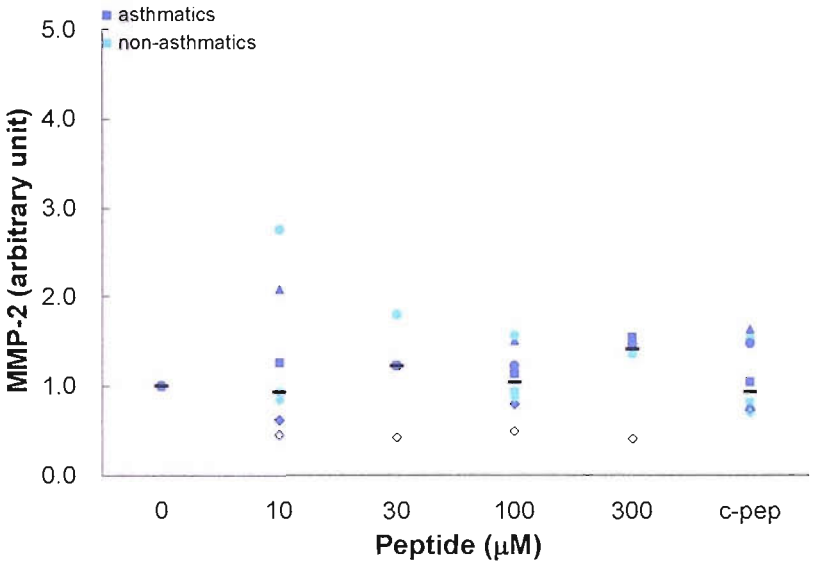
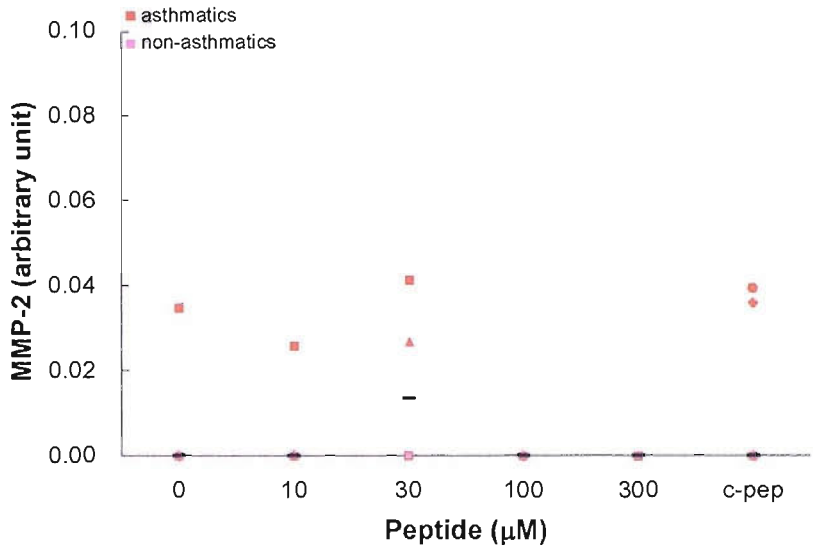


Fig. 3.6.12

Effect of the PAR-2 agonist peptide, SLIGKV-NH_2 and its control peptide (c-pep; at 100 μM), LSIGKV-NH_2 on release of proMMP-2 by (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. ProMMP-2 production was measured in cell supernatants by zymography. Median values are shown for 4 to 10 separate experiments. *P<0.05 and**P<0.01 compared with the response with no stimulus by Dunnett's test.

A



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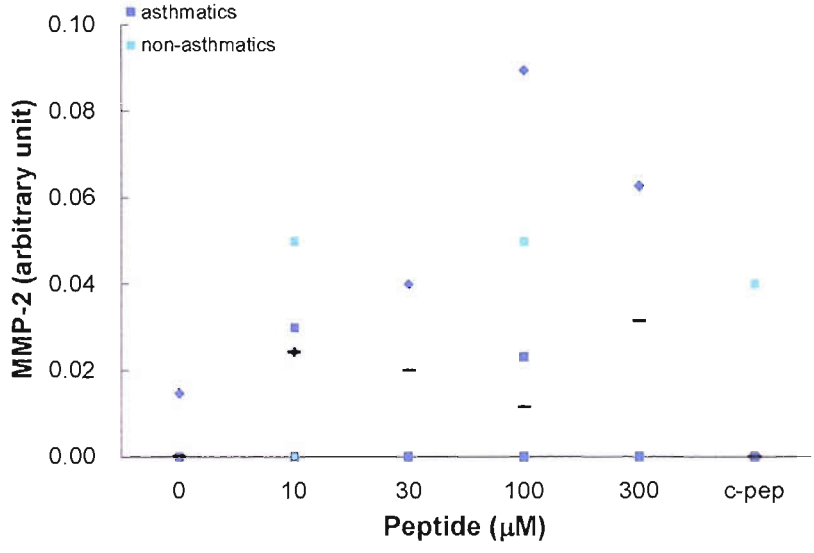


Fig. 3.6.13

Effect of the PAR-2 agonist peptide, SLIGKV-NH_2 and its control peptide (c- pep; at 100 μM), LSIGKV-NH_2 on release of activated MMP-2 by (A) fibroblasts and (B) myofibroblasts 24 h after stimulation. Activated MMP-2 production was measured in cell supernatants by zymography. Median values are shown for 4 to 10 separate experiments.

3.6.3 Characterisation of collagen types and TIMP

The effect of tryptase on expression of mRNA for the ECM components, collagen Iα, collagen IIIα, TIMP 1 and TIMP 2 was investigated by RT-PCR 6 h after addition using cDNA from primary cultures of fibroblasts and myofibroblasts. APRT was used to normalise the band intensity.

A distinct band was observed just above the 200 bp marker with both of the amplification conditions used with primers for collagen (expected value for collagen I: 214 bp and for collagen III: 207 bp) (Fig. 3.6.14). With both amplification conditions with primers for TIMP, there was a clear band observed between the 100 and 200 bp markers (expected value for TIMP 1: 170 bp and for TIMP 2: 155 bp). The presence of a single RT-PCR product showed that the primers and thermocycling conditions were appropriate in this system. Expression of mRNA for collagen Iα, collagen IIIα, TIMP 1 and TIMP 2 in the primary cultures of fibroblasts was confirmed.

The type of collagen synthesised by the fibroblasts and myofibroblasts in response to stimulation with tryptase was explored using RT-PCR to detect mRNA for types I and III collagen. Both collagen type I and III were produced by the fibroblast and myofibroblast preparations (Fig. 3.6.15). Expression of mRNA for collagen type I was not altered by addition of tryptase to fibroblasts, although addition of tryptase at 30 mU/ml to myofibroblasts appeared to increase expression with some preparations. Levels of mRNA for collagen type III were increased by tryptase to a greater extent in fibroblasts and myofibroblasts than was collagen type I in the same preparations.

The potential for the generation of TIMP in response to tryptase stimulation was examined by investigation of mRNA for TIMP1 and 2. For both of these inhibitors,

mRNA was expressed in fibroblasts and myofibroblasts (Fig. 3.6.16). There was a trend for increased expression of mRNA for TIMP 1 and TIMP 2 in response to tryptase stimulation in fibroblasts and myofibroblasts, though this did not reach statistical significance in these studies.

Summary

Tryptase was found to induce an increased release of collagen from fibroblasts ($P<0.01$) and myofibroblasts ($P<0.05$) but the peptide agonist of PAR-2 did not alter collagen synthesis significantly under the same conditions. The type of collagen synthesised by fibroblasts and myofibroblasts in response to tryptase was determined to be type III collagen rather than type I collagen. Increased amounts of MMP-2 were released in response to tryptase from fibroblasts ($P<0.05$) and myofibroblasts ($P<0.01$). The peptide agonist of PAR-2 stimulated an increase in MMP-2 release by fibroblasts ($P<0.01$) but not from myofibroblasts.

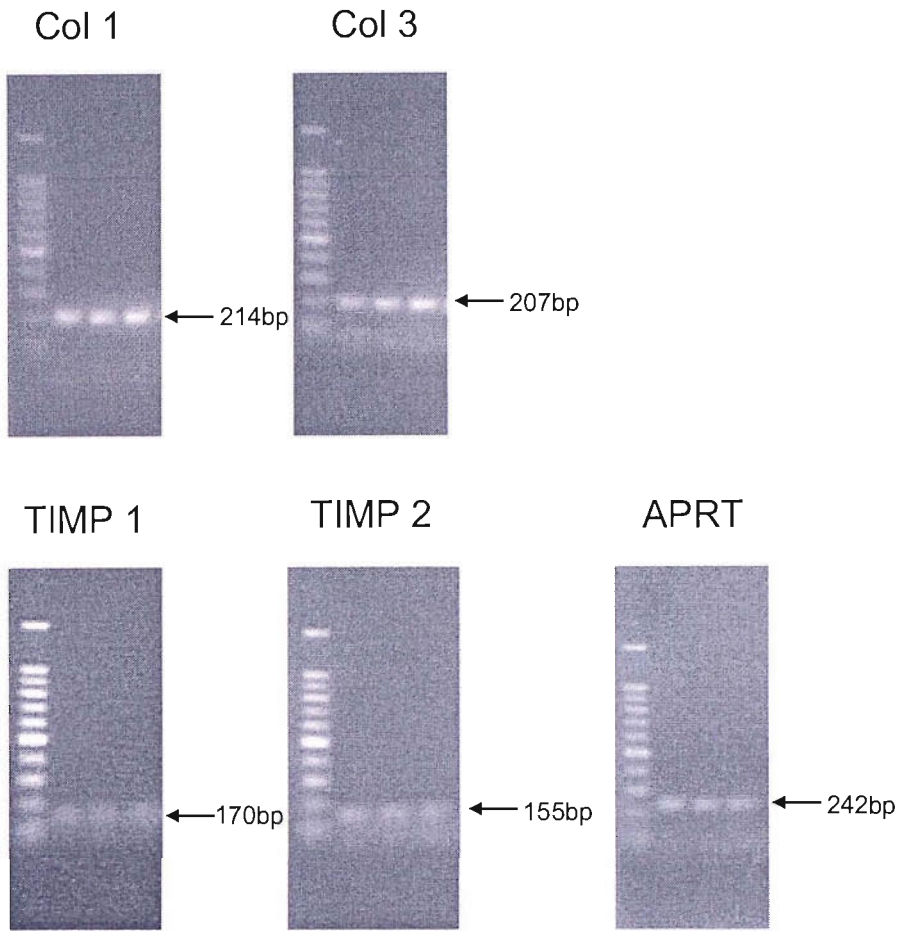


Fig. 3.6.14

Expression of collagen I, collagen III, TIMP1 and TIMP2 in preparations of fibroblasts stimulated with tryptase for 6 h. RT-PCR products were run on a 1.5% agarose gel. APRT was included as a house keeping gene. These gels are representative of these obtained in three separate experiments with different preparations of fibroblasts.

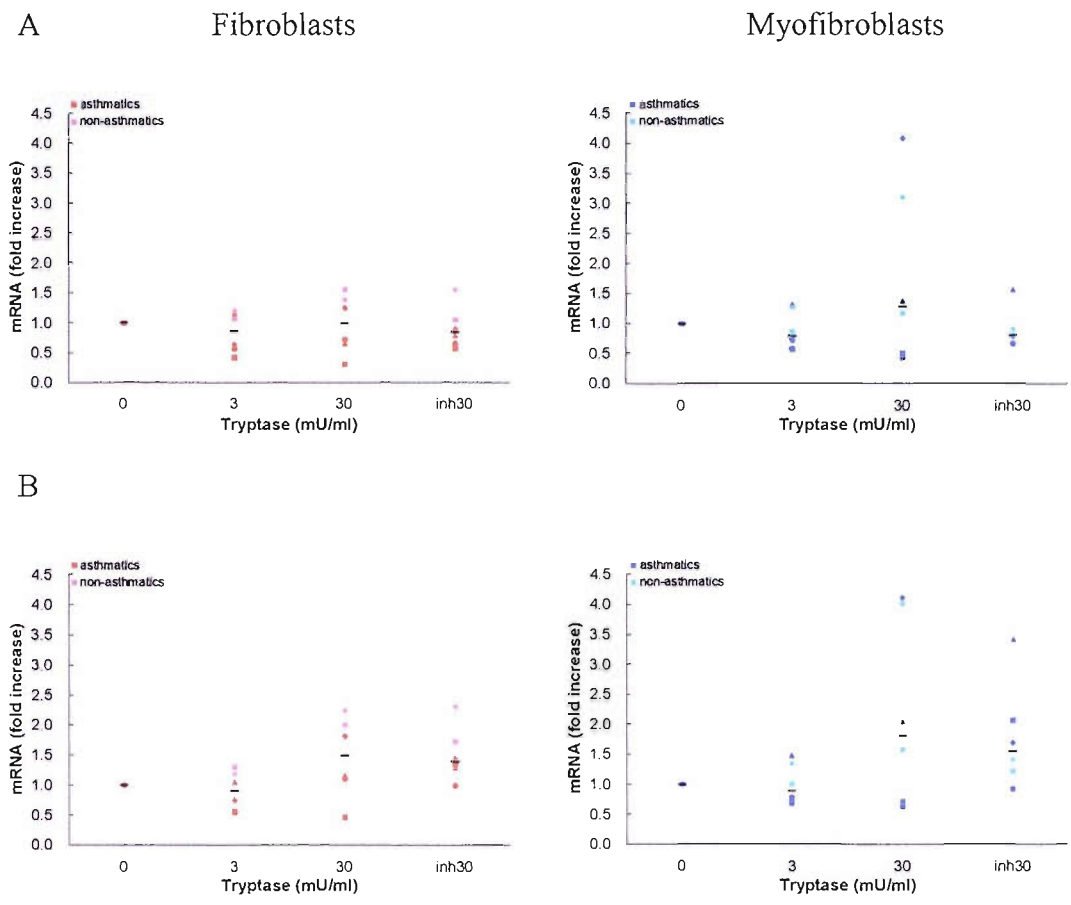


Fig. 3.6.15

Effect of tryptase and heat inactivated tryptase (inh-) on expression of mRNA for (A) collagen I and (B) collagen III by fibroblasts and myofibroblasts 6 h after stimulation, as measured by RT-PCR. Median values are shown for 6 separate experiments.

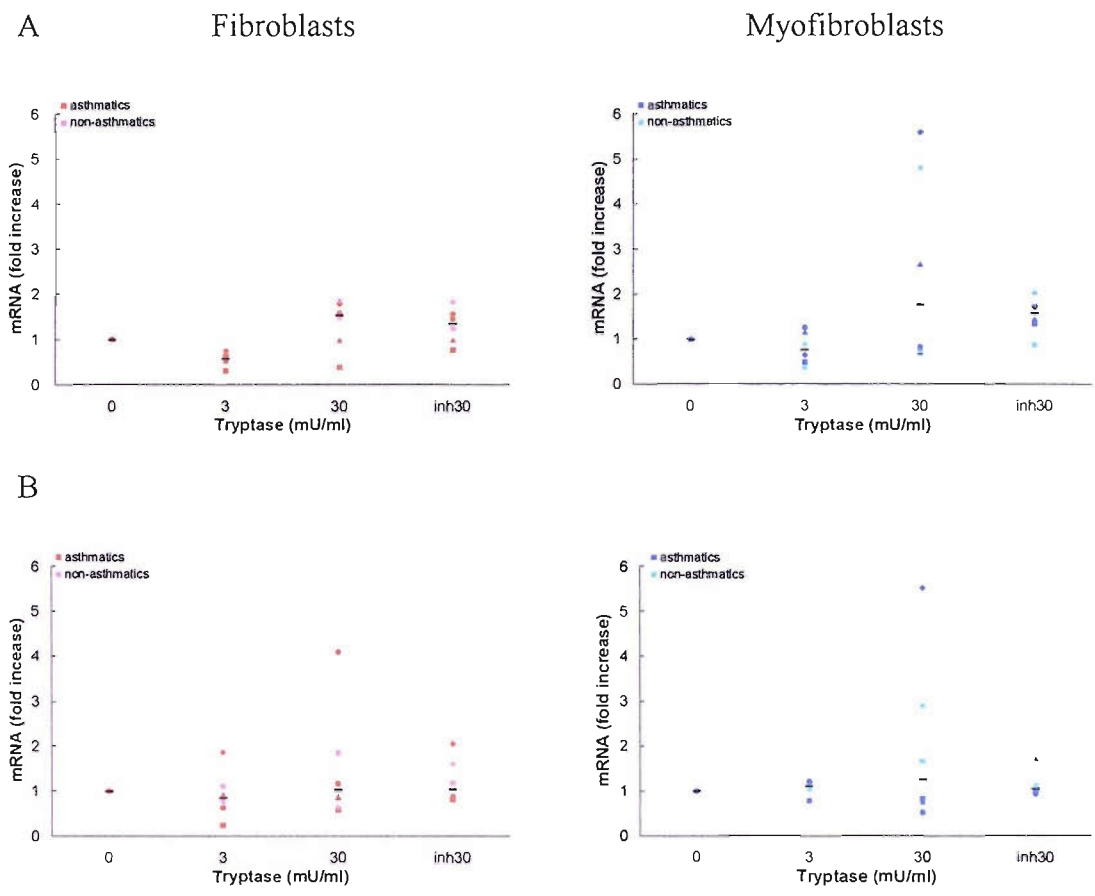


Fig. 3.6.16

Effect of tryptase and heat inactivated tryptase (inh-) on expression of mRNA for (A) TIMP1 and (B) TIMP2 by fibroblasts and myofibroblasts 6 h after stimulation as measured by RT-PCR. Median values are shown for 6 separate experiments.

Chapter 4

Discussion

4 Discussion

These studies represent the first systematic investigations of the actions of mast cell tryptase and other agonists of PAR-2 on primary cultures of human lung fibroblasts and myofibroblasts. There was considerable heterogeneity in the responsiveness of cell preparations, but in various systems, tryptase was found to induce biological effects on these cells. This major mast cell product could contribute to processes of inflammation and tissue remodelling in bronchial asthma and other conditions. Some of the actions of tryptase were dependent on an intact catalytic site, and suggest a role for PAR-2, while for others there was little evidence for this mechanism.

Employing preparations of tryptase of high purity was essential for these studies. The recombinant β-tryptase isolated from a *Pichia pastoris* expression system was characterised in detail and was demonstrated to have similar enzymatic and physicochemical properties to tryptase purified from human lung tissue. The same preparation was employed for consistency throughout these studies.

The differences between the recombinant tryptase and the native enzyme in some of the physicochemical properties may have been a consequence of post-translational processing. The recombinant tryptase required NaCl at a concentration of 4.0 M for elution from heparin, while human lung tryptase could be eluted with 1.0 M NaCl. The stronger binding of recombinant tryptase to heparin may reflect a different pattern of glycosylation. This is also likely to contribute to the slight difference in molecular mass observed on polyacrylamide gel electrophoresis with silver staining and on western blotting in this and in a previous study of recombinant tryptase (Niles *et al.* 1998). Differences in charge and molecular mass in tryptase from human tissue have

been attributed to differences in glycosylation, as deglycosylation studies with peptide N-glycanase (PNGase) F have been found to induce a reduction of approximately 3 to 8 kDa in the molecular weight (Peng *et al.* 2003). There is also the potential for differences in amino acid sequence to contribute to the heterogeneity, but much of that which is stored and released by mast cells is likely to be the β form.

Studies involving heat inactivation of tryptase also revealed differences between native and recombinant preparations, with the latter proving more stable. Thus, heat inactivation at 94°C for 5 min resulted in complete abolition of the activity of human tryptase but only 65% of that of the recombinant tryptase. Again differences in glycosylation are the most likely explanation. Standardisation of preparations of tryptase so that the material is representative of that which is secreted form mast cells in vivo represents a challenge, and it remains to be determined the extent to which glycosylation may provide a means for the regulation of tryptase function. Nevertheless, the preparation of recombinant tryptase did share enzymatic properties with that purified from human lung tissue, and it could be inhibited by the protease inhibitors, leupeptin as well as by the selective tryptase inhibitor APC1390.

The percentage of tryptase estimated to be enzymatically active was very high in all preparations of purified tryptase. Where calculations indicated a percentage greater than 100%, the overestimation may have been due to the concentration of the 4-methylumbelliferone stock solution used to produce the standard curve being inaccurate, or to a shift in extinction coefficient (Jameson *et al.* 1973) owing to underestimation of protein, or possibly to both.

Human lung fibroblasts were successfully transformed into cells of a myofibroblast phenotype after incubation with TGF-β_2 for 72 h, using the procedure described by

Richter *et al.* (2001). The TGF-β_2-induced myofibroblasts clearly possessed a myofibroblast phenotype, and expressed α-smooth muscle actin and vimentin, but not smooth muscle myosin as has been described by Low (1999). An advantage in using primary cultures of TGF-β-induced fibroblasts is that the transformation from fibroblasts to myofibroblasts has occurred for the cell population at the same time, and it is possible to investigate differences between the two cell phenotypes in well characterised, closely related cell populations. Primary cultures of myofibroblasts obtained directly from tissue could represent an alternative source of cells for study instead of using TGF-β stimulated fibroblasts. However, with primary cultures of cells with the myofibroblast phenotype, it is generally not clear to what extent the cells may represent myofibroblasts, or fibroblasts that have differentiated into myofibroblasts in culture.

TGF-β_2 has been used to induce myofibroblasts in investigations designed to imitate the remodelling that occurs during wound healing. Levels of TGF-β and especially TGF-β_2 are increased by epithelial injury (Zhang *et al.* 1999b), and TGF-β_2 is likely to be a natural stimulus for inducing a phenotypic change in fibroblasts in the airways. The myofibroblast phenotype induced by TGF-β_1 has been reported to be unstable, and removal of TGF-β to result in decreased expression of α-smooth muscle actin (Arora & McCulloch 1999). However, in the present studies, removal of TGF-β_2 from the system did not lead to a reduction in numbers of cells of the myofibroblast phenotype after a period of 48 h. Stimulation of myofibroblasts with TGF-β_2 for a further 48 h did not induce myosin expression. TGF-β_2 added to cells may have affected the performance of the mitogenesis assay, as TGF-β can regulate apoptosis of fibroblasts and myofibroblasts (Zhang & Phan 1999) which may interfere with the system. In this study, to avoid any effect that exogenous TGF-β_2 may have on cell

responses, this growth factor was removed from cultures of myofibroblasts before addition of the stimulus.

The transformed myofibroblasts varied greatly in the degree to which they expressed smooth muscle actin. It is thus difficult to assess by immunocytochemistry the proportion of cells that may have been transformed into myofibroblasts. There were no significant differences in response between fibroblasts and myofibroblasts examined in these studies. Myofibroblasts which express different quantities of smooth muscle actin may not behave in a uniform way, and it is possible that the myofibroblast cultures contain not just a population of myofibroblasts, but also fibroblasts and perhaps cells with an intermediate phenotype. Such heterogeneity is also likely to occur in the lung in health and disease, though how closely the profile in cell culture may mimic that in the physiological situation is difficult to assess. It is possible that some of the effects of tryptase and other agonists on the myofibroblast cultures are a consequence of actions on fibroblasts present in these cultures. For this reason, the findings with the myofibroblast cultures have been compared with those of the corresponding fibroblast cultures.

4.1 Tryptase as a mediator of fibroblast and myofibroblast mitogenesis and proliferation

Tryptase was able to induce dose-dependent alterations in [^{3}H]-thymidine incorporation in both fibroblast and myofibroblast cultures. A bell-shaped response was noted, possibly on account of negative feedback mechanisms that could induce receptor desensitisation. As has been suggested by some previous reports (Cairns & Walls 1997, Akers *et al.* 2000, Frungieri *et al.* 2002), tryptase could represent an important stimulus for mitogenesis. Although effective at inducing fibroblast and myofibroblast mitogenesis, tryptase did not induce an increase in cell numbers under the conditions studied. This was somewhat surprising as increases in response to tryptase have been reported previously with the MRC-5 human fibroblast cell line (Cairns & Walls 1997), with primary cultures of human adult parenchymal lung fibroblasts (Akers *et al.* 2000), and with a human foetal foreskin fibroblast cell line (Frungieri *et al.* 2002). However, this has not been investigated with primary cultures of human lung myofibroblasts, and heterogeneity in cell preparations may have contributed to the inability to induce cell proliferation in the present studies. In this respect, there is a parallel with the report that tryptase can stimulate mitogenesis but not cell proliferation in HUVEC (Compton *et al.* 1998). It is possible that additional stimuli are necessary for tryptase to trigger proliferation in the primary cultures of fibroblasts and myofibroblasts studied.

There was considerable heterogeneity between cell preparations in the degree to which cell mitogenesis was induced in response to the various stimuli. The nature of such heterogeneity is difficult to assess. Corteling *et al.* (2003) have reported that passage number could affect the degree of mitogenesis in primary cultures of guinea-pig tracheal smooth muscle cells in response to tryptase and other PAR-2 agonists. In the present studies, passage number was not found to be associated with variation in the

mitogenic response with tryptase. Differences in cell responses to tryptase have been reported with other cell types from different subjects. Thus, for example, eosinophils isolated from asthmatic subjects have shown quite different responses to tryptase in the release of granule mediators, with one third of the population appearing to be unresponsive (Vliagoftis *et al.* 2004). The issue as to whether the responsiveness of fibroblasts and myofibroblasts from asthmatics differed from those of non-asthmatics was investigated in the present studies, but no evidence for altered responses to tryptase were noted under the conditions employed.

Heat-inactivation of tryptase reduced the enzymatic activity, but it did not alter the mitogenic actions of tryptase. Though the majority of studies have indicated proteolytic actions of tryptase on cells, it has been suggested that tryptase may alter cell function also through non-proteolytic mechanisms. Thus, Brown *et al.* (2002) have reported that an increase in DNA synthesis induced by human lung tryptase on human airway smooth muscle cells was not affected by irreversible inhibition of catalytic activity by the serine protease inhibitor pAPMSF. In the present studies, a failure to inactivate tryptase fully could provide another explanation, perhaps as a consequence of the relative resistance of the recombinant tryptase to heat-inactivation.

The actions of tryptase on mitogenesis were reduced by preincubating the enzyme with the tryptase inhibitor APC 1390. At the concentrations employed in this study, leupeptin did not significantly inhibit the mitogenic actions of tryptase but there was a trend for this. The studies with tryptase inhibitors suggest that an intact catalytic site is required for tryptase-provoked mitogenesis of fibroblasts and myofibroblasts, and PAR-2 deserves particular attention as a substrate in the system.

An antagonist of PAR-2 is not available. However, the finding that the effects of tryptase on mitogenesis could be mimicked by the addition of the peptide agonist

SLIGKV-NH_2 suggests that the mitogenic actions may be mediated by activation of PAR-2. These findings are consistent with previous reports that tryptase and PAR-2 agonist peptides can stimulate cell mitogenesis in a dose dependent manner in lung fibroblasts (Akers *et al.* 2000), and in airway smooth muscle cells (Berger *et al.* 2001a). Moreover, recombinant β tryptase has been reported to induce mitogenesis in a PAR-2 expressing murine lymphoid cell line, but not in cells expressing PAR-2 that had been modified by an amino acid substitution at the cleavage site rendering them unresponsive to proteolytic activation (Mirza *et al.* 1997).

Somewhat surprisingly, though mitogenesis was stimulated by tryptase in myofibroblasts, this was not observed with the PAR-2 agonist peptide. It is possible that the concentration of the agonist peptide employed was not high enough to provoke PAR-2 stimulation in myofibroblasts. The peptide agonist is relatively weak as an investigational tool, and can be susceptible to degradation. A role for tryptase as a stimulus of myofibroblast mitogenesis remains unclear, and it could possibly be independent of PAR-2.

Calcium flux was induced by the PAR-2 agonist peptide SLIGRL-NH_2 confirming that the PAR-2 expressed on these cells was functional. The result was verified by PAR-2 desensitisation studies and by the lack of response to the non-PAR-2-activating peptide added as a control. There was evidence also of functional PAR-1 expression detected using the peptide agonist, but not of PAR-3 or PAR-4. There is currently no peptide agonist for PAR-3 available, so PAR-3 expression was examined indirectly by investigating the ability of thrombin to stimulate a response after desensitising the cells to other PAR.

In these studies, the preparations of lung myofibroblasts expressed functional PAR-2 as assessed by calcium flux in response to peptide agonists of PAR-2 and trypsin.

There has been some controversy over the extent to which PAR-2 may be expressed by fibroblasts, and until recently tools for the detection of PAR-2 have been limited. However, with the advent of the new PAR-2 specific monoclonal antibody, P2A, an improved method for immunocytochemical detection of PAR-2 is possible. Using this antibody, expression of PAR-2 was detected by immunocytochemistry in cultures of myofibroblasts as well as in fibroblasts. In addition to surface expression, there was strong cytoplasmic expression suggesting the presence of an intracellular pool of PAR-2.

There appeared to be constant expression of mRNA for PAR-2 in fibroblasts and myofibroblasts as assessed by RT-PCR over a period of 48 hours. PAR-2 expression has not been investigated previously in lung myofibroblasts, but this receptor has been detected in cells with similar characteristics in other tissues. Progressive transformation of rat hepatic stellate cells to myofibroblasts in culture has been reported to be associated with increased mRNA for PAR-2 as assessed by RT-PCR and northern blotting, and also with increased expression of PAR-2 in western blotting (Gaca *et al.* 2002). The expression of mRNA for PAR-2 has been reported to be constant in primary cultures of guinea pig tracheal smooth muscle cells over different passages (Corteling *et al.* 2003). Masamune *et al.* (2005) have observed PAR-2 expression in rat pancreatic stellate cells by western blotting, and detected mRNA for this receptor. Interestingly, expression of PAR-2 was not detected in freshly isolated cells in that study, and was observed only after the cells had been cultured.

The failure of both recombinant and purified lung tryptase to stimulate calcium flux and hence to activate PAR-2, appears to be at variance with previous reports of tryptase acting as an agonist for this receptor. Most studies that have appeared to indicate that tryptase can act through PAR-2, have been based simply on the observation of PAR-2 expression in the cells employed, and on the finding that actions

of tryptase can be mimicked by PAR-2 agonist peptides. However, tryptase as well as trypsin and PAR-2 agonist peptides have been reported to induce increases in levels of intracellular calcium in primary cultures of keratinocytes (Steinhoff *et al.* 1999), human bronchial smooth muscle cells (Schmidlin *et al.* 2001), and bovine aortic endothelial cells (Sendo *et al.* 2003), all cells that have been found to express PAR-2. Calcium flux was investigated in myofibroblast preparations (which in these studies will have contained fibroblasts also, approximately 15% of the cells present). In future studies, the effects on calcium flux in non-TGF-β exposed fibroblasts should be tested with tryptase and PAR-2 agonists.

The idea that tryptase can induce calcium flux through PAR-2 activation has been strengthened by studies in which keratinocytes have been desensitised to tryptase by preincubation with a PAR-2 agonist peptide or trypsin (Schechter *et al.* 1998). Moreover, Berger *et al.* (2001b) have found that desensitisation of airway smooth muscle cells with tryptase could block induction of an increase in intracellular calcium in response to PAR-2 agonists, and vice versa. The induction of calcium flux by tryptase required enzymatic activity. In contrast, addition of tryptase to non-PAR-2 expressing cells such as platelets has been reported to be without effect on intracellular calcium levels (Molino *et al.* 1997).

The failure of tryptase to stimulate the activation of PAR-2 has been reported by Compton *et al.* 2001, who did not see any effect on calcium flux in cells expressing rat PAR-2. Moreover, Weidinger *et al.* (2003) have recently reported that though enzymatically active tryptase can inhibit the motility of human spermatozoa that express PAR-2, it did not alter intracellular calcium levels in these cells. Furthermore, treatment of spermatozoa with pertussis toxin (a blocker of Gi proteins) did not inhibit the effects of tryptase on motility, suggesting the potential for non-PAR-2 mediated pathways for tryptase action.

Tryptase has been found to induce responses in cells by a catalytic mechanism apparently in the absence of PAR-2. Although Vliagoftis *et al.* (2004) did not detect expression of PAR-2 in eosinophils by flow cytometry, tryptase provoked the release of eosinophil peroxidase from these cells. A PAR-2 agonist peptide failed to reproduce this response in these cells. Evidence for a non-PAR-2 dependent pathway for tryptase actions has come also from studies of tryptase-induced DNA synthesis in primary cultures of guinea pig tracheal smooth muscle cells maintained up to the second passage (Corteling *et al.* 2003). The actions of tryptase on mitogenesis were reduced by the inhibitor leupeptin, and although expression of PAR-2 on these cells was suggested by detection of specific mRNA, a PAR-2 agonist peptide did not induce DNA synthesis.

In spite of the various reports to the contrary, there is evidence that tryptase can indeed act through PAR-2. Lung tryptase can induce [^{3}H]-thymidine incorporation and cell proliferation, as measured by the MTS procedure in primary cultures of human airway smooth muscle cells (Berger *et al.* 2001a). The effects of tryptase on these cells were abolished by preincubating with protease inhibitors and by heat-inactivating the enzyme. Application of pertussis toxin to the cells inhibited the mitogenic actions of tryptase, and a PAR-2 agonist peptide mimicked the actions of tryptase. Thus, these mitogenic and proliferative responses of cells to tryptase are likely to be mediated by PAR-2. Human recombinant tryptase has been reported to induce the expression of mRNA for IL-8 from human peripheral blood eosinophils on which PAR-2 had been detected by FACS (Temkin *et al.* 2002). This was decreased in the presence of antisera to which PAR-2 blocking effects were ascribed, supporting the idea that the actions of tryptase were mediated by PAR-2 in this system. TNF-α release from a human astrocytoma cell line has been reported to be induced by trypsin, a PAR-2 agonist peptide and by tryptase (Kim *et al.* 2002). Each of these three stimuli have

been found to induce the depolarisation of guinea pig submucosal neurons (in which mRNA for PAR-2 was detected) (Reed *et al.* 2003). Alm *et al.* (2000) have reported that tryptase purified from human lung or skin can cleave murine PAR-2 tagged with insulin C-peptide to allow quantification by ELISA expressed in an insect cell line, though the potency of tryptase in this system was only some 10% of that of pancreatic trypsin (Alm *et al.* 2000).

Conflicting accounts of the ability of tryptase to activate PAR-2 could be due to differences in the PAR-2 expressed on the cells. Glycosylation could restrict access of tryptase to the PAR-2 cleavage site. Compton *et al.* (2001) have reported that the failure of tryptase to activate PAR-2 could be overcome by treatment with neuraminidase or tunicamycin, or by site-direct mutation of human embryonic kidney cells or rat kidney epithelial cells transfected with PAR-2. The cleavage of deglycosylated PAR-2 by tryptase was confirmed by the detection of the peptide cleaved from PAR-2 using immunocytochemistry and HLPC analysis. Heparin in a ratio of 2: 1 with tryptase was found to inhibit PAR-2 cleavage by tryptase, suggesting heparin may interrupt the interaction of tryptase with PAR-2. Alteration of the position of the glycosylation sites of human PAR-2 in a Chinese hamster ovary fibroblast cell line by site directed mitogenesis (so that glycosylation was removed) was found to increase the sensitivity to tryptase cleavage (Compton *et al.* 2002a). This was also forced to reduce the sensitivity to other PAR-2 agonists as assessed by calcium flux measurements. Such studies have been extended to a rat aortic ring model in which it has been reported that tryptase could induce relaxation after neuraminidase treatment of the tissues, whereas such a pretreatment was not necessary for a response with trypsin or with a peptide agonist of PAR-2 (Compton *et al.* 2002b).

A polymorphic variant of PAR-2 has been described with an amino acid substitution in the second extracellular loop, the agonist recognition site of PAR-2 (Compton *et al.*

2000). The mutant receptor showed marked differences in sensitivity to various PAR-2 agonists as assessed by measurement of intracellular calcium levels. Genetic variation could provide an additional explanation for the variable responsiveness of PAR-2 to tryptase.

The lack of reliable tools can make the study of PAR-2 mediated proceses a challenge. Thus, trypsin and tc-LIGRLO-NH_2 which have been widely employed as PAR-2 agonists may induce various biological responses independently of PAR-2 activation. Trypsin has been found to induce increased PGE_2 release from human airway smooth muscle cells, and increased expression of COX-2, as well as mRNA for COX-2, but such responses were not induced by tryptase or by a PAR-2 agonist peptide (Chambers *et al.* 2003). The release of β-hexosaminidase has been reported from rat peritoneal mast cells in responses to tc-LIGRLO-NH_2, but not to trypsin, tryptase, or other PAR-2 agonists (Stenton *et al.* 2002). Corteling *et al.* (2003) have reported that tryptase induced DNA synthesis in primary cultures of guinea-pig tracheal smooth muscle cells could be reduced by the inhibitor leupeptin, but a PAR-2 agonist peptide did not induce DNA synthesis in these cells.

The present studies indicate that tryptase can induce fibroblast and myofibroblast mitogenesis. This activity of tryptase seems to depend on an intact catalytic site. The peptide agonist of PAR-2 could mimic the mitogenic actions of tryptase in fibroblasts but not in myofibroblasts, even though both expressed PAR-2. Clear evidence for a role for PAR-2 in tryptase-induced mitogenesis was not obtained. Neither tryptase nor the PAR-2 agonist peptide were able to stimulate the proliferation of cells, suggesting that stimuli other than those required for DNA synthesis, may be required for cell division.

4.2 Pro-inflammatory actions of tryptase on fibroblasts and myofibrobalsts.

Although cytokine synthesis and release from fibroblasts and myofibroblasts has been studied in response to other stimuli such as TNF-α and IL-1β (Zhang *et al.* 1996a, Zhang *et al.* 1996b, Rogler *et al.* 2001) this is the first investigation of the effects of tryptase and PAR-2 stimulation in these cells. IL-6, IL-8 and GM-CSF were selected as being of particular interest as these cytokines are known to be released from fibroblasts and myofibroblasts, and they could play major roles in processes of inflammation and remodelling in asthma.

Both trypsin and a peptide agonist of PAR-2 stimulated cytokine IL-6 and IL-8 release from primary cultures of fibroblasts and myofibroblasts. These responses were not elicited with tryptase at the concentrations used in these studies. However, when a wider concentration range of tryptase was employed in studies with the MRC-5 cell line, the higher concentrations of tryptase were found to increase cytokine release from these cells. It is possible that at high concentrations, tryptase can disturb cell attachment and induce cytokine release indirectly, but the concentrations used were not high enough to detach cells from the culture vessels.

Peak expression of mRNA for cytokines was observed 6 to 12 h before the detection of cytokines in the cell supernatants. TNF-α used as a positive control stimulus induced increased expression of mRNA and cytokine release with similar kinetics for the fibroblast and myofibroblast preparations. The temporal association between mRNA expression and cytokine release suggests that the cytokines detected in supernatants may be newly synthesised in response to the stimulus rather than released from cytokine stores in the cells.

There was considerable variation between responses even with the same stimulus. This can be explained by individual differences between the donors of cell cultures. The cells employed in these studies were derived from a mixed population with a range of different ages and both sexes represented. No differences in cell responses were observed between the asthmatic and non-asthmatic populations, though the limited numbers of cells available did not allow this comparison to be made for measurements of cytokine release in response to the various stimuli.

Aggarwal *et al.* (1995) have reported that differences in cell passage number (passage 23 with 70) can be associated with a decline in TNF-induced fibroblast proliferation responses as well as in the production of IL-6 and IL-8 by fibroblasts. Particular care was taken in the present studies to employ cells of similar passage number for all comparisons, but the possibility of differences in cell age contributing to the variation cannot be excluded.

TNF-α, added as a positive control stimulus induced significant changes in expression of mRNA for IL-6 and IL-8 under the conditions employed, but this was not seen with tryptase or with PAR-2 agonists. Release of GM-CSF and expression of mRNA for this cytokine was extremely low compared with that of the other cytokines studied. Compared with endothelial cells that are recognised as a major source of this cytokine (Krishnaswamy *et al.* 1999), fibroblasts produce GM-CSF in relatively small quantities. In the present studies levels of GM-CSF in supernatants were below the limit of detection.

GAPDH has been employed as a house keeping gene in studies of the expression of numerous different genes by quantitative real time reverse transcription polymerase chain reaction (Johnson *et al.* 2000), and GAPDH has been used previously in studies of mRNA expression in fibroblasts (Takahashi *et al.* 2000). There have been

suggestions that GAPDH may be unstable and thus unsuitable as a reference house keeping gene (Bond *et al.* 2002). One study involving a comparison of GAPDH, β-actin, cyclophilin and 28SrRNA as internal standards in quantifying RNA levels, concluded that GAPDH may give highly variable signals under conditions of hypoxia (20-70% variation) (Zhong & Simons 1999). However, for semi-quantitative analysis of gene expression, GAPDH has shown the least variation in a range of human tissues compared with that with other house keeping genes, such as β-actin whose expression may be upregulated or downregulated under different experimental conditions (Kondo *et al.* 1994, Medhurst *et al.* 2000).

A problem with cDNA amplification arose unexpectedly in the present studies, and there were some difficulties in obtaining intact cDNA from extracted total RNA from samples stimulated with tryptase. The total RNA measured (at 260 nm) and its quality (260 nm/280 nm) were not different from those of other stimulated cells, but there was no amplification of products by PCR, even of the house-keeping gene. Intensive washing of cells appeared to solve the problem, but the reason for the failure to obtain PCR products remains unresolved. As only those samples stimulated with active tryptase and not those with the protease inhibitor or with heat-inactivated tryptase were affected, it would appear to be dependent on an intact catalytic site. As the initial wash should have been sufficient to remove most of the tryptase present, and phenol in the RNA extracting solution should have denatured any protein present at that stage of extraction, it is unlikely that tryptase acted directly on the RNA. There have been no reports of tryptic enzymes acting on nucleic acid.

PAR-2 stimulation was found to induce the release of inflammatory cytokines in primary cultures of human lung fibroblasts and myofibroblasts 24 h after stimulation. Tryptase may also stimulate the release of proinflammatory cytokines from cells via processes either dependent or independent of PAR-2 activation. The present findings

suggest that mast cell degranulation can, through release of tryptase, stimulate inflammatory changes in fibroblasts and myofibroblasts that are manifested up to 48 h after the initial triggering event.

Investigation of processes of cell signalling should help to unravel mechanisms whereby tryptase can stimulate alterations in cell behaviour. Should tryptase act through PAR-2 action, then one would expect cytokine release in response to trypsin and peptide agonists of PAR-2 to provoke similar signals. Numanami *et al.* (2003) have reported that inhibitors of serine protease can interfere with the production of chemotactic cytokines by human lung fibroblasts in response to TNF-α and IL-1β stimulus, indication the potential for confounding factors in studies of the cellular action of tryptase and PAR-2 agonists, clearly there is the need for a broad range of investigational approach to be adopted.

Mast cells have been reported to have the capacity to contribute directly to the cytokine network in wound repair though the release of IL-4 and other cytokines, which can themselves stimulate fibroblast proliferation (Trautmann *et al.* 2000). This is the first report of a mast cell protease inducing proinflammatory actions in fibroblasts and myofibroblasts though there have been previous studies of tryptase and of agonists of PAR-2 inducing cytokine release from other cells. Thus, it has been shown that tryptase can stimulate IL-8 release from cells of an airway epithelial cells (Cairns & Walls 1996), and from an endothelial cell line (Compton *et al.* 1998), and IL-6 and IL-8 release from eosinophils (Temkin *et al.* 2002), and stem cell factor from airway smooth muscle cells (Berger *et al.* 2003). Peptide agonists of PAR-2 have been reported to stimulate the release of GM-CSF from human airway epithelial cells (Vlisgoftis *et al.* 2001), IL-6 and IL-8 from dermal endothelial cells (Shpacovitch *et al.* 2002), IL-8 from gingival fibroblasts (Uehara *et al.* 2003).

The generation and release of IL-6, IL-8 and GM-CSF from fibroblasts and myofibroblasts could have important consequences, as these are major pro-inflammatory cytokines. IL-8 can play key roles in the migration of inflammatory cells, and GM-CSF can induce the activation, survival and adhesion of inflammatory cells such as macrophages, eosinophils and neutrophils (Hamilton 2002). Furthermore, it has been suggested that these cytokines can have actions on fibroblasts consistent with remodelling. Thus, IL-6 can exert suppressive effects on fibroblast proliferation. (Shahar *et al.* 1996), induce synthesis of procollagen, inhibit the spontaneous apoptosis of these cells, and also inhibit mitogenic activity via the MAPK/tyrosine kinase pathway in lung fibroblasts. (Scaffidi *et al.* 2002). These various actions can link processes of inflammation and remodelling.

4.3 Tryptase as a stimulus for tissue remodelling.

There have been several reports of tryptase having actions on fibroblasts consistent with roles in remodelling. The present studies confirm and extend this concept, and indicate for the first time that this major mast cell product can interact with myofibroblasts, a key cell involved in tissue remodelling. Tryptase was found to stimulate altered release and synthesis of key components of the ECM and also of matrix metalloproteases from myofibroblasts as well as from fibroblasts. In recent years, there has been a growing awareness of the importance of remodelling as well as of inflammation in the pathogenesis of asthma. The contribution of tryptase as a stimulus for the deposition of components of the ECM from myofibroblasts below the basement membrane deserves consideration in the asthmatic lung.

Tryptase was found to induce an increased release of collagen from fibroblasts and myofibroblasts as assessed by measurement of [^{3}H]-proline collagen. This finding is in accord with previous studies that have indicated that tryptase can stimulate type I collagen from a human lung fibroblast cell line (MRC-5) (Cairns & Walls 1997) and from dermal fibroblasts (Abe *et al.* 1998). The type of collagen synthesised by fibroblasts and myofibroblasts in response to tryptase was determined by PCR to be like type III collagen rather than type I collagen. This is at variance with the previous report that MRC-5 fibroblasts synthesise predominantly collagen type I, and little collagen type III in response to tryptase (Cairns & Walls 1997). However, bands for PCR products of both collagen type I and III were observed on gels at all the time points tested. Heat-inactivation of tryptase did not alter the response of cells significantly, suggesting that the stimulation of collagen synthesis and release may not be dependent on the catalytic activity of tryptase.

Under the conditions of the present studies there was no significant difference between fibroblasts and myofibroblasts in the degree of collagen synthesis induced by tryptase. Human myofibroblasts have been reported to express more collagen and MMP than fibroblasts (Ramos *et al.* 2001). Epithelial cells from asthmatic subjects (which can release greater amounts of active TGF-β1 than those from non-asthmatic subjects), have been reported to stimulate the release of collagen type III from myofibroblasts but not collagen type I (Hastie *et al.* 2002).

The lack of effect of heat inactivation on tryptase-induced collagen synthesis by fibroblasts and myofibroblasts would argue against a role for PAR-2 in this process. In keeping with this, the peptide agonist of PAR-2 did not alter collagen synthesis significantly.

The presence of gelatinase activity was detected by zymography in supernatants of both fibroblasts and myofibroblasts. Myofibroblasts from the human lung have previously been reported to release MMP-2 with a molecular wight of 72 kDa, and MMP-9 of 92 kDa (Fukuda *et al.* 1998). The high intensity band detected with an apparent molecular weight of 72 kDa in the present studies will be pro MMP-2, and at 67 kDa, MMP-2 in activated form. There was no detectable MMP-9 band observed even in supernatants from myofibroblasts, though there were faint bands that could be observed by the human eye (but which were not detected with the resolution of the imaging equipment available).

The quantities of MMP-2 detected in cell supernatants in response to tryptase were highly variable. MMP-2 was found to be present in cells in the pro-form and both the pro-form and the active form could have been newly generated by the cells in response to tryptase. The formation of the active form may be dependent on the quantity of the pro-form available. Collagen disk contraction by rat fibroblasts, taken as a measure

of wound healing, has been reported to be delayed with increased age in rats, and increasing age is associated with the release of greater quantities of MMP-2 from fibroblasts (Ballas & Davidson 2001). The extent to which release of MMPs from human subjects may be related to age remains to be investigated, but it is possible that this could contribute to the considerable variation in the response of cells from different donors.

It has been reported that MMP-2 and fibronectin are substrates for tryptase and are degraded by this protease (Lohi *et al.* 1992). In the present studies the increased amounts of activated MMP-2 induced in response to tryptase are likely to be a consequence of the actions of tryptase on the cells and not direct degradation of proMMP-2 by tryptase. Tryptase has been reported to activate latent rheumatoid synovial collagenase (Gruber *et al.* 1988), and there are reports of the activation of MMP-3 by dog tryptase but not of MMP-2 or MMP-9 (Lees *et al.* 1994, Johnson *et al.* 1998). Activated mast cells have been found to stimulate production of MMP-1, MMP-3 and MMP-2 by endometrial stroma cells (Zhang *et al.* 1998). However, it has been reported that mast cell tryptase did not alter basal levels of MMP-1, MMP-2, MMP-3, MMP-9 or TIMP-1 in dermal fibroblasts, or alter expression of mRNA for these proteins (Zhang *et al.* 1999c). Tryptase could exert both direct and indirect effects on components of the ECM, that are mediated through catalytic and non-catalytic actions of this mast cell product.

The production and activation of collagenase in response to PAR-2 stimulation differed between fibroblasts and myofibroblasts. With fibroblasts there was a significant increase in pro-MMP-2 synthesis in response to the peptide agonist of PAR-2, but this was not seen with myofibroblasts. This observation suggests there may be a greater contribution for fibroblasts than for myofibroblasts in controlling the balance of ECM components in response to PAR-2 activation. The active form of

MMP-2 was rarely observed following PAR-2 simulation, though it was clearly induced in response to tryptase. These findings, again suggest that different mechanisms are involved with tryptase and PAR-2 stimulation. Interestingly even with the fibroblast cultures (in which there was induction of collagenase), there appeared to be preparations of responding and non-responding cells.

Levels of TIMP-2 appeared to be affected more than those of TIMP-1 in response to tryptase stimulation in both fibroblasts and myofibroblasts, though measurement of the band densitometry of end point PCR should be regarded as a semi-quantitative technique. Real time PCR should provide a more precise method of mRNA quantification than end point PCR for mRNA quantification (Schmittgen *et al.* 2000). The amount of MMP and TIMP production and their relative activities are important as the MMP-9/TIMP-1 ratio has been reported to correlate with airflow obstruction in asthma (Vignola *et al.* 1998). An interest in carrying out these studies was to investigate potential alterations in the MMP-9/TIMP-1 ratio between the cell types in response to the different stimuli, but the small quantities of MMP-9 and TIMP present did not allow this issue to be addressed.

In the present studies it has been established that tryptase can induce the synthesis of collagen and increase MMP-2 production in primary cultures of human lung fibroblasts and myofibroblasts. In the asthmatic airways, this mast cell product could contribute to an imbalance in ECM homeostasis through PAR-2 independent pathways that could possibly be more important than PAR-2 dependent processes in this system. The ability of PAR-2 activation to stimulate MMP-2 production, but not collagen production by fibroblasts would seem likely to increase the rate of collagen degradation, a mechanism that would run counter to the processes of collagen deposition associated with the pattern of remodelling seen in asthmatic airways. Tryptase on the other hand could stimulate the release of ECM components from

fibroblasts and myofibroblasts. These actions of tryptase would seem to be maintained for a period of several days after the initial exposure of cells to this protease, and could have been preceded by direct actions on extracellular substrates.

A correlation has been reported between levels of tryptase and of MMP-2, MMP-9 and TGF-β1 but not with levels of TIMP-1 in nasal polyp tissue homogenates (Lee *et al.* 2003). The present studies suggests there may be a causal relationship between tryptase release and the release of MMP, though further studies with a broader range of approaches will be required to understand the nature and the extent to which such processes may occur in asthmatic airways.

4.4 Conclusions

Tryptase has the ability to provoke a range of actions in fibroblasts and myofibroblasts. This major mast cell product was found to induce mitogenic responses and the release of cytokines, as well as to alter the composition of the ECM through the production of collagens and activation of collagenases. Some of the effects of tryptase on primary cultures of fibroblasts and myofibroblasts could be reproduced using a peptide agonist of PAR-2, but this was not seen consistently. Moreover, PAR-2 agonists had effects which were not seen with tryptase (Fig.4.4).

The presence of PAR-2 on primary cultures of lung fibroblasts and myofibroblasts was detected by immunocytochemistry, and mRNA for this receptor by PCR. Moreover, the response of fibroblasts and myofibroblasts to stimulation with PAR-2 agonists indicates expression of functional PAR-2. The present studies strongly suggest that mechanisms other than activation of these receptors may be involved in tryptase-mediated processes on fibroblasts and myofibroblasts. Systematic comparison of the actions of tryptase and peptide agonists of PAR-2 has revealed important differences in response. These findings would appear to suggest that the situation is more complex than has been suggested in previous studies where the effects of tryptase on fibroblasts have been interpreted purely in terms of activation of PAR-2 (Akers *et al.* 2000).

The lack of an effective antagonist hampers investigation of the contributions of PAR-2, especially given the observations that catalytically active tryptase does not invariably activate this receptor. Important questions remain over the potential for heterogeneity in PAR-2 as well as in tryptase, and the conditions for optimal receptor activation. In the present studies heterogeneity in fibroblasts and myofibroblasts, and particularly that between preparations from different human donors is likely to have

contributed to variability in responses, despite attempts to carefully standardise procedures.

The apparent inability of heat-treatments or of pre-incubating the enzyme with protease inhibitors to reduce some of the effects of tryptase on fibroblasts and myofibroblasts calls attention to the potential for non-proteolytic mechanisms. This possibility has been mooted previously by Brown *et al.* (2002), who noted that protease inhibitors failed to inhibit tryptase-induced mitogenesis in airway smooth muscle cells. A number of other proteases are recognised for their ability to mediate biological processes by non-catalytic mechanisms, and it will be important for this issue to be examined more fully for tryptase.

Whatever the mechanisms whereby tryptase may modulate cell function and the extent to which PAR-2 activation may be involved, it is clear that this major secretory product of human mast cells can stimulate diverse effects on a variety of cell types. The mitogenic actions of tryptase on fibroblasts and myofibroblasts, have been observed also with epithelial cells (Cairns & Walls 1996), endothelial cells (Blair *et al.* 1997) and airway smooth muscle cells (Berger *et al.* 2001a). The ability of tryptase to stimulate cytokine release from fibroblasts and myofibroblasts is consistent with previous reports of release of cytokines and growth factors by epithelial cells (Cairns & Walls 1996), endothelial cells (Compton *et al.* 1998), eosinophils (Temkin *et al.* 2002) and airway smooth muscle cells (Berger *et al.* 2003). The observation of MMP-2 release from fibroblasts and myofibroblasts in the present studies has some parallels with the reported release of MMP from epithelial cells (Vliagoftis *et al.* 2000) and supports the concept that tryptase can participate in processes of tissue remodelling. The ability of tryptase to stimulate also the release of collagens from fibroblasts and myofibroblasts found in these studies, and reports that this protease can activate certain MMP (Gruber *et al.* 1989), further underscores the potential for

tryptase to alter the composition of the ECM. The release of mast cell tryptase into the airways could lead to inflammatory changes and alterations in tissue structure within minutes as well as having effects many hours after the initial triggering events.

Some of the effects of tryptase on fibroblasts and myofibroblasts seen in the present studies have been less striking than have been observed with certain other cell types or other models, but the overall findings do strengthen the concept of tryptase as a mediator of inflammation and remodelling. Of particular significance could be the potential of tryptase as a participant in both of these processes, and it could provide a link between inflammation and remodelling.

Future work

Tryptase deserves attention as a stimulus for the development of chronic disease. Though, some of the effects on fibroblasts and myofibroblast appeared less potent than the EGF and TNF-α employed a s positive control stimuli, it is difficult to make accurate comparisons based on the local concentrations of these various stimuli. Certainty in the asthmatic lung, it is well established that there may be high levels of tryptase. It is not possible to reproduce *in vitro*, the complex situation *in vivo*, where tryptase is likely to be present with other stimuli of cell function. Future work is required to investigate the potential for interaction using models involving co-culture of fibroblasts and myofibroblasts with mast cells and other cell types in order to investigate the effect of tryptase in a more relevant setting. The possibility that tryptase may exert a priming role, or interact synergistic way with other mediators also requires investigation.

In the present studies the preparation of human lung and recombinant tryptase were found to be having broadly similar chemical and biological properties, though there were some differences observed. However, apparent discrepancies between these findings and those of some previous published reports raise the possibility of heterogeneity in preparations of tryptase employed. Future studies should focus on this issue, and in particular the effects of different sequences of tryptases should be examined.

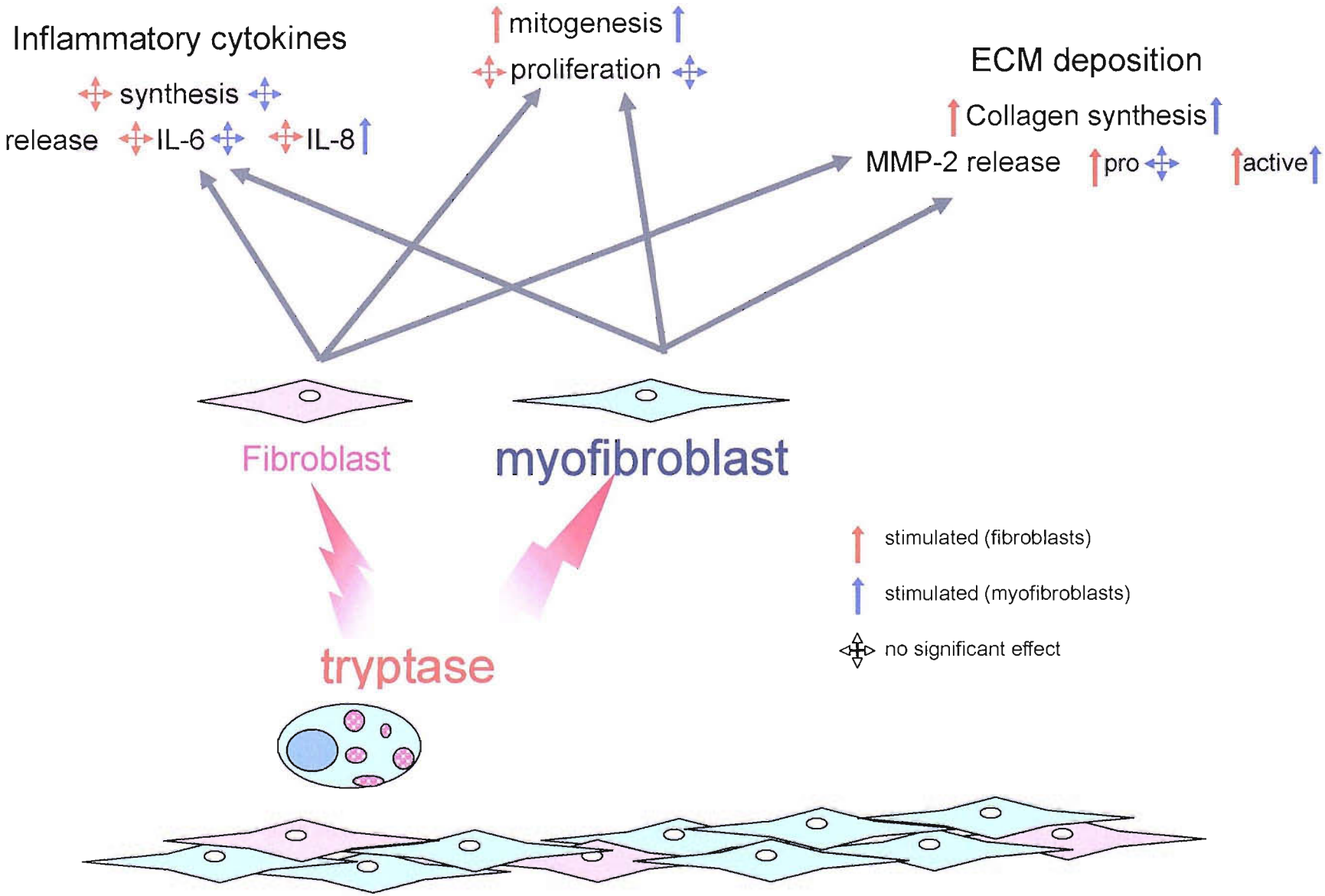
The possibility that tryptase can alter cell function independently of PAR-2 activation requires further study. The lack of an effective antagonist for PAR-2 restricts the scope of investigations of the contributions of PAR-2, and the development of suitable tools will be important in allowing this issue to be addressed. Meanwhile, PAR-2 knock-out mice or PAR-2 transfected cell lines can be used to advantage. It would be instructive to examine the effects of tryptase on cells transfected to allow to expression of different forms of PAR-2 that could be functional and non-functional, or possess glycosylation site differences. Detailed investigation of processes of cell signalling should also help to unravel the mechanisms whereby tryptase can stimulate alterations in cell behaviour. Involvement of GPCR can be investigated by inhibiting the signal transduction of G protein using pertussis toxin, and down stream events should be tested systemically using specific inhibitors.

PAR-2 activation has been reported to induce the release of PGE_2 and COX-2 from airway smooth muscle cells (Chambers *et al.* 2003), and PGE_2 can inhibit local immune responses and increase blood flow, and this could facilitate faster resolution of local inflammation (Cocks *et al.* 1999). Such a protective role of PAR-2 activation in inflammation should further investigated in models involving fibroblasts and myofibroblasts, as well as the pro-inflammatory action.

The extent of which fibroblasts from non-asthmatic subjects may differ from those of asthmatic subjects was not investigated in depth in these studies on account of the small sample numbers available. It will be important to re-examine this issue as larger numbers of samples are available, a link between cell function and disease could be important for an understanding of the contributions of tryptase and PAR-2 in asthma.

Tryptase represents an attractive target for therapeutic intervention in asthma and other respiratory conditions. Inhibitors of tryptase would be expected to inhibit processes of tissue remodelling (an area of unmet medical need), as well as to have anti-inflammatory actions. The availability of potent and selective inhibitors of tryptase should shed light on the natural substrates of this abundant mast cell protease, and perhaps allow a better assessment of non-catalytic actions. The findings of the present studies suggest that the administration of tryptase inhibitors and antagonists of PAR-2 will have quite different effects in patients.

A


Inflammatory cytokines
synthesis
release IL-6 IL-8
mitogenesis
proliferation
ECM deposition
Collagen synthesis
MMP-2 release pro active
Fibroblast
myofibroblast
tryptase
stimulated (fibroblasts)
stimulated (myofibroblasts)
no significant effect

B

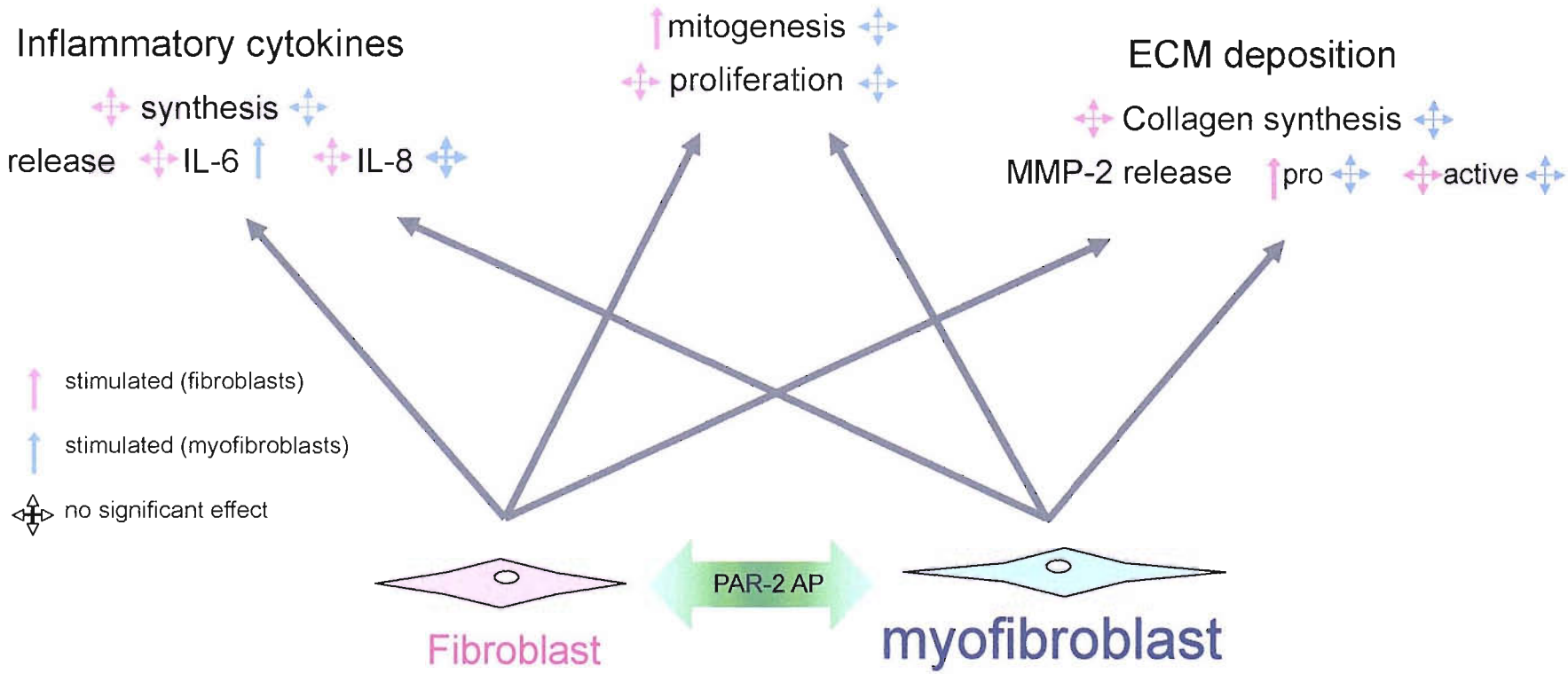


Fig. 4.4

Potential actions of (A) tryptase and (B) peptide agonist of PAR-2 on fibroblasts and myofibroblasts and the consequences for inflammation and remodelling in the lung.

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Appendices

Appendix 1

Current ∝ Light Intensity

$I = I_o e^{-\alpha \ell c}$

Beer-Lambert Law

$$OD = \log_{10}\left(\frac{I_o}{I}\right) = \varepsilon \ell c$$

$$\varepsilon = \frac{\alpha}{2.303}$$

Kinetics

$OD = \varepsilon \ell c$

$$\frac{d}{dt}(OD) = \varepsilon \ell \frac{dx}{dt}$$

$$c = \frac{x}{V}$$

$$\frac{d(OD)}{dt} = \frac{\varepsilon \ell}{V} \cdot \frac{dx}{dt}$$

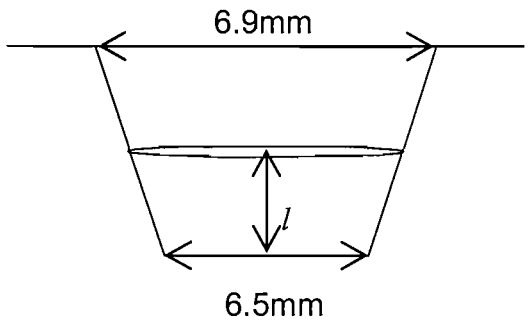
$$\frac{dx}{dt} = \frac{V}{\varepsilon \ell} \cdot \frac{d(OD)}{dt}$$

$$\ell = \frac{V}{A} = \frac{V}{\pi r^2}$$

$$\frac{dx}{dt} = \frac{1.0 \times 10^{-4} \ell}{8800 \ell \ mol^{-1} cm^{-1} \left(\frac{0.1 cm^3}{\pi (0.33 cm)^2}\right)} \cdot \frac{d(OD)}{dt}$$

$$= 3.888 \times 10^{-8} mol \cdot \frac{d(OD)}{dt}$$

Well of a 96- well plate



Enzymatic Activity

$1\ U = 1 \mu mol/\min$

$1\ katal = 1 mol/s$

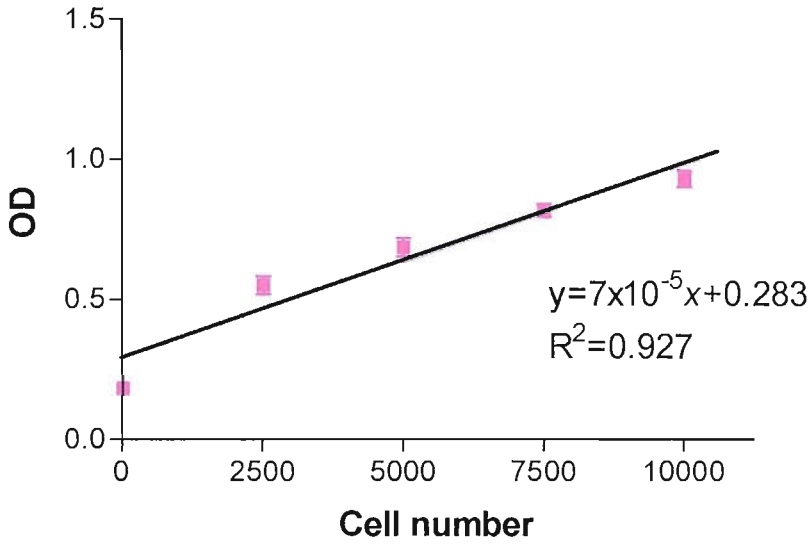
$$\frac{enzyme\ activity}{unit\ vol} = \frac{3.888 \times 10^{-8} \times \frac{1\ U}{10^{-6} mol/\min}}{0.01 ml} \cdot \frac{d(OD)}{dt}$$

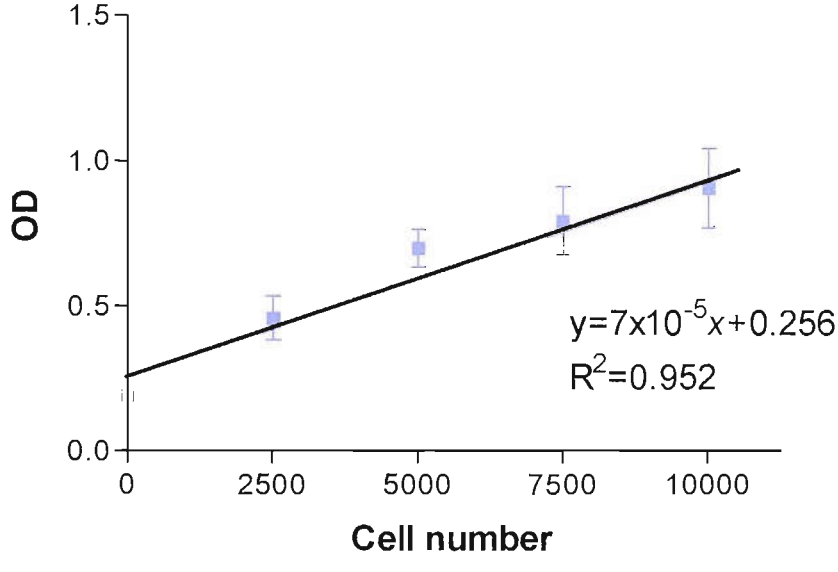
$$= 3.888 U\ \min\ ml^{-1} \cdot \frac{d(OD)}{dt}$$

$$\frac{d(OD)}{dt} as \frac{mOD}{\min}$$

$$\frac{enzyme\ activity}{ml} = 3.888 mU\ \min\ ml^{-1} \cdot \frac{mOD}{\min}$$

Appendix 2





A standard curve to show a correlation between a formazan formation (absorbance at 490nm) with fibroblast cell number for (A) fibroblasts and (B) myofibroblasts in MTS assay. Mean ± SEM are shown for three separate experiments.