The Activation of Lung Fibroblasts by Human Mast Cell Tryptase:
A role for protease activated receptor-2 (PAR-2)

Thesis submitted in fulfilment of the degree of Doctor of Philosophy.
University of London
by
Ian A Akers

Cardiopulmonary Biochemistry and Respiratory Medicine
Royal Free and University College Medical School
University College, London
The Rayne Institute,
5 University Street
London WC1E 6JJ

January, 2001
“Research is the process of going up alleys to see if they are blind”

Marston Bates (1906-74) American writer, author
Abstract

One of the features of asthma is the remodelling of the airway wall with increased deposition of extracellular matrix molecules, in particular collagen and proteoglycans, in the lamina reticularis beneath the epithelial basement membrane. The mechanism of this deposition is currently unknown and current therapies have little effect on this aspect of asthma pathology. Fibroblasts and myofibroblasts, which are thought to be major producers of extracellular matrix molecules, are present within the lamina reticularis, however the mechanisms by which these cells are activated are unclear. Within the asthmatic airway there are also increased numbers of activated mast cells that produce a range of mediators capable of modulating fibroblast function. One such mediator, tryptase, is a known fibroblast mitogen and profibrotic agent. The mechanism by which this occurs is also unclear, although recent evidence suggests that tryptase is able to activate protease activated receptor (PAR)-2, a member of a family of 7-transmembrane, G-protein coupled receptors activated by enzymatic cleavage. The overall aim of this thesis was to examine fibroblast mitogenesis and extracellular matrix production in relation to airway remodelling associated with asthma. In doing so, I addressed the hypothesis that mast cell tryptase modulates fibroblast function by the activation of PAR-2 present on the cell surface. To address this hypothesis the effects of tryptase, trypsin, a known activator of PAR-2, and small polypeptide activators of PAR-2, SLIGKV and SLIGRL, were examined on fibroblast proliferation, procollagen and proteoglycan metabolism. All four agonists induced lung parenchymal and airway fibroblast proliferation. The two enzymes were more potent than the two activating peptides. The mitogenic responses of the enzymes were dependent of their catalytic activity. It was found that lung parenchymal and airway fibroblasts expressed PAR-2 mRNA, using reverse transcriptase polymerase chain reaction, and PAR-2 protein was localised to the cell surface by immunohistochemistry using polyclonal antibodies. The same antibodies inhibited trypsin- and partially inhibited tryptase-induced fibroblast proliferation, demonstrating that both enzymes activated PAR-2. It was unclear whether PAR-2 was present on fibroblasts in
the airway. Therefore using PAR-2 antibodies and a cell cytoskeletal marker for smooth muscle F-actin, phalloidin, it was possible to co-localise PAR-2 and actin filaments suggesting that PAR-2 is expressed on myofibroblast-like cells in human bronchus. Finally, results using a procollagen $\alpha2(I)$ promoter linked to a luciferase reporter gene demonstrated that both tryptase and the PAR-2 activating peptides were able to stimulate procollagen gene promoter activity. However, neither agonist induced increases in the steady-state levels of $\alpha1(I)$-procollagen mRNA or procollagen protein metabolism assessed by measurement of hydroxyproline. This inconsistency may be due to known effect of tryptase to induced the release, and activate, matrix metalloproteinases that are capable of degrading newly formed and/or deposited procollagen. Tryptase and trypsin had variable effects on large and small molecular weight proteoglycan metabolism in human lung fibroblast cell cultures. The data presented in this thesis demonstrate that tryptase mediates its effects on fibroblast mitogenesis, at least partly via the activation of PAR-2 and is consistent with the hypothesis that tryptase may play an important role in fibroblast proliferation and matrix deposition in the airways of asthmatic patients.
Table of Contents

ABSTRACT ................................................................................................................................... 3

TABLE OF CONTENTS ............................................................................................................. 5

ACKNOWLEDGEMENTS ....................................................................................................... 11

LIST OF FIGURES ................................................................................................................ 12

LIST OF TABLES ................................................................................................................... 16

ABBREVIATIONS .................................................................................................................. 18

1 CHAPTER 1 ......................................................................................................................... 22

1.1 PREAMBLE. AN INTRODUCTION TO ASTHMA ................................................................. 22

1.1.1 An historical perspective of asthma ................................................................................. 23

1.1.2 Clinical features of asthma ................................................................................................. 25

1.1.3 Epidemiology: Prevalence, morbidity and mortality of Asthma ................................ 27

1.2 PATHOPHYSIOLOGY OF ASTHMA ..................................................................................... 29

1.2.1 Structural features of the human bronchial airway ............................................................ 29

1.2.2 Structural changes in airways of asthmatic patients ......................................................... 32

1.2.3 Functional effects of extracellular matrix deposition in the airway wall ..................... 36

1.3 THE ROLE OF RESIDENT CELLS IN PATHOLOGY OF ASTHMA ................................. 39

1.4 FIBROBLASTS IN THE AIRWAYS ...................................................................................... 44

1.4.1 Extracellular Matrix within the airways ............................................................................. 47

1.4.1.1 Collagens ................................................................................................................................. 47

1.4.1.2 Collagen synthesis ................................................................................................................ 50

1.4.1.3 Collagen degradation ........................................................................................................... 51

1.4.1.4 Proteoglycans and glycoproteins ..................................................................................... 53

1.5 INFLAMMATORY CELLS, CYTOKINES, GROWTH FACTORS AND AIRWAY REMODELLING ........................................... 57

1.6 MAST CELLS ..................................................................................................................... 60

1.6.1 Mast cells in lung disease ................................................................................................. 61

1.6.2 The interaction of mast cells and fibroblasts .................................................................... 63

1.6.3 Mediators released by activated mast cells ................................................................. 66

1.6.3.1 Histamine ............................................................................................................................ 66
2.6.2.3 Transfer to nylon membrane ........................................................................................................... 132
2.6.2.4 Prehybridisation .................................................................................................................................... 133
2.6.2.5 Labelling probe ...................................................................................................................................... 134
2.6.2.6 Post-hybridisation washes and imaging ............................................................................................. 134
2.6.3 Determination of procollagen protein metabolism .................................................................................. 135
2.6.3.1 Cell culture conditions ....................................................................................................................... 135
2.6.3.2 Protein extraction and hydrolysis ........................................................................................................ 136
2.6.3.3 Measurement of hydroxyproline by reverse-phase HPLC ................................................................. 137
2.6.3.3.1 Sample derivatisation .......................................................................................................................... 137
2.6.3.3.2 Instrumentation and chromatographic conditions ............................................................................. 138
2.6.3.3.3 Quantification of hydroxyproline content............................................................................................ 141
2.6.3.3.4 Calculation of procollagen synthesis, production and degradation rates ........................................ 143

2.7 STATISTICAL ANALYSIS ....................................................................................................................... 144
2.7.1 Statistical analysis of cell proliferation assay .......................................................................................... 144
2.7.2 Statistical analysis for extracellular matrix production data .................................................................... 144

3 RESULTS .................................................................................................................................................. 145
3.1 EFFECT OF HUMAN LUNG TRYPTASE ON HUMAN LUNG FIBROBLAST PROLIFERATION ................................................................. 145
3.1.1 Effect of recombinant human tryptase on human foetal lung fibroblast proliferation 150
3.1.2 Effect of exposure time of tryptase on human foetal lung fibroblast proliferation 151
3.1.3 Effects of tryptase on isolated human lung parenchymal, airway and dermal fibroblast proliferation 152

3.2 INHIBITION OF TRYPTASE-INDUCED FIBROBLAST PROLIFERATION ............................................. 154
3.2.1 Effect of heat inactivation on tryptase-induced human foetal lung fibroblast proliferation ......................... 155
3.2.2 Effect of proteolytic inhibitors on tryptase-induced cleavage of specific peptide substrate ....................... 155
3.2.3 Effect of secretory leukocyte proteinase inhibitor on tryptase-induced human foetal lung fibroblast cell proliferation ........................................................................................................ 159
3.2.4 Effect of lactoferrin on tryptase-induced human foetal lung fibroblast proliferation ................................ 161

3.3 EXPRESSION AND LOCALISATION OF PAR-2 ON FIBROBLASTS ...................................................... 163
3.3.1 PAR-2 mRNA expression in human lung and dermal fibroblasts .......................................................... 163
3.3.1.1 Detection of other protease activated receptors by RT-PCR ................................................................. 167
3.3.2 Immunolocalisation of PAR-2 receptor protein ....................................................................................... 169
3.3.2.1 Immunolocalisation of PAR-2 in isolated human lung and dermal fibroblasts .... 169
3.3.2.2 Characterisation of PAR-2 antibodies ................................................................. 170

3.4 EFFECT OF PROTEASE-ACTIVATED RECEPTOR-2 ACTIVATION ON HUMAN LUNG
FIBROBLAST PROLIFERATION ......................................................................................... 174

3.4.1 Effect of trypsin on human foetal lung fibroblast proliferation ......................... 175
3.4.2 Effects of PAR-2 activating peptides on human lung fibroblast proliferation .... 175
3.4.3 Human lung parenchymal and airway fibroblast proliferation .......................... 176
3.4.4 Effect of neutral endopeptidase inhibitors on PAR-2 AP-induced fibroblast
proliferation .................................................................................................................. 178
3.4.5 Effect of highly selective PAR-2 AP, tc-LIGRO-NH₂ on human lung airway
fibroblast proliferation .................................................................................................. 179
3.4.6 Effect of trypsin and PAR-2 activating peptides on human dermal fibroblasts.... 180
3.4.7 Effect of trypsin and PAR-2 activating peptides on dermal fibroblast proliferation.
181

3.5 EFFECT OF RAT PAR-2 ANTISERA ON TRYPES- AND TRYPsin-INDUCED HUMAN
FOETAL LUNG FIBROBLAST PROLIFERATION .......................................................... 183

3.6 LOCALISATION OF PAR-2 IN AIRWAY BIOPSY SAMPLES FROM NORMAL NON-
ASTHMATIC PATIENTS ................................................................................................. 186

3.6.1.1 Localisation of PAR-2 in human bronchus ......................................................... 187
3.6.1.2 Localisation of PAR-2 in bronchial biopsies from patients with asthma ......... 191
3.6.1.3 Localisation of PAR-2 in fibroblasts isolated from bronchial biopsy samples.... 194

3.7 THE EFFECTS OF TRYPES AND PROTEASE ACTIVATED RECEPTOR ACTIVATING
PEPTIDES ON EXTRACELLULAR MATRIX METABOLISM ......................................... 196

3.7.1 Effect of tryptase and PAR-2 activating peptide, SLIGKV on procollagen α2(I)
gene promoter activity .................................................................................................. 197
3.7.2 Effect of tryptase, SLIGKV, and TGFβ on steady state levels of α1(I) procollagen
mRNA .................................................................................................................................. 200
3.7.3 Effects of tryptase on collagen synthesis and degradation by human foetal lung
fibroblasts ......................................................................................................................... 203
3.7.4 Effect of PAR-2 activating peptide on procollagen metabolism in human foetal
lung fibroblasts ............................................................................................................... 208

4 DISCUSSION ............................................................................................................. 212

4.1 PROLIFERATION STUDIES .................................................................................... 212

4.1.1 Tryptase-induced fibroblast proliferation ............................................................. 212
4.1.1.1 Inhibition of tryptase-induced fibroblast proliferation ........................................ 216
4.1.2 Tryptase-induced proliferation of dermal fibroblasts ......................................... 221
4.2 LOCALISATION AND FUNCTION OF PROTEASE ACTIVATED RECEPTOR-2........... 223

4.2.1 PAR-2 expression by RT-PCR ................................................................. 223
4.2.2 Localisation of PAR-2 by immunohistochemistry .................................. 224
4.2.2.1 Fibroblast cell cultures .......................................................................... 224
4.2.2.2 Location of PAR-2 in normal human airways ......................................... 226
4.2.2.3 PAR-2 expression in human airway fibroblasts ..................................... 229
4.2.3 Mitogenic effects of PAR-2 activating peptides ........................................... 231
4.2.4 The effect of anti-rat PAR-2 antisera (B5) on the mitogenic responses to trypsin
    and tryptase ....................................................................................................... 233

4.3 EFFECT OF TRYPTASE AND PAR-2 ACTIVATING PEPTIDES ON EXTRACELLULAR
    MATRIX PRODUCTION ..................................................................................... 236
4.3.1 The effects of tryptase, trypsin, and PAR-2 activating peptides on procollagen
    metabolism .......................................................................................................... 236

4.4 IMPLICATIONS OF THESE FINDINGS FOR DISEASE ...................................... 243
4.4.1 The potential role of mast cells and mast cell tryptase in lung disease pathology 243
4.4.2 Fibroblast association with sub-epithelial thickening .................................. 246
4.4.3 A role for tryptase and PAR-2 activation in bronchoconstriction .................. 251
4.4.4 The potential role of tryptase and PAR-2 in inflammation .............................. 255
4.4.5 PAR-2 and airway secretions ....................................................................... 257

4.5 SUMMARY ....................................................................................................... 258

4.6 FUTURE STUDIES ............................................................................................ 259

5 REFERENCES ...................................................................................................... 261

6 APPENDIX .......................................................................................................... 296
6.1 PATIENT DETAILS ............................................................................................ 296
6.1.1 Adult human lung and airway fibroblasts ..................................................... 296
6.1.2 Lung tissue for PAR-2 and fibroblast localisation ........................................ 297
6.2 PUBLICATIONS ASSOCIATED WITH THIS WORK ........................................ 299
Acknowledgements

I would like to express my sincerest gratitude to my supervisor, Dr Robin J McAnulty, for his continuous support, guidance and enthusiasm, and for his friendship throughout my PhD.

I would also like to thank Professor Geoffrey J Laurent for allowing me the opportunity to spend three years within his Department and for his support and help throughout my studies. I am also extremely grateful to all my fellow students who made my time within the Lab so enjoyable and for providing many memorable moments. In particular, I would like to thank Carmel, Louise, Maddy, Jenny, Olivier and Richard, all who started their studies about the same time as me and have all since gained their Doctorates - excellent friends.

I also wish to extend my thanks to Glaxo Wellcome who, over the years have provided me with both opportunity and the financial support that has allowed me to further my research interests. In particular, Dr Robert Coleman, for persuading me to push for the opportunity to do a post-graduate degree, to Dr Malcolm Johnson for his unwavering support over the years and to Dr Shahin Sanjar, my industrial co-supervisor. I would also like to pass on my thanks to Professor John Wilson, and his wife, Dr Nerina Harley, for their friendship, for their enthusiasm and their continuos support.

Thank you also to Professor William Roche and Dr Anton Paige for allowing me to spend an extend amounts of time within their Departments at Southampton University, and to Professor Morley Hollenberg for his hospitality and constructive comments throughout the three years. I am also grateful for the gift of a numbers of research tools, without which I would not have been able to perform many of the studies – Dr David Andrews, Mr Neil Waslidge, Professor Morley Hollenberg, Dr Franscesco Ramirez, Professor Gareth Howells.

Love and thanks to my Mum, Dad and Sister, and her family, for their support not only for the last four years but also for the many years before, encouraging me to fulfil my dreams.

Finally, and most importantly, to Anne, Kate, Stuart and Richard, I cannot express how grateful I am for your love, patience and support - without you none of this would have been possible.
## List of figures

### Chapter 1

| Figure 1.1 | A schematic diagram of the normal bronchial airway | 31 |
| Figure 1.2 | Photomicrograph of an airway from an asthmatic patient | 32 |
| Figure 1.3 | Comparison of the airway wall structure from a normal and an asthmatic patient | 34 |
| Figure 1.4 | A schematic diagram showing the effects of structural changes on the airway lumen | 38 |
| Figure 1.5 | Cell cycle | 46 |
| Figure 1.6 | Collagen structure and metabolism | 49 |
| Figure 1.7 | Schematic representation of matrix molecules found within the bronchial airway | 55 |
| Figure 1.8 | Solid-surface and ribbon representation of tetrameric structure of tryptase | 76 |
| Figure 1.9 | A schematic diagram of the structure of protease activated receptors | 84 |

### Chapter 2

| Figure 2.1 | Culture of explanted human airway tissue | 98 |
| Figure 2.2 | Characterisation of human foetal lung fibroblasts | 103 |
| Figure 2.3 | Characterisation of human dermal fibroblasts | 104 |
| Figure 2.4 | Positive staining for antibodies used in fibroblast characterisation studies | 105 |
| Figure 2.5 | Culture of human lung fibroblasts | 106 |
| Figure 2.6 | Effects of the presence and absence of bovine serum albumin and human holo-transferrin on human foetal lung fibroblast growth | 109 |
| Figure 2.7 | Determination of Km and Ki for tryptase in the presence of BABIM | 114 |
| Figure 2.8 | Calculation of IC50 values for benzamidine and antipain | 115 |
| Figure 2.9 | Apparatus used for capillary transfer of RNA from agarose gel to nylon membrane | 133 |
| Figure 2.10 | Hydroxyproline derivatisation with NBD-Cl | 138 |
| Figure 2.11 | Graph showing the composition of the elution buffer in respect to the percentage of buffer B | Page 139 |
| Figure 2.12 | Separation of hydroxyproline by reverse phase HPLC | Page 141 |
| Figure 2.13 | Effects of increasing concentrations of hydroxyproline standard solutions on absorbance measured by HPLC | Page 141 |

### Chapter 3

<p>| Figure 3.1 | The mitogenic effects of tryptase on human foetal lung fibroblasts | Page 147 |
| Figure 3.2 | Mean concentration effect curve of tryptase on human foetal lung fibroblast proliferation | Page 148 |
| Figure 3.3 | Effect of rh-tryptase on human foetal lung fibroblast proliferation | Page 151 |
| Figure 3.4 | Time course of mitogenic effects of tryptase on human foetal lung fibroblasts | Page 152 |
| Figure 3.5 | Effects of tryptase on fibroblast isolated from adult human lung and skin. | Page 154 |
| Figure 3.6 | Effect of heat inactivated tryptase on human foetal lung fibroblast proliferation | Page 155 |
| Figure 3.7 | The effect of serine protease inhibitors on tryptase-induced proliferation of human foetal lung fibroblasts | Page 157 |
| Figure 3.8 | The effect of bis (5-amidino-2-benzimidazolyl) methane (BABIM) on tryptase-induced proliferation of human foetal lung fibroblasts | Page 158 |
| Figure 3.9 | The effect of [N-(1-hydroxy-2-naphtoyl)-L-arginy]-L-prolinamide hydrochloride (APC366) on tryptase-induced human foetal lung fibroblast proliferation | Page 160 |
| Figure 3.10 | The effect of secretory leukocyte proteinase inhibitor (SLPI) on tryptase-induced proliferation of human foetal lung fibroblasts | Page 161 |
| Figure 3.11 | The effect of lactoferrin on tryptase-induced human foetal lung fibroblast proliferation | Page 162 |
| Figure 3.12 | Expression of PAR-2 mRNA by human fibroblasts | Page 164 |
| Figure 3.13 | Sequencing of PAR-2 RT-PCR product | Page 166 |
| Figure 3.14 | Expression of mRNA for PAR-1, -3, -4 in lung and dermal fibroblasts | Page 168 |
| Figure 3.15 | Localisation of PAR-2 immunoreactivity in lung and dermal fibroblasts | Page 171 |
| Figure 3.16 | Characterisation of the rabbit anti-rat PAR-2 antisera | 173 |
| Figure 3.17 | Effect of trypsin on human foetal lung fibroblast proliferation | 175 |
| Figure 3.18 | Effect of protease-activated receptor (PAR)-2 activating peptides SLIGKV and SLIGRL on human foetal lung fibroblast proliferation | 176 |
| Figure 3.19 | Effect of PAR-2 activating peptides, SLIGKV and SLIGRL on human parenchymal and airway fibroblast proliferation | 177 |
| Figure 3.20 | Effect of neutral endopeptidase inhibitors on PAR-2 activating peptide-induced human foetal lung fibroblast proliferation | 178 |
| Figure 3.21 | Effect of tc-LIGRLO-NH₂ on human fibroblast proliferation | 180 |
| Figure 3.22 | Effect of trypsin and PAR-2 activating peptides on human dermal fibroblast proliferation | 182 |
| Figure 3.23 | Effect of PAR-2 antisera on tryptase- and trypsin-induced human foetal lung fibroblast proliferation | 184 |
| Figure 3.24 | Effects of PAR-2 antisera on time dependent responses of tryptase on human foetal lung fibroblasts | 185 |
| Figure 3.25 | Auto-fluorescence of human airway tissue | 187 |
| Figure 3.26 | Localisation of PAR-2 bronchial epithelium | 188 |
| Figure 3.27 | Localisation of PAR-2 in bronchial sub-mucosal glands | 188 |
| Figure 3.28 | Co-localisation of PAR-2 and fibroblast F-actin fibres in human airway | 189 |
| Figure 3.29 | PAR-2 localisation to a fibroblast located beneath the epithelial basement membrane | 190 |
| Figure 3.30 | Localisation of PAR-2 in human airway biopsies from asthmatic patients | 192 |
| Figure 3.31 | Characterisation of spindle-shaped cells in human airway biopsy sections | 193 |
| Figure 3.32 | Localisation of PAR-2 in airway fibroblasts from asthmatic and non-asthmatic airways | 194 |
| Figure 3.33 | Effect of tryptase and PAR-2 activating peptides on the human procollagen α2(I) gene promoter | 199 |
| Figure 3.34 | Effect of tryptase and TGFβ on α1(I) procollagen mRNA expression | 201 |
| Figure 3.35 | Effect of SLIGKV on α1(I) procollagen mRNA expression | 202 |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.36</td>
<td>Effect of 24h treatment with tryptase on procollagen metabolism by human foetal lung fibroblasts</td>
<td>206</td>
</tr>
<tr>
<td>3.37</td>
<td>Effect of 48h treatment with tryptase on procollagen metabolism by human foetal lung fibroblasts</td>
<td>207</td>
</tr>
<tr>
<td>3.38</td>
<td>Effect of 24h treatment with PAR-2 activating peptide, SLIGKV, on procollagen metabolism by human foetal lung fibroblasts</td>
<td>209</td>
</tr>
<tr>
<td>3.39</td>
<td>Effect of 48h treatment with PAR-2 activating peptide, SLIGKV, on procollagen metabolism by human foetal lung fibroblasts</td>
<td>210</td>
</tr>
</tbody>
</table>

**Chapter 4**

**Figure 4.1** Electronmicrograph of subepithelial collagen and fibroblast from airway of mild asthmatic patient | 237 |
List of Tables

Chapter 1

Table 1.1 Mediators and adhesion molecules expressed by activated resident cells implicated in airway remodelling 43
Table 1.2 Collagens known to be present in the airway. 48
Table 1.3 Mediators reported to be increased in asthma which have been shown to promote fibroblast migration, proliferation and matrix production 59
Table 1.4 A comparison of the number and the state of activation of mast cells in the airways of asthmatic and non-asthmatic subjects 62
Table 1.5 Summary of the mediators constitutively expressed, or newly synthesised in the different phenotypes of human mast cells after activation 65
Table 1.6 Summary of the cellular functions of the cytokines, chemokines and growth factors released by activated mast cells 69
Table 1.7 Selected range of mast cell tryptase inhibitors 81
Table 1.8 Sequence of the new N-terminal sequence of human and rodent (mouse and rat) PAR's after cleavage by specific enzymes 86
Table 1.9 PAR-2 antibodies 92
Table 1.10 Protease-activated receptor-2 expression in human tissue 92

Chapter 2

Table 2.1 Antibodies used in characterisation of fibroblast cell lines 101
Table 2.2 Inhibitors used to inhibit the catalytic activity of tryptase 112
Table 2.3 PCR components 120
Table 2.4 Schedule for processing airway biopsies using an automated carousel-based processor 126
Table 2.5 Rehydration schedule for biopsy specimens 127
Table 2.6 Schedule for dehydration of immunostained biopsy sections 128
Table 2.7 Chromatographic conditions for the separations of hydroxyproline by reverse-phase liquid high pressure chromatography (HPLC) 139
Table 2.8 Reproducibility of HPLC measurements 142
### Chapter 3

| Table 3.1 | The inter-batch variation of tryptase-induced mitogenic responses on human foetal lung fibroblasts | 149 |
| Table 3.2 | The intra-batch variation of tryptase-induced mitogenic responses on human foetal lung fibroblasts | 149 |
| Table 3.3 | Effect of 24h treatment of tryptase on human foetal lung fibroblast proliferation | 204 |
| Table 3.4 | Effect of 48h treatment with tryptase and TGFβ on human foetal lung fibroblast proliferation | 204 |
| Table 3.5 | Effect of 24h treatment of SLIGKV on human foetal lung fibroblast proliferation | 208 |
| Table 3.6 | Effect of 48h treatment of SLIGKV on human foetal lung fibroblast proliferation | 211 |

### Chapter 4

| Table 4.1 | Procollagen production data corrected for cell counts | 241 |
| Table 4.2 | Measurements of thickness of lamina reticularis in normal control and asthmatic subjects | 248 |
| Table 4.3 | The effects of asthma treatment on subepithelial thickening in airways of asthmatic patients | 249 |
Abbreviations

Ala Alanine
ANOVA Analysis of variance
APC Antigen presenting cells
APC366 \([N\cdot(1\text{-hydroxy-2-naphthoyl})\cdotL\text{-arginyl\cdotL-prolinamide hydrochloride}]\)
ARDS Adult respiratory distress syndrome
Arg Arginine
ATCC American tissue culture collection
AUC Area under curve
BABIM Bis (5-amidino-2-benzimidazolyl)methane
BAL Bronchoalveolar lavage
BAPNA \(\alpha\text{-N-benzoyl-D,L-Arg p-nitroanilide}\)
BOOP Bronchiolitis obliterans organising pneumonia
BPD Bronchopulmonary dysplasia
BSA Bovine serum albumin
Ca\(^{2+}\) Calcium ions
CAT Chloramphenicol O-acetyltransferase
cDNA Complimentary Deoxyribonucleic acid
cDNA Chromosomal deoxyribonucleic acid
CFA Cryptogenic fibrosing alveolitis
CGRP Calcitonin gene related peptide
cm\(^2\) Centimetres
CO\(_2\) Carbon dioxide
COPD Chronic obstructive pulmonary disease
DAB 3,3'-diaminobenzidine
DFP Diisopropylfluorophosphate
DMEM Delbecco's modified eagles medium
DMSO Dimethylsulphoxide
dpm Disintegration's per minute
DPX A mixture of distyrene, a plasticiser (tricresyl phosphate) and xylene, a mountant for histology
ECP Eosinophil cationic protein
EDTA Ethylene diaminetetraacetic acid
ELISA Enzyme linked immunosorbent assay
EPO Eosinophil peroxidase
EST Expressed sequence tags
FACIT  Fibril-associated collagens with interrupted triple helix
FceRI  High affinity IgE receptor
FceRII  Low affinity IgE receptor
FEV₁  Forced expiratory flow in 1 second
FITC  Fluorescein isothiocyanate
FRC  Functional residual capacity
FVC  Forced vital capacity
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
Gly  Glycine
GM-CSF  Granulocyte-macrophage colony stimulating factor
GP  General practitioner
Grp  Glucose-regulated protein
GVHD  Graft versus host disease
HEPES  4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
HFL-1  Human foetal lung fibroblasts-1
HPLC  High pressure liquid chromatography
hr  hours
Hsp  Heat shock protein
HUVEC  Human umbilical vein endothelial cell
IC  Inspiratory capacity
ICAM  Intercellular adhesion molecule
IFNγ  Interferon-γ
Ig  Immunoglobulin
IGF-1  Insulin growth factor-1
IL  Interleukin
IPF  Interstitial pulmonary fibrosis
IPV  Inspired reserve volume
kDa  Kilodaltons
Ki  Enzyme inhibitory constant
LDTI  Leech-derived tryptase inhibitor
LSIGKV  Leucine-serine-isoleucine-glycine-lysine-valine
LSIGRL  Leucine-serine-isoleucine-glycine-arginine-leucine
LT  Leukotriene
M  Molarity
MBP  Major basic protein
MC<sub>CT</sub>  Mast cells containing chymase and tryptase
MC<sub>T</sub>  Mast cells containing tryptase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>Mandin-Darby canine kidney epithelial cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar (1 x 10^3 M)</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar (1 x 10^6 M)</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NBD-Cl</td>
<td>7-chloro-4-nitrobenz-2-oxa-1,3,diazole chloride</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>NHLI</td>
<td>National Heart &amp; Lung Institute</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NK₂</td>
<td>Neurokinin receptor-2</td>
</tr>
<tr>
<td>nM</td>
<td>Nonomolar (1 x 10^9 M)</td>
</tr>
<tr>
<td>NSE</td>
<td>No significant effect</td>
</tr>
<tr>
<td>NT</td>
<td>Not tested</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PAR-AP</td>
<td>Protease activated receptor activating peptide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC₂₀</td>
<td>Concentration of agent which reduces airflow by 20% from baseline</td>
</tr>
<tr>
<td>PCP</td>
<td>Procollagen C-endopeptidase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PGF₃α</td>
<td>Prostaglandin F₃α</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>rh-tryptase</td>
<td>Recombinant human-tryptase</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SLIGKV</td>
<td>Serine-leucine-isoleucine-glycine-lysine-valine</td>
</tr>
<tr>
<td>SLIGRL</td>
<td>Serine-leucine-isoleucine-glycine-arginine-lysine</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SSP</td>
<td>Statutory sick pay</td>
</tr>
<tr>
<td>TAME</td>
<td>p-tosyl-L-Arg methyl ester</td>
</tr>
<tr>
<td>tc-LIGRLO-NH₂</td>
<td>trans-cinnomoyl leucine-isoleucine-glycine-leucine-ornithine-NH₂</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TV</td>
<td>Tidal volume</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
Chapter 1

1.1 Preamble. An Introduction to asthma

Asthma has been recognised for centuries as a major disease within the civilised world since the early 1900’s. Within the last 20-30 years asthma has become an ever increasing burden on the health services of many countries around the world. Although, it is now possible to diagnose asthma and treat the symptoms in the majority of cases, the underlying causes for this disease are uncertain. In addition in a sub-group of patients the symptoms are not adequately controlled by the current therapies leading to what has been described as ‘steroid-resistant’ asthma, with these patients having to be maintained on high dose oral steroids. In addition, the airway obstruction seen in severe chronic asthmatic patients (status asthmaticus) is resistant to bronchodilating agents such as the β2-adrenoceptor agonists. The reasons for these chronic changes are unclear but may involve changes in the numbers and types of resident cells seen within the airway wall, infiltration of inflammatory cells and structural remodelling of the airway tissue. These changes include an increase in the number of cells within the epithelium, in particular, the number of goblet and serous cells. Upon activation these cells can produce large amounts of mucus which, in a severe asthmatic attack, can block the airways (mucus plugging). There are also increased amounts of bronchial smooth muscle, which demonstrate increased reactivity to a number of specific and non-specific stimuli, leading to severe, irreversible bronchoconstriction (bronchial hyperreactivity). There is also an increase in the deposition of extracellular matrix within the lamina reticularis beneath the epithelial basement membrane and within the adventitial tissue of the airway wall (airway fibrosis). This thesis will investigate one aspect of this structural re-modelling of the airways, the sub-epithelial airway fibrosis. In particular, the role of tryptase, a mediator released by inflammatory mast cells, that are known to be activated in the airways of asthmatic patients, and the mesenchymal cells, fibroblasts and myofibroblasts, cells known to be associated with the production of structural proteins during development.
and injury repair. The proliferation of fibroblasts/myofibroblasts and the production of extracellular matrix following their activation by mast cell mediators are not unique to asthma, and therefore their interactions in other fibrotic diseases will be considered. The following section gives an historical perspective of asthma leading to a discussion of the pathophysiological changes seen in the airways of asthmatic patients.

1.1.1 An historical perspective of asthma

Bronchial asthma has been known and written about for over 4000 years. The “Fire Emperor” of China, Shen-Nung (2838-2698 B.C.) may have been the first person to describe the symptoms of a disease similar to asthma. He recognised the therapeutic nature of a Chinese herbal medicine from *ma-huang*, a plant from which ephedrine (adrenaline) was extracted and added to wine. Interestingly, a second reference to a ‘first description’ of asthma was in the ancient medical annals of internal medicine, *Nei Ching* ascribed to the third of China’s mythological emperors – Huang Ti, the “Yellow Emperor” (2698-2598 B.C.).

The Emperor said “Man is afflicted when he cannot rest and when his breathing has a sound (is noisy)- or when he cannot rest and his breathing is without any sound. He may rise and rest (his habits of life may be) as of old and his breathing noisy; he may have his rest and his exercise and his breathing is troubled (wheezing, panting): or he may not get any rest and be unable to walk about and his breathing is troubled. There are those who do not get a rest and those who rest and yet have troubled breathing…..”

An excerpt taken from Vieth I. Huang Ti Nei Ching Su Wen, The Yellow Emperor’s Classic of Internal Medicine: Williams & Wilkins, 1949 pp 252-253

From his writings, Hippocrates (460-375 B.C.) seems to have studied the disease using the Greek, *ασθμα*, meaning panting to describe what is now known as asthma. Also, early manuscripts by Galen (circa 130-200 A.D.) and Paracelsus (1493-1541) both described the production of mucus in the lungs of asthmatics. A comprehensive study of asthma in ancient medicine can be found in a 1992 review by Cohen (Cohen, 1992).
In a review by Donald (Donald, 1971) the following definition of asthma was quoted from the first English medical textbook, *The compleat System of Physic* by Salmon in 1686.

"A difficult respiration, sometimes with and sometimes without fever, sometimes with a noise and sometimes without, arising from an obstruction of the bronchia and cells of the lungs...............The bronchia are most hurt in this disease"

This text also mentioned the work of Galen and Paracelsus with the quote:

"......the original of this Disease to Phlegm falling on the lungs"

Prior to this, Helmont (1579-1644) had described asthma as

"......Asthma seizes one suddenly just as if a Rope were tied about a Man's neck"

In addition he wrote

"......the cause of this Disease is a peculiar Poison which affects the Lungs by its property"

Thus, Helmont had made the observations that asthma involves the obstruction of the airways, restricting airflow and also described the property and actions of allergens upon the lung.

However, only relatively recently has progress been made in understanding the mechanism of asthma. In the late 1800's Dr Henry Hyde Salter described asthma as

"......paroxysmal dyspnea of a peculiar character generally periodic with healthy respiration between attacks"

suggesting the reversible nature of bronchoconstriction in the asthmatic patient (Salter, 1868) a description still used in clinical asthma today. The first description of inflammation as a feature of asthma was by William Osler (Osler, 1898). However, it was not until the introduction of fibre-optic bronchoscopy that asthma was seen, in terms of the modern definition of inflammation, as an inflammatory disease. Prior to this, studies on asthma had been restricted to case reports of patients who had died of their disease (Ellis, 1908;
Fraenkel, 1900; Schmidt, 1892). These were the first reports of airway remodelling - thickening of the region below the subepithelial basement membrane, epithelial damage, goblet cell hyperplasia with the presence of excessive luminal secretions, and the infiltration of inflammatory cells into the airway wall. This will be discussed further in section 1.3.

With the identification of the pathophysiological changes in the asthmatic airways it is important to put this into context with clinical features of the disease. The following section therefore explores these features in more detail and includes a brief overview of asthma epidemiology.

1.1.2 Clinical features of asthma

Asthma is a syndrome with a number of clinical and pathological features leading to a shortness of breath. The clinical diagnosis of asthma is difficult, as the symptoms are common to a number of lung diseases including Chronic Obstructive Pulmonary Disease (COPD), in particular bronchitis and emphysema (American Thoracic Society, 1987; Ciba Symposium, 1959). There have been several published guidelines on the diagnosis and management of asthma. In particular an early publication by the American Thoracic Society (ATS) defined and classified asthma in terms of other chronic lung diseases in 1962, which was modified and updated in 1987 (American Thoracic Society, 1962; American Thoracic Society, 1987). The British Thoracic Society (BTS), in association with other eminent Medical Societies, published their guidelines to the diagnosis and treatment of asthma in 1993, with an extensive update in 1997 (British Thoracic Society et al., 1993; The British Thoracic Society et al., 1997).

The classical symptoms are an intermittent or daily shortness of breath (dyspnea), a productive cough, chest tightness or wheezing due to repeated bronchoconstriction which either reverses spontaneously or by the use of bronchodilating agents, such as the β2-
adrenoceptor agonists salbutamol or terbutaline. The given clinical term is 'reversible bronchoconstriction'. Clinicians also use $\beta_2$-adrenoceptor treatment diagnostically to establish that patients have reversible airway obstruction due to smooth muscle contraction rather than blocked airways due to mucus plugging. There is an increase in airways responsiveness (i.e. bronchoconstriction) to provoking agents such as histamine, methacholine, adenosine, isotonic saline or cold air. This is known as bronchial hyper-responsiveness and is measured by evaluating the concentration of a particular agent that induces a 20% decrease in airflow, measured as Forced Expiratory Flow in 1 sec ($FEV_1$), and is termed the $PC_{20}$. In most patients the symptoms tend to be worse at night leading to disturbed sleep, or they are woken early in the morning with a shortness of breath - '4 AM early morning dip'.

On lung function testing there is a pronounced diurnal variation in Peak Expiratory Flow Rate (PEFR) which is the maximum flow velocity (L/min) of a forced expiration from total lung capacity. To allow a diagnosis of asthma, airflow and lung volume measurements in patients are compared to age, sex and height matched normal controls, they include

- a reduced $FEV_1$ due to airway restriction or obstruction
- an approximate 120% increase in the Functional Residual Capacity (FRC) and/or Residual Volume (RV) due to air trapping.
- a reduction in the Forced Vital Capacity (FVC) due to a combination of airway restriction and air trapping.
- an $FEV_1/FVC$ ratio of less than 75%, indicating a greater airway constriction component to the functional reduction in airflow.

Although clinicians have a battery of tests to aid in the diagnosis of asthma, at best they give a good indication but do not provide conclusive proof, due to the similarity of the symptoms with other lung diseases. In addition to the physiological and functional changes seen in
asthma, clinicians can use pathological and cytological examinations to aid their diagnosis. The introduction of bronchial biopsies, the histological evaluation of the pathological changes, the use of bronchoalveolar lavage (BAL) and induced sputum, demonstrate the consistent finding of structural remodelling and increased numbers of inflammatory cells within the airways of asthmatics (See Section 1.6). These types of studies have also been useful in the investigations of asthma epidemiology, which are briefly reviewed in the next section.

1.1.3 Epidemiology: Prevalence, morbidity and mortality of Asthma

Many different definitions of asthma have been used in epidemiological studies, the most widely used being “a history of wheeze”. Values of prevalence range from between 1% to 35% depending on the country being studied, with the majority of epidemiological studies in childhood asthma. In the recent UK National Asthma Campaign’s “Asthma Agenda” it was reported that 38% of school children miss more than one week at school per year, with 8% missing more than a month (National Asthma Campaign, 1999). Many more children having to curtail their daily activities due to the severity of their disease. Over 20 years from 1971 to 1991/92 there has been a 4-5 fold increase in the number of children (aged 0-15 years) consulting General Practitioners (GP) about their asthma (Department of Health, 1995).

The prevalence in adults is difficult to define due to age-related increases of different respiratory diseases with symptoms similar to that of asthma. 15-20% of adults suffer from wheeze but less than 5% suffer from night-time breathlessness or reversible airflow limitation – characteristic symptoms of asthma. In the 1958 Birth Cohort study, 4% of 23 year olds reported having suffered from asthma, whereas by the age of 33, this had increased to 8% (Burney, 1997; Sears, 2000).
From 1968 to 1985 there has been a steady increase in the numbers of patients admitted to hospital with a main diagnosis of asthma (Department of Health, 1995). Similar changes have been reported in Australia, New Zealand and United States of America (Moore et al., 1996). In England during 1992, asthma caused in excess of 1600 deaths accounting for 0.3% of all deaths (Department of Health, 1995). These deaths were mainly people over the age of 65 (>60%) whereas only 2% were in children under the age of 15. Since 1970 the mortality rates for males and females under the age 54 years have decreased, whereas, there were increases in the 54-75 and 75 and over age groups. However, data from the 1992 – 1997 suggest that asthma mortality is falling by approximately 6% a year among people between 5 – 64 years, with a slightly lower rate of decline for people over the age of 65 years (Department of Health, 1995).

With the large and increasing numbers of patients who are presenting with asthma, the cost to the Health Service is also increasing. It is estimated that 1 in 7 children (aged 2-15) and at least 1 in 25 adults (16 and over) have asthma in the United Kingdom which is equivalent to 3.4 million people, of which 640,000 (~20%) have severe asthma. It has also been calculated that asthma costs the National Health Service (NHS) in excess of £1000 million a year (~£300 per patient per year) (National Asthma Campaign data, 1999).

In summary, it appears there is an increase in the prevalence and morbidity of asthma in all age groups throughout the world. Overall, the mortality rate due to asthma is declining. With the spiralling costs adding to the overall burden on the health systems, new approaches to the treatment of asthma have to be sort. All the therapeutic approaches to date have concentrated on the relief of asthma symptoms (bronchodilators) and the reduction in airway inflammation (steroids). Even with optimal use of these treatments there is a small, but significant group of patients in which it is difficult to control their symptoms, as they do not respond to treatment. In particular, they do not respond to high dose inhaled or orally administered steroids, leading to these patients being termed ‘steroid-resistant’. With the
introduction of new technologies over the last 20 years (bronchoscopy, bronchial biopsies) it has been possible to study the changes in airway structure associated with the development of asthma, thus allowing new approaches to be used to gain a better understanding of the disease.

The following section will describe the pathophysiology of asthma. To allow a better understanding of these changes I will first describe the normal structural features of the human airway, followed by a description of the structural changes seen in the asthmatic airway and the consequential changes in lung function.

1.2 Pathophysiology of Asthma

1.2.1 Structural features of the human bronchial airway

The trachea divides into two main bronchi, which further subdivide into segmental bronchi that enter the left and right lungs. The trachea and bronchi are supported by surrounding cartilage, however, as the bronchi subdivide further into bronchioles the cartilage is lost. The terminal bronchioles give rise to respiratory bronchioles and finally the alveoli (Hegele and Hogg, 1996).

The bronchiole wall consists of a mucosal lining (epithelium), a basement membrane, subepithelial connective tissue (lamina reticularis), a sub-mucosal layer (smooth muscle) and adventitia (Figure 1.1, Kuhn, 1988). The proximal airway epithelium consists mainly of basal cells, pseudo-stratified, ciliated, columnar epithelial cells, clara cells, goblet (mucus), and serous cells, with more cuboidal, non-ciliated epithelial cells in the distal airways (Kuhn, 1988, Tavakoli et al., 1997).

The basal and epithelial cells are attached to a basement membrane which consists of lamina rara and lamina densa (Figure 1.1c) which contain matrix molecules, including collagen
types IV, VII and XV, the glycoproteins, laminin, nidogen (entactin), fibronectin, SPARC (BM-40) and the heparan sulphate-linked proteoglycan, perlecan (Yurchenco and Schittny, 1990). Type V collagen is also found in small amounts within the basement membrane (Roche et al., 1989). It is the distribution and density of these molecules which gives rise to the characteristic features of the lamina rara and lamina densa. This membrane is 80-90 nm thick and provides a scaffold for attachment of cells and regulates the surrounding milieu by controlling the flux of small and large molecules (Yurchenco and Schittny, 1990).

Within the lamina reticularis there are fibroblast-like cells which continuously metabolise extracellular matrix molecules to maintain the structural integrity of the airway wall (Figure 1.1b & c, see Section 1.5 Fibroblasts in the airways, (Brewster et al., 1990, Redington et al., 1998)). These cells and the extracellular matrix molecules are in continuum with the smooth muscle cells and other resident cells in the sub-mucosa (Figure 1.1b, Cutz et al., 1978, Roche et al., 1989, Minshall et al., 1997, Redington et al., 1998).

Within the sub-mucosal layer there are bronchial glands that contain both serous and mucus-secreting cells that are in communication with the airway lumen (Rogers, 2001). The numbers of glands decreases within successive generations of airways (Whimster et al., 1984). In contrast, the amount of smooth muscle increases with successive airways. In the upper, large airways the amount of smooth muscle is only 5% of the total airway wall, whereas in the lower membranous bronchioles it is 20% of the total wall thickness (Hegele and Hogg, 1996). The outer adventitial layer of the wall consists of bundles of collagen, arterial and venous blood vessels, lymphatics and nerves (Figure 1.1b, (Hegele and Hogg, 1996)). This outer adventitial layer of the airway connects with the parenchymal tissue of the alveoli and is important in keeping the airway lumen open by applying forces to the outside of the airway wall (See Section 1.2.3, Mead, 1961, Pare et al., 1997).
Figure 1.1. A schematic diagram of the normal bronchial airway. 
a) shows the overall structure of the bronchial with the epithelium lining the airway lumen, surrounded by the sub-mucosal layer and finally the adventitia extending to the alveolar attachments. b) shows, in greater detail, the mucosa, lamina reticularis (also called lamina propria), sub mucosa and adventitia with the extensive matrix molecule connections between the cells and the structural components within the bronchial wall. c) highlights the matrix molecule connections between the fibroblasts in the lamina reticularis and the basement membrane.
1.2.2 Structural changes in airways of asthmatic patients

In asthmatics there are major changes in the airways with chronic inflammation, mucosal thickening, including goblet cell hyperplasia and metaplasia leading to mucus plugging of the airways (Lamb, 1990, Dunnill et al., 1969). In addition there is a thickening of the lamina reticularis; increased amounts of smooth muscle with a diffuse fibrosis extending out into the interstitium and parenchymal tissue (Minshall et al., 1997). Recent reports have described an increase in vascularity, suggesting a mechanism for the airway wall oedema and plasma exudation (Wilson and Stewart, 1999, Wilson, 2000). Figure 1.2 demonstrates these features in a histological section of an airway of a patient who had died of their asthma. The following section describes, in more detail, the structural changes seen in the airways of asthmatic patients.

Figure 1.2 Photomicrograph of an airway from an asthmatic patient. This figure show the histological features of the asthmatic airway including epithelial sloughing (EC), intraluminal mucus (M), fibrotic lesion in the subepithelial region (lamina reticularis, LR), increased amounts of smooth muscle (SM), enlarged bronchial glands (G) and inflammatory cells throughout the airway wall. Adventitial tissue (A) is shown in connection with the lung alveoli (alv). (Reprinted from Hegele RG & Hogg JC (1996) The pathology of asthma In: Severe asthma. Pathogenesis and Clinical Management. Pp61-76. Series: Lung Biology in Health and Disease. Vol 86 Eds. Szeftler SJ & Leung DYM by courtesy of Marcel Dekker Inc.)
Huber and Koessler published the first comprehensive account of the pathology of bronchial asthma (Huber and Koessler, 1922). They described an increase in the overall thickness of the airway wall, including increases in the amount of smooth muscle, the numbers of inflammatory cells and the amount of mucus within the lumen of the airways. In morphological studies, the airway wall was found to be significantly thicker in asthmatic compared with non-asthmatic patients (James et al., 1989) and Carroll and colleagues demonstrated increased wall thickness in both the large and small airways in fatal asthma, but only in the small airways in non-fatal asthma (Carroll et al., 1993).

This increase has also been noted in other airway diseases such as chronic obstructive pulmonary disease (Bosken et al., 1990; Crepea and Harman, 1955; Pare et al., 1991), bronchiectasis, bronchitis, and tuberculosis (Crepea and Harman, 1955). The thickening results from an increase in the thickness of the mucosa (James et al., 1989, Laitinen et al., 1985), lamina reticularis (Jeffery et al., 1989; Jeffery, 1991; Jeffery et al., 1992; Roche et al., 1989), and sub-mucosal layers of the airway (Cutz et al., 1978; Dunnill, 1960; Huber and Koessler, 1922; James et al., 1989; Jeffery, 1991, Figure 1.3). Changes in the sub-mucosa are likely to involve both smooth muscle cell hyperplasia (Hossain and Heard, 1970) and hypertrophy (Ebina et al., 1990).
It was initially thought that the changes in the lamina reticularis were a consequence of the thickening of the epithelial basement membrane, due to increased collagen deposition (Dunnill, 1960; Glynn and Michaels, 1960; Houston et al., 1953; Huber and Koessler, 1922).

However, using ultrastructural microscopy, the thickening was localised to the lamina reticularis, with the lamina rara and the lamina densa of the basement membrane remaining unchanged (Jeffery et al., 1989; Jeffery, 1991; Roche et al., 1989). These changes appear to be an early event in the pathology of asthma, as increases in the thickening of the lamina reticularis are seen in children with asthma (Cutz et al., 1978) and in-patients with mild disease (Jeffery et al., 1989).

It was postulated that the increased thickness of the lamina reticularis was due to the activation of the epithelial cells (Jeffery et al., 1989) that have been shown to secrete collagen in-vitro (Dodson and Hay, 1971; Simon et al., 1993). However, the composition of
this layer was shown to consist predominantly of collagen types I, III and V, fibronectin (Roche et al., 1989; Wilson and Li, 1997) and elastin (Gabbrielli et al., 1994), molecules that are generally thought to be produced by fibroblasts, rather than epithelial cells. Also, no laminin was identified within the lamina reticularis, but was found within the 'true' basement membrane, again, suggesting that the matrix deposition in the sub-epithelial layer was not associated with epithelium (Roche et al., 1989).

In addition to the increase in collagen, fibronectin and elastin, other extracellular matrix components are increased within the lamina reticularis of asthmatic airways. For example, hyaluronan, versican, biglycan and decorin are increased in the airways of patients with severe asthma (Roberts, 1995). Recently, the deposition of lumican, biglycan and versican were shown to be increased in the airway walls of mild, atopic asthmatic subjects when compared with that seen in normal controls (Huang et al., 1999) and also increased levels of proteoglycans have been demonstrated in the sputum from asthmatic subjects (Shute et al., 1998). In support of these findings, recent reports demonstrate increased proteoglycan production from bronchial fibroblast cell lines cultured from the airways of asthmatic patients (Tremblay et al., 1998a). Due to the hydrophilic nature of these molecules, it can be speculated that an increased fluid content within the sub-mucosa may further thicken the airway wall. Tenascin, a glycoprotein normally found in embryogenesis and tumour tissue, is expressed in lamina reticularis of airways from chronic and seasonal asthmatic subjects, whereas there is little or no expression in the airways of normal control subjects (Laitinen et al., 1997). The authors of this study suggest that the increased expression of tenascin is part of a wound repair and/or remodelling process within the damaged airways of the asthmatic.

An increase in the size and number of vessels (vascularity) within the bronchial wall has been reported (Li and Wilson, 1997). It is postulated that vasodilatation, induced by inflammatory mediators, and the increase in plasma protein leak (oedema) from these vessels
would add to the thickness of the airway wall. This increase in fluid content within the airway wall would be enhanced by the hydrophilic nature of the proteoglycans.

Thus in summary, the increase in the thickness of the airway wall due to the deposition of extracellular matrix molecules, increased smooth muscle mass, increased numbers of blood vessels, tissue oedema are all likely to play a role in controlling airway lumen size and the subsequent restriction of airflow to and from the alveoli. The following section discusses further the role of these changes in controlling airflow and the subsequent impact on the lung function in the asthmatic patient.

1.2.3 Functional effects of extracellular matrix deposition in the airway wall

Both the airways and the parenchyma contribute to the elastic recoil of lung tissue. Mead hypothesised that the parenchyma is able to stabilise the alveoli and terminal bronchioles, by generating a recoil force (interdependence), thus preventing airway collapse (Figure 1.4a) (Mead, 1961, Pare et al., 1997). A decrease in elastic recoil may have profound effects on the airway contractility. This hypothesis was extended to suggest that the adventitial tissue around the airway is an important determinant of the magnitude of bronchoconstriction (Hoppin, Jr., 1995; Mead et al., 1970; Robinson et al., 1992). Several groups have hypothesised that the load exerted by the lung parenchyma in the normal airways must be overcome by the smooth muscle to enable the airway to contract (Figure 1.4a, Hoppin, Jr., 1995; Macklem, 1995; Robinson et al., 1992). Excessive extracellular matrix deposition outside the smooth muscle layer, within the adventitial tissue surrounding the airway (peribronchial fibrosis), would uncouple these opposing forces allowing the smooth muscle to contract more easily (Figure 1.4c, Bramley et al., 1994). The increase in the deposition of extracellular matrix within the mucosal layer and lamina reticularis would also have consequences for airway constriction. A thickened mucosal layer, compared to normal,
causes an increase in the narrowing of the lumen for any given degree of smooth muscle contraction (Figure 1.4b, Paré and Bai, 1996).

An important normal physiological response to smooth muscle contraction is the folding of the mucosa, which restricts lumenal narrowing. However, in the inflamed airway, deposition of the extracellular matrix within the lamina reticularis would limit the number of folds generated, thus allowing a further reduction in lumenal diameter (Figure 1.4 b & c, Lambert, 1991).
Airway remodelling could be a consequence of the activation of cells within the normal airway. The initial acute activation of cells, such as the epithelial cell and macrophage, by known and unknown factors could be seen as a homeostatic or defence mechanism in protecting the airway from inhaled particles or gases. However, the chronic stimulation by
long term exposure to allergens, noxious chemicals/pollutants or viruses may result in inappropriate release of cytokines, chemokines or enzymes, inducing tissue and cellular damage. This implies an interaction between local resident cells and inflammatory cells within the airway. The following two sections review the cell types that could be involved in this proposed mechanism, highlighting the interaction between the resident fibroblast/myofibroblasts and the inflammatory mast cells.

1.3 The role of resident cells in pathology of asthma

Initial autopsy studies from patients who had died of asthma, clearly showed a the loss of epithelial cells, copious quantities of mucus containing Charcot-Leydon crystals of major basic protein from eosinophils and smooth muscle hyperplasia and hypertrophy (Cardell and Pearson, 1959; Craige, 1941; Earle, 1953; Kountz and Alexander, 1928; Macdonald, 1933). In addition, sputum from asthmatic patients contain Curshmann's spirals (condensed spirals or whorls arising from mucus casts of the smaller airways), clumps of shed epithelial cells (Creola bodies) and Charcot-Leydon crystals.

For the purposes of this overview, macrophages, epithelial cells, smooth muscle cells and fibroblasts/myofibroblasts are termed 'resident cells', which are present in both the normal and the asthmatic airway. Macrophages are the major cell type in BAL from both normal and asthmatic lung. These cells, which are derived from monocyte precursors, are phagocytic for particulate material including bacteria and viruses. They are able to present antigen to T-lymphocytes and participate directly in the allergic response due to the expression of low affinity IgE receptor (FcεRII). The numbers of macrophages in BAL increase after allergen challenge, which is thought to be due to the migration into the airways of monocytes from the peripheral circulation, where they differentiate into macrophages. When these cells are activated by particle ingestion, antigens and other locally produced
mediators such as interleukin-1 and interferon-γ, they release a wide range of mediators which may have profound effects of inflammatory cell infiltration and tissue remodelling (Table 1.1, see Daftary et al., 1998 for review).

Within both the normal and asthmatic airway, the epithelium is an important site involved in the defence of the underlying tissue from noxious chemicals and particulate matter. However, when the epithelium is injured this barrier is lost, which in asthma, is seen as abnormal or damaged cells, with fragile attachments to other epithelial cell types (basal, goblet, serous, ciliated cells) leading to large areas of cell loss (desquamation). This exposes the underlying sub-mucosal cells (fibroblasts, smooth muscle, nerves and blood vessels, Figure 1.1) to activation by inhaled particles. In addition, these 'active' epithelial cells produce pro-inflammatory cytokines, peptide and lipid mediators, which have profound effects on inflammatory cell proliferation, differentiation, migration and activation (Table 1.1), and the generation of degradative enzymes (For a more extensive reviews see Devalia and Davies, 1993; Holgate et al., 1999; Knight et al., 1994).

It is well established that the contractile phenotype of the airway smooth muscle cell has profound effects of the lumenal diameter, increasing airflow resistance with the subsequent decrease in lung function (see Section 1.3.3). The smooth muscle of the first and second order bronchi in the normal airway are similar to tracheal smooth muscle, whereas the fourth to the seventh are clearly different (Daniel et al., 1986). The size and arrangement of the muscle bundles are different as is the appearance of the myofilaments. Also there is an increase in the numbers of mast cells associated within the smooth muscle cells of the lower airways (Rodger, 1992).

In general each airway smooth muscle cell is innervated and the cell to cell connections are poor. Due to the low numbers of gap junctions between cells the electrical activity does not pass between cells and induce spontaneous contractions. However, in asthmatic smooth
muscle cells there is an increase in action potential activity with the subsequent spontaneous contractions being associated with increases in responsiveness of the asthmatic airways (Akasaka et al., 1975).

Recently the contractile and relaxant properties of smooth muscle from asthmatic patients have been reviewed (Black, 1997, Seow et al., 1998). If the smooth muscle from these patients is abnormal it could be due an increase in the contractile activity of the tissue or a decrease in the ability to relax. Under experimental conditions this could be demonstrated by an increase in the maximum response to a particular agonist or a leftward shift in the agonist dose-response curve (i.e. an increase in agonist potency), a decrease in the maximal response to the relaxant agonist or a rightward shift of the dose-response curve (i.e. a decrease in potency).

A number of studies have been reported, however, the conclusions are unclear. For example, in two studies using smooth muscle tissue from patients who had died in status asthmatic or who had mild disease, histamine sensitivity was decreased (Whicker et al., 1988, Goldie et al., 1986). Whereas, in a third study of asthmatic tissue demonstrated an increase in histamine sensitivity (Cerrina et al., 1989). Although these studies were able to show changes in reactivity to histamine they were unable to show any increase in sensitivity to a range of other agonists including cholinergic agonists (Whicker et al., 1988, Goldie et al., 1986), leukotrienes and prostaglandins (Dahlen et al., 1980), allergen or anti-human IgG (Bjorck and Dahlen, 1993). However, de Jonste and colleagues were able to demonstrate marked increases in reactivity to histamine, leukotriene C4 and methacholine (de Jongste et al., 1987). In addition, in mild to moderate asthmatic patients it is possible to show an increase in sensitivity to adenosine (Bjorck et al., 1992).

To further add to the confusion, several studies that were able to show increases in in-vivo hyperresponsiveness could not demonstrate increases reactivity in-vitro (de Jongste et al.,
Finally, a series of studies by the same authors showed an increase in reactivity to histamine in tracheal smooth muscle but not in lower sub-segmental bronchi (Bai, 1991, Bai, 1990).

It may be possible to explain these differences in responses seen in-vivo and in-vitro by suggesting that removing the tissue from the lung removes the cells from a source of a mediator that is required for inducing hyperresponsiveness. Also a number of these studies were performed on post-mortem tissue obtained >12h post death. It is therefore possible that the tissue viability may have been affected. However, the differences seen are not the same for all agonists, thus suggesting that the differences may be dependent on the agonist used. Finally, the studies from de Jonste and colleagues suggest that the responses seen may be dependent on which level of the airway was investigated. It is thought that the increases in airways resistance in asthmatics is at the level of the conducting airways, many of the studies to date have used a variety of different sized airways depending on the source of the tissue e.g. tracheal smooth muscle or airways <2 mm in diameter.

However, more recent research has focused on its synthetic phenotype in which the cell is able to produce pro-inflammatory cytokines and chemotactic agents, cellular adhesion molecules, growth factors (Table 1.1), and increased amounts of extracellular matrix molecules (Hirst, 1996; Johnson and Knox, 1997). It has been postulated that this synthetic phenotype of smooth muscle cell, in addition to its hyperplastic and hypertrophic responses, can enhance epithelial and sub-mucosal tissue remodelling in the airway.
Table 1.1 Mediators and adhesion molecules expressed by activated resident cells implicated in airway remodelling

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Lipid mediators</th>
<th>Cytokine/Chemokine</th>
<th>Growth factors</th>
<th>Adhesion molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>LTB₄, TXA₂, PAF,</td>
<td>IL-1, IL-6, GM-CSF,</td>
<td>TGFβ</td>
<td>ICAM-1, CD44</td>
</tr>
<tr>
<td>Epithelial cell</td>
<td>LTB₄, LTC₄, PGE₂</td>
<td>IL-1, IL-6, IL-8, GM-CSF,</td>
<td>PDGF, IGF-1, EGF, FGF-2, TGFβ, endothelin-1</td>
<td></td>
</tr>
<tr>
<td>Smooth muscle cell (synthetic phenotype)</td>
<td>PGE₂,</td>
<td>IL-6, IL-8, RANTES</td>
<td>PDGF-BB, IGF-1, FGF-2</td>
<td>CD44, VCAM-1, ICAM-1</td>
</tr>
</tbody>
</table>

LT - Leukotriene, TXA₂ - Thromboxane A₂, PAF - platelet activating factor, PGE₂ - prostaglandin E₂, IL - interleukin, RANTES - regulated upon activation, normal T-cell expressed and secreted, GM-CSF, Granulocyte-macrophage colony-stimulating factor, TGFβ, Transforming growth factor β, TNFα, Tumour necrosis factor α, PDGF - platelet derived growth factor, IGF-1, Insulin-like growth factor 1, FGF - fibroblast growth factor, EGF - epidermal growth factor, ICAM-1 - intercellular adhesion molecule -1, VCAM-1 - vascular cell adhesion molecule-1

The fibroblast/myofibroblast play an important role in the morphogenesis and development of the airway (see Jeffery, 1998; McGowan, 1992 for reviews). There appears to be a close relationship between epithelial cells and fibroblast/myofibroblasts in the airway. In a three-dimensional, in-vitro, co-culture system in which myofibroblasts were embedded in collagen gels and bronchial epithelial cells were cultured on the gel surface, the damage of the epithelial cells induced myofibroblast proliferation (Zhang et al., 1999). The majority of the mitogenic effects were inhibited by blockade of fibroblast growth factor-2, platelet derived growth factor, insulin-like growth factor-1, transforming growth factor-b and endothelin-1, suggesting these mediators are important in tissue remodelling following epithelial damage. This interaction has been termed the epithelial-mesenchymal trophic unit (Evans et al., 1999). The next section discusses the role of fibroblasts and myofibroblasts in maintaining the structural integrity of the normal airway wall and in tissue remodelling seen in asthma.
1.4 Fibroblasts in the airways

Compared to other mesenchymal and resident cells, fibroblasts possess the greatest capacity to produce extracellular matrix molecules (see Tremblay et al., 1995). They appear flattened with a stellate appearance, and an average diameter of 28 μm and a thickness of 0.55 μm. (Evans et al., 1993). They are localized to the lamina reticularis, forming a sheath around the airways (Figure 1.1b, Evans et al., 1993). The cells contain prominent rough endoplasmic reticulum, ribosomes and Golgi apparatus, reflecting their high metabolic activity and pores in the cell membrane.

A cell of similar phenotype to fibroblasts, but morphologically larger and containing a higher proportion of cytoplasm, is the myofibroblast (Gabbiani et al., 1971; Krishna et al., 1998; Skalli et al., 1989). These cells have been identified within the lamina reticularis of normal airways (Brewster et al., 1990; Gizycki et al., 1997) and can be distinguished from fibroblasts by the prominent amounts of contractile filaments or stress fibres containing α-smooth muscle actin (Brewster et al., 1990; Sappino et al., 1990), the partial covering with a basal lamina, and an increase in rough endoplasmic reticulum (Gizycki et al., 1997). They are found in multicellular strands and in close apposition with other cells (Henry and Campbell, 1996). Myofibroblasts numbers increase subsequent to tissue injury (Darby et al., 1990; Mitchell et al., 1990).

In normal, uninjured tissue fibroblasts are sparsely distributed throughout the connective tissue matrix. In wound healing and tissue remodelling, fibroblast functions such as proliferation and chemotaxis are controlled by the interaction of a number of factors (both stimulatory and inhibitory) released locally by several different cell types (see Table 1.1). Within a few hours, an injured site is infiltrated by fibroblast, which are then stimulated to proliferate by growth factors released from surrounding inflammatory cells. During cell culture, an exponentially growing population of fibroblasts goes through cell division, on
average, every 18 to 20 hours. The period between each cell division, mitosis, is called the cell cycle. The following section gives a brief review of the cell cycle. For more comprehensive reviews see Brooks and La Thangue, 1999 and Neufeld and Edgar, 1998.

The cell cycle is divided into phases (see Figure 1.5). The initial phases, G₁, is controlled by the synthesis and degradation of cyclins D and E within the cell and are referred to as the start cyclins. The exposure of cells to competence factors, such as platelet-derived growth factors or fibroblast growth factors renders cells competent to progress through G₁ towards the second phase, S phase or DNA synthesis. However to allow the progression to S phase the cells must also be stimulated with progression factors such as insulin-like growth factor (IGF-1) or epidermal growth factor (EGF). These competent and progression factors stimulate the expression of D-type cyclins during early G₁ and E-type cyclins during late G₁, allowing the progression into S Phase.

Throughout DNA synthesis, cyclin A is produced and towards the later stages of this phase cyclin B₁ is expressed, which is maintained during the third phase, G₂ or pre-mitosis, being maximally expressed at the transition of G₂ to the M phase or the mitotic phase. If cells are deprived of growth factors or they have achieved confluent density of the culture plate surface, the cells pass into a fifth phase, G₀ or resting phase, in which cells can remain for extended periods of time waiting for growth factor stimulation so that they can re-enter G₁.

The average cell cycle time varies among different cell types. However, the combined time for S, G₂ and M phases is consistent at between 10-12 h. Within this time frame, S Phase is consistently about 8 hours and M phase lasts 30 min to 1hr. The total length of the cell cycle is controlled by the time the cell is in G₁ and depends on the exposure to progression and competence factors. Cells stimulated to exit G₀ and to re-enter G₁ show a characteristic 12-
15hr delay before the onset of DNA synthesis, whereas cells continuously cycling only show a 6 hr delay.

Figure 1.5 Cell cycle. This figure shows the 5 phases G₀, G₁, S, G₂, and M and their interaction of the competence and progression factors, and the cyclins that lead to cell proliferation

The source of myofibroblasts at sites of tissue injury is uncertain, both differentiation from fibroblasts and smooth muscle cells have been proposed (Gabbiani, 1996; Phan, 1996). Transforming growth factor-β (TGFβ) can induce smooth muscle-actin expression in fibroblasts, thus giving the cell a myofibroblast-like phenotype (Desmouliere et al., 1993). Myofibroblasts cultured from granulating wounds are capable of reverting back to a fibroblast phenotype (Oda et al., 1990) demonstrating that these cells may not be terminally differentiated, and therefore, under the influence of appropriate mediators can change phenotype. An intermediate cell phenotype has been reported which appears to possess characteristics of both smooth muscle cell and fibroblasts, suggesting that cells from the
smooth muscle bundle have migrated into the lamina reticularis, and under the influence of inflammatory mediators, differentiated into a myofibroblast-like cell (Gizycki et al., 1997).

The location of the fibroblast/myofibroblast within the airway wall and the activation of these cells may play an important role in the remodelling of the airway. There are no studies to date that have measured the production and degradation rates of collagen in the airways of asthmatics, although recent studies implicating the fibroblast in proteoglycan deposition within the asthmatic airway have been published (Huang et al., 1999; Tremblay et al., 1998a). Therefore, a more in-depth investigation of these processes using isolated, in-vitro cultured cells, would enhance the understanding of the role of the fibroblast/myofibroblast in this process. Section 1.5.1 gives a brief background to the collagen and proteoglycan metabolism.

1.4.1 Extracellular Matrix within the airways

1.4.1.1 Collagens

There are at least 19 different collagens, of which, 11 have been shown to be present in the lung (Table 1.2). These collagens are classified into three groups - the fibril-forming types (I, II, III, V, XI), the non-fibril-forming types (IV, VI, VII, VIII) and the FACIT (fibril-associated collagens with interrupted triple helices, XII, XIV).

Collagen types I and III are produced by fibroblasts (Hance et al., 1976; Sage et al., 1979). It has been proposed that type I collagen provides the tensile strength to all flexible surfaces of the lung, whilst type III contributes to the tissue compliance (Burgeson, 1987). They also provide a structural scaffold to which resident cells attach via specific integrins. The \( \alpha \)-chains of these collagens has a triple helical domain that forms 95% of the molecule (see Figure 1.6). Once secreted into the interstitial space, they form long, thin cable like structures covalently bonded to homologous regions of neighbouring fibrils. These fibrils
exist as copolymers with common associations between collagens I - V, I - VI, III - VI and III - VII (Amenta et al., 1988; Chambers and Laurent, 1997; Timpl and Engel, 1987) for review]. As an example type VI collagen consists of alternating filamentous and beaded regions in which the α1(VI) and α2(VI) chains are known to contain a number of collagen binding sites and RGD sequences suggesting that collagen type VI participates in matrix-matrix interactions and cell-matrix interactions (Timpl and Engel, 1987).

<table>
<thead>
<tr>
<th>Type</th>
<th>Supranolecular structure</th>
<th>Function</th>
<th>Distribution in the airway wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibril</td>
<td>Structural component, tensile strength</td>
<td>Lamina reticularis, sub-mucosa, adventitia</td>
</tr>
<tr>
<td>II</td>
<td>Fibril</td>
<td>Structural component, tensile strength</td>
<td>Bronchial and tracheal cartilage</td>
</tr>
<tr>
<td>III</td>
<td>Fibril</td>
<td>Structural component, compliance</td>
<td>Lamina reticularis, sub-mucosa, adventitia</td>
</tr>
<tr>
<td>IV</td>
<td>Non-fibrillar 3-dimensional network</td>
<td>Molecular sieving, cell support</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>V</td>
<td>Fibril</td>
<td>Regulation of type I collagen fibrillogenesis</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
<tr>
<td>VI</td>
<td>Beaded filament</td>
<td>Cell adhesion to matrix</td>
<td>Associated with type I and III collagen, interstitium</td>
</tr>
<tr>
<td>VII</td>
<td>Fibril</td>
<td>Anchors basement membrane to matrix</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
<tr>
<td>VIII</td>
<td>Filamentous lattice (short chain)</td>
<td>Mechanical strength</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>IX</td>
<td>FACIT</td>
<td>Regulation of type II collagen fibrillogenesis</td>
<td>Cartilage, associated with type II collagen</td>
</tr>
<tr>
<td>XI</td>
<td>Fibril</td>
<td>Regulation of type II collagen fibrillogenesis</td>
<td>Cartilage, associated with type II collagen</td>
</tr>
<tr>
<td>XV</td>
<td>Multiplexin</td>
<td>Anchors basement membrane to matrix</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
</tbody>
</table>

FACIT, fibril-associated collagens with interrupted triple helix; Multiplexin, multiple triple-helix domains and interruptions.
Figure 1.6  Collagen structure and metabolism. This schematic representation shows the transcription of the collagen gene, its translation and post-translational modifications. The transcription occurs within the nucleus prior to translation in the rough endoplasmic reticulum in the cytoplasm. Signal peptides are cleaved within the endoplasmic reticulum. Further post-translational modifications occur within the golgi apparatus, including hydroxylation of proline and lysine residues, by prolyl 4-hydroxylase and lysyl hydroxylase, respectively, glycosylation of the lysine residues by galactosyl transferase and glucosyl transferase, formation of di-sulphide bonds and finally the formation of triple helices. The procollagen molecules are packaged in secretory vesicles. As the molecules are secreted the C- and N-terminal peptides are cleaved by procollagen C-endopeptidases and procollagen N-endopeptidases, respectively. The removal of these pro-peptides reduces the collagen solubility by >1000-fold in the extracellular space, allowing the formation of collagen fibrils. The structure is stabilised by the formation of cross-links by lysyl oxidase. Collagen is constantly being degraded both intracellularly and extracellularly. Procollagen can be degraded intracellularly within the endoplasmic reticulum, Golgi apparatus or with lysosomes. Extracellular collagenases cleave at specific points within the molecule leaving the molecule open to degradation by other neutral proteases in the extracellular space. (Figure adapted from Laurent, 1987)
1.4.1.2 **Collagen synthesis**

Collagen biosynthesis is comprehensively reviewed by Bateman and colleagues (Bateman *et al.*, 1996). The principle steps are outlines in the following paragraphs and schematically represented in Figure 1.6.

Collagen production begins in the nucleus with transcription of collagen genes to type specific mRNA transcripts. Translation of mRNA produces pre-procollagen α-chains containing large extension peptides at both ends and an N-terminal hydrophobic signal sequence designating the molecules for secretion. The signal peptides are cleaved in the rough endoplasmic reticulum by signal peptidase. There is evidence that several classes of molecular chaperones, including heat-shock protein (Hsp) 47 and glucose-regulated protein (Grp) 78 and 94, regulate procollagen processing in the endoplasmic reticulum. These proteins play important roles in binding procollagen molecules that have inappropriate secondary structures (folding) preventing their secretion, thus only allowing cells to produce procollagen molecules that can achieve the correct secondary structure.

Further post-translational modifications occur in the Golgi apparatus. This includes hydroxylation of proline residues in the Y position of the Gly-X-Y triplet sequence by prolyl-4-hydroxylase and lysine residues in the X position by lysyl hydroxylase. Some proline residues in the X position are hydroxylated in the 3-position of the pyrrole ring by prolyl-3-hydroxylase. The process of proline hydroxylation, for which ascorbic acid is an essential cofactor, is almost specific to collagen. Elastin, lung surfactant apolipoprotein A and D, mannose-binding protein, C1q component of complement and acetylcholinesterase also contain hydroxyproline. However, their relative scarcity and small amounts of hydroxyproline they contain has led to the measurement of hydroxyproline being used as an index of collagen content. Hydroxylysine residues are glycosylated by galactosyl transferase.
and glucosyl transferase. Finally an N-linked carbohydrate group is added to each C-propeptide by the oligosaccharyl transferase complex.

Interchain and intrachain disulphide bond formation by protein disulphide isomerase and the cis-trans isomerisation of prolyl peptide bonds by peptidyl-prolyl cis-trans isomerase initiates the formation of the triple helix. In the Golgi apparatus procollagen molecules are packed into secretory vesicles. As the molecules are secreted the C- and N-terminal peptides are cleaved by specific proteases to yield the triple helical collagen molecule. The C-terminal propeptides of α1(I), α2(I) and α1(II) chains are cleaved at the Ala-Asp bond at the C-telopeptide/C-propeptide junction by procollagen C-endopeptidases (PCP, EC 3.4.24.19) (Kessler et al., 1996). Recently, the analysis of the cDNAs for PCP suggests that up to six alternatively spliced products may exist. To date, however, two PCPs have been isolated from the culture media of mouse (80kDa) and chick fibroblasts (110kDa). These two PCP's are products of the same gene and are referred to as bone morphogenetic protein-1 (BMP-1) (or pCP1) and mTld (or pCP2) respectively (Kessler, 1999). The processing of procollagen by PCP, is enhanced by procollagen C-proteinase enhancer (PCPE), a glycoprotein that binds to the C-propeptide of procollagen type I protein (Adar et al., 1986; Kessler et al., 1990). The N-terminal propeptides of procollagen types I and II are cleaved by procollagen N-endopeptidase (EC 3.4.24.14), whereas the N-terminal peptide of procollagen III is cleaved by procollagen III N-endopeptidase. The cleavage of the C-terminal peptides is crucial to fibril formation by reducing the solubility of the collagen molecule by >1000-fold. The processed collagen molecules form into fibrils, which are stabilised by the formation of cross-links by lysyl oxidase.

1.4.1.3 Collagen degradation

The fibroblast/myofibroblast plays a key role in the maintenance of the airway extracellular matrix. Collagens are continually synthesised and degraded in the normal lung, with average
turnover rates estimated to be 10% per day in lungs of young adult rats and rabbits (Laurent, 1982, Laurent et al., 1988; McAnulty and Laurent, 1987). These rates decrease with age, but proceed at relatively rapid rates throughout life (Mays et al., 1991). Furthermore, the balance of intracellular and extracellular breakdown pathways also changes with age. In the lungs of young animals about 30% of newly synthesised collagen is degraded intracellularly within minutes of its synthesis (Laurent and McAnulty, 1983; McAnulty and Laurent, 1987) but this proportion increases with age to about 80% (Mays et al., 1991). In addition, the rates of these processes change when the lung is injured. For example, in experimental models of pulmonary fibrosis the proportion of newly synthesised collagen degraded intracellularly decreases, contributing to the increased collagen deposition in the injured lung (Laurent and McAnulty, 1983).

The rate of extracellular degradation is thought to depend on the degree of cross-linking and in older animals the mature collagen fibrils may be protected from degradation. Nevertheless, extracellular collagens are susceptible to breakdown and are degraded rapidly both in growing and diseased tissue. This is accomplished by a family of metalloproteinases, which are produced by both resident cells (fibroblasts, epithelial and endothelial cells) and inflammatory cells (activated macrophages and neutrophils) (O'Connor and FitzGerald, 1994).

There are at least fifteen zinc- and calcium-dependant metalloproteinases that have the capacity to breakdown a wide range of extracellular matrix proteins (Murphy and Docherty, 1992; Woessner, Jr., 1991; Wojtowicz-Praga et al., 1997). They are important in embryonic tissue development, cell migration, inflammation and wound healing. The actions of the metalloproteinases is tightly regulated by a diverse group of anti-proteinases including the tissue inhibitors of metalloproteinases (TIMPs), which are synthesised and secreted by
activated mesenchymal cells, and circulating anti-proteinases such as α2-macroglobulin, α1-antitrypsin, α2-antiplasmin and SLPI.

1.4.1.4 Proteoglycans and glycoproteins

Proteoglycans are synthesised by a variety of cell types including fibroblasts (Lories et al., 1986), type II epithelial cells (Sahu et al., 1980) and pulmonary arterial endothelial cells (Benitz and Bernfield, 1990). Mediators such as platelet-derived growth factor (PDGF) and TGFβ are capable of stimulating proteoglycan production by fibroblasts (Castor et al., 1983; Tiedemann et al., 1997).

The core proteins of eighteen different proteoglycan molecules have been reported. Figure 1.7 shows the structure of three proteoglycan molecules found in airway tissue, perlecan, decorin and versican. Each core protein is linked to a variety of glycosaminoglycan side chains that consist of alternating galactosamine and glucuronic/iduronic acid units (chondroitin/dermatan sulphate), alternating glucosamine and glucuronic/iduronic acid units (heparin and heparan sulphate) or alternating glucosamine and galactose units (keratan sulphate). Their functions include matrix hydration, modulation of collagen fibre formation, cell-matrix and cell-cell interactions and the binding of growth factors (Fosang and Hardingham, 1996).

Decorin and biglycan (Figure 1.7) are two small chondroitin/dermatan sulphated proteoglycans, which are found associated with type I and type VI collagens, in the lamina reticularis and smooth muscle bundles of the sub-mucosa within airways (Bidanset et al., 1992; Roberts, 1995; Roberts and Paramo, 1992). These molecules can bind polypeptide growth factors e.g. TGFβ (Yamaguchi et al., 1990) protecting them from denaturation and proteolytic degradation (Moscatelli, 1988; Sommer and Rifkin, 1989). It has therefore been
suggested that these proteoglycans can act as a reservoir for growth factors, storing them until their release by proteases during tissue remodelling.

Versican, a large aggregating chondroitin sulphate proteoglycan (Figure 1.7) is localised around the smooth muscle bundles in the sub-mucosa of the airway wall, in association with hyaluronan (Roberts and Burke, 1994). It is thought to change the mechanical properties of the airways in a similar manner to aggrecan in cartilage, by regulating the fluid (osmotic) balance within the airway tissue (see Roberts, 1995).

Perlecan is a heparan sulphate-linked proteoglycan. It is found in all basement membranes and gives the structure a fixed negative charge, which is important for the filtration properties of the membrane. It is also able to bind to other extracellular matrix molecules such as laminin and collagen type IV, and is an attachment substrate for cells. Heparan sulphate proteoglycans, as a group of molecules, have been implicated in the control of cell growth, activation and chemotaxis (see Gallagher, 1996 and the references within). They have been shown to bind TGFβ, (similar to decorin) and fibroblast growth factors (FGF-1 FGF-2, FGF-4 and FGF-7). It was found that for the FGF molecules it is an essential co-factor for expression of biological activity. In addition, the chemokines IL-8 and hepatocyte growth factor (HGF) also have requirements for high affinity binding to heparan sulphate to express activity.
Collagen IV forms a network of fibrils within the basement membrane providing support to epithelial cells, but also acting as a molecular sieve or barrier for particulate matter. Collagen types I and III are triple-helical fibrils, that allow tissue to have high tensile strength and compliance respectively. They also provide the structural scaffold within the lamina reticularis and adventitia to which resident cells attach via specific integrins. Decorin is a small chondroitin/dermatan sulphated proteoglycan that is associated with collagen types I and VI. It is known to bind to polypeptide growth factors protecting them from degradation. Versican and perlecan are large chondroitin sulphate proteoglycans which are associated with regulation of the osmotic balance in the airway. Perlecan, named due to the string of globular domains in the polypeptide region of the molecule, also has a high negative charge enhancing the filtration properties of the basement membrane. This molecule also binds to other extracellular matrix molecules, providing attachment sites for resident and inflammatory cells.

In summary, fibroblasts and myofibroblasts respond to cytokines and growth factors by proliferating and producing a variety of extracellular matrix molecules, the degradation of which is tightly regulated by both intracellular and extracellular proteinases and their
inhibitors. When this tight regulation breaks down, as seen in the collagen diseases (e.g., osteogenous imperfecta) or in inflammatory diseases (e.g., asthma, rheumatoid arthritis, COPD or lung fibrosis) tissue function is compromised. The increased thickness of the lamina reticularis in the asthmatic airway has been correlated with increased numbers of fibroblasts/myofibroblasts (Brewster et al., 1990; Gabbrielli et al., 1994; Glynn and Michaels, 1960). Furthermore, in a recent study, increased numbers of myofibroblasts were observed in the lamina reticularis of mild asthmatic patients after antigen challenge (Gizycki et al., 1997). From these studies it was suggested that myofibroblasts were responsible for deposition of the increased extracellular matrix. However, other cell types within the airway wall are capable of producing extracellular matrix molecules and are also likely to be involved in remodelling. For example, smooth muscle cells are capable of producing similar amounts of procollagen to fibroblasts (Dabbagh et al., 1998).

Many of these mediators described as being released by resident cells, are also released by inflammatory cells (see Barnes, 1996; Holgate, 1993 for reviews). It is now believed that there is a major inflammatory component to the bronchial obstruction and airways hyper-responsiveness in asthma. Djukanovic and colleagues reviewed the published data and reported increases in inflammatory cells (eosinophils, macrophages, lymphocytes and mast cells) in BAL and bronchial biopsy from asthmatic and non-asthmatic patients (Djukanovic et al., 1990a). They also reported studies demonstrating additional increases in neutrophils in BAL and bronchial biopsies after allergen challenge in atopic asthmatics. Using bronchoscopy and BAL, a correlation was found between the increase in the numbers of inflammatory cells, in particular mast cells and eosinophils, and the severity of asthma. In addition, a variety of inflammatory mediators are found elevated in BAL from asthmatic subjects (Table 1.3). The following sections will briefly discuss the involvement of the inflammatory cells and their mediators in airway remodelling.
1.5 **Inflammatory cells, cytokines, growth factors and airway remodelling**

The first comprehensive report of the cellular components of the airways in asthma compared to other airway diseases was by Dunnill (Dunnill, 1960). During the 1960's and 1970's, even though inflammation was indicated as a confounding factor in asthma, it was still thought only to relate to patients who had died of their asthma. Whereas, patients with milder forms of the disease, it was largely thought to be due to changes in amounts and reactivity of the airway smooth muscle. With the introduction of the bronchoscope, the sampling of the airways by BAL and small bronchial biopsies highlighted the infiltration and activation of inflammatory cells and the structural changes at all stages of the disease (Barnes, 1996). Many of the inflammatory cells, in particular T-lymphocytes, monocytes/macrophages, eosinophils, neutrophils and mast cells are intimately involved in the pathophysiology of asthma.

In last decade or so the eosinophil has been extensively studied in the context of asthma. Evidence shows that the number of these cells are increased in this disease, which correlated with disease severity, and they are capable of producing many of the mediators which have been implicated in asthma (Table 1.3, Kumar and Busse, 1998). Similarly, there is considerable support for a role for lymphocytes in asthma (see Corrigan, 1998 for review). They have been associated with the local cell-mediated immune responses within the airways. The numbers of activated lymphocytes within the bronchial mucosa are correlated with eosinophil numbers and disease severity.

Table 1.3 shows a variety of cytokines, polypeptide growth factors and lipid mediators released by inflammatory cells in asthma which are known to activate mesenchymal cells (Chambers and Laurent, 1997; McAnulty and Laurent, 1995). In the context of airway remodelling, the mesenchymal cells include the resident fibroblasts, myofibroblasts and smooth muscle cells, which are the major cell types producing extracellular matrix molecules...
during development and maintenance of the airway wall, and tissue repair after injury. It is therefore important that any investigations into the mechanisms underlying the structural changes within the airway involved the study of these cells.
Table 1.3  Mediators reported to be increased in asthma which have been shown to promote fibroblast migration, proliferation and matrix production.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Likely Source</th>
<th>Directed migration</th>
<th>Enhanced proliferation</th>
<th>Matrix production</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Mφ, TC, BC, MC, Fb, Ep, En, Neut,</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Goldring and Krane, 1987; Libby et al., 1985)</td>
</tr>
<tr>
<td>IL-4</td>
<td>TC,</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Feghali et al., 1992; Monroe et al., 1988; Postlethwaite et al., 1992; Sempowski et al., 1994)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mo, Mφ, MC, Fb, Ep, En,</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Duncan and Berman, 1991; Grossman et al., 1989)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Mφ, TC, MC, Fb, Ep, Eos,</td>
<td>↑</td>
<td>NT</td>
<td>↓</td>
<td>(Rubbia-Brandt et al., 1991)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Mφ, MC, Eos</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Leibovich et al., 1987; Postlethwaite and Seyer, 1990; Duncan and Berman, 1989; Solis-Herruzo et al., 1988)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Mφ, Fb, Ep,</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Cambrey et al., 1995; Goldstein and Polgar, 1982; McAnulty et al., 1992)</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Mo, Mφ, Fb, MC, Ep, Eos</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Fine and Goldstein, 1987; Ignotz and Massague, 1986; Khalil and Greenberg, 1991; Lyons and Moses, 1990; McAnulty et al., 1991; Pentinen et al., 1988; Postlethwaite et al., 1987; Tiedemann et al., 1997; Westergren-Thorsson et al., 1990; Westergren-Thorsson et al., 1993a)</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fb, En, SMC</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>(Butt et al., 1995a; Kondo et al., 1993)</td>
</tr>
<tr>
<td>ECP</td>
<td>Eos</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>(Birkland et al., 1994; Herrnas et al., 1992; Pincus et al., 1987; Shock et al., 1991)</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>Mo, Mφ, Bo, MC, Eos, Neut</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Mensing and Czarnecki, 1984; Phan et al., 1988; Rieger et al., 1990)</td>
</tr>
<tr>
<td>ET-1</td>
<td>Mφ, Fb, Ep, En</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Dawes et al., 1996; Kahaleh, 1991; Peacock et al., 1992)</td>
</tr>
<tr>
<td>Tryptase</td>
<td>MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Cairns and Walls, 1997; Gruber et al., 1997; Hartmann et al., 1992; Ruoss et al., 1991)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>plasma</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Chambers et al., 1998; Chen and Buchanan, 1975; Dawes et al., 1993; Gray et al., 1990)</td>
</tr>
</tbody>
</table>

Table 1.3 cont.
Asthma is now considered to be an inflammation driven disease with involvement of eosinophil, macrophages, lymphocytes, and to a lesser degree neutrophils. The mast cell has also been shown to play a pivotal role, both in the transient bronchoconstriction and the chronic inflammatory phase of the disease (Schulman, 1993). With the established role of mast cells and their mediators in allergy and asthma, it is important to obtain a better understanding of the interaction of its mediators with fibroblasts and myofibroblasts within the diseased airway.

1.6 Mast cells

Mast cells are found in every connective tissue of nearly all the organs in the body (see Scott and Kaliner, 1993). In the late 1880’s Paul Ehrlich first described them as cells that are stained metachromatically with basic dyes, which bind ionically to proteoglycans within the granules (Ehrlich, 1877). They appear most frequently in the skin, gastrointestinal tract and upper (nose) and lower (lung) respiratory tract (Schulman, 1993). In general they localise around blood vessels, in particular the adventitia of arteries, lymph vessels and nerves (Schulman, 1993). It is estimated that mast cells are less than 1% of the total number of cells in the lung and <0.5 – 3% of the cells recovered from BAL. About 50% of the mast cells are located in the alveolar septa and the remainder localised to the mucosa and submucosa of the bronchi and bronchial airways (Schulman, 1993).

<table>
<thead>
<tr>
<th>Fibrinogen &amp; fibrinopeptides</th>
<th>plasma</th>
<th>NT</th>
<th>↑</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropeptides</td>
<td>Sensory nerves</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
</tr>
<tr>
<td>Histamine</td>
<td>Bo, MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

(Gray et al., 1990)  
(Harrison et al., 1995)  
(Hatamochi et al., 1991; Jordana et al., 1988)

Mo, monocyte, MΦ, macrophage, TC, T cell, BC, B cell, Bo, basophil, MC, mast cell, Fb, fibroblast, Ep, epithelial cell, En, endothelial cell, Eos, eosinophil, Neut, neutrophil, SMC, smooth muscle cell, IL, Interleukin, GMCSF, Granulocyte-macrophage colony-stimulating factor, IGF-1, Insulin-like growth factor 1, TGFβ, Transforming growth factor β, TNFα, Tumour necrosis factor α, ECP, Eosinophil cationic protein, ET-1, Endothelin-1, FGF-2, Fibroblast growth factor 2, ↑ increased function, ↓ decreased function, NT- not tested
The origin of the mast cell is debatable. Using murine models, mast cells are derived from pluripotent haematopoietic cells in the bone marrow. In humans, mast cells can be differentiated from pluripotent CD34⁺ progenitor cells (Galli et al., 1993, Kirshenbaum et al., 1991, Metcalfe et al., 1997). The development and differentiation of mast cells is controlled by stem cell factor (c-kit ligand) and its receptor c-kit tyrosine kinase receptor, in addition to IL-3 and IL-4 which controls cell proliferation (Galli et al., 1993, Galli, 1990, Metcalfe et al., 1997). The cells enter the circulation as precursor with no distinguishing features. They migrate to specific tissues, where, under the influence of T lymphocytes, they differentiate into specific mast cell phenotypes i.e. the development of electron-dense cytoplasmic granules containing histamine, glycosaminoglycans and the proteases tryptase and chymase (Metcalfe et al., 1997).

In man, different mast cell phenotypes can be distinguished by the protease content in their cytoplasmic granules. Cells that contain only tryptase in their granules have been designated MCᵣ, those containing both tryptase and chymase are called MCᵣc, whilst those containing only chymase, MCᵣc (Li et al., 1996). Approximately 90% of the cells in the lung are of the MCᵣ phenotype, whereas in the skin and small intestine the majority of mast cells are of the MCᵣc phenotype (Weidner and Austen, 1993). The MCᵣc type only accounts for a few percent in the lung and skin, but between 7-18% in the gastrointestinal tract mucosa (Weidner and Austen, 1993).

1.6.1 Mast cells in lung disease

There is an increase in the number of mast cells in the airways of extrinsic (atopic) and intrinsic (late onset, non-atopic) asthmatic patients (Flint et al., 1985a, Wardlaw and Kay, 1985, Ying et al., 1997). Significant inverse correlations have been demonstrated between the percentage of mast cells in BAL fluid and lung function (Flint et al., 1985b). Early studies of asthma focused on release of mast cell mediators capable of causing
bronchoconstriction which could be inhibited primarily by the use of antihistamines (H$_1$ blockers) or the beta adrenoceptor agonists, isoprenaline, salbutamol or terbutaline.

Correlations were also found between histamine content of the BAL fluid and spirometry suggesting the mast cells in the airways were activated (Agius et al., 1985, Casale et al., 1987). Data obtained using mast cells from both extrinsic and intrinsic patients suggest they are more likely to degranulate than those obtained from non-atopic, control patients. Table 1.4 reviews a series of reports in which the numbers and the activation state of airway mast cells were compared in asthmatic and non-asthmatic patients.

Table 1.4 A Comparison of the number and the state of activation of mast cells in the airways of asthmatic and non-asthmatic subjects.

<table>
<thead>
<tr>
<th>References</th>
<th>Numbers in BAL fluid</th>
<th>Numbers in mucosa/airway wall</th>
<th>Activation state</th>
<th>Correlation to lung function</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cutz et al., 1978)</td>
<td>NT</td>
<td>NC</td>
<td>√</td>
<td>NT</td>
</tr>
<tr>
<td>(Flint et al., 1985b)</td>
<td>↑</td>
<td>NT</td>
<td>↑</td>
<td>√</td>
</tr>
<tr>
<td>(Kirby et al., 1987)</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
<td>√</td>
</tr>
<tr>
<td>(Casale et al., 1987)</td>
<td>↑</td>
<td>NT</td>
<td>↑</td>
<td>NT</td>
</tr>
<tr>
<td>(Wardlaw et al., 1988)</td>
<td>↑</td>
<td>NT</td>
<td>↑</td>
<td>√</td>
</tr>
<tr>
<td>(Beasley et al., 1989)</td>
<td>NT</td>
<td>NC</td>
<td>↑</td>
<td>NT</td>
</tr>
<tr>
<td>(Djukanovic et al., 1990b)</td>
<td>NT</td>
<td>X</td>
<td>↑</td>
<td>X</td>
</tr>
<tr>
<td>(Ferguson et al., 1992)</td>
<td>↑</td>
<td>NT</td>
<td>↑</td>
<td>√</td>
</tr>
<tr>
<td>(Gibson et al., 1993)</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>√</td>
</tr>
<tr>
<td>(Pesci et al., 1993a)</td>
<td>NT</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
</tr>
<tr>
<td>(Koshino et al., 1995)</td>
<td>NT</td>
<td>↑</td>
<td>NT</td>
<td>√</td>
</tr>
<tr>
<td>(Laitinen et al., 1993)</td>
<td>NT</td>
<td>↑</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT - Not Tested, NC - No Change, ↑ increase in asthmatic compared to non-asthmatic, √ - significant correlation between mast cell number or mast cell mediator in BAL fluid of asthmatic compared to non-asthmatic, X - no correlation. The correlation to lung function refers to either the numbers of mast cells or the increase in measurement of histamine or tryptase in BAL fluid compared to spirometric measurements or bronchial hyperresponsiveness.

One notable study, demonstrated an increase in mast cell degranulation in the airways of asthmatic children compared to non-asthmatic controls, suggesting that the change in mast
cell activity is an early event in the development of disease (Cutz et al., 1978). However, an increase in mast cell numbers and/or activation does not always correlate with reduced lung function. In the study by Beasley and co-workers, they were not able to demonstrate a significant correlation between mast cell numbers and functional changes within the study group, although individual values tended towards a decrease in lung function (Beasley et al., 1989).

1.6.2 The interaction of mast cells and fibroblasts

Lung tissue from patients with asthma, COPD and interstitial pulmonary fibrosis, IPF (also known as cryptogenic fibrosing alveolitis, CFA) have increased numbers of mast cells which are often found in close apposition to fibroblasts (Chanez et al., 1993; Fortoul and Barrios, 1990; Heard et al., 1992; Hunt et al., 1992; Kawanami et al., 1979; Miadonna et al., 1994; Pesci et al., 1991; Pesci et al., 1993b; Rankin et al., 1987). In asthmatic subjects, increased numbers of mast cells noted in bronchoalveolar lavage have also been related to the 'subepithelial thickening' in bronchial biopsies (Djukanovic et al., 1990b; Djukanovic et al., 1992; Ferguson et al., 1992; Tomioka et al., 1984; Walls et al., 1990).

In IPF (CFA) patients there are increased numbers of activated mast cells in the alveolar septum and sub-pleura ((Hunt et al., 1992; Kawanami et al., 1979)) and around the airways (Hunt et al., 1992). In patients with adult respiratory distress syndrome (ARDS) the number of mast cells are not increased in the early stages of the disease, but are during the latter stages (Liebler et al., 1998). Activated mast cells have also been shown to be present at the sites of fibrotic lesions in a number of other respiratory diseases - bronchopulmonary dysplasia (BPD) (Lyle et al., 1995), farmer's lung disease, bronchiolitis obliterans organising pneumonia (BOOP) (Pesci et al., 1993b) and bronchiectasis (Sepper et al., 1998). The interaction between mast cells and fibroblast has been studied in a number of other diverse conditions including Graft versus Host Disease (GVHD), liver fibrosis (Okazaki et al.,
1998), scleroderma (Chanez et al., 1993) wound healing and keloids (see Hebda et al., 1993).

Mast cells synthesise and release many mediators, both protease and non-protease in nature, some are preformed and stored and others generated upon activation by de-novo synthesis (Table 1.5).
Table 1.5 Summary of the mediators constitutively expressed, or newly synthesised in the different phenotypes of human mast cells after activation.

<table>
<thead>
<tr>
<th>Mediators</th>
<th>MC&lt;sub&gt;T&lt;/sub&gt;</th>
<th>MC&lt;sub&gt;TC&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteases</strong></td>
<td>Tryptase</td>
<td>Tryptase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chymase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathepsin G-like</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carboxypeptidase A</td>
</tr>
<tr>
<td><strong>Preformed (stored)</strong></td>
<td>Elastase, β-hexosaminidase, β-glucuronidase, aryl sulphatase</td>
<td>Histamine</td>
</tr>
<tr>
<td><strong>Proteases</strong></td>
<td></td>
<td>Heparin, Chondroitin sulphate E,</td>
</tr>
<tr>
<td><strong>Autocoid</strong></td>
<td></td>
<td>IL-1β, IL-3, IL-4, IL-10, TNFα</td>
</tr>
<tr>
<td><strong>Proteoglycans</strong></td>
<td></td>
<td>IL-8</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>SCF, M-CSF, VEGF</td>
<td></td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Synthesised (de-novo)</strong></td>
<td>MMP-9</td>
<td>IL-6, IL-12, IL-13, IL-16</td>
</tr>
<tr>
<td><strong>Proteases</strong></td>
<td></td>
<td>MCP-1, MIP-1α, MIP-1β, RANTES, I-309</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td>TGF-β, NGF, GM-CSF, PDGF, FGF-2, VIP</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;, LTC&lt;sub&gt;4&lt;/sub&gt;, PGD&lt;sub&gt;2&lt;/sub&gt;, PAF</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


In addition to the epithelial cell barrier, the location of the mast cell in the mucosal layer of the airway led to the hypothesis that this cell forms part of the first line of defence against inhaled antigens. After an initial exposure to foreign material, antigen presenting cells (APC) primarily dendritic cells and macrophages, process the antigen to express specific epitopes on their surface in association with major histocompatibility complex class II (MHCII). The interaction of the APC, T helper lymphocytes and B cells leads to the production of specific IgE by the B cell. The IgE binds to selective high affinity receptors (Fc<sub>ε</sub>RI) on mast cells, thus sensitising the cells. Upon a subsequent exposure to the specific antigens, the IgE molecules on the surface of the mast cell are cross-linked causing degranulation and the release of the stored mediators.
1.6.3  Mediators released by activated mast cells

When mast cells are activated they degranulate, releasing preformed stored mediators from granules, as well as the de novo synthesis and release of a number of other mediators (see Table 1.5). The following section briefly reviews both the stored and synthesised mediators released by cells, and highlights their potential functions in asthma and airway remodelling.

1.6.3.1 Histamine

Histamine is the best known and most studied granule associated mediator. There is approximately 3-8 pg of histamine per cell comprising 5-10% of the mast cell granule (Scott and Kaliner, 1993). Histamine has many proinflammatory effects on a variety of cells that are mediated by three subtypes of histamine receptors, H₁, H₂ and H₃ (Cavanah and Casale, 1993). The H₁ receptor subtype induces increases in plasma protein leak and oedema from post capillary venules in the large airways, neurogenic reflexes, smooth muscle contraction and nasal mucus production; all reported pathological features in the asthmatic airway (Cavanah and Casale, 1993). The main role for activation of H₂ receptor subtype is in the control of gastric acid secretions from parietal cells and is not thought to produce major responses in the lung, although H₂ mediated effects are reported to inhibit T lymphocytes and B cell activity, leukocyte function and induce vasodilatation (Del Valle and Gantz, 1997, Weltman, 2000). Also, in pulmonary fibrosis histamine, acting via H₂ receptor, induces pulmonary fibroblast proliferation (Jordana et al., 1988). H₃ receptors inhibit cholinergic neurotransmission and the non-adrenergic, non-cholinergic bronchoconstriction induced by neurokinin A and other neuropeptides (see Cavanah and Casale, 1993 for review).
1.6.3.2 Proteoglycans

Upon mast cell activation, in conjunction with histamine, the proteoglycans, heparin and chondroitin sulphate E are released. Proteoglycans are composed of a central protein core of repeating serine and glycine residues with extended, un-branched carbohydrate side chains – glycosaminoglycans. The glycosaminoglycan polymers in heparin are repeating \( \alpha-1,4 \)-linked disaccharides of a uronic acid, either glucuronic or iduronic, in a \( \beta-1,4 \) linkage to glucosamine. This polymer links to the protein backbone by a ser-xyl-gal-gal-glucoronate linkage (see Fosang and Hardingham, 1996 for review). Heparin forms approximately 65% of the proteoglycans in the granules. It is a highly negatively charged molecule and is crucial in the binding of the positively charged molecules (histamine and the neutral proteases) in the storage granule. Following mast cell activation, histamine and the acid hydrolases are released from heparin, whereas the neutral proteases, tryptase and chymase, maintain their association. This continued binding to heparin preserves the catalytic activity of these enzymes (see Section 1.8.2 for a review of this process).

Heparin is thought to have a number of activities independent of the binding to neutral proteases, the best known being its anticoagulant activity. This is by the binding to antithrombin III and the inhibition of thrombin, factor XII, XIa, Xa and IXa. In addition, heparin can prevent fibroblast and smooth muscle proliferation (Westergren-Thorsson et al., 1991; Westergren-Thorsson et al., 1993b), and has potential anti-inflammatory and immunomodulatory activity (see Kilfeather and Page, 1997 for review).

Chondroitin sulphate E is composed of a similar protein backbone to heparin, but has repeating glycosaminoglycan units of \( \beta-1,4 \)-linked mono-sulphated disaccharides of glucuronic or iduronic acid in \( \beta-1,3 \) linkage to galactosamine. It forms 35% of the mast cell granule proteoglycan content. The biological activity of chondroitin sulphate E is less well studied than heparin, although it does possess anticoagulant activity, but is much weaker.
than heparin. It also has the potential to bind to the neutral serine protease, tryptase, having a similar effect to heparin in maintaining its catalytic activity (Lindstedt et al., 1998; Pereira et al., 1998).

1.6.3.3 Cytokines, chemokines and growth factors

Human mast cells express a range of cytokines, chemokines and growth factors that act at specific cell surface receptors to control cell proliferation, differentiation, haemopoiesis, immune and inflammatory responses (Metcalfe et al., 1997, Redington et al., 1995, Gordon et al., 1990). Some of these functions are shown in Table 1.6. Many of these have direct and indirect effects (highlighted in Table 1.6) on fibroblast function and may play a role in airway remodelling.
Table 1.6  Summary of the cellular functions of the cytokines, chemokines and growth factors released by activated mast cells.
Mediators in bold and underlined are known to have effects on fibroblast function.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Function</th>
<th>Mediator</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td>Acute phase protein, pyrogenic, enhances fibroblast proliferation and chemotaxis</td>
<td>MCP-1</td>
<td>Chemokine for monocytes</td>
</tr>
<tr>
<td>IL-3</td>
<td>Colony stimulating factor for mast cells, granulocytes, macrophages</td>
<td>MIP1α</td>
<td>Chemoattractant for monocytes, T cells and eosinophils</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>Activation of B cells, activates fibroblasts</td>
<td>MIP-1β</td>
<td>Chemoattractant for monocytes and T cells, binds β1 integrin</td>
</tr>
<tr>
<td>IL-5</td>
<td>Eosinophil differentiation and survival factor</td>
<td>RANTES</td>
<td>Chemoattractant for basophils and eosinophils</td>
</tr>
<tr>
<td>IL-6</td>
<td>Acute phase protein</td>
<td>I-309</td>
<td>Chemoattractant</td>
</tr>
<tr>
<td>IL-8</td>
<td>Chemoattractant for neutrophils, B and T cells</td>
<td><strong>TGFβ</strong></td>
<td>Controls mesenchymal cell proliferation (mitogenic and antiproliferative), differentiation and stimulates ECM production</td>
</tr>
<tr>
<td>IL-10</td>
<td>Suppresses Th1 responses</td>
<td>PDGF</td>
<td>Mitogenic and chemoattractic for mesenchymal cells</td>
</tr>
<tr>
<td>IL-12</td>
<td>Growth factor for T cells</td>
<td><strong>FGF-2</strong></td>
<td>Mitogenic for mesenchymal cells, angiogenic and a competence factor</td>
</tr>
<tr>
<td>IL-13</td>
<td>Induces cytokine production</td>
<td>NGF</td>
<td>Stimulates B cells, nerve growth factor</td>
</tr>
<tr>
<td>IL-16</td>
<td>T cell chemoattractant</td>
<td>VEGF</td>
<td>Mitogenic for endothelial cells indices vascular permeability</td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
<td>Cytotoxic, pyrogenic, cell proliferation and differentiation, eosinophil activation, activates fibroblasts</td>
<td>VIP</td>
<td>Vasodilator and bronchodilator</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>Controls production, differentiation and function of granulocytes, monocytes, macrophages and chemoattract for fibroblasts</td>
<td>PAF</td>
<td>Stimulates neutrophils, monocytes, macrophages, induced vascular permeability and vasodilator</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Stimulation of macrophage colonies</td>
<td><strong>LTB4</strong></td>
<td>Chemokinetic and chemoattractic for neutrophils goblet cell mucus gland hypersecretion</td>
</tr>
<tr>
<td>SCF</td>
<td>Stimulation of mast cell proliferation</td>
<td><strong>LTC4</strong></td>
<td>Increases vascular and bronchial tone, induced vascular permeability goblet cell mucus gland hypersecretion</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>PGD2</strong></td>
<td>Chemokinetic and chemoattractic for neutrophils, goblet cell mucus gland hypersecretion</td>
</tr>
</tbody>
</table>
1.6.4 Mast cell Proteases

Chymase and tryptase are the major proteases within the mast cell granules, however, there are other neutral proteases that include the acid hydrolases - β-hexosaminidase, β-glucuronidase, and arylsulphatases, the metalloexopeptidase - carboxypeptidase A, and cathepsin G-like enzymes (Schwartz et al., 1981a, Schwartz and Austen, 1981, Reynolds et al., 1992, Goldstein et al., 1987). Although the function of these enzymes, in the context of airway remodelling is uncertain, it has been suggested that they may contribute to epithelial cell denudation and degradation of connective tissue proteins in the airway. Elastase (EC 3.4.21.37), although synonymous with neutrophils and macrophages, has been identified in the granules of mast cells (Meier et al., 1989). In addition to its ability to cleave elastin, it also cleaves collagen types I, II, III, IV, VIII, IX and XI, fibronectin, laminin and proteoglycans. It is also able to activate pro-MMP-1 (procollagenase), pro-MMP-3 (prostremelisyn) and pro-MMP-2 and pro-MMP-9 (72kD and 92kD progelatinases) (Bieth, 1998). The levels of this enzyme in mast cells are relatively low (70-170 ng/10^6 cells) compared to those found in neutrophils (3000 ng/10^6 cells) (Schulman, 1993). Also, after mast cell activation, the enzyme binds more avidly within the granule, thus only a fraction of the total elastase enzyme in the cell is released. Therefore, it is doubtful that elastase released from mast cells will have a significant functional role in lung homeostasis.

Chymase (EC 3.4.21.39) is found in mouse, rat, dog and human mast cells. It is a chymotrypsin-like enzyme with a molecular weight of 26-32 kDa including a number of glycosylation sites. It cleaves proteins and polypeptides with aromatic residues in the P1 site (potency: phenylalanine>tyrosine>tryptophan). It is present in MC\textsubscript{TC} and MC\textsubscript{C} type mast cells (Weidner and Austen, 1993, Li et al., 1996). Human mast cell chymase is activated within the cell by the cleavage of an N-terminal dipeptide by the action of dipeptidyl-peptidase I (EC 3.4.14.1). It is stored within granules, at acidic pH bound to heparin, which reduces it activity, and becomes fully active upon release into a neutral to alkaline
extracellular environment. It is able to cleave extracellular matrix proteins (Briggaman et al., 1984), activate TGF-β and matrix metalloproteinases (Saarinen et al., 1994), potentiate plasma protein leak (He and Walls, 1998), stimulate submucosal gland cell secretion (Sommerhoff et al., 1992), generate extravascular angiotensin II (Urata et al., 1990), and control complement-mediated inflammation - all processes involved with the inflammatory response and extracellular matrix remodelling in the asthmatic airway.

Tryptase, a trypsin-like serine protease is also found in the granules of human mast cells. Not only is this enzyme used in the sub-typing of mast cells, it is also used as a diagnostic test to demonstrate mast cell activation (Schwartz et al., 1987a). In BAL and sputum from atopic asthmatics there was no significant difference in the basal levels between the atopic and normal groups (Wenzel et al., 1988). However, when this group of patients were challenge with the appropriate allergen there was a significant increase in the levels of tryptase in the BAL from the aptopic group (Wenzel et al., 1988). Similar data has been obtained in other studies of atopic patients (Bousquet et al., 1991; Broide et al., 1991; Louis et al., 1997). The levels of tryptase range from 0—2 ng/ml in the normal non-atopic subject to >6 ng/ml in asthmatic patients. Heaney and colleagues compared the concentration of tryptase in BAL in atopic asthmatics, atopic non-asthmatics and non-atopic, non-asthmatics (Heaney et al., 1998). They were able to demonstrate raised basal levels of tryptase in the atopic asthmatic group compared to the control groups. However they were not able to demonstrate significant increases in tryptase in BAL following allergen challenge. Ennis and colleagues demonstrated similar data in that they were unable to show a significant difference between groups of children with or without asthma following antigen challenge (Ennis et al., 1999). Contrary to this data, Svensson and colleagues in patients with seasonal allergic rhinitis, could induce increases in tryptase in nasal secretions and BAL following both nasal and bronchial allergen challenge, respectively (Svensson et al., 1995).
In a study of sputum from asthmatic patients, tryptase was detectable in 9/51 patients, which included 5 atopic and 4 intrinsic asthmatic cases, but was undetectable in normal healthy volunteers (Bettiol et al., 1999). In the 9 patients with raised tryptase levels, these authors were able to demonstrate a weak correlation between the level of tryptase in the sputum and the number of eosinophils, suggesting tryptase may have a role in eosinophil chemotaxis in some asthmatic patients. Tryptase has been measured in serum from atopic asthmatic patients with mild to moderate disease at concentrations similar to that seen in BAL and sputum (Swystun et al., 2000). However, no data was presented for the levels of tryptase in the serum of normal, non-asthmatic controls. This study demonstrated similar data to that of Bettiol and coworkers, with an association between increased levels of tryptase in serum with increased eosinophilia following 10-day treatment with the \( \beta_2 \)-adrenoceptor agonist, salbutamol (Swystun et al., 2000).

Tryptase has been used as a marker of allergic rhinitis. The levels in nasal lavage from atopic individuals were higher than non-atopic individuals and in symptomatic compared with asymptomatic seasonal allergic rhinitics (Jacobi et al., 1998; Klimek et al., 1999; Wilson et al., 1998).

All these studies have measured levels of tryptase in BAL, sputum or nasal lavage, whereas the activation of the mast cell would occur within the airway or nasal wall. Thus, the local concentration of tryptase in these tissues would be much greater than found in the lumenal secretions from these airways. It has been postulated that after mast cell activation tryptase, unlike histamine, takes several hours to diffuse through tissue due to complex molecular structure and its interaction with proteoglycans (Schwartz, 1990, also see Section 1.8.2).

Similar increases in the levels of tryptase are seen in BAL of other lung and airway diseases such as farmer’s lung disease (Miadonna et al., 1994; Pesci et al., 1991; Pesci et al., 1993b), pulmonary fibrosis (Fortoul and Barrios, 1990; Heard et al., 1992; Hunt et al., 1992;
Kawanami et al., 1979; Rankin et al., 1987), bronchiolitis obliterans organizing pneumonia (Pesci et al., 1996), fibrosing alveolitis associated with scleroderma (Chanez et al., 1993) allergic alveolitis (Walls et al., 1991), sarcoidosis (Eklund et al., 1993) and bronchiectasis (Sepper et al., 1998). Therefore, the activation of mast cells and the release of tryptase in the lung and airways suggests that this enzyme may have a role in the airway remodelling, especially since it has been shown to be chemotactic and mitogenic for fibroblasts (Cairns and Walls, 1997; Gruber et al., 1997; Hartmann et al., 1992; Ruoss et al., 1991), stimulates type I collagen production in human lung fibroblasts (Cairns and Walls, 1997) and increases procollagen mRNA levels in dermal fibroblasts (Gruber et al., 1997). The following section will review the structure, function and inhibition of tryptase in the context of airway remodelling.

1.7 Tryptase

All types of mast cells contain tryptase (EC3.4.21.59), a trypsin-like serine protease with a sequence homology of 40% to vertebrate trypsin (EC3.4.21.4). It forms 23% of the total cellular protein of these cells (Schwartz et al., 1981b). Skin mast cells contain approximately 35 pg per cell, whereas lung mast cells only contain 10 pg per cell. In its active form it is a tetrameric protein with a molecule weight of approximately 130-140kD. It has a limited range of substrates compared to trypsin, although the preferred cleavage sites of C-terminal side of arginine and lysine residues are similar. In addition, unlike trypsin, the endogenous protease inhibitors $\alpha_2$-macroglobulin and $\alpha_1$-antitrypsin do not inhibit tryptase.

1.7.1 Genetic and molecular structure of tryptase

In the human mast cell from lung and skin there are two types of tryptase – $\alpha$ & $\beta$, which can be subdivided into at least 4 related tryptases – $\alpha$, $\beta_1$, $\beta_2$, $\beta_3$ (Miller et al., 1989; Miller et al., 1990; Vanderslice et al., 1990). All the forms of $\beta$-tryptase are 98-99% homologous,
whereas the α form is 91% homologous to the β form and has been suggested to be a product of a different gene (Miller et al., 1989; Miller et al., 1990). However, the genes for both α and β forms of tryptase reside on chromosome 16p13.3 (Miller et al., 1990; Pallaoro et al., 1999). The complete sequences of human α and βI-tryptase cDNA have been determined (Miller et al., 1989; Miller et al., 1990) and shown to have 1143 and 1142 bases, respectively, which code for leader sequences of 30 amino acids and catalytic sequences of 244 amino acids with two glycosylation sites (α) and 245 amino acids with one glycosylation site (β).

1.7.2 Protein structure

Tryptase monomers are produced in precursor forms. It is hypothesised that the monomeric β-tryptase is auto-processed to an intermediate, at acidic pH in the presence of heparin, by cleavage between Arg$^{13}$ and Val$^{12}$ in the leader sequence. The N-terminal activation dipeptide is then cleaved by dipeptidyl peptidase I, at acid pH in the absence of heparin, to produce an inactive monomer.

The crystalline structure of human mast cell β-tryptase has been identified as a tetramer, composed of 4 monomers arranged in a flat square ring (Figure 1.8, (Pereira et al., 1998; Sommerhoff et al., 1999)). Each monomer has a molecular weight of 29-31kDa, with the tetramer being 134 kDa. The discrepancy in the molecular weights is due to variable glycosylation (Harvima et al., 1999; Little and Johnson, 1995). The tetramer is bound to heparin, which stabilises the structure due to its negative charge binding to positively charged regions of the monomers (Figure 1.8). This enzyme is stored within the granule in its active form (Sakai et al., 1996). Each monomer has a catalytic site consisting of Ser$^{195}$, His$^{57}$ and Asp$^{102}$ that face a central pore (Figure 1.8). Substrate specificity is conferred by the size of this central pore, in conjunction with each monomers' catalytic site having a
unique sequence of amino acids which provides ‘a projecting canopy’ to restrict substrate entry (Pereira et al., 1998; Sommerhoff et al., 1999).

The mechanism for processing α-tryptase appears to be different since in the leader peptide of the precursor the Arg$^{-3}$ of β-tryptase is replaced by Gln$^{-3}$, which prevents auto-processing. It has been speculated that this is the reason why the α-tryptase is not stored within the granules, but is constitutively secreted from the cell (Sakai et al., 1996). Consistent with this hypothesis α-tryptase is the major form of the enzyme found in the serum of normal patients and subjects with mastocytosis whereas during anaphylaxis the predominant form is β-tryptase (Schwartz et al., 1995).
Figure 1.8. Solid-surface and ribbon representations of tetrameric structure of tryptase. In panel A, tryptase is depicted as four monomers (labelled A-D) arranged at the corners of a flat square. The blue and red patches are positively and negatively charged residues respectively. In panel B, the tetramer is depicted as a ribbon consisting of monomers A (dark blue), B (red), C (green) and D (gold). Monomer A is shown with a leech-derived tryptase inhibitor molecule (light blue) modelled in the active site. Whereas, monomers B, C and D have a 4-amidinophenylpyruvic acid molecule (yellow) in their active sites. (Reprinted by permission from Nature 392: 306-311 (1998) copyright (2000) Macmillan Magazines Ltd).

A number of tryptases have been cloned, purified and characterised including guinea-pig lung tryptase, (Mceuen et al., 1996), monkey lung tryptase (Robinson and Muller, 1997) bovine liver mast cell tryptase (Fiorucci et al., 1992; Jolly et al., 1999), sheep intestinal mast
cell tryptase (Pemberton *et al.*, 1997), rat skin tryptase (Braganza and Simmons, 1991), rat peritoneal mast cell tryptase (Ide *et al.*, 1995), mouse mast cell protease-6 (Reynolds *et al.*, 1991), mouse mast cell protease-7 (McNeil *et al.*, 1992), gerbil intestinal mast cell tryptase (Murakumo *et al.*, 1995). More recently another human tryptase has been identified and cloned which has a transmembrane spanning domain similar to the mouse transmembrane tryptase (Wong *et al.*, 1999). It is ~50% homologous to the known human tryptases (α, βI, βII, and βIII) with a unique feature of a 48-residue membrane spanning domain suggesting that this tryptase is membrane bound, which could have important implications for cell activation.

1.7.3 Functional activity of tryptase

Tryptase has a selective range of substrates many of which appear to be small peptides in nature. The neuropeptides, vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM) and calcitonin gene related peptide (CGRP) are inactivated by tryptase (Vanderslice *et al.*, 1990) suggesting a possible role in the modulation of the bronchodilating mediators released from airway nerves. In addition, tryptase has been shown to cleave high molecular weight kininogen releasing bradykinin and lysyl-bradykinin, which are potent vasodilator and bronchoconstrictor agents (Kozik *et al.*, 1998a).

One of the early substrates identified for tryptase was fibrinogen. Fibrinogen is composed of three polypeptide chains termed Aα, Bβ and γ (Herrick *et al.*, 1999). Tryptase cleaves specific sites within both the α- (Arg\(^{572}\)) and β- chains (Lys\(^{21}\)) which has been reported to lead to a reduction in thrombin-induced blood clotting (Thomas *et al.*, 1998). Tryptase can cleave the extracellular matrix proteins fibronectin (Lohi *et al.*, 1992) and collagen type IV (Kielty *et al.*, 1993) and activate pro-matrix metalloproteinase-3 (proMMP-3, prostromelysin) which then activates proMMP-1 (procollagenase, Gruber *et al.*, 1989). In a recent report tryptase was also suggested to activate proMMP-8 (neutrophil procollagenase) in
bronchiectasis patients (Sepper et al., 1997). In addition is can cleave the 72kD gelatinase (MMP-2) to a 62kD form, with both forms retaining their gelatinolytic activity (Lohi et al., 1992). These data suggest direct and indirect roles for tryptase in tissue remodelling in chronic inflammatory conditions such as asthma, bronchiectasis, atopic dermatitis and psoriasis. Tryptase has also been implicated in wound healing by its activation of single chain urinary type plasminogen activator (uPA or urokinase, Stack and Johnson, 1994).

1.7.4 Cellular effects

Tryptase is a potent mitogen for lung and dermal fibroblasts (Abe et al., 1998; Cairns and Walls, 1997; Hartmann et al., 1992; Pohlig et al., 1996; Ruoss et al., 1991) epithelial cells (Cairns and Walls, 1996) and smooth muscle cells (Brown et al., 1995). It is chemotactic for eosinophils, neutrophils, lymphocytes and fibroblasts (Gruber et al., 1997; He et al., 1997; Peng et al., 1997; Walls et al., 1995), although the neutrophil infiltration may be due to tryptase-induced increase in IL-8 (Cairns and Walls, 1996; Compton et al., 1998; Compton et al., 1999). In addition to the increased chemotactic activity, tryptase may enhance cell migration by the up-regulation of ICAM-1 expression (Cairns and Walls, 1996).

Tryptase has been shown to enhance histamine-induced constriction of non-sensitised and sensitised human isolated bronchi (Berger et al., 1999; Johnson et al., 1997). It was also suggested that tryptase can increase the numbers of mast cells within the airway wall, in particular within the smooth muscle layer (Berger et al., 1999). In animal models, inhalation of human mast cell tryptase in allergic sheep can induce bronchoconstriction and airway hyperresponsiveness by the release of mast cell histamine (Molinari et al., 1996). In the same allergic sheep model, the early and late phase bronchoconstriction responses induced after treatment with the antigen, ascaris suum, were inhibited by the selective tryptase inhibitors APC366, bis(5-amidino-2-benzimidazolyl)methane (BABIM, Clark et al., 1995)
and lactoferrin (Elrod et al., 1997), and also partially inhibited by secretory leukocyte protease inhibitor (SLPI), a potent, non-selective inhibitor of tryptase (Wright et al., 1999).

Tryptase may also have a significant role in inflammation. When administered into the skin of guinea pigs, tryptase induced an accumulation of neutrophils and eosinophils (He and Walls, 1997). In addition, when injected into the peritoneum of mice, tryptase induced a profound neutrophilia (He et al., 1997). Both responses were inhibited by the non-selective tryptase inhibitor, leupeptin, and by heat inactivation of the enzyme, suggesting that an intact catalytic site is required. In a model of delayed-hypersensitivity in the mouse skin, a novel tryptase-inhibitor reduced antigen-induced oedema and numbers of neutrophils (Combrink et al., 1998). Finally, in the allergic sheep model, the selective inhibitor, APC366, inhibited the late phase airway inflammation (Molinari et al., 1996).

In combination with the changes in lung function and pro-inflammatory effects, tryptase has been implicated in structural remodelling. In addition to the activation of MMPs which promote the degradation of extracellular matrix proteins (see above), tryptase can stimulate the expression of collagen type α1 (I) mRNA (Gruber et al., 1997), and protein in dermal and lung fibroblasts (Abe et al., 1998; Cairns and Walls, 1997).

The potential diverse effects of tryptase in the lung makes the inhibition of this enzyme a therapeutic target for a range of pulmonary diseases including asthma and various forms of pulmonary fibrosis. A number of approaches used to develop potential selective and potent therapeutic agents are discussed in the following section.
1.7.5 Inhibitors of tryptase

The common protease inhibitors within serum fail to inhibit the catalytic effects of tryptase (Alter et al., 1990; Smith et al., 1984). Hence, endogenous tryptase activity is either uncontrolled or is controlled by an, as yet unidentified, inhibitor or mechanism. In in-vitro experiments, tryptase is inactivated over time by dissociation into its monomeric form (Kozik et al., 1998b; Selwood et al., 1998) or by the loss of heparin from the tetramer, which destabilises the complex (Schechter et al., 1995; Schwartz and Bradford, 1986). Two molecules have been identified from neutrophil granules that have been shown to inhibit tryptase activity by displacing heparin. Lactoferrin, a 78 kDa cationic protein found in the primary secretory granules of neutrophils, is a member of the transferrin class of proteins. It contains a heparin-binding site, which is thought to compete for heparin binding to tryptase, therefore disrupting and inactivating the enzyme (Elrod et al., 1997). Similarly, protamine, another cationic protein containing a high proportion of Arg, Ser and Ala amino acid residues, inhibits tryptase by competing for the heparin binding site (Rice et al., 1998).

Myeloperoxidase (MPO), is a 118 kDa cationic protein also found in the primary granules of neutrophils. In addition to its known antimicrobial activity, MPO can degrade heparan sulphate glycosidic links. Cregar and co-workers were able to show that MPO was a potent inhibitor of tryptase in-vitro (Cregar et al., 1999). It was therefore postulated that neutrophil granular proteins might endogenously regulate tryptase enzyme activity. The enzyme heparinase also inhibits tryptase in-vitro (Rice et al., 1998). The recently identified naturally occurring protease inhibitor SLPI (Thompson and Ohlsson, 1986) has also been demonstrated to inhibit tryptase enzyme activity in-vitro (Wright et al., 1999). However, recent computational chemical and structural data would suggest that the SLPI molecule is unable to enter the binding pocket of tryptase and therefore is unlikely to be a direct inhibitor of tryptase (Sommerhoff et al., 1999).

Due to the potential pathological role that tryptase may have, the identification of a small, non-peptide molecular inhibitor of tryptase is a priority for many research groups. Rice and
colleagues recently reviewed several different chemical series of non-peptide inhibitors of tryptase (Rice et al., 1998). Table 1.7 shows a selection of tryptase inhibitors with some selectivity data against two other proteases, trypsin and thrombin.

Table 1.7  Selected range of mast cell tryptase inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Selective (s) / non-selective (ns)</th>
<th>Tryptase</th>
<th>Trypsin</th>
<th>Thrombin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antipain</td>
<td>ns</td>
<td>96% @10,000nM</td>
<td>NT</td>
<td>NT</td>
<td>(Katunuma and Kido, 1990)</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>ns</td>
<td>87% @10,000nM</td>
<td>NT</td>
<td>NT</td>
<td>(Katunuma and Kido, 1990)</td>
</tr>
<tr>
<td>BABIM ^</td>
<td>s</td>
<td>2500</td>
<td>18800</td>
<td>3700</td>
<td>(Rice et al., 1998)</td>
</tr>
<tr>
<td>BABIM − Zn2+</td>
<td>s</td>
<td>5</td>
<td>90</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>BABIM + Zn2+</td>
<td>s</td>
<td>22000</td>
<td>33000</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Pentamindine</td>
<td>ns</td>
<td>1200</td>
<td>2300</td>
<td>NT</td>
<td>(Caughley et al., 1993)</td>
</tr>
<tr>
<td>APC366</td>
<td>s</td>
<td>330</td>
<td>162</td>
<td>415</td>
<td>(Clark et al., 1995)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>s</td>
<td>24</td>
<td>&gt;100000</td>
<td>&gt;100000</td>
<td>(Elrod et al., 1997)</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>s</td>
<td>16</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>(Cregar et al., 1999)</td>
</tr>
<tr>
<td>(IC50)</td>
<td></td>
<td>(IC50)</td>
<td></td>
<td>(IC50)</td>
<td></td>
</tr>
<tr>
<td>SLPI</td>
<td>s</td>
<td>580</td>
<td>23600</td>
<td>NSE</td>
<td>(Wright et al., 1999)</td>
</tr>
<tr>
<td>Leech derived tryptase inhibitor (LDTI)</td>
<td>ns</td>
<td>1.4</td>
<td>0.9</td>
<td>NT</td>
<td>(Sommerhoff et al., 1994)</td>
</tr>
</tbody>
</table>

NSE - no significant effect, NT - not tested BABIM - bis(5-amidino-2-benzimidazolyl)methane, SLPI - secretory leukocyte protease inhibitor

^ Note: An important feature of one class of tryptase inhibitors, 'BABIM-like', is that the addition of zinc ions (Zn^2+) enhances its potency and selectivity by 500-fold and 20-fold respectively (Katz et al., 1998).

One compound from the list in Table 1.7, APC366 (Axys Pharmaceuticals Inc), has been tested in an allergen challenge model in sheep (Clark et al., 1995). The compound caused a non-significant reduction in the early phase response and a significant reduction in the late phase response. It also completely inhibited the antigen-induced airway hyperresponsiveness to carbachol 24h following antigen challenge. This compound has
progressed through to phase IIa clinical trials, where data has been reported, although, second and third generation compounds are now currently in development. In 20 mild to moderate asthmatic subjects, 2.5 or 5.0 mg of APC366, given three times a day for a total of 13 doses, improved both the late response and hyperresponsiveness to antigen challenge compared to baseline (Zhang and Timmerman, 1997). In a second randomised, double blind cross-over study with 16 mild to moderate asthmatic, APC366 or placebo was given 3 times a day for 4 days. Lung function was studied on day 4 after an antigen challenge. In subjects treated with APC366, there was an 18% decrease in the early phase response following antigen challenge, a significant improvement (33%) in the area under the curve (AUC) during the late phase response and a 21% improvement in FEV₁ during the late response (Krishna et al., 1998; Rice et al., 1998). These 'proof of concept' studies were encouraging, however studies with more potent compounds and larger study groups are required. This data would suggest that inhibitors of tryptase can modulate the effects of antigen challenge in asthmatics but there is currently no information on their effects on airway remodelling.

In addition to the inhibitory effects of lactoferrin, SLPI and APC366, the non-selective serine protease inhibitors, antipain, BABIM and benzamidine have been used to inhibit the cellular effects (mitogenesis and extracellular matrix production) of tryptase (Cairns and Walls, 1996; Cairns and Walls, 1997; Caughey et al., 1993; Compton et al., 1998; Gruber et al., 1997; Ruoss et al., 1991; Sturzebecher et al., 1992; Walls et al., 1992). However, the data presented in some of these studies failed to demonstrate complete inhibition of the tryptase-induced responses. The majority of the responses to tryptase could be via direct activation of the cell following the binding of the enzyme to a cell surface receptor inducing catalytic-dependent or independent responses. Alternatively, it could be indirectly mediated via the release of growth factors following cellular activation or the cleavage and activation of growth factors from the extracellular matrix. In an attempt to explain the mitogenic effects of other proteases, a role was suggested for protease receptors on the cell surface membrane. These receptors were termed 'protease-activated receptors' or PAR's. The following section
reviews the role of PAR’s in cellular activation, highlighting the role of protease-activated receptor-2 (PAR-2).

**1.8 Protease-activated receptors (PAR)**

One of the earliest reports of protease-induced cell replication was by Simms and Stillman (Simms and Stillman, 1936) who were able to demonstrate that trypsin induced chick fibroblast proliferation. For an in-depth discussion of protease-induced cell proliferation and differentiation, readers are referred to an excellent review by Scher (Scher, 1987).

**1.8.1 PAR-1, PAR-3 and PAR-4**

The first reported protease-induced mitogenesis of human fibroblasts was by Pohjanpelto in 1977 (Pohjanpelto, 1977), who reported the induction of primary human fibroblast mitogenesis in serum-free medium with thrombin, trypsin or plasmin. This work strongly suggested the catalytic sites of these enzymes were needed to induce their effects but no cellular mechanisms were proposed. In 1991 Vu and colleagues cloned and sequenced a receptor for thrombin, 'the thrombin receptor', in human platelets and vascular endothelial cells (Vu et al., 1991b; Vu et al., 1991a). This receptor was proposed as a member of a new family of 7-transmembrane G-protein coupled receptors with an extended N-terminal domain (Figure 1.9) containing a thrombin cleavage site, LDPR (41/S 42)FLLRN (where / denotes the cleavage site). The cleavage releases 41 amino acids revealing a new N-terminus, the so called 'tethered ligand', which is hypothesised to act back upon the receptor in the region of the second extracellular loop to induce cell activation (see Figure 1.9). Mutations around this site render the receptor unresponsive to thrombin, however they could still be activated by a synthetic peptide, homologous to the new N-terminal sequence (Vu et al., 1991b). This receptor was later termed protease-activated receptor-1 (PAR-1).
Figure 1.9  A schematic diagram of the structure of protease activated receptors. The structure of the PAR’s has not been fully elucidated. However, from the amino acid sequence of the protein, it would appear to consist of 7 transmembrane spanning domains (TM 1-7), with three extracellular loops (I-III), a long extracellular N-terminal domain and an intracellular C-terminal with binding sites for G-protein coupling. The N-terminal domain contains an enzyme-specific cleavage site, which, when cleaved reveals a new N-terminal sequence, the so-called tethered ligand. This new sequence interacts with a proposed binding site in extracellular loop II. This induces a conformational change in the receptor, allowing G-protein phosphorylation and activation of intracellular signalling.

In PAR-1 knockout mice, thrombin was not able to stimulate fibroblasts but could still activate platelets, suggesting a second thrombin receptor. PAR-3 was subsequently cloned and sequenced in rat platelets, and found to be expressed in human bone marrow and mouse megakaryocytes (Ishihara et al., 1997). This receptor has 27% homology with PAR-1 and is cleaved in a similar manner within the extracellular N-terminal domain (...TLPIK \textsuperscript{38}/T \textsuperscript{39}FRGA…). However, in contrast to other PAR’s, the proposed tethered ligand for PAR-3
(TFRGAP) fails to activate its receptor, to date the reason for this is unclear (Ishihara et al., 1997).

In the search for other members of the PAR family of receptors, Xu and colleagues identified a partial cDNA sequence from an expressed sequence tag (EST) database that had 34% homology with a transmembrane region of PAR-2. A full-length cDNA clone was identified, cloned, sequenced and designated as PAR-4 (Xu et al., 1998).

Thrombin-induced activation of human platelets is predominately mediated by PAR-1 with perhaps a small component of PAR-3 activation (Schmidt et al., 1998). Whereas, in the mouse, thrombin’s effects are mediated via PAR-3, with little to no contribution from PAR-1 (Kahn et al., 1998a). In PAR-3 knockout mice, thrombin is able to induce weak platelet aggregation suggesting yet another thrombin receptor present within these cells. Following a similar approach to Xu and colleagues, Kahn and co-workers identified a conserved region of PAR cDNA, from which a full-length cDNA was obtained. This was cloned, sequenced and found to be identical to PAR-4 reported by Xu and colleagues. PAR-4 has 30% homology to PAR-3 and is activated by thrombin to reveal a new N-terminal domain, the hallmark of protease activated receptors. The tethered ligand, GYPGQV, is able to activate the PAR-4 receptor (Table 1.8, Kahn et al., 1998a). It is highly expressed in lung, liver, pancreas, small intestine, thyroid and testis (Xu et al., 1998). The PAR-4 genes are located on human chromosomes 19p12 and the mouse syntenic region 8B3.3 (Kahn et al., 1998b).
Table 1.8  Sequence of the new N-terminal sequence of human and rodent (mouse and rat) PAR’s after cleavage by specific enzymes (red arrow shown in Figure 1.9). Synthetic peptides mimicking the PAR’s tethered ligand sequences can activate PAR’s by binding to the receptor in the region of extracellular loop II, without the need for enzyme cleavage. The exception to this is PAR-3 peptide sequence shown in Italics.

<table>
<thead>
<tr>
<th>Species</th>
<th>PAR-1</th>
<th>PAR-2</th>
<th>PAR-3</th>
<th>PAR-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>SFLLRN</td>
<td>SLIGKV</td>
<td>TFRGAP</td>
<td>GYPGQV</td>
</tr>
<tr>
<td>Rodent</td>
<td>SFFLRN</td>
<td>SLIGRL</td>
<td>SFNGCP</td>
<td>GYPGKF</td>
</tr>
</tbody>
</table>

1.8.2 PAR-2

The fourth member of the protease activated receptor family is PAR-2. This receptor is distinct from PAR-1, -3, -4, in that it is not activated by thrombin. PAR-2 was the second protease activated receptor which was in fact, identified by chance while screening a mouse genomic library with primers to the second and sixth transmembrane domains of the Neurokinin-2 (NK-2) receptor (Nystedt et al., 1994; Nystedt et al., 1995b; Nystedt et al., 1995a). It was identified as a 7-transmembrane G-protein coupled receptor, which displays a 30% homology to human PAR-1 and 28% to mouse PAR-1. A probe from the 3' exon of the mouse PAR-2 used to screen a human genomic library identified a human PAR-2 homologue (Bohm et al., 1996a; Nystedt et al., 1995b) which had a protein sequence of 397 amino acids and a 83% homology with the mouse PAR-2. The mouse and the human PAR-2 genes are similar in structure. They are divided into two exons, separated by an intron of variable length, the first coding for a signal peptide and the second coding for the mature protein (Kahn et al., 1996; Kahn et al., 1998b). The gene is located on chromosome 5q13 in the human and 13D2 in the mouse (a syntenic region of the mouse genome, Guyonnet, V et al., 1998; Kahn et al., 1996; Kahn et al., 1998b; Nystedt et al., 1995b; Schmidt et al., 1997) within a cluster of protease activated receptor (PAR-1, -2, -3) genes.
The N-terminal domain of both the rodent and human PAR-2 contains a cleavage site for trypsin \((\ldots \text{SKGR}^{36/37}\text{LIG}\ldots, \text{Figure 1.9})\), which upon cleavage leads to cellular activation (Bohm et al., 1996a; Nystedt et al., 1994; Nystedt et al., 1995b) by a proposed interaction of the new tethered ligand with a tripeptide sequence within extracellular loop II (Al Ani et al., 1999a). If the cleavage site is mutated to \(\ldots\text{SKGR}^{36/p37}\text{LIG}\ldots\), trypsin is unable to activate the receptor (Nystedt et al., 1994).

1.8.2.1 \textit{Intracellular signalling induced by PAR-2 activation}

The intracellular signalling induced by PAR-2 activation appears to be a complex interaction of a number of diverse pathways. Studies within enterocytes and cells transfected with human PAR-2, indicate that the receptor is coupled to \(G_{q}\alpha\) and \(G_{q}\alpha\). The phosphorylation of \(G_{q}\alpha\) activates phospholipase C (PLC) \(\beta\), which induces phosphotidylinositol hydrolysis to release inositol triphosphate and diacylglycerol, leading to \(\text{Ca}^{2+}\) mobilisation (Bohm et al., 1996a; Bohm et al., 1996b; Corvera et al., 1997; Kong et al., 1997; Santulli et al., 1995). In addition, the activation of PAR-2 in enterocytes causes the release of arachidonic acid and subsequent generation of \(\text{PGE}_{2}\) and \(\text{PGF}_{1\alpha}\), suggesting the activation phospholipase A\(_2\) (PLA\(_2\)) and the constitutively expressed cyclo-oxygenase (COX)-1 or its induced form COX-2. Activation of PAR-2 has also been shown to activate PLC and PLA\(_2\) in endothelial cells (Mirza et al., 1996; Molino et al., 1997a). In a human pancreatic cancer cell line, MIA PaCa-2 cells, PAR-2 activation caused an increase in intracellular \(\text{Ca}^{2+}\), phosphotidylinositol metabolism and protein kinase C (PKC) activation (Kaufmann et al., 1998). A similar intracellular signalling pathway was found after PAR-2 activation in human keratinocytes (Wakita et al., 1997).

Using the PAR-2 activating peptides, the activation of PAR-2 in smooth muscle cells and enterocytes activates the mitogen-activated protein (MAP) kinase pathway by stimulating the extracellular signal-related kinase (ERK)-1 and ERK-2, and p38 (Belham et al., 1996). In
kirsten murine sarcoma virus-transformed rat kidney epithelial cells and a rat enterocyte cell line, hBRIE380, PAR-2 activating peptides caused increases in ERK-1 and -2 (DeFea et al., 2000). In this same series of studies it was shown that following PAR-2 stimulation, ERK-1 stimulated cell activation was dependent on the receptor being internalised, and forming a complex with β-arrestin, raf-1 and the activated ERK-1 (DeFea et al., 2000). Professor Bunnett’s Group at UCLA reported in two recent reviews, unpublished data indicating that the activation of PAR-2 by trypsin and the activating peptides in enterocytes causes the induction of c-fos and COX-2 (Dery et al., 1998; Dery and Bunnett, 1999). The stable transfection of mouse 293 cells, which do not normally express PAR-2, with mouse PAR-2 cDNA, gave a cell line in which PAR-2 was coupled to pertussis toxin-sensitive G-proteins. The activation of which caused the stimulation of c-fos and the tyrosine phosphorylation of SHP2, a src homology-2 domain-containing protein-tyrosine phosphatase(Yu et al., 1997). Thus suggesting PAR-2 may be coupled to a signalling pathway that modulates the src family of kinases associated with mitogenesis.

More recent evidence has been published in abstract form demonstrating that in human skin epithelial cells transfected with human PAR-2, trypsin induced activation of the stress-activated protein kinases, c-Jun N-terminal kinases (JNK) and p38 MAP kinase, whereas no effect was seen in control cells(Kanke et al., 2000). In the same cell line the activation of PAR-2 by trypsin also stimulated the activation of NFκB DNA-binding and associated nuclear translocation. Trypsin also induced a transient loss of IκBα, suggesting that PAR-2 activation is linked to the NFκB-IκB signalling pathway(MacFarlane et al., 2000).

Therefore, in summary it would appear that PAR-2 is linked to the G-protein signalling pathway with subsequent activation of PLC, PLA₂ and PKC. It is also able to activate the MAP kinase pathway, the stress-activated kinase pathway and the NFκB activation pathway. These signalling pathways have not been fully elucidated and therefore further studies are
needed. However, with the number of diverse pathways implicated, there is clearly potential for cross-activation between them.

1.8.2.2  **PAR-2 desensitisation and inactivation**

Following activation of PAR-2 by enzymatic cleavage or with the activating peptides, PAR-2 is inactivated by desensitisation, followed by internalisation and subsequent degradation, a mechanism common to other 7 transmembrane G-protein coupled receptors (see review (Bohm et al., 1997)). Upon activation, PAR-2 G proteins are phosphorylated by G protein receptor kinases and uncoupled by the binding of β-arrestin. This receptor complex is internalised by means of clathrin coated vesicles, which after losing their clathrin, form early endosomes. The receptor is de-phosphorylated within the endosome, allowing the dissociation of β-arrestin and the transfer of the receptor to lysosomes where it is degraded (Bohm et al., 1997; DeFea et al., 2000; Dery et al., 1999). Re-sensitisation of the cell is by mobilisation of PAR-2 stores from the Golgi apparatus and de-novo receptor synthesis(Bohm et al., 1996b).

1.8.2.3  **Activation of PAR-2 by enzymatic cleavage**

After the discovery of PAR-2 the prototype serine protease used to activate PAR-2 was trypsin (Nystedt et al., 1994; Nystedt et al., 1995b; Nystedt et al., 1995a). The activation was assessed by measurement of intracellular Ca\(^{2+}\) mobilisation. Subsequently trypsin has been shown to activate a wide range of cells that either constitutively express or were transfected with PAR-2 (see Dery et al., 1998 for review). The expression of trypsin and trypsinogen mRNA in cells other than the pancreas suggest that trypsin-like enzymes may play a role in activation of PAR-2 in sites where pancreatic trypsin would not be expected to be present (Brattsand and Egelrud, 1999; Cocks et al., 1999a; Koshikawa et al., 1997; Yasuoka et al., 1997). Tryptase, a serine protease with trypsin-like activity has been shown to activate PAR-2 in rat colonic myocytes (Corvera et al., 1997), guinea pig myenteric
neurones (Corvera et al., 1999), a murine lymphoid cell line (Mirza et al., 1997) and keratinocytes (Steinhoff et al., 1999).

Other serine proteases have been tested to evaluate their potency at cleaving the arginine-serine bonds in the N-terminal sequence of PAR-2 and by using Ca\textsuperscript{2+} mobilisation assays. Thrombin, urokinase and tissue kallikrein failed to cleave a peptide sequence homologous to that seen in PAR-2. Whereas, Plasmin, Factor Xa, and plasma kallikrein, did cleave the peptide but at a much slower rate than trypsin. Tryptase was shown to cleave the peptide over a similar concentration range to trypsin (Molino et al., 1997b). One important aspect of these studies was that both trypsin and tryptase were able to cleave the peptide sequence between lysine and valine residues at positions equivalent to residues 41 and 42 in PAR-2, leading the authors to suggest that prolonged exposure to the agonist could inactivate the receptor. However, the cleavage rate was only 10% of that of the primary cleavage point (Molino et al., 1997b). Using a fusion protein containing the peptide sequence of the extracellular domain of PAR-2, Altrogge and Monard demonstrated that trypsin can cleave at the proposed cleavage site, whereas, thrombin, activated protein C, plasmin, urokinase, tissue plasminogen activator elastase and chymotrypsin failed to induce cleavage (Altrogge and Monard, 2000). Fox and colleagues proposed that trypsin, tryptase and acrosin, a serine protease from the head of sperm, were possible activators of PAR-2, whereas thrombin, Factor Xa, plasmin, tissue kallikrein and granzyme A were not. (Fox et al., 1997).

In addition to the activation of PAR-2 by serine proteases, an arginine-specific protease has been identified as an activator of PAR-2. Gingipain-R from Porphyromonas gingivalis was able to induced Ca\textsuperscript{2+} mobilisation due to PAR-2 cleavage on human neutrophils (Lourbakos et al., 1998). Finally, data presented at a recent American Thoracic Society Meeting suggested that proteases found in house dust mite extract may activate PAR-2 (Chambers et al., 2000).
1.8.2.4  Activation of PAR-2 by activating peptides

As discussed earlier in Section 1.9.1, the cleavage of the extracellular domain of protease activated receptors exposes a new tethered ligand which interacts with the receptor. Peptides containing the first six amino acids of the new N-terminal sequence of human PAR-2, SLIGKV, or the rodent PAR-2, SLIGRL, have been used to stimulate the receptor without the need for enzymatic cleavage (Lerner et al., 1996; Magazine et al., 1996; Nystedt et al., 1994; Nystedt et al., 1996). Both these peptides exhibit a high degree of selectivity for PAR-2 compared with other protease-activated receptors. However, they are potentially open to enzymatic degradation, thus a highly selective and potent PAR-2 agonist was designed, trans-cinnamoyl-LIGRLO (Saifeddine et al., 1998; Vergnolle et al., 1998; Vergnolle et al., 1999). These agonists have been used in bioassays to identify the expression of PAR-2 in a range of tissues and cells. Whilst similar activities of PAR-2 activating peptides suggest a role for activation of PAR-2 in particular cell functions, they do not provide proof. One important aspect of the pharmacological characterisation of PAR-2 is the current lack of selective antagonists for this receptor.

1.8.2.5  Tissue localisation of PAR-2

A range of antibodies has been generated to PAR-2, the majority against the N-terminal domain of the receptor, with two generated against C-terminal sequences (Table 1.9). Using these antibodies, PAR-2 has been localised in wide range of tissues including gastrointestinal tract, skin and lung (Table 1.10).
Table 1.9 PAR-2 antibodies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide antigen sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S14.8.2</td>
<td>Rabbit anti-human</td>
<td>26^TNRSKGRSLIGKVDGTSHVTGKVTVETVE^35</td>
</tr>
<tr>
<td>PAR-2C</td>
<td>Rabbit anti-human</td>
<td>37^SLIGKVDGTSHVTGKGVC^53</td>
</tr>
<tr>
<td>SAM.11</td>
<td>Mouse anti-human</td>
<td>37^SLIGKVDGTSHVTGKGVC^50</td>
</tr>
<tr>
<td>Anti-PAR-2</td>
<td>Rabbit anti-human</td>
<td>33^SKGRSLIGKVDGTSHVTGKGVC^51</td>
</tr>
<tr>
<td>PAR-2 (N-19)</td>
<td>Goat anti-human</td>
<td>37^SLIGKVDGTSHVTGKGVC^55</td>
</tr>
<tr>
<td>B5</td>
<td>Rabbit anti-rat</td>
<td>30^GPNSKGRLIGRLDT^46</td>
</tr>
<tr>
<td>PAR-2 (S-19)</td>
<td>Goat anti-mouse</td>
<td>39^SLIGRLETQYFDTKG1GPVC^57</td>
</tr>
<tr>
<td>C-terminal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAR-2 (C-17)</td>
<td>Goat anti-human</td>
<td>36^CRSVRTVKQMQVSLTSKHS^380</td>
</tr>
<tr>
<td>RAB 9717</td>
<td>Rabbit anti-mouse</td>
<td>C^394SVST^399</td>
</tr>
</tbody>
</table>

Table 1.10 Protease-activated receptor-2 expression in human tissue

<table>
<thead>
<tr>
<th>PAR-2 localisation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver, kidney, pancreas, prostate, small intestine colon and leukocytes</td>
<td>(Nystedt et al., 1995b)</td>
</tr>
<tr>
<td>Keratinocytes, endothelial cells, hair follicles, sweat glands, 'dermal dendritic-like cells'</td>
<td>(Steinhoff et al., 1999)</td>
</tr>
<tr>
<td>Enterocytes, intestinal smooth muscle, lung epithelium, lung smooth muscle, prostate, vascular smooth muscle, brain</td>
<td>(D'Andrea et al., 1998)</td>
</tr>
<tr>
<td>Keratinocytes, basal cells, endothelial cells</td>
<td>(Hou et al., 1998)</td>
</tr>
<tr>
<td>Aorta, intestinal smooth muscle, vascular smooth muscle, keratinocytes, sweat glands, intestinal epithelium,</td>
<td>(Molino et al., 1998)</td>
</tr>
<tr>
<td>Airway epithelium, airway fibroblasts, bronchial smooth muscle</td>
<td>(Cocks et al., 1999a)</td>
</tr>
</tbody>
</table>

In summary, despite the on-going research on the pathology and etiology of asthma, no conclusive mechanism has been defined to explain the excessive extracellular matrix deposition, in particular collagen and proteoglycans, within the airways of asthmatic patients leading to a thickening of the lamina reticularis. It is known that upon activation, fibroblasts can produce both collagen and proteoglycans.
Section 1.8 suggests a role for the serine protease, tryptase, in asthma. In particular, it’s ability to induce a fibrotic response, by directly activating fibroblasts and myofibroblasts, and it’s modulation of connective tissue breakdown. Tryptase also enhances bronchoconstriction, vasodilatation, angiogenesis, inflammatory cell chemotaxis and activation. All these responses strongly suggest that tryptase plays an important role in airway reactivity, tissue inflammation and remodelling in asthma.

The mechanism whereby tryptase induces fibroblast and myofibroblast activation is unclear, however, it is known that it requires an intact catalytic site. In Section 1.9 it is postulated that tryptase induces responses in other cells by activation of the protease activated receptor, PAR-2. Therefore a number of questions need to be addressed. Firstly it is not known whether there are PAR-2 present on the cell surface of human parenchymal lung or airway fibroblasts. Secondly it is not known whether tryptase or the known PAR-2 activator trypsin would activate these receptors. Thirdly it is not known whether the activation of PAR-2 on these cells would induce fibroblast proliferation or extracellular matrix production. These are particularly important questions to address given the potential for the development of selective tryptase inhibitors and PAR-2 antagonists as therapeutic agents for this disease.

1.9 The aims of the thesis

1.9.1 Hypothesis

The overall aim of this thesis was to examine the roles of tryptase and the protease activated receptor-2 (PAR-2) in the activation of fibroblasts and myofibroblasts from human lung parenchymal tissue and airways. In meeting this aim, I will address the questions whether PAR-2 is expressed by lung fibroblasts and whether tryptase can activate these receptors to induce fibroblast mitogenesis and extracellular matrix production.
1.9.2 Specific aims

The specific aims are outlined below.
Firstly, to establish fibroblast cell lines from human lung parenchyma, airway, and skin by explant culture and the development of an in vitro proliferation assay using confluent cultures to demonstrate that tryptase and trypsin can induce fibroblast proliferation.
Secondly to use RT-PCR and immunohistochemistry to determine if human lung parenchymal and airway fibroblasts express PAR-2
Thirdly to establish whether PAR-2 plays a role in tryptase induced proliferation.
Fourthly, to establish whether tryptase can induce procollagen in human lung fibroblasts and whether PAR-2 plays a role in these responses.

This first chapter (Chapter 1: Introduction) has established the background to this thesis. The next (Chapter 2: Methods) will describe in detail the methods and techniques used to address the above aims. Chapter 3 (Results) gives an account of the results, while the final chapter (Chapter 4: Discussion) will discuss the findings in relation to the hypothesis of the role of tryptase and PAR-2 in activation of human lung parenchymal and airway fibroblasts, and to the published literature on their potential function in airway remodelling in asthma.
Chapter 2

Methods

The chapter will be divided into three main sections that will describe the techniques and protocols used to:

1) Characterise the mitogenic effects of tryptase and the PAR-2 activating peptides.
2) The immunohistochemical location of PAR-2 in isolated human lung fibroblasts and samples of human bronchus.
3) To study the effects of tryptase and the PAR-2 activating peptides on extracellular matrix synthesis in human lung fibroblasts.

2.1 Cell culture

For all the cell culture studies Dulbecco’s Modified Eagles Medium (DMEM), penicillin/streptomycin, fungizone (amphotericin B), and Trypsin-EDTA solutions were obtained from Life Technologies (Paisley, Scotland). Newborn calf serum (NCS) was obtained from Imperial Laboratories (Andover, England) of which the same batch was used throughout all cell cultures studies. Phosphate buffered saline (PBS) was obtained from Oxoid (Basingstoke, England). Cell culture plastic ware was obtained from Marathon, (London) or Nunc, Life Technologies (Paisley, Scotland) unless otherwise stated.

2.1.1 Established cell line

Human foetal lung fibroblasts (HFL-1), at passage 12 or 13, were purchased from the American Tissue Culture Collection (ATCC) and stored in a liquid Nitrogen Cell Bank. For
all experiments, HFL-1’s were used between passage 15-23 as the cells demonstrated progressively poor growth beyond passage 23.

2.2 Cell lines established from Tissue explant

2.2.1 Human lung parenchymal, airway and dermal fibroblasts

Fibroblast cell lines were established from lung tissue obtained from patients undergoing resection of localised lung tumours (See Appendix 1 for patient details). For parenchymal lung fibroblasts, tissue was carefully cut into pieces of <1mm³ which were placed into 10 cm diameter petri dishes (Corning Costar, Cambridge, MA, USA) with 2ml of DMEM containing penicillin (100U/ml), streptomycin (100µg/ml), fungizone (amphotericin B, 2.5µg/ml), and 10% NCS. The petri dishes were incubated at 37°C in a humidified atmosphere of air containing 10% CO₂. When the tissue had firmly attached to the plastic surface, a further 8 ml of culture medium was slowly added to the petri dish and incubated as before. The media was changed every 7 days. For airway fibroblasts, the airways were carefully dissected free of parenchymal connective tissue and <1mm³ pieces of tissue were cultured as for the parenchymal tissue. Samples of skin obtained from patients undergoing breast reduction surgery were dissected free of fatty tissue and 1mm³ pieces placed, dermal side down, into petri dishes containing 2 ml of DMEM as described above and cultured.

After approximately 3 weeks of culture, fibroblasts were seen growing out from the explanted tissues (Figure 2.1A & B). These were cultured for at least a further 2 weeks, until confluent islands of cells were observed (Figure 2.1C). The growth media was removed and replaced with 2 ml of trypsin (0.05% w/v)/EDTA (0.02% w/v) mix solution and incubated at 37°C for 90 sec. The culture dish was examined with an inverted phase contrast light microscope (Olympus TCK-2, Olympic Optical Company) to confirm that the cells had detached and appeared rounded. In addition to causing the detachment of the cells, trypsin could cause the activation of PAR-2 as described in Section 1.8.2.3. However it has been
suggested that the following activation, internalisation with subsequent degradation of the acted receptor, de-novo PAR-2 is expressed on the cell surface within 60-90 mins (Bohm et al., 1996b). The trypsin was neutralised by the addition of 10 ml of DMEM supplemented with 5% NCS. The cultures were passaged with a split ratio of 1:2 into sterile 75 cm² flasks containing culture media without fungizone. Thereafter, confluent cultures were passaged every 5-6 days with a split ratio of 1:4. Experiments were performed with cells between passages 2-8 as the cells demonstrated progressively poor growth following passage 8. To obtain a continuous supply of cells, cultures at passage 2 or 3 were stored using the following protocol.
Figure 2.1  Culture of explanted human airway tissue. Tissue obtained from human lung resections for cancer diagnosis was cleaned free of connective tissue, cut into 1mm pieces and cultured for up to 3-4 weeks. Panel A and B show the growth of fibroblasts from the explanted tissue after 3 and 5 weeks respectively. Panel C show the culture after 7 weeks culture.
Cells cultures were stored at passage 2 and 3 in liquid nitrogen cell banks for later use. In brief, cells were resuspended as above with trypsin/EDTA solution. The suspension was centrifuged at 300g at room temperature and the supernatant discarded. The cells were resuspended in 1 ml of 20% NCS in DMEM followed by slow addition of 1 ml of 20% NCS and 20% DMSO in DMEM to give a final concentration of 20% NCS and 10% DMSO in DMEM. The DMSO was used to reduce the formation of ice crystals and subsequent cell damage upon freezing and thawing. The cell suspensions were placed at -80°C overnight and transferred to a liquid nitrogen cell bank (-196°C) until use.

Cells were rapidly thawed at 37°C for 3 min, followed by the addition of 10 ml 5% NCS in DMEM and thoroughly mixed. The suspensions were centrifuged at 300 x g and the supernatant discarded to remove the DMSO. The cell pellet was resuspended in 1 ml fresh 5% NCS in DMEM and transferred to 75cm² flasks containing 10 ml DMEM with penicillin (100U/ml), streptomycin (100µg/ml) and 5% NCS. The cells were cultured at 37°C in humidified air containing 10% CO₂ and passaged as above when they became visually confluent (every 6-7 days).

All cell cultures were tested for Mycoplasma infection using the Mycoplasma Gen-Probe Rapid Detection System Kit (Laboratory Impex) and/or Mycoplasma Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. No mycoplasma infections were detected in any cell lines used in these studies.

2.3 Immunohistochemical characterisation of cell lines

Fibroblast cell lines obtained from ATCC (HFL-1) and the cell lines established from tissue explants were characterised immunohistochemically to confirm their purity following the initial passage of the cell batch. Each cell line used in the study was cultured in 75 cm² cell culture flasks. When visually confluent the cell medium was removed and discarded and the cells washed in 10 ml of PBS. Cell suspensions were obtained as stated in Section 2.2.1, the
numbers of cells were counted using a Neubauer haemocytometer (BDH/Merck. Lutterworth, UK). Cells were seeded onto 8-well chamber slides (Lab-Tek permanox, Nunc Inc, Life Technologies LTD, Paisley, Scotland) at a density of $1 \times 10^4$ cells/well. The slides were incubated at 37°C in humidified air with 10% CO$_2$ for 48-72 hr until they were 70-80% confluent.

The cell culture medium was discarded and the cells fixed with ice-cold methanol for 30 min. The methanol was removed and the cells washed three times in PBS for 5 min. To reduce the non-specific binding of the secondary antibody, each well was preincubated with 1:20 dilution of normal goat or swine serum, (Dako, UK) for 20 min at room temperature (RT) depending on whether the anti-rabbit or anti-mouse secondary antibodies were raised in goat or swine. Following incubation, the serum was removed and the cells incubated for 60 min in an humidified chamber with primary antibodies raised against human cell marker proteins at the concentrations indicated in Table 2.1. In dermal fibroblasts, the $\alpha$-smooth muscle actin filaments were stained using rhodamine phalloidin (Molecular Probes, Eugene, Oregon, USA). Cells were also incubated with non-specific IgG or PBS alone.
Table 2.1  Antibodies used in characterisation of fibroblast cell lines

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Animal primary antibody raised in</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α-smooth muscle actin (Sigma)</td>
<td>Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>Human myosin (Sigma)</td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>Human Vimentin (Sigma)</td>
<td>Mouse</td>
<td>1:40</td>
</tr>
<tr>
<td>Human pan-cytokeratin (Sigma)</td>
<td>Mouse</td>
<td>1:300</td>
</tr>
</tbody>
</table>

FITC = fluorescein isothiocyanate

Following incubation the cells were washed three times in PBS for 5 min and then incubated with the specific secondary antibody conjugated to fluorescein isothiocyanate (FITC) (see Table 2.1) for 60 min in an humidified chamber. After three 5 min washes with PBS, the chambers were removed and cover slips mounted using an antifade glycerol/PBS based mountant, AF1 (Citiflour Products, Canterbury, England). Slides were evaluated using a Zeiss Axiophot Fluorescence microscope. Photographs were taken of the slides using an attached side camera with Fugichrome Provia 1600 Daylight film.

Staining with antibodies to cytokeratin and myosin was negative indicating that the cultures did not contain significant numbers of epithelial or smooth muscle cells (Figure 2.2 & 2.3). Greater than 95% of cells stained positively for vimentin and between 20-30% of cells were also positive for α-smooth muscle actin confirming the fibroblast/myofibroblast phenotype of the cells lines (Figure 2.2 & 2.3). The phenotype of the cells did not form a highly architected culture demonstrating a characteristic ‘hill and valley’ appearance, suggesting that these cells were not smooth muscle cells (Hirst, 1997). Although a percentage of the fibroblast cells demonstrated a positive staining for α-actin, they failed to show any staining to myosin. It is generally accepted that only smooth muscle cells stain for both actin and myosin (Hirst, 1997). This is demonstrated in the positive controls in which rat smooth
muscle cells (A10) stained for both myosin and α-actin, whereas the fibroblasts only stained for α-actin (Figure 2.4). In addition, the cells were not considered to be endothelial-like cells as they did not have the phenotypical 'cobble stone' appearance of confluent endothelial cells (Takahashi et al., 1990).

Positive staining for the cell marker proteins was established by Dr Louise Reynolds and Dr Maddy Parsons (Centre for Cardiopulmonary Biochemistry and Respiratory Research, UCL using Mardin-Darby canine kidney epithelial cells (MDCK), rat smooth muscle cells (A10) and human cardiac fibroblasts for cytokeratin, myosin, vimentin, and α-smooth muscle actin (Figure 2.4).
Figure 2.2  Characterisation of human foetal lung fibroblasts.
Cells were cultured in 8 well chamber slides until 50-60% confluent. The cells were fixed with ice-cold methanol for 30 min. Cells were immunohistochemically stained with α-smooth muscle actin (A), vimentin (B), myosin (C), pan-cytokeratin (D), non-specific IgG (E) or PBS alone (F).
Figure 2.3  Characterisation of human dermal fibroblasts. Cells were grown to 50-60% confluence in 8 well chamber slides. Cells were fixed in ice-cold methanol for 30 min and stained for α-smooth muscle actin using rhodamine phalloidin (A), vimentin (B), myosin (C) or non-specific IgG.
<table>
<thead>
<tr>
<th></th>
<th>Human cardiac fibroblasts</th>
<th>Rat smooth muscle cells (A10)</th>
<th>MDCK epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>vimentin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>myosin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>cytokeratin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**Figure 2.4** Positive staining for antibodies used in fibroblast characterisation studies. Cells were grown to 50-60% confluence and fixed in ice-cold methanol. Human cardiac fibroblasts, rat smooth muscle cells (A10) and MDCK epithelial cells were stained for vimentin, myosin, α-smooth muscle actin and cytokeratin.
2.4 Fibroblast proliferation studies

2.4.1 Pilot studies

2.4.1.1 Incubation medium

To establish the culture conditions for the fibroblasts, pilot studies were performed using different conditions. HFL-1 cells were trypsinised (trypsin (0.05\% w/v)-EDTA (0.02\% w/v) mix) and for studies using sub-confluent cultures, seeded into 96-well microtitre plates (Nunc, Life Technologies, Paisley, Scotland) at $3 \times 10^3$ cells per well in 100 \( \mu l \) of DMEM containing antibiotics and NCS, as described in Section 2.2.1, for 24 hr. For studies using confluent cultures, cells were plated at $1 \times 10^4$ cells in growth media and incubated for 4-5 days. With the cells at sub-confluence (10-20\%, Figure 2.5A) or at visual confluence (100\%, Figure 2.5B), the media was replaced with 100\( \mu l \) of fresh incubation media of either serum-free DMEM, or serum-free DMEM with two supplements to maintain the osmotic and iron balance within the cells - bovine serum albumin (BSA, 1mg/ml, Sigma, Poole, UK) and human holo-transferrin (1\( \mu g/ml \), Sigma. Poole, UK). The cells were incubated for 24-72hr at 37\(^\circ\)C in humidified air with 10\% CO\(_2\). Cell numbers were assessed by the use of a spectrophotometric assay.

![Figure 2.5 Culture of human lung fibroblasts](image)

Cells were added to 96 well culture plates at a density of $3 \times 10^3$ cells per well and cultured for 24 h (sub-confluent, A) or at a density of $1 \times 10^4$ cells per well and cultured for 4-5 days (confluent, B).
2.4.1.2 **Cell proliferation (spectrophotometric) assay**

The assay used has been previously described (Oliver *et al.*, 1989). Briefly, the media was removed and the plates washed in PBS. The cells were fixed in formal saline (10% v/v formalin in 0.15 M NaCl) for at least 30 min. The formal saline was removed and the plates blotted dry. Cells were stained with 100μL/well methylene blue (1% w/v in 0.01 M borate buffer) for 30 min. The excess dye was removed by washing in 0.01M borate buffer (pH 8.5) using a plate washer (Denley Instruments Cellwash, Life Science International, Basingstoke, England). The bound dye was then eluted from the cells by addition of 100μL of acidified alcohol (0.01 M HCl/ethanol, 1:1, v/v) and the absorbance measured at 650nm using a microplate spectrophotometer (Titretek Multiscan MCC/340 MK II, Flow Laboratories Ltd, Rickmansworth, England).

The absorbance reading obtained for each well is directly related to cell number (Oliver *et al.*, 1989). The changes in cell number in each well were calculated using the equation below. The mean and standard errors of the means were obtained for each treatment and compared to the mean absorbance of wells treated with media alone. Changes in fibroblast cell number were confirmed by direct cell counting using a haemocytometer.

\[
\% \text{ changes from mean control absorbances} = \left( \frac{X - Y}{Y} \right) \times 100 \quad \text{equation 1}
\]

Where \(X\) is the absorbance of each individually treated well, \(Y\) is the mean of the absorbance of the \(n\) number of control wells.

These studies demonstrated that both sub-confluent and confluent fibroblasts cultures were maintained in serum-free media containing BSA and transferrin over a 72 h period (Figure 2.6). In the absence of BSA and transferrin there was no growth seen in either the sub-confluent or confluent fibroblast cell cultures over the 72h incubation period (figure 2.6 A &
B). However, there was a significant increase in fibroblast growth in the sub-confluent cultures over 72h in the presence of BSA and holo-transferrin cultures \((p<0.01, \text{Figures 2.6A})\), whereas there was no growth in the confluent cultures. Therefore, in all subsequent proliferation studies were performed at confluence, with BSA \((1 \text{ mg/ml})\) and transferrin \((1 \mu\text{g/ml})\) included in the serum-free incubation media.
Figure 2.6  Effect of the presence (+) and absence (-) of bovine serum albumin and human holo-transferrin on human foetal lung fibroblast cell growth. Panel A shows cells plated at $3 \times 10^4$ cells per well, in serum-free media, and cultured for 24h at 37°C in 10%CO$_2$ in air. Media was replaced with fresh media, with and without BSA (1mg/ml) and holo-transferrin (1μg/ml) and incubated for up to 72h. Panel B shows cells plated at $1 \times 10^5$ cells per well in 5%NCS in DMEM and incubated for 4-5 days at 37°C in 10%CO$_2$ in air. At visual confluence the media was replaced with fresh media with and without BSA (1mg/ml) and holo-transferrin (1μg/ml) and incubated for up to 72h. Cell proliferation was assessed by spectrophotometric analysis. Data is a mean of three separate experiments. Within each experiment each data point is a mean of 6 values.
2.4.1.3 Sub-confluent and confluent cell cultures.

In further pilot studies, to establish the degree of cell confluency required to obtain robust measures of cell proliferation, tryptase-induced cell proliferation of HFL-1 was assessed in sub-confluent (10-20%) and confluent (100%) cultures obtained as described above. In sub-confluent cultures, tryptase at concentrations ≤ 3.5mU/ml caused a small mitogenic response, however at concentrations > 3.5 mU/ml tryptase caused cell detachment. In studies with confluent cultures, cell detachment was not seen until concentrations >80 mU/ml were used. In parallel control plates, cell numbers were assessed at the time of agonist addition and at the end of the incubation 48 hours later. There was an approximate 10% decrease in cell numbers as determined by direct cell counting using a haemocytometer ($T_0$: 76328 ± 3841 c.f. $T_{48}$: 67109 ± 5258), however, this was not significant (p=0.18).

Previous studies have demonstrated that tryptase was able to induce cell proliferation of sub-confluent and confluent cultures by measuring increases in cell numbers and $^\text{H}$-thymidine incorporation (Abe et al., 1998; Cairns and Walls, 1997; Ruoss et al., 1991). Therefore, with the data from confluent cultures demonstrating no increase in cell numbers over a 72h incubation period in the presence of BSA and transferrin (Figure 2.6), the previously published data demonstrating tryptase-induced fibroblast proliferation of confluent cultures, and the pilot studies measuring proliferation over a wider range of concentrations of tryptase in confluent cultures, all subsequent studies were performed in confluent cultures using the protocol described in Section 2.4.1.4.

2.4.1.4 Effect of tryptase and trypsin on fibroblast proliferation

Studies were performed on HFL-1, adult human lung parenchymal and airway fibroblasts, and dermal-derived fibroblast cell lines. When the cells were visually confluent the media was replaced with 100μl of serum-free media (incubation media) containing, BSA (1mg/ml) and holo-transferrin (1μg/ml) and incubated for a further 24h. The media was replaced with...
100μl of fresh incubation media containing tryptase (0.7 - 70 mU/ml) or trypsin (Sigma, 1 - 20nM). In pilot experiments cells were incubated with appropriate dilutions of the buffer mixture used to store the stock concentrations of tryptase (10 mM 2-[N-Morpholino]ethansulfonic acid, (MES, Sigma, Poole, UK), 300 mM sodium chloride (Sigma, Poole, UK), 0.02 mM heparin (Sigma, Poole, UK), and 0.02% sodium azide(Sigma, Poole, UK). These dilutions of the buffer had no significant effect on cell proliferation over 48h incubation time period. Human mast cell tryptase was purchased from Bioprocessing Inc., Scarborough, ME, USA. Its activity was expressed in milliUnits (mU), and was defined as the amount of enzyme which gave a change of 1 absorbance unit per minute at 410nm using n-benzyl-DL-arginine-pNA as the substrate. The batches of tryptase used in these studies had specific activities ranging from 5275 - 6089 mU/mg of total protein. A positive control (5% NCS) was added to cells to ensure they were able to respond to growth factors. All treated cultures were incubated at 37°C for 24 – 72h in humidified air containing 10% CO₂. Cell proliferation was assessed as in Section 2.4.1.2.

### 2.4.1.5 Recombinant human tryptase

In addition to the tryptase isolated from cadaveric human lungs, recombinant human tryptase (rh-tryptase) was also tested in the proliferation assay. rh-Tryptase, a gift from Dr A Niles (Promega Corporation, Madison, WI, USA), is an active mature enzyme expressed in *Pichia pastoris* and purified by affinity chromatography (Niles et al., 1998).

### 2.4.1.6 Time dependent induction of fibroblast proliferation by tryptase and trypsin

To further characterise the mitogenic responses of tryptase and trypsin, lung fibroblasts were exposed to the agonists for varying lengths of time, up to 48 hr, after which the agonist was removed and replaced with incubation media alone. Cell numbers were examined, after a
total of 48 hr incubation, by spectrophotometric analysis (see Section 2.4.1.2) after 5, 10, 30 and 60 min, 2, 4, 8, 24 and 48h incubations with the enzymes.

2.4.2 Inhibition of tryptase-induced cell proliferation

2.4.2.1 Enzyme inhibitors

To assess the requirement of the catalytic activity of tryptase to induce fibroblast proliferation, a selection of serine protease inhibitors, with varying degrees of selectivity for tryptase, were used to inhibit the catalytic site (Table 2.2).

Table 2.2 Inhibitors used to inhibit the catalytic activity of tryptase

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Selectivity of compound for tryptase inhibition</th>
<th>Concentration range (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antipain</td>
<td>Non-selective</td>
<td>0.1-100</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>Selective</td>
<td>0.1-1000</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>Selective</td>
<td>0.1-10</td>
</tr>
<tr>
<td>BABIM</td>
<td>Selective</td>
<td>0.0037-10</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Unknown</td>
<td>0.1-100</td>
</tr>
<tr>
<td>SLPI</td>
<td>Unknown</td>
<td>0.1-100</td>
</tr>
<tr>
<td>APC366</td>
<td>Selective</td>
<td>0.1-10</td>
</tr>
</tbody>
</table>

2.4.2.2 Assay for inhibition of tryptase activity

Tryptase activity and its inhibition by serine protease inhibitors were assayed using the synthetic peptide substrate Tosyl-Gly-Pro-Arg-p-Nitroanilide. Stock solutions of substrate and inhibitors, Antipain, Benzamidine and BABIM, were dissolved in DMSO. Twenty microlitres of a sub-maximal concentration of tryptase (17mU/ml final concentration) was added to the reaction mixture containing 50µl of Tris 10mM, NaCl 120mM at pH7.8, 20µl of substrate (final concentration 400 µM, stock solution 2 mM) and 10µl of inhibitor, giving final concentrations of antipain, 0.1 – 100µM, BABIM, 0.001 – 10 µM, or benzamidine, 1 – 1000 µM. The reaction volume was 100µl with a final DMSO concentration of 1.8%. Absorbance was measured at 405 nm with a 630/650 nm reference filter.
A Km was obtained for tryptase of 4.93 nM with a Vmax of 73.02 activity units (mOD/min) (Figure 2.7A). BABIM was found to be a mixed type inhibitor of tryptase with apparent decreases in Vmax and increases in Km with increasing concentrations of BABIM (Figure 2.7A). A Ki for BABIM against tryptase was calculated to be 18nM (Figure 2.7B). For comparative purposes, IC50 values were calculated for BABIM, benzamidine and antipain and found to be 0.0286 μM, 483μM and 7.1μM, respectively.

The results are expressed as a percentage inhibition of tryptase-induced cleavage of the synthetic peptide to obtain IC50 values (the concentration of the inhibitor that causes 50% reduction in enzyme activity) and dissociation constant for inhibitor binding to the enzyme (Ki) using the equation of Cheng and Prusoff (Figure 2.8, (Cheng and Prusoff, 1973). Both calculations were performed using GraphPad Prism version 3.00 for Windows 95 (GraphPad Software, San Diego, California USA). In section 2.4.2.3, three serine protease inhibitors (antipain, BABIM and benzamidine, all at 100μM) were used to inhibit tryptase-induced mitogenesis. At this concentration the percent inhibitions of tryptase-induced cleavage of the synthetic peptide substrate by antipain, BABIM and benzamidine were 87.8 ± 3.3%, 93.4 ± 2.5% and 24 ± 13.0%, respectively confirming that these compounds are able to inhibit the catalytic activity of tryptase. These studies were kindly performed by Mr Neil Waslidge, Enzyme Pharmacology, Glaxo Wellcome R&D Ltd.
Figure 2.7 Determination of Km and Ki for tryptase in the presence of BABIM. Panel A shows the Michaelis-Menton plot of substrate concentration ([S] expressed as μmol/L) against velocity (v) expressed as changes in optical density (OD) units per min. Panel B shows the calculation of the Ki (using the equation of Cheng and Prusoff, Cheng and Prusoff, 1973) and IC50 for BABIM. Values are mean and SEM obtained from four experiments that were performed in triplicate.
Figure 2.8  Calculation of IC50 values for Benzamidine and Antipain. Data expressed as percentage inhibition of control tryptase activity. Data is expressed as means and SEM from three experiments, performed in triplicate. If error bars are not shown they are within the symbol. IC50 calculated as the dose of inhibitor causing 50% reduction in enzyme activity.
2.4.2.3 *Serine protease inhibitors*

A single concentration of tryptase (17mU/ml), which gave an approximate 70% of the maximal achievable response was incubated with a range of concentrations of serine protease inhibitors (see Table 2.2) at 37°C for 60 min prior to the addition of the mixtures to confluent HFL-1 cultures. In addition to the use of serine protease inhibitors, studies were performed in which tryptase was heated to 65°C for 60 min in an attempt to denature the protein prior to its incubation with the cells. The cultures were incubated for a further 48 h and cell proliferation assessed by spectrophotometric assay (see Section 2.4.1.2).

2.4.2.4 *PAR-2 neutralising antibodies*

Confluent cell cultures were obtained as previously described. The media was replaced with fresh serum-free incubation media containing a range of concentrations of a rabbit anti-rat PAR-2 antisera (1:10000 - 1:1000 dilution, see Section 2.5.2 for details) and incubated for 1 hr at 37°C. The media was aspirated and replaced with single concentrations of tryptase or trypsin in serum-free, antibody free media, for varying lengths of time (5 min to 48 hr). After each exposure time the agonist containing media was replaced with fresh serum-free incubation media. Proliferation was assessed after a total incubation time of 48 hr.

2.4.3 *Effect of PAR-2 activating peptide on fibroblast proliferation*

To establish whether PAR-2 activating peptide’s were able to induce fibroblast proliferation, confluent cultures of HFL-1, human lung parenchymal and airway fibroblasts, and dermal fibroblasts were incubated with a range of concentrations of human or rat PAR-2 activating peptide’s or control peptide sequences. Human PAR-2 activating peptide, SLIGKV (serine-leucine-isoleucine-glycine-lysine-valine) and the control peptides, LSIGKV (leucine-serine-isoleucine-glycine-lysine-valine) and LSIGRL (leucine-serine-isoleucine-glycine-arginine-
leucine) were kindly synthesised by Professor Robert Mecham, Washington University School of Medicine, St Louis, MO, USA. All peptides were in the free carboxylate form. The synthetic peptides were prepared by conventional solid phase synthesis on an Applied Biosystems model 431A synthesiser using FastMoc chemistry. The sequence and purity of the peptides were confirmed by electron spray mass spectrometry. Rat PAR-2 activating peptide (SLIGRL, serine-leucine-isoleucine-glycine-arginine-leucine) was purchased from Neosystems Laboratoire, Strasbourg, France.

In addition to the use of the endogenous peptide activators of PAR-2 from human and rat, a chemically modified peptide, demonstrating a high degree of selectivity and potency for PAR-2 receptor activation (Al Ani et al., 1999b; Roy et al., 1998), was also tested in this assay. The peptide, trans-cinnamoyl-LIGRLO-NH₂ (tc-LISRLO-NH₂), was a gift from Professor Morley Hollenberg, (Protein Synthesis Core Facility, Department of Medicinal Biochemistry, University Calgary, Canada). It was synthesised in a similar manner to the previous peptides except that the C-terminal was aminated.

Fibroblasts were grown to confluency as in Section 2.4.1.3. The media was changed to a serum-free media containing BSA (1mg/ml) and transferrin (1μg/ml) and incubated for 24hr. The media was replaced with media (100 μl) containing a range of concentrations of SLIGKV, SLIGRL, LSIGKV or LSIGRL (0.1-1mM) and the cells were incubated for 48hr. Cell proliferation was assessed by spectrophotometric assay as described in Section 2.4.1.2.

2.4.3.1 Inhibition of neutral endopeptidase

As PAR-2 activating peptides are short peptides, to ensure the agonists were not being degraded by endogenous enzymes (e.g. neutral endopeptidase, NEP) during the 48hr incubation period, studies were performed using three structurally diverse inhibitors of NEP.
Bestatin, Thiorphan and Phosphoramidon. The cells were incubated with 50µl of a single concentration (10µM) of an inhibitor for 60 min prior to the addition of 50µl of PAR-2 AP giving a final range of agonist concentrations (0.1-1mM) in a total volume of 100µl. Cell proliferation was assessed by spectrophotometric assay as described in Section 2.4.1.2.

2.5 Expression, localisation and activation of PAR-2 on fibroblasts

Two methods were used to characterise the expression and surface localisation of PAR-2 in fibroblasts (1) the use of reverse transcriptase polymerase chain reaction (RT-PCR) to demonstrate fibroblasts expression of PAR-2 mRNA, (2) immunohistochemistry, to show the localisation of the receptor within the isolated cells, lung and airway tissue. RT-PCR was performed on total RNA extracted from human lung, airway and dermal fibroblasts. Immunohistochemistry was performed on cultured human lung, airway and dermal fibroblasts. In addition, studies were performed using fibroblasts derived from bronchial biopsies and tissue from whole bronchus obtained from patients undergoing autopsy who had died from non-respiratory related conditions.

2.5.1 Expression of PAR-2 mRNA in fibroblasts

2.5.1.1 Isolation of RNA from human foetal lung, adult airway and dermal fibroblasts

Human foetal lung, adult airway and adult dermal fibroblasts were grown to confluence in T75 cell culture flasks as described in Section 2.2. Total RNA was extracted from the cells using TRizol™ (Gibco BRL, Life Technologies, Paisley) according to the manufacturers instructions. Briefly, 1.5 ml TRizol™ was added to each cell culture flask and incubated for 15 min at room temperature. RNA separation was achieved by the addition of 300 µl chloroform (BDH/Merck, UK), after mixing of the sample for 15 sec, it was allowed to stand
for 3 min at room temperature. The sample was centrifuged at 12,000 x g (Beckman GS-15R, Palo Alto, USA) for 15 min at 4°C. The aqueous layer containing the RNA was transferred to a fresh eppendorf tube and mixed with 500 µL isopropyl alcohol (Sigma, Poole, UK) and incubated at room temperature for 10 min. The sample was centrifuged at 12,000 x g for 10 min at 4°C and the supernatant discarded. The remaining pellet was washed twice with 1 ml 70% ethanol, vortexed and centrifuged at 7500 x g for 5 min at 4°C. The RNA pellet was allowed to air dry for 10-15 min and re-suspended in 20µl of diethyl pyrocarbonate (DEPC, Sigma, Poole, UK)-treated Milli-Q (MQ) water (Milli-RO 12 plus and Milli-Q plus PF, Millipore Ltd, Watford, UK). The solution was heated to 60°C for 10 min, to ensure the RNA was fully dissolved, prior to RNA quantification using a spectrophotometer. Samples were frozen at -80°C until analysed.

2.5.1.2 Spectrophotometric evaluation of nucleic acids

The quantification of purified nucleic acid samples was performed by a Beckman DU-64 spectrophotometer (Beckman, Palo Alto, USA). Nucleic acids absorb light maximally at 260 nm. At this wavelength 40µg/ml of RNA will have an absorbance of 1. The presence of contaminating protein in the sample is measured at 280 nm. Calculation of the ratio of absorbance at 260 and 280 nm (A260/A280) was performed to estimate the purity of the preparation. A ratio of 1.8 or greater was judged ideal.

2.5.1.3 Reverse transcription (RT)

First strand cDNA was synthesised by reverse transcription of the RNA using ‘Superscript™ Pre-amplification system for First Strand cDNA Synthesis’ (Gibco BRL, Life Sciences, Paisley) according to the manufacturers instructions. For each sample 5µg of total RNA was added to 1 µl of Oligo RNA12-18 and the volume was made up to 12µl with DEPC-treated
MQ water. Each sample was incubated at 70°C for 10 min followed by quenching on ice for 1 min. For each reaction, a RT mixture was prepared by the addition of 2 µl of 10x PCR buffer, 2 µl 25 mM MgCl₂, 1 µl 10 nM dNTP mix to 2 µl 0.1M dithiothreitol (DTT). 7 µl of this RT mixture was added to the 12 µl of the sample mixture and incubated at 42°C for 5 min. Finally 1µl of the enzyme, Superscript II reverse transcriptase, was added to the mixture, giving a final reaction volume of 20µl, and incubated at 42° for 50 min. The reaction was terminated by heating the mix to 70°C for 15 min followed by quenching on ice. After a brief centrifugation, 1 µl of RNase H was added to the reaction mix and incubated at 37°C for 20 min to digest the RNA template from the cDNA:RNA hybrid molecule.

2.5.1.4 Polymerase chain reactions (PCR)

Polymerase chain reaction (PCR) amplification was performed using the following reaction mixture for each sample.

Table 2.3 PCR components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR Buffer</td>
<td>5</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>3</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1</td>
</tr>
<tr>
<td>Primer set 1</td>
<td>1</td>
</tr>
<tr>
<td>Primer set 2</td>
<td>1</td>
</tr>
<tr>
<td>Taq DNA polymerase (2.5 units)</td>
<td>0.5</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>36.5</td>
</tr>
</tbody>
</table>

The oligonucleotide primers for human PAR receptors used in this study were obtained from Life Technologies (Paisley, Scotland). PAR-1, forward 5' - CATGGCCCATCCTTGTTGTCATCC-3'; reverse 5' - CGGTGGCAAATGCGGAAGAGC-3'; PAR-2, forward 5' - GTTGATGGCACATCCCACGTCC-3, reverse 5' - GTACAGGGCATAGACATGGC-3'; PAR-3 5' - CTGATAACAATGCATCTACCTCC-3'
Two microlitres of the first strand DNA from the RT Reaction was added to 48 µl of the PCR reaction mix and two drops of mineral oil added to the surface of the solution to stop evaporation during the repeated cycling process. The mixture was subjected to heating for 1 min at 95°C followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 min and 72°C for 2 min using a thermal cycler (PTC-200 Peltier, MJ Research, USA). During the final cycle the mixture was heated to 72°C for 5 min and then kept at 4°C before being subjected to gel electrophoresis.

2.5.1.5 Gel electrophoresis

The quality of the PCR product was checked by 1.5% agarose gel electrophoresis. 2.25g of electrophoresis grade agarose (Sigma, Poole, UK) was added to 150 ml of 1x TAE buffer (Tris/EDTA) and heated in a microwave (Sharp Compact, R-2V16) until dissolved. When the molten agarose had cooled, 15 µl of ethidium bromide was added and thoroughly mixed. The agarose solution was poured into a 10 x 6.5 cm casting tray (BIORAD, Hemel Hempstead, UK) containing an appropriate sized gel comb and allowed to set for 30 min. For loading of the gel, 10 µL of the sample was mixed with 1 µl of loading buffer (Sigma, Poole, UK) and briefly centrifuged prior to loading on the gel. In addition, to assist in product identification a 1kb ladder (Life Technologies, Paisley, Scotland) was also added. The gel was run at 90 volts for 45 min and the resulting bands visualised under a UV illuminator and images recorded using a Polaroid camera.
2.5.1.6 Identification of RT-PCR products

The initial product identification was done using the size marker and comparison with the expected product size (PAR-1 505 bp, PAR-2 813 bp, PAR-3 528 bp and PAR-2 635 bp). The signal obtained in the PCR amplification using the PAR-2 cDNA was compared with the product (452 bp) generated from a glyceraldehyde-3 phosphate dehydrogenase (GAPDH) PCR control primer pair (forward, 5’-ACCACAGTCCATGCCATCAC-3’; reverse, 5’-TCCACCACCTGTTGCTGTA-3’)) by densitometry using an Image Master 1D gel and film scanner (Pharmacia, UK). The identity of the PAR-2 RT-PCR product was confirmed by sequencing using AmpliTaq DNA polymerase FS (Perkin-Elmer) fluorescently labelled dye-terminator chemistry and the use of an Applied Biosystems Inc. 377 PRISM automated sequencer, which was kindly performed by Dr Michael Hill (Centre for Cardiopulmonary Biochemistry, UCL).

2.5.2 Immunohistochemical localisation of PAR-2

PAR-2 has been identified in a number of tissues within the body (Cocks et al., 1999a; D'Andrea et al., 1998; Steinhoff et al., 1999), however, it is unclear whether these receptors are present on the cell surface of fibroblasts. Therefore, using standard immunohistochemistry and confocal microscopy techniques, the localisation of PAR-2 was investigated in isolated human lung, airway and dermal fibroblasts and sections obtained from human airway tissue. The antibody used was a rabbit anti-rat PAR-2 serum (B5) which was raised against a peptide that spans the rat PAR-2 trypsin cleavage/activation site: \(^{30}\text{GPNSKGR/SLIGRLD}^{46}\text{T}-\text{TGGC}\) coupled to keyhole limpet hemocyanin (/, trypsin cleavage site; YGGC for coupling). The antiserum was a kind gift from Professor Morley Hollenberg (Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Canada).
2.5.2.1 *Fibroblast cell lines*

Confluent cultures of human foetal lung, adult lung parenchymal and airway fibroblasts, and, dermal fibroblasts were obtained using techniques described in Section 2.1. Studies were also performed using fibroblasts derived from airway biopsies of patients with asthma. These cells were a kind gift from Dr Jamila Chakir (Laval Hospital Research Centre, Quebec, Canada).

To ensure that PAR-2 was not activated by treatment with the trypsin-EDTA mix, single cell suspensions were obtained from the confluent culture by non-enzymatic methods using cell dissociation fluid (Sigma, Poole, UK). Cells were seeded into 8-well chamber slides (Lab-Tek permnox, Nunc Inc, Life Technologies LTD, Paisley, Scotland) at a density of 1 x 10^4 cells/well in 400μl of DMEM containing 5% NCS. The cells were incubated at 37°C in a humidified atmosphere of air containing 10% CO₂ for 48 hr. The cells were fixed with 4% paraformaldehyde for 3 minutes at room temperature. The fixed cells were washed in PBS, treated with normal swine serum (Dako, UK) for 20 min followed by three 5-min PBS washes. The cells were incubated with rabbit anti-rat PAR-2 antisera (1:1000 dilution) for 16 hours at 4°C. Normal rabbit serum (1:1000, Sigma, Poole UK) or rabbit IgG (20ng/ml, Sigma, Poole,UK) were used in place of the primary antisera as negative controls. The cells were washed thoroughly and then incubated with a secondary swine anti-rabbit IgG conjugated to FITC for 60 min at room temperature. After three 5 min washes with PBS, the chambers were removed and cover slips mounted using an antifade glycerol/PBS based mountant, AF1. The cells were visualised by confocal microscopy (Leica UK Ltd., Milton Keynes, England) equipped with a multiline 750mW air-cooled Omnichrome argon-krypton laser. Cells were viewed using x63 water immersion lens with appropriate pinhole settings using a 512 x 512 image format. Digital images were obtained with a Polaroid Digital Palette HR6000 (Leica UK Ltd., Milton Keynes, England) and photomicrographs were obtained using Polaroid Presentation Chrome (PC-135-24) 35mm (ASA100).
2.5.2.2 Localisation of PAR-2 in human bronchial biopsy samples

Human airway tissue was obtained from 1st or 2nd order bronchi at autopsy, from patients who had died from non-respiratory-related conditions. It was snap frozen and stored in liquid nitrogen until use. The use of these tissues was kindly offered to us by Professor WR Roche (Department of Pathology, University of Southampton).

2.5.2.2.1 Tissue cutting

Upon use, the tissue was removed from the tissue bank and allowed to thaw for 2 min at room temperature. The tissue was transferred to a metal stage using OCT compound (Tissue-Tek, Raymond Lamb, London, UK) and frozen in place by submerging in liquid nitrogen. The tissue was placed at -20°C in a cryostat (Cryocut 1800, Reichet-Jung). The stage was placed in the chuck of the cryostat and orientated to obtain transverse tissue sections.

To establish whether it would be best to use thin or thick sections, 7 and 60 μm thick sections were obtained and placed on APES-treated slides (Surgipath Microscope Slides, Raymond Lamb, London, UK) at room temperature. The slides were allowed to dry at room temperature for 30 min, prior to storing at -20°C overnight wrapped in aluminium foil.

2.5.2.2.2 Fixation and staining

The slides were allowed to thaw at room temperature for 15 min prior to fixation with either 4% paraformaldehyde (15 min - 7μm sections, 30 min - 60μm sections) or ice-cold anhydrous acetone (15 min at -20°C). The slides were washed in Tris buffered saline (TBS) containing 1% BSA. Each slide was treated with normal swine serum (1:20 dilution) for 20
min. The excess was removed and the rabbit anti-rat PAR-2 antisera, diluted in TBS containing 1% BSA and 0.1% triton X-100, was applied to the slides. The slides were incubated in an humidified chamber at room temperature for 1 hr or at 4°C for 16h.

After incubation the slides were thoroughly washed in TBS containing 1% BSA (three 5 min washes). All the slides were incubated with swine anti-rabbit IgG conjugated to FITC for 1h in a dark humidified chamber at room temperature. After thorough washing (three 5 min washes) the slides were mounted in antifade (AF1) mounting solution, covered with a coverslip and stored in the dark at 4°C.

2.5.2.3 Co-localisation studies

Some slides were used for co-localisation studies using the rabbit anti-rat PAR-2 antisera and rhodamine phalloidin (Sigma, Poole, UK) for the staining of F-actin microfilaments. After the incubation with the PAR-2 antisera, the slides were washed and incubated with rhodamine phalloidin (1:100 dilution) for 60 min in a dark humidified chamber. The slides were again washed thoroughly, and incubated with the swine anti-rabbit IgG conjugated to FITC and mounted in antifade mountant as above.

2.5.2.4 Slide analysis

The tissues were visualised by confocal laser scanning microscopy using a Leica TCS NT microscope similar to that used in Section 2.5.2.1. Images were stored to disk and either printed to a Lexmark 1100 Colour jet-printer or photographs taken using Kodak Elite 100 ASA.
2.5.2.5  *Biopsy specimens from normal and asthmatic subjects*

Biopsies from the airways of normal and asthmatic patients undergoing diagnosis for other respiratory conditions were kindly offered to us from Dr Tudor Toma, Spitalul Clinic Universitar de Pneumoftiziologic Iasi, Romania. The biopsies were fixed in 4% paraformaldehyde, washed in 50% alcohol and then stored in 70% alcohol at 4°C until they were embedded in paraffin using an automated carousel processor. The fixed biopsies were placed through a series of increasing concentrations of alcohols, and two washes in xylene prior to wax embedding (Table 2.4).

<table>
<thead>
<tr>
<th>Container</th>
<th>Fluid</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70% alcohol</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>95% alcohol</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>100% alcohol</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>100% alcohol</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>100% alcohol</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>100% alcohol</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>100% alcohol / xylene</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>xylene</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>xylene</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>Wax</td>
<td>2.5</td>
</tr>
<tr>
<td>11</td>
<td>Wax</td>
<td>4</td>
</tr>
</tbody>
</table>

2.5.2.5.1  *Cutting sections*

Prior to cutting the sections the wax blocks were placed on ice. Five micrometre sections were cut, removed from the cutting blade and placed on to a slide with 20% alcohol and then transferred to a water bath containing distilled water at 42-45°C. The sections were transferred to a clean slide pre-coated with 3-aminopropyltriethoxysilane (APES) and allowed to air dry. The slides were then heated to 60°C for 30 min.
2.5.2.5.2 Immunostaining protocol

The slides were dewaxed in 100% xylene for 3 min. This was repeated twice more followed by washing in 50% xylene/50% alcohol for 1 min. The slides were rehydrated by washing in decreasing concentrations of alcohol.

<table>
<thead>
<tr>
<th>Alcohol concentration (%)</th>
<th>Wash time (min)</th>
<th>Number of washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>90</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

The endogenous peroxidase activity within the biopsy specimen was blocked by incubation of each slide with 3% hydrogen peroxidase for 3 min, followed by 3 five minute washes with PBS containing 1% bovine serum albumin (PBS-BSA). To reduce non-specific binding the slides were incubated with normal goat serum (1:20 dilution) in a humidified chamber for 20 min.

Sections were incubated in an humidified chamber with anti-rat PAR-2 antisera overnight (16 h) at 4°C, human anti-α-smooth muscle antibodies or human anti-vimentin antibodies for 1 h at room temperature. A secondary biotinylated antibody was applied to the slides for 60 min followed by incubation with streptavidin-alkaline phosphatase with 3 five min washes with PBS-BSA between each incubation. The antibody binding was visualised using the phosphatase substrate, 3,3′-diaminobenzidine (DAB, Sigma, Poole, UK, 600 μg/ml).

The sections were counter-stained with Mayers-Haemotoxylin for 7 sec, washed in tap water for 3 min, briefly exposed to acidified alcohol for 6 sec followed by further washes in Scotts tap water (5 min) and running tap water (1 min). The slides were dehydrated using the
schedule shown in Table 2.6, and mounted in DPX (BDH Merck, Litterworth, UK), with a cover slip.

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Wash time (min)</th>
<th>Numbers of washes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol 50%</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Alcohol 70%</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Alcohol 100%</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Xylene 100%</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

The slides were viewed using an Axioscope20 microscope. Photographs were taken using Fuji Provia ASA 1600 Daylight film.

2.6 Measurement of procollagen gene expression and metabolism

As alluded to in the introduction collagen molecules consist of three polypeptide chains coiled into a triple left-handed helix. The polypeptide composition of the helix depends on the type of collagen. Fibroblasts are capable of producing large amounts of extracellular matrix in response to activation by a variety of inflammatory mediators. They principally produce collagen type I (Hance et al., 1976; Sage et al., 1979) consisting of \( \alpha 1(I) \) and \( \alpha 2(I) \) polypeptide chains, which are expressed by different genes. These cells are also capable of producing collagen type III (Hance et al., 1976).

To investigate the effects of tryptase and the PAR-2 AP on procollagen gene expression and metabolism by human lung fibroblasts, three approaches were used:

1. Assessment of procollagen gene expression by using a reporter gene assay
2. The quantification of steady state levels of procollagen type I mRNA by Northern analysis
3. The quantification of procollagen protein by measuring hydroxyproline by high pressure liquid chromatography (HPLC).
2.6.1 Evaluation of procollagen α2(I) gene activation

The activation of procollagen α2(I) gene by tryptase and PAR-2 AP was studied by the transfection of human foetal lung fibroblasts with a 3.5kb construct of the human procollagen α2(I) gene promoter (Boast et al., 1990) that was kindly made available to us by Dr Francesco Ramirez (Mount Sinai School of Medicine, New York, USA). The promoter construct was originally derived from a 3.5kb genomic sub-clone that spanned from position -3500 to +58 of the human procollagen α2(I) promoter (Boast et al., 1990), which had subsequently been excised, linked to a firefly luciferase reporter gene (Inagaki et al., 1994) and sub-cloned into the expression vector pcDNA3.1.

2.6.1.1 Preparation of fibroblasts for transfection

Human foetal lung fibroblasts were seeded into 12 well tissue culture plates (1x10⁴ cells/well) in DMEM containing 5% NCS, penicillin (100U/ml) and streptomycin (100μg/ml). The cells were incubated for 3-4 days at 37°C in 10% CO₂ in air and allowed to reach approximately 80% visual confluence. The incubation medium was removed and the cells washed in serum-free DMEM.

2.6.1.2 Preparation of transfection media

The DNA construct was complexed with an α₂β₁ integrin-targeting peptide (Hart et al., 1997) and Lipofectin reagent (Gibco BRL, Life Technologies, Paisley, UK) as previously described (Hart et al., 1998). These studies were kindly performed by Dr Pat Garcia (Cardiopulmonary Biochemistry and Respiratory Medicine, UCL). Lipofectin reagent is a liposome formulation containing a cationic lipid and dioleoyl phosphatidylethanolamine suitable for the transfection of nucleic acids in tissue culture (Felgner et al., 1987).
However, for these studies lipofectin was used at a concentration that does not form liposomes.

For a single well, the transfection media was obtained by the mixing the appropriate ratios of lipofectin, integrin-targeting peptide and DNA construct in the following order:

- 0.75μl Lipofectin reagent in 100μl of Optimem (Sigma, Poole, UK)
- 40μl integrin-targeting peptide (stock solution 100μg/ml)
- 1μg DNA in 100μl Optimem

and complexes allowed to form for 60 min at room temperature. The final volume was adjusted to 400μl with Optimem. The volumes were increased depending on the numbers of wells tested.

2.6.1.3 Transfection and agonist stimulation

The serum-free media was replaced with transfection media (400μl) and incubated at 37°C for 6hr. The media was replaced with DMEM containing 10% NCS, penicillin (100U/ml), streptomycin (100μg/ml) and incubated for 16hr at 37°C. Finally, the media was changed to a serum-free media prior to the addition of tryptase (0.7 – 17.6 mU/ml), trypsin (10nM), SLIGKV (1mM), LSIGKV (1mM), TGFβ (1ng/ml) or thrombin (25nM) and the cells incubated for 24hr at 37°C before assessment of luciferase activity.

2.6.1.4 Luciferase activity assay

Luciferase activity was assayed using Luciferase Assay Kit (Promega Corporation, Madison, WI, USA). In brief, the cells were washed twice in cold PBS and lysed with 100μl Lysis Buffer and incubated at room temperature for 15 min. The cells were harvested by scraping
and the extracts centrifuged for 10 min at 15000 x g at 4°C to remove cell debris. The luciferase activity was measured immediately in a 10 μl sample of the cell extract in 100μl of luciferase assay buffer using a luminometer (Turner Designs 20/20). The data was expressed in relative light units (RLU) per well.

2.6.2 Expression of procollagen type I mRNA (Northern Analysis)

Human foetal lung fibroblasts were seeded into 6 cm petri-dishes (Marathon, London) at a density of 1.5 x 10⁵ cells per plate, in DMEM containing 5% NCS, penicillin (100U/ml), streptomycin (100μg/ml) and incubated at 37°C in 10% CO₂ in air. The cells were allowed to reach visual confluence at which point the media was changed to a serum-free incubation media supplemented with BSA (1mg/ml), human holo-transferrin (1μg/ml), proline (0.2mM) and ascorbic acid (50μg/ml) and incubated for a further 24 hr. The media was replaced with fresh incubation media containing tryptase, SLIGKV, LSIGKV, TGFβ or media alone. The cells were incubated for 24 hr and the total RNA within the culture was extracted.

2.6.2.1 Total RNA extraction

RNA was extracted from the cells using TRIzol™ as described previously (Section 2.5.1.1) with modifications to the volumes or reagents used. Samples were frozen at −80°C until analysed.

2.6.2.2 Agarose gel electrophoresis

A 1 % gel was prepared by dissolving 1.1g of electrophoresis grade agarose (Sigma, Poole, UK) in 94.6 ml DEPC-treated MQ water) by heating in a microwave. The solution was allowed to cool to approximately 60°C before the addition of 5.5 ml of 20 x 3-[N-
morpholino]propanesulphonic acid (MOPS, Sigma, Poole, UK) and 9.9 ml formaldehyde (Sigma, Poole, UK). The agarose solution was poured into a 10 x 6.5 cm casting tray (Bio-Rad, Hemel Hempstead, UK) containing an appropriate sized gel comb with 10 slots and allowed to polymerise for 30 min.

RNA Ladder (1μg/ml, Boehringer Mannheim, Germany), and the RNA samples were thawed on ice. Each sample, containing 5μg RNA was made up to 10μl with DEPC-treated water and 3 μl of loading buffer (deionised formamide, 62,5%(v/v), formaldehyde, 1.14M, bromophenol blue, 200μg/ml, xylene cyanole, 200μg/ml, MOPS-EDTA-sodium acetate at 1.25X working concentration and ethidium bromide, 50μg/ml, Sigma, Poole, UK). In a separate eppendorf tube, 10μl of RNA ladder was added to 3μl of loading buffer. All the samples were heated to 65°C for 10 min, briefly centrifuged using a bench centrifuge (Micro Centaur, MSE, UK) and stored on ice. The gel was submerged in an electrophoresis tank (Bio-Rad, Hemel Hempstead, UK) containing 250 ml of 1 x MOPS, the comb removed and pre-run for 10 min at 85 volts (Power Pac 3000, Bio-Rad, Hemel Hempstead, UK).

Samples (13 μl) were loaded on the gel, and run at 85 volts for 2.5 hr. The gel was then placed in 2 x Sodium citrate/Sodium Chloride solution (SSC, Sigma, Poole, UK) and the resulting bands visualised under a UV illuminator.

2.6.2.3 Transfer to nylon membrane

The gel was washed twice in 2 x SSC for 10 min. The apparatus for the transfer to a nylon membrane was assembled as shown in Figure 2.9. A suitable container was partially filled with 20x SSC. A single piece of Whatmann 3MM paper (Whatmann, UK) was placed over a stage and soaked in 20x SSC ensuring there was no trapped air bubbles. Each end was immersed in the 20x SSC so that it acted as a wick. The gel was placed on to the wick with
the RNA facing down. A single piece of Hybond-N (nylon) membrane was placed on the gel again ensuring no air bubbles were trapped. Three wet pieces of Whatmann 3MM paper were placed on top of the membrane followed by three dry pieces. A stack of paper towels were placed on top of the Whatmann paper followed by a glass plate and a metal weight (~250g). The apparatus was left overnight. When the apparatus was dismantled a corner was cut off the membrane for identification purposes. It was washed in 20x SSC and an image was taken of both the gel and the membrane with a phosphoimager (FLA-3000G, Fuji Photo Film Co., Ltd, Tokyo, Japan) using Image Reader Software (Fuji Photo Film Co. Ltd, Tokyo, Japan) assess transfer efficiency. The membrane was cross-linked with UV Transilluminator (1200J).

![Apparatus used for capillary transfer of RNA from agarose gel to nylon membrane](image)

### Figure 2.9. Apparatus used for capillary transfer of RNA from agarose gel to nylon membrane

#### 2.6.2.4 Prehybridisation

A prehybridisation solution (25 ml) was prepared containing 5x SSC, 5X Denhardt's solution and 0.5% sodium dodecysulphate (SDS, Sigma, Poole, UK) to a 50 ml polypropylene tube (Falcon, Marathon, UK) giving. 150µl of salmon sperm was heated to 100°C for 5 min and 100µl added to the mix. The membrane was placed in a hybridisation tube (Hybaid HB-OV-BS) and 12.5 ml of the prehybridisation solution added. The tube was placed in a hybridisation oven (Bachofer, Reutlingen, Germany) at 65°C for 2 hr. The remaining prehybridisation solution was maintained at 65°C for use later.
2.6.2.5 *Labelling probe*

The procollagen type I probe (Hf677, American Tissue Culture Collection) was radiolabelled using \(^{32}\)P-d-CTP with a Amersham Megaprime DNA Labelling Kit (Amersham Pharmacia Biotech Inc) following the manufacturers instruction. In brief, 5 \(\mu\)l of a primer solution was added to 25ng procollagen I probe DNA and 24 \(\mu\)l of DEPC-treated water. The solution was heated to 100°C for 5 min, quenched on ice and briefly centrifuged. After the addition of 10\(\mu\)l of labelling buffer, 3 \(\mu\)l of klenow enzyme and 3 \(\mu\)l of \(^{32}\)P-d-CTP (specific activity ~400Ci/nmol), the solution was incubated for 60 min at 37°C. The reaction was stopped by the addition of 10 \(\mu\)l of EDTA, giving a final volume of 60 \(\mu\)l.

Unincorporated nucleotides were removed from the probe mixture by the use of a spin column (ProbeQuant G50 micro column (Amershan Pharmacia Biotech Inc). The column was vortexed to ensure re-suspension of resin, centrifuged at 750 x g for 2 min and supernatant discarded. The radioactive probe mixture was placed on the resin and centrifuged at 750 x g for 1 min. The column was discarded and the supernatant, which included the DNA probe, was denatured by heating to 100°C for 5 min followed by quenching on ice for 30 sec.

The prehybridisation solution from the hybridisation tube was replaced with fresh prehybridisation solution. The radiolabelled probe (60\(\mu\)l) was added to the hybridisation tube and incubated for 16 hr at 65°C in the hybridisation oven.

2.6.2.6 *Post-hybridisation washes and imaging*

Following incubation, the hybridisation solution was decanted off and the membrane washed with 20 ml of a low stringency wash solution (2 x SSC and 0.1% SDS). This solution was
replaced with fresh solution and incubated for 5 min at 65°C. The membrane was washed with a higher stringency wash solution (0.5x SSC, 0.1% SDS) for 20 min at 65°C. The membrane was blotted dry, wrapped in saran wrap, placed against an imaging plate (IP Stage, Fuji Photo Film Co., Ltd, Tokyo, Japan) and kept at room temperature for 1-3 hr. The plate was placed on a FLA 3000G Phosphoimager and the resulting image analysed by densitometry using ScienceLab Software. Alternatively the membrane was placed with a Kodak x-ray film in an autoradiography cassette at -80°C for 1-3 hr. The film was developed automatically.

2.6.3 Determination of procollagen protein metabolism

Procollagen protein metabolism in HFL-1 cells was determined by measuring hydroxyproline in ethanol-insoluble protein and in low molecular weight degradation products (ethanol soluble fraction) derived from newly synthesised procollagen degraded during the culture period. This method allows the measurement of picamolar amounts of hydroxyproline by reverse-phase high pressure liquid chromatography (HPLC) (Campa et al., 1990; McAnulty et al., 1991).

2.6.3.1 Cell culture conditions

Human foetal lung fibroblast cells were grown to visual confluence in DMEM with 5% NCS as previously described (Section 2.4.1.3). Cells were resuspended and seeded into 12 well plates at a density of $1 \times 10^4$ cells/well in 5% NCS in DMEM. When confluent the media was changed to a preincubation media containing DMEM, proline (0.2mM) and ascorbic acid (50μg/ml), bovine serum albumin (1mg/ml) and holo-transferrin (100μg/ml). After 24 hr incubation, the media was replaced with fresh preincubation media with or without, trypatase (17 mU/ml), the PAR-2 activating peptide, SLIGKV (1mM) or TGFβ1 (1ng/ml).
The cells were incubated for 48 h at 37°C with a further addition of ascorbic acid (50µg/ml) at 24h. Following incubation the cell cultures were frozen until assayed. When the agonists were first added, a control plate (T₀) was frozen for measurement of basal levels of hydroxyproline. Parallel plates were set up to evaluate the effects of the agonists on cell proliferation under these assay conditions.

2.6.3.2 Protein extraction and hydrolysis

Procollagen synthesis was measured by the method of McAnulty et al. (McAnulty et al., 1991) with more recent modifications (McAnulty et al., 1995). In brief, after thawing, cells were removed from the base of each well by scraping and aspirated. The well was washed with 1 ml PBS and combined with the original aspirate. The total aspirate was mixed with ethanol to a final concentration of 67% and the protein precipitated at 4°C overnight. The extracted protein mixture was filtered using a 0.45µm filter (type HV, Millipore Ltd, Watford, UK) and a vacuum filtration unit (Millipore Ltd, Watford, UK). The supernatants were collected and stored at room temperature. The filters were placed in Pyrex hydrolysis tubes and allowed to dry. Two millilitres of 6M HCl was added to each hydrolysis tube and then hydrolysed at 110°C for 16 hours. The hydrolysates were mixed with ~30mg of charcoal (BDH Chemicals, Poole, UK) and filtered using a 0.65µm type DA filter (Millipore Ltd, Watford, UK). The resulting filtrates were stored at room temperature.

The ethanol soluble fraction containing small peptides and free amino acids were evaporated to dryness on a Dri-Block Sample Concentrator (DB-3 SC-3 Techne). 2ml of 6M HCl was added to the resulting solid and processed in a similar manner to the extracted protein samples.
2.6.3.3 Measurement of hydroxyproline by reverse-phase HPLC

2.6.3.3.1 Sample derivatisation

100µl of the filtered hydrolysate was transferred to a 1.5 ml eppendorf tube, covered with perforated parafilm and evaporated to dryness under vacuum with a sample concentrator (Savant Speedvac plus AR SC110 AR, Life Sciences, UK). The dried samples were re-dissolved in 100 µl of MQ water and buffered with 100µl of 0.4M potassium tetraborate, pH 9.5, (Sigma, Poole, UK) and mixed with 100 µl of 36 mM 7-chloro-4-nitrobenz-2-oxa-1,3, diazole (NBD) chloride (Figure 2.10, Sigma, Poole, UK) in methanol to a final concentration of 12mM NBD-Cl. Each sample was protected from the light and incubated for 20 min at 37°C. The reaction was stopped by acidification with the addition of 50µL of 1.5M HCl. 150µL of a concentrated solution of HPLC running buffer A (167 mM sodium acetate in 26% aqueous acetonitrile, pH 6.4) was added and the samples were filtered using a low dead volume, 0.22µm pore size, type GV filter (Millipore Ltd, Watford, UK). A 100µl aliquot was then injected on to the HPLC Column and eluted with an acetonitrile gradient (see below). The hydroxyproline content was assessed by comparison of the area under the curves (AUC) obtained with the samples and hydroxyproline standards that had been prepared in an identical manner.
Figure 2.10 Hydroxyproline derivatisation with NBD-Cl

2.6.3.3.2 *Instrumentation and chromatographic conditions*

The derivatised samples were separated on a Beckman System Gold HPLC System (Beckman Instruments, High Wycombe, UK) coupled with an autosampler. The HPLC column used was a reverse-phase cartridge column (LiChroCART LiChrosopher 250 mm length x 4 mm diameter, 5 μm particle size, 100-RP-18, BDH/Merck) coupled directly to a pre-column (LiChrosorb, 4 mm x 4 mm, 5 μm particle size 100 RP-18, BDH/Merck). The column was maintained at 40°C in a integral heated column oven. At the start of each HPLC analysis, running buffers (A and B, defined in Table 2.7) were degassed with helium and the HPLC system equilibrated in buffer A for 40 min. Figure 2.11 shows the composition of the elution buffer with respect to the percentage of buffer B. The first HPLC runs consisted of three derivatised standards of 50pmol hydroxyproline solutions.
Table 2.7  Chromatographic conditions for the separations of hydroxyproline by reverse-phase liquid high pressure chromatography (HPLC)

<table>
<thead>
<tr>
<th>Column</th>
<th>LiChrospher. 100 RP-18, 250 x 4 mm. 5µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>Buffer A - 5% acetonitrile (v/v)</td>
</tr>
<tr>
<td></td>
<td>50mM sodium acetate. pH 6.4</td>
</tr>
<tr>
<td></td>
<td>Buffer B - 75% acetonitrile (v/v)</td>
</tr>
<tr>
<td></td>
<td>25% MQ water</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.00 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Detection</td>
<td>495 nm</td>
</tr>
<tr>
<td>Gradient</td>
<td>Time (min) %B</td>
</tr>
<tr>
<td></td>
<td>0  0</td>
</tr>
<tr>
<td></td>
<td>5  5</td>
</tr>
<tr>
<td></td>
<td>6  80</td>
</tr>
<tr>
<td></td>
<td>12 80</td>
</tr>
<tr>
<td></td>
<td>12.5 0</td>
</tr>
<tr>
<td></td>
<td>25 0</td>
</tr>
</tbody>
</table>

Figure 2.11  Graph showing the composition of the elution buffer in respect to the percentage of buffer B
NBD-Cl derivatised samples and standards were eluted with an acetonitrile gradient. Changing the relative proportions of each running buffer with time generated increasing concentrations of acetonitrile. The chromatographic conditions are shown in Table 2.7. Post-column detection was achieved by monitoring absorbance at 495 nm using a flow-through cell with a deuterium light source. The signal was processed using the System Gold Software Package (Beckman Instruments, UK) and displayed on an IBM PC. The column eluent was discarded. The total running time for each sample was 25 min. Figure 2.12 shows a typical chromatogram obtained from a sample following 24h incubation with media. Figure 2.13 shows the effect of increasing concentrations of hydroxyproline standard solutions on the absorbance values obtained by HPLC.
Figure 2.12 Separation of hydroxyproline by reverse phase HPLC
Figure shows typical chromatograms obtained for a sample fraction obtained after 24 h treatment with media alone following derivatisation with NBD-Cl and reverse-phase HPLC.

Figure 2.13 Effects of increasing concentrations of hydroxyproline standard solutions on absorbance measured by HPLC. Solutions containing known concentrations of hydroxyproline (10-500pMole) were derivatised with NBD-Cl and then passed through reverse-phase HPLC.

2.6.3.3.3 Quantification of hydroxyproline content

The hydroxyproline content of each sample was determined by comparison with the mean AUC obtained from the chromatograms of 3 separate aliquots of 50 pMole hydroxyproline solution.
To demonstrate the reproducibility of the HPLC measurements, the same hydroxyproline standards (50pM) was tested in triplicate, on four separate occasions (Table 2.8). There was no significant difference between the values as measured by ANOVA (P value = 0.5204). The coefficient of variation for each group is shown in Table 2.8. All the individual group values are less than 10%, and when all the values are combined, the value is less than 8% indicating the measurements are reproducible from day to day.

**Table 2.8  Reproducibility of HPLC Measurements.** Table shows the coefficient of variation for HPLC measurements made on the same standard samples (50pMole of hydroxyproline) on four separate occasions. The values are given in pMole hydroxyproline.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>57.09</td>
<td>54.06</td>
<td>50.5</td>
<td>49.83</td>
</tr>
<tr>
<td></td>
<td>49.26</td>
<td>58.52</td>
<td>54.59</td>
<td>48.24</td>
</tr>
<tr>
<td></td>
<td>47.99</td>
<td>50.5</td>
<td>51.15</td>
<td>51.9</td>
</tr>
<tr>
<td>Mean</td>
<td>51.45</td>
<td>54.36</td>
<td>52.08</td>
<td>49.99</td>
</tr>
<tr>
<td>SD</td>
<td>4.928</td>
<td>4.018</td>
<td>2.198</td>
<td>1.825</td>
</tr>
<tr>
<td>CV</td>
<td>9.58%</td>
<td>7.39%</td>
<td>4.22%</td>
<td>3.67%</td>
</tr>
</tbody>
</table>

As the cell monolayer contains a small amount of procollagen and any residual serum within the incubation media may contain hydroxyproline, the amount of hydroxyproline present in the combined culture medium and cell layer at the start of incubation (t₀) was determined in both the ethanol -soluble and -insoluble fractions and then subtracted from the sample values. The results are expressed as percentage increases in the amounts of hydroxyproline per well compared to media treatment alone.
Due to possible changes in cell numbers during the incubation period, parallel cell culture plates were set-up which were treated in exactly the same way as for the procollagen assay, and changes in cell numbers determined by direct cell counting using a haemocytometer. Hydroxyproline concentrations were expressed both in nmol per well and nmol per $10^6$ cells.

Hydroxyproline measured in the ethanol-insoluble fraction was taken as an index of procollagen production, whereas the hydroxyproline in the ethanol soluble fraction represents hydroxyproline derived from procollagen synthesised and subsequently degraded during the incubation period.

### 2.6.3.3.4 Calculation of procollagen synthesis, production and degradation rates

Procollagen production was calculated from the quantity of hydroxyproline present in the ethanol-insoluble fraction. The total amount of hydrolysate used was 2ml. 100µl of this hydrolysate was dried, re-suspended and derivatised with NBD-Cl in a total volume of 500µl. 100µl of this solution was loaded on to the HPLC. The software generated an AUC for each chromatographic peak associated with hydroxyproline (Peak Area) and related this to the standard hydroxyproline peaks, which is given in arbitrary numbers (HPLC number). To calculate the concentration of hydroxyproline in the original sample the following calculation was used:

$$\text{Total hydroxyproline (pMole/well)} = \text{HPLC Number} \times 5 \times \left( \frac{2000}{100} \right) \quad \text{equation 2}$$

Total procollagen synthesis was represented by the sum of hydroxyproline in protein (ethanol insoluble fraction) and the ethanol soluble fraction (equation 3). The proportion of
newly-synthesised procollagen degraded was calculated from the quantity of hydroxyproline in the ethanol-soluble fraction as a proportion of the total hydroxyproline and expressed as a percentage (equation 4).

\[
Total \ hydroxyproline \ synthesised \ (\text{pMole}) = (x + y) \quad equation \ 3
\]

\[
\% \ newly \ synthesised \ procollagen \ degraded = \left( \frac{x}{(x+y)} \right) \times 100 \quad equation \ 4
\]

where \( x \) denotes the ethanol soluble fraction and \( y \) denotes ethanol insoluble fraction (protein)

### 2.7 Statistical Analysis

#### 2.7.1 Statistical analysis of cell proliferation assay

For the cell proliferation assays the variation between data sets was tested using ANOVA and the significance was tested using unpaired t tests with Bonferroni modification for multi-comparison of data (Fisher and van Belle, 1993).

#### 2.7.2 Statistical analysis for extracellular matrix production data

For data generated in the extracellular matrix production studies, mean ± SEM of measurements were calculated, and variation between data sets tested using One-way ANOVA with student t test. Differences were considered significant when \( p<0.05 \). Coefficient of variation testing was performed on repeated hydroxyproline measurements using GraphPad Prism version 3.00 for Windows 95 (GraphPad Software, San Diego, California USA).
Chapter 3

3 Results

3.1 Effect of human lung tryptase on human lung fibroblast proliferation

Human lung tryptase has previously been shown to be a potent mitogen for endothelial cells (Blair et al., 1997), epithelial cells (Cairns and Walls, 1995), airway smooth muscle (Brown et al., 1995), keratinocytes (Pohlig et al., 1996), and lung fibroblasts (Cairns and Walls, 1997; Hartmann et al., 1992; Ruoss et al., 1991). However, it was necessary to confirm the mitogenic effects of tryptase on fibroblasts derived from human lung and airways. Figure 3.1 shows the effect of tryptase on human foetal lung fibroblasts proliferation over 48h.

The 48h incubation of cells with media containing bovine serum albumin and holo-transferrin induced a small but significant decrease (-14.4%) in absorbance units (Control, 1.409 ± 0.04 absorbance units; 48h incubation, 1.21 ± 0.05 absorbance units, P<0.01, n=29). This decrease was paralleled in direct cell counting experiments in which a small decrease in cell numbers was obtained (-12.1%), however, this was not significant (control 76328 ±3841; 48h incubation, 67109 ± 5258, p=0.18, n=8). In all proliferation studies a positive control (5% NCS) was included in the assay to establish the viability of the cells. This control induced a consistent and repeatable increase in absorbance within the spectrophotometric assay (32.1 ± 1.91% compared to media control, n=12). If the control cells failed to respond to 5% NCS the assay was discarded.

Tryptase stimulated proliferation in a concentration-dependent manner. Proliferation was induced at concentrations of 0.7 mU/ml and above, with a maximal stimulation of approximately 50% obtained at 35 mU/ml. Growth was assessed spectrophotometrically following elution of the methylene blue dye by acidified ethanol from cells as described in Section 2.4.1.2. Data from the spectrophotometric assay are shown as absorbance readings.
(Figure 3.1A) and as percentage change from the mean absorbance reading obtaining for cells treated with media alone (Figure 3.1B). Increasing absorbance units is an indication of an increase in cell numbers present in each well. Figure 3.1C shows a correlation between increases in absorbance and increases in cell numbers, with a linear regression of 0.9265. This data is consistent with previously published data demonstrating that increases in spectrophotometric absorbance correlates with fibroblast cell numbers (Oliver et al., 1989).

Similar data was obtained by Dr Olivier Blanc-Brude within our laboratory in which spectrophotometric absorbance readings for fibroblast proliferation correlated with $({}^3$H)-Thymidine incorporation (data not shown). Figure 3.2 shows a mean concentration response curve to tryptase between 0.7 and 70mU/ml from 8 separate experiments.
Figure 3.1  The mitogenic effects of tryptase on human foetal lung fibroblasts. Panel A shows the data from the spectrophotometric assay as mean absorbance units. Cells were incubated with or without increasing concentrations of tryptase. Growth was assessed spectrophotometrically as described in Section 2.4.1.2. Panel B shows the same data as mean percentage changes in absorbance following incubation with increasing concentrations of tryptase compared to cells incubated with media alone. Each point is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 8 separate experiments. Panel C show a correlation of increases in absorbance with increases in cell numbers.
Throughout the course of this thesis a number of batches of tryptase were purchased and used. Therefore it is important to establish a consistency between batches (inter-batch variation) and within batches (intra-batch variation). Table 3.1 and 3.2 demonstrated the consistent mitogenic responses obtained to the same concentration of tryptase (17 mU/ml) from different batches and within those batches respectively. The analysis of variance between the four batches had an overall P value of 0.777. The multiple comparison analysis between each group (Bonferroni" multiple comparison test) demonstrated no significant difference (p>0.05). The analysis of variance of the tryptase-induced mitogenic responses within each batch was not significantly different (P>0.05). The overall analysis of variance for the responses within each batch was not significant (3j 1996FP, P = 0.7609; 3j 2296FP, P = 0.325; 796262, P = 0.319; 796271, P = 0.09).

Figure 3.2. Mean concentration-effect curve of tryptase on human foetal lung fibroblast proliferation. Cells were incubated with tryptase at concentrations between 0.7 and 70 mU/ml for 48 h. Results are shown as a mean ± SEM from 8 experiments. Within each individual experiment each point was a mean of 6 observations. * P<0.01 compared with media control.
Table 3.1  The inter-batch variation of tryptase-induced (17mU/ml) mitogenic responses on human foetal lung fibroblasts.  
Data shown as percentage increase above media control.

<table>
<thead>
<tr>
<th>Batch Number</th>
<th>3I 1996FP</th>
<th>3J 2296FP</th>
<th>796262</th>
<th>796271</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>34.8</td>
<td>31.3</td>
<td>35.6</td>
<td>37.2</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>5.25</td>
<td>1.86</td>
<td>3.09</td>
<td>5.03</td>
</tr>
</tbody>
</table>

Table 3.2  The intra-batch variation of tryptase-induced (17mU/ml) mitogenic responses on human foetal lung fibroblasts.  
Data shown as percentage increase above media control.

<table>
<thead>
<tr>
<th>Batch Number</th>
<th>Expt. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>3I 1996FP</td>
<td>34.8</td>
</tr>
<tr>
<td>Mean</td>
<td>3.25</td>
</tr>
<tr>
<td>3J 2296FP</td>
<td>33.7</td>
</tr>
<tr>
<td>Mean</td>
<td>2.72</td>
</tr>
<tr>
<td>796262</td>
<td>33.9</td>
</tr>
<tr>
<td>Mean</td>
<td>1.71</td>
</tr>
<tr>
<td>796271</td>
<td>37.2</td>
</tr>
<tr>
<td>Mean</td>
<td>5.03</td>
</tr>
</tbody>
</table>

The effect of tryptase on HFL-1 proliferation was confirmed by direct cell counting. Values were generally higher than for the spectrophotometric assay. Tryptase at concentrations of 3.5 and 17.6 mU/ml increased cell numbers above those seen in parallel control plates by ~25 and 70% respectively (control, 124,200 ± 12,800; 3.5 mU/ml, 156,700 ± 32500 cells; 17.6 mU/ml, 210,000 ± 11,100 cells).

Concentrations of tryptase above 35 mU/ml, caused disruption of the cell monolayer and cell rounding. This is believed to be due to the trypsin-like effects of tryptase, cleaving 'cell-
plastic' and 'cell-cell' attachments. Therefore, all future studies used concentrations of tryptase that caused up to 60-70% of the maximum measurable response in this model.

The human isolated tryptase used for these assays was obtained from a commercial source. Although the suppliers had specifically isolated tryptase from lung mast cells they could not guarantee the purity of the sample. This question was partially answered in the previous section (section 3.1) in which consistent responses were obtained between different batches of tryptase. Further evidence that the mitogenic response was not due to an impurity within the isolated sample is demonstrated in the next section in which the mitogenic effects of a recombinant human tryptase were studied.

3.1.1 Effect of recombinant human tryptase on human foetal lung fibroblast proliferation

To further address the questions as to whether the mitogenic effects seen with isolated human lung tryptase was due to an impurity, a series of proliferation assays were performed with a recombinant human tryptase (a gift from Dr Andrew Niles Promega, Corporation, USA). The rh-tryptase was provided in protein concentration units rather than in activity units. However, using the protein concentrations provided by Europa Bioproducts Ltd, and an assumed molecular weight of 134kDa for the tetrameric molecule, the concentrations of the isolated human lung tryptase used were similar to the concentrations of rh-tryptase protein used. For comparative purposes, 0.8nM rh-tryptase corresponds to 0.71mU/ml of isolated human lung tryptase, 4nM to 3.5mU/ml, 20nM to 17.6 mU/ml and 40nM to 35.3 mU/ml. In addition, the activity of the rh-tryptase against two specific tryptase substrates, was at least 130% more active than mast cell tryptase isolated from human lung (Personal communication – Dr Andrew Niles, Promega Corporation).

Figure 3.3 demonstrates a similar profile of activity of the rh-tryptase compared to the isolated form. rh-Tryptase induced proliferation at concentrations above 0.032 nM. A
maximal response was observed at 4 nM, with concentrations of 20 and 40 nM causing no further increases. If a comparison is made between the proliferative responses of the commercially available tryptase (assumed molecular weight 134,000 and an average specific enzyme activity of 10,000 mU/mg of protein) and rh-tryptase, there is an approximate 10-fold difference in potency, with rh-tryptase being the more potent. These higher concentrations of rh-tryptase caused disruption of the cell monolayer and the rounding of cells.

![Figure 3.3. Effect of rh-tryptase on human foetal lung fibroblast proliferation.](image)

Cells were incubated with rh-tryptase at concentrations between 0.032 and 40 nM for 48 h. Each point is a mean ± SEM of 6 observation from a single experiment. Similar results were obtained in 3 separate experiments.

3.1.2 Effect of exposure time of tryptase on human foetal lung fibroblast proliferation

The mitogenic response of tryptase was further characterised by studying the effects of increasing the time the fibroblasts were exposed to a single concentration of the mitogen (17.6 mU/ml). There appeared to be two phases of mitogenic activity with tryptase. Mitogenic activity was seen following only 5 min exposure to tryptase (Figure 3.4). No significant increase was seen when the exposure time was extended to 15 min, 60 min or 4h. However, further increases were seen after 24 and 48h incubation (Figure 3.4). Therefore, in
these experiments 5 min exposure to tryptase resulted in a large proportion of the mitogenic effects seen at 48hr.

To summarise the data presented in the last two sections (Sections 3.1.1 and 3.1.2), it was confirmed that tryptase was able to induce concentration-dependent increases in proliferation of human foetal lung fibroblasts. The following section investigates the effects of tryptase on two other human lung fibroblast cell lines derived from the lung parenchyma and the airways. In addition, the effects of tryptase were studied on a non-lung fibroblast cell line derived from human skin.

3.1.3 Effects of tryptase on isolated human lung parenchymal, airway and dermal fibroblast proliferation

Tryptase also caused concentration-dependent increases in the proliferation of fibroblasts derived from adult human lung parenchyma and airways over a similar concentration range to that seen with foetal lung fibroblasts (Figure 3.5A &B). Tryptase stimulated proliferation
of adult parenchymal fibroblasts to an extent similar to that observed in foetal lung fibroblasts. The airway fibroblasts appeared to be more responsive to tryptase, with responses to 17mU/ml being approximately 3-fold higher than those observed for parenchymal cells (Figure 3.5A & B). Tryptase also induced dermal fibroblast proliferation over the same concentration range with responses similar to those seen with parenchymal fibroblasts (Figure 3.5C).

To summarise this section, a method has been developed for measuring the mitogenic effects of enzymes on fibroblasts using a spectrophotometric assay. Using this assay, the serine protease, tryptase was found to induce concentration-dependent increases in proliferation of foetal lung fibroblasts, which was confirmed by direct cell counting. In addition, tryptase was shown to induce proliferation of adult parenchymal lung and airway fibroblasts, and a non-lung fibroblast cell line derived from human skin. The responses to tryptase were reproducible between and within different batches. Also, the use of a recombinant human tryptase produced similar mitogenic effects to that of tryptase isolated from human lung. The purity of the isolated human lung tryptase is questionable and may contain factors that could induce fibroblast proliferation, however, with the reproducibility of different batches of tryptase and the similar effects of the recombinant protein, it would suggest the effects were due to the enzyme alone. To further confirm the mitogenic activity was due to the enzyme, the following section assesses the effects of enzyme inhibition on tryptase-induced fibroblast proliferation.
Figure 3.5 Effect of tryptase on fibroblasts isolated from adult human lung and skin. Confluent cell cultures of human lung parenchymal (A), airway (B) and dermal fibroblasts (C) were treated with tryptase (0.7 - 17.6 mU/ml) for 48h. Data are means ± SEM of 6 observations from a single experiment. Data were reproduced in at least 4 separate experiments.

3.2 Inhibition of tryptase-induced fibroblast proliferation

The mitogenic effects of tryptase have been attributed to the enzymes’ catalytic activity. Serine protease inhibitors, both selective- and non-selective for tryptase, have been shown to inhibit tryptase-induced mitogenesis of lung fibroblasts (Cairns and Walls, 1997; Ruoss et al., 1991), endothelial cells (Blair et al., 1997) and smooth muscle cells (Brown et al., 1995). Therefore to demonstrate if the catalytic activity of tryptase is required for its mitogenic activity on lung fibroblasts and to provide further evidence of the specificity of the effect,
tryptase was inactivated prior to its addition to confluent cell monolayers and assessment of cell proliferation.

3.2.1 Effect of heat inactivation on tryptase-induced human foetal lung fibroblast proliferation.

The addition of tryptase, equivalent to 0.7 and 3.5 mU/ml, after heat inactivation (65°C for 60 min), failed to induce cell proliferation. These effects were significantly different from the non-inactivated controls, and were not significantly different from media controls. The effects of the higher concentration of tryptase (17.6 mU/ml) were inhibited by approximately 50% (Figure 3.6).

![Figure 3.6](image)

**Figure 3.6** Effect of heat inactivated tryptase on human foetal lung fibroblast proliferation. Three concentrations of tryptase were inactivated by heating to 65°C for 60 min prior to addition to confluent monolayers. Proliferation was assessed after 48 h in all cases. Data are means ± SEM of 6 observations from a single experiment. Similar data was obtained in a repeated study.

3.2.2 Effect of proteolytic inhibitors on tryptase-induced cleavage of specific peptide substrate

The effect of proteolytic inhibition on tryptase is shown in figures 3.7 and 3.8. The non-selective serine protease inhibitor, antipain, and the relative selective inhibitor of tryptase, benzamidine, caused concentration-dependent inhibition of tryptase-induced human foetal lung fibroblast proliferation ($p<0.01$). At the highest concentration used (100 μM) antipain...
and benzamidine inhibited the response to tryptase by 68 ± 17.7 and 70 ± 5.8% respectively. The addition of the inhibitors alone to the cells had no significant effect on basal cell proliferation (p>0.05, Figure 3.7).

The selective tryptase inhibitor, bis (5-amidino-2-benzimidazolyl) methane (BABIM) caused concentration dependent inhibition of the tryptase-induced human foetal lung fibroblast proliferation (p<0.01). The highest concentration (100μM) caused a 100 ± 6.8% inhibition. The addition of this concentration to cells alone had no effect on basal cell proliferation (p>0.05, Figure 3.8).

As demonstrated in Section 2.4.2.2, at the highest concentrations used in these proliferation assays, antipain, BABIM and benzamidine (100μM) caused 87.8 ± 3.3%, 93.4 ± 2.5% and 24 ± 13.0%, inhibition of tryptase-induced cleavage of a synthetic peptide substrate, respectively. Thus, confirming that these compounds are able to inhibit the catalytic activity of tryptase.
Figure 3.7 Effect of serine protease inhibitors on tryptase-induced proliferation of human foetal lung fibroblasts. Tryptase (17.6 mU/ml) was incubated with antipain (1-100 μM, A) or benzamidine (1-100 μM, B) for 60 min at 37°C before addition to cells. Controls included tryptase preincubation with medium in the absence of inhibitors and with inhibitors in the absence of tryptase. Cell proliferation was assessed after a subsequent 48h incubation. Data shown are means ± SEM of 6 observations from an individual experiment. Similar results were obtained in at least 4 further experiments.
Figure 3.8 Effect of bis (5-amidino-2-benzimidazolyl) methane (BABIM) on tryptase-induced proliferation of human foetal lung fibroblasts. Tryptase (17.6 mU/ml) was incubated with BABIM (1-100 μM) for 60 min at 37°C before addition to cells. Controls included tryptase preincubation with medium in the absence of the inhibitor and with the inhibitor in the absence of tryptase. Cell proliferation was assessed after a subsequent 48h incubation. Data shown are means ± SEM of 6 observations from an individual experiment. Similar results were obtained in at least 4 further experiments.

The selective tryptase inhibitor, [N- (1-hydroxy- 2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride] (APC366, (Clark et al., 1995; Rice et al., 1998)), was a gift from Dr David Andrews at Glaxo Wellcome R&D Ltd, Stevenage, UK. The compound displays a different mode of action to the other serine protease inhibitors tested by having a kinetically slow and irreversible multi-step inhibition mechanism (Rice et al., 1998). Therefore the protocol was changed to accommodate this. Tryptase was incubated with increasing concentrations of APC366 (1-100 μM) for 1, 2 or 3 h prior to addition to confluent monolayers of human foetal lung fibroblasts. Cell proliferation was assessed spectrophotometrically at 48h, as previously described in Section 2.4.1.2.

The inhibition of tryptase-induced fibroblast proliferation was progressive with time. In these studies a maximal inhibition was seen after 3h incubation (Figure 3.9). The incubation
of cells in the presence of APC366 (100µM) alone had no effect on basal cell proliferation (Figure 3.9).

3.2.3 Effect of secretory leukocyte proteinase inhibitor on tryptase-induced human foetal lung fibroblast cell proliferation

Evidence presented at the American Thoracic Society meeting in New Orleans May 10-15, 1996 suggested that secretory leukocyte proteinase inhibitor (SLPI) was an inhibitor of tryptase (Forteza et al., 1997). These findings were further supported by data demonstrating the inhibition of tryptase-induced increases in airways resistance in an animal model by SLPI (Robinson et al., 1996).

Figure 3.10 demonstrates the effect of SLPI on tryptase-induced human foetal lung fibroblast cell proliferation. SLPI (1-100µM) was incubated with tryptase (17.6 mU/ml) for 1h at 37°C for 60 min prior to addition to the cells. SLPI appeared to partially inhibit tryptase-induced cell proliferation at all concentrations tested, however these responses did not reach significance (Figure 3.10, P>0.05).
Figure 3.9  Effect of [N-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride] (APC366) on tryptase-induced human foetal lung fibroblast proliferation. Tryptase was incubated with increasing concentrations of APC366 for 1h (A), 2h (B) or 3h (C) at 37°C before addition to the cells. Controls included tryptase preincubation with medium in the absence of the inhibitor and with the inhibitor in the absence of tryptase. Cell proliferation was assessed after a subsequent 48h incubation. Values are means ± SEM of 6 observations from a single experiment. Similar results were obtained in at least 3 further experiments. NT denotes that APC366 100µM was not tested at the 1h and 2h time points.
Figure 3.10  Effect of secretory leukocyte proteinase inhibitor (SLPI) on tryptase-induced proliferation of human foetal lung fibroblasts. Tryptase (17.6 mU/ml) was incubated with SLPI (1-100 μM) for 60 min at 37°C before addition to cells. Controls included tryptase preincubation with medium in the absence of the inhibitor and with the inhibitor in the absence of tryptase. Cell proliferation was assessed after a subsequent 48h incubation. Data shown are means ± SEM of 6 observations from an individual experiment. Similar results were obtained in at least 3 further experiments.

3.2.4 Effect of lactoferrin on tryptase-induced human foetal lung fibroblast proliferation

Lactoferrin has been shown to be a potent and selective inhibitor of tryptase enzyme activity with a Ki of 24nM (Elrod et al., 1997). It was also shown to reduce the late phase response and inhibit airway hyperresponsiveness to carbachol after antigen challenge in the allergic sheep model (Elrod et al., 1997). Lactoferrin at a concentration 400-fold greater than its proposed Ki for inhibition of tryptase (10μM), failed to inhibit tryptase-induced cell proliferation in this assay (Figure 3.11).
Figure 3.11  Effect of lactoferrin on tryptase-induced human foetal lung fibroblast cell proliferation. Tryptase (17.6 mU/ml) was incubated with lactoferrin (1-10 μM) for 60 min at 37°C before addition to cells. Controls included tryptase preincubation with medium in the absence of the inhibitor and with the inhibitor in the absence of tryptase. Cell proliferation was assessed after a subsequent 48h incubation. Data shown are means ± SEM of 6 observations from an individual experiment. Similar results were obtained in at least 3 further experiments.

To summarise the data presented in this section, tryptase-induced increases in proliferation were inhibited by the serine protease inhibitors antipain, BABIM and benzamidine at concentrations that inhibit its catalytic activity. This would suggest that the mitogenic effects of are predominantly dependent on the catalytic activity of the enzyme. The proposed endogenous inhibitors of tryptase, SLPI and lactoferrin failed to significantly inhibit tryptase-induced fibroblast proliferation. The evidence that tryptase-induced proliferation requires its proteolytic activity suggests that a member of the protease activated receptor (PAR) family may be involved in the response. It has been postulated that tryptase may activate PAR-2 (Corvera et al., 1997; Fox et al., 1997; Mirza et al., 1997; Molino et al., 1997b). However, there is currently no evidence that lung fibroblasts express PAR-2. Therefore, the following section investigates whether human foetal lung, adult parenchymal and airway fibroblasts express PAR-2 mRNA and if the PAR-2 protein is localised to the cell surface, comparing this with the expression in dermal fibroblasts.
3.3 Expression and localisation of PAR-2 on fibroblasts

The role of PAR-2 in cell proliferation is controversial, with activation of PAR-2 in human vascular endothelial cells and keratinocytes causing proliferative and anti-proliferative effects, respectively (Derian et al., 1997; Mirza et al., 1996). The role of PAR-2 in tryptase-induced fibroblast proliferation is currently unknown, although it has been suggested that dermal and arterial fibroblasts do not express PAR-2 mRNA (Belham et al., 1996; Santulli et al., 1995; Schechter et al., 1998). To address this, I have examined the expression and localisation of PAR-2 in human lung fibroblasts with RT-PCR and immunohistochemical staining. I have also included, as a comparison, data obtained with dermal fibroblasts.

3.3.1 PAR-2 mRNA expression in human lung and dermal fibroblasts

RT-PCR primers targeted to human PAR-2 amplified a product corresponding to the predicted size (813 bp) indicating the presence of mRNA in human foetal lung fibroblasts and adult airway fibroblasts (Figure 3.12). The levels of PAR-2 mRNA in human foetal lung and adult airway fibroblasts were compared by densitometry and found to similar (Figure 3.12). RT-PCR was also performed on total RNA extracted from human dermal cells and compared to the levels seen in the lung fibroblasts. A similar PCR product was detected but at a much lower intensity (Figure 3.12). RT-PCR with primers to GAPDH gave a product of similar intensity in all cell types (Figure 3.12) indicating the consistent loading of the agarose gels.
Figure 3.12 Expression of PAR-2 mRNA by human fibroblasts.
The upper panel (a) shows the amplification by RT-PCR of a product (813 bp) with primer sequence for human PAR-2 from mRNA prepared from human foetal lung and adult airway and dermal fibroblasts. As a comparison, primers for human GAPDH were used to obtain RT-PCR product of 452 bp with mRNA prepared from the same cultures. The lower panel (b) shows the intensity of each band of PAR-2 (solid bars) and GAPDH (open bars) as measured by densitometric scanning. Data is shown from a single experiment, similar results were obtained from a repeated study.
The RT-PCR PAR-2 product was sequenced using an Applied Biosystems 377 PRISM automated sequencer (Figure 3.13). The sequence produced was compared to known nucleotide sequences by searching the GenEMBL database using BLASTN 2.0.8 search program (http://www.ncbi.nlm.nih.gov/blast/, (Altschul et al., 1997)). The resulting alignment had a 98% identity with the human protease-activated receptor-2 mRNA sequence (U34038), confirming the presence of PAR-2 mRNA in these lung fibroblasts.
Figure 3.13  Sequencing of PAR-2 RT-PCR product. The PCR product was separated on a 1% agarose gel and visualise to ensure the product was a single clear band. The remaining PCR product was sequenced by cycle sequence method using AmpliTaq DNA polymerase FS (Perkin-Elmer) fluorescently labelled dye-terminator chemistry and the use of an Applied Biosystems 377 PRISM automated sequencer. The sequence produced was compared to known nucleotide sequences by searching the GenEMBL database using BLASTN 2.0.8 search program and shown to be 98% homologous to human PAR-2. This data was repeated on a separate independent sample.
3.3.1.1 Detection of other protease activated receptors by RT-PCR

The expression of PAR-1, PAR-3 and PAR-4 mRNA was also investigated using RT-PCR in lung, airway and dermal fibroblasts. Figure 3.14 demonstrates that both the human foetal lung and the adult human airway fibroblast, in addition to PAR-2, express PAR-1, PAR-3 and PAR-4. Although the expression of PAR-4 appears to be weaker than the other receptor types. Dermal fibroblasts express PAR-1, PAR-3 and PAR-4 mRNA (Figure 3.14).
Figure 3.14 Expression of mRNA for PAR-1, -3- 4 in lung and dermal fibroblasts. Figure shows a representative experiment demonstrating the expression of mRNA for PAR-1, -3 and -4 and GAPDH in human foetal lung (A), adult airway (B) and dermal fibroblasts (C). The intensity of PCR product band was measured by densitometric scanning. Similar results were obtained in 2 further experiments.
3.3.2 Immunolocalisation of PAR-2 receptor protein

The last section demonstrated that the human lung fibroblasts are able to express the mRNA for PAR-2. However, this is does not prove that these cells are able to express the PAR-2 receptor protein on their cell surface. I therefore used immunohistochemistry to try and localise PAR-2 on the cell surface of human lung and dermal fibroblasts using a rabbit anti-rat PAR-2 antisera which is known to cross react with human PAR-2 (Corvera et al., 1997; Kong et al., 1997).

3.3.2.1 Immunolocalisation of PAR-2 in isolated human lung and dermal fibroblasts

Human foetal lung, adult lung parenchyma and adult airway fibroblasts were fixed for 3 min in 4% paraformaldehyde and incubated with anti-rat PAR-2 antisera and FITC labelled secondary antibody. The cells were examined using confocal microscopy under ×63 water immersion objective giving a final magnification of ×630. Fluorescent images were sequentially obtained at 1 μm steps through the fibroblasts at an emission wavelength of 488 nm.

PAR-2 was localised to the cell surface of human foetal lung, adult parenchymal lung and airway fibroblasts (Figure 3.15). PAR-2 was clearly localised to the cell surface as demonstrated by a greater intensity of staining around the perimeter of the cells in the confocal images (Figure 3.15A-C). There was also some intracellular staining seen. Since the images seen in Figure 3.15 are optical slices of cells generated by confocal microscopy, it is possible that the antiserum is staining cytoplasmic organelles. Figure 3.15E demonstrates no non-specific staining with rabbit IgG conjugated to FITC. However, due to the level at which these optical images were generated through the cell, the confocal microscope may be
imaging all the fluorescently labelled PAR-2 cell surface receptors. It is possible that this intracellular staining is non-specific, however the control data shown in Section 3.3.2.2 (Figures 3.16 A – E), demonstrates that, at the concentration of antisera used, there appears to be little to no non-specific staining.

Weak, sparse staining was observed in two different human adult dermal fibroblast cell lines (Figure 3.15D). No distinct staining was observed around the perimeter of the cell, however, similar to the lung fibroblasts there appeared to be some intracellular staining.

3.3.2.2 Characterisation of PAR-2 antibodies

As part of the characterisation of the anti-rat PAR-2 antisera in these studies, a series of controls were performed including:

1. Elimination of non-specific staining by the addition of swine serum to the incubation media
2. Addition of normal rabbit serum in the absence of primary PAR-2 antisera
3. Addition of the appropriate dilution of rabbit IgG
4. Pre-absorption of the primary antibody with the peptide antigen used to generate the antisera.
5. Pretreatment of cells with activators of PAR-2 followed by subsequent staining with the anti-rat PAR-2 antisera.

In this series of studies confocal images were taken at 1µm intervals through the cell (at least 8 optical slices) and the images reconstructed electronically to visualise the fluorescent staining throughout the cell. Fluorescent staining was absent in control preparations with normal rabbit serum (1:1000 dilution, data not shown) or rabbit IgG 20 ng/ml (Figure 3.16A). Pre-absorption of the anti-rat antisera completely inhibited the staining of the fibroblasts (Figure 3.16B).
Figure 3.15 Localisation of PAR-2 immunoreactivity in lung and dermal fibroblasts. Cells were fixed for 3 min in 4% paraformaldehyde. Human foetal lung fibroblasts (A), adult lung parenchymal (B) and airway (C) fibroblasts, and adult dermal fibroblasts (D) were incubated with anti-rat PAR-2 antisera for 16 h overnight at 4°C. Panel E demonstrates the lack of non-specific binding using rabbit IgG antibodies conjugated to FITC. Immunoreactivity was visualised by FITC labelling and images obtained by confocal microscopy. Arrows denote the localisation of PAR-2 to the cell surface. Final magnification, x630.

Upon activation, PAR-2 is internalised and cell surface immunoreactivity is lost (Bohm et al., 1996b; DeFea et al., 2000). To demonstrate the antibodies used in this study were specific for PAR-2, an experiment was performed in which the cells were pretreated with tryptase (17.6 mU/ml), trypsin (20 nM) or media alone for 30 min. Subsequently, these cells were incubated with the rabbit anti-rat PAR-2 antisera and visualised using fluorescent microscopy (Figure 3.16). The cells that had been pretreated with tryptase and trypsin had
visibly reduced fluorescent staining compared to cell treated with media alone, suggesting that treatment with both serine proteases induced the activation and internalisation of PAR-2.

Further characterisation of the PAR-2 staining in fibroblasts was obtained by comparison of the rabbit anti-rat PAR-2 antisera staining with that of an rabbit anti-human PAR-2 polyclonal antibody, obtained from Dr Gareth Howells (London School of Dentistry). Similar fluorescent staining patterns are obtained with both antibodies, suggesting they are binding to the same or similar proteins on the fibroblast cell surface (Figure 3.16F).
Figure 3.16  Characterisation of the rabbit anti-rat PAR-2 antisera. Panels A and B show control photomicrographs of lung fibroblasts demonstrating the lack of fluorescent staining (FITC) after incubation with non-specific rabbit IgG antibodies, in place of the anti-rat PAR-2 antisera (A), or the anti-rat PAR-2 antisera after pre-absorption with the specific PAR-2 peptide sequence for 60 min prior to addition to the cells (B). Panel C shows the staining with the anti-rat PAR-2 antisera after 30 min exposure media alone, whereas panels D and E show the reduced staining after exposure to tryptase (17.6 mU/mL, D) or trypsin (20nM, E). Panel F demonstrates the staining of human foetal lung fibroblast with a human PAR-2 polyclonal antibody. Representative photomicrographs are shown of computer reconstructed images generated from combining 8 x 1μm optical slices through the cells. Similar data was obtained from at least 4 experiments using independent fibroblast cell cultures. The final magnification was ×630.
This section has demonstrated that lung fibroblasts can express PAR-2 mRNA, which is translated into PAR-2 protein which can be localised to the fibroblast cell surface using both a rabbit anti-rat PAR-2 antisera and a rabbit anti-human PAR-2 polyclonal antibody. In addition, it would appear that the incubation of these cells with tryptase and trypsin induces a reduction in the antibodies binding to cell surface proteins as seen by a reduction in the fluorescent labelling of the cell surface, suggesting both these enzymes activate PAR-2. To further characterise PAR-2 on lung and dermal fibroblasts, the following section investigates the mitogenic effects of the known activators of PAR-2, trypsin and the PAR-2 activating peptides.

### 3.4 Effect of protease-activated receptor-2 activation on human lung fibroblast proliferation

Trypsin has been shown to induce cell proliferation in a range of different cell types, including fibroblasts (Pohjanpelto, 1977; Scher, 1987; Simms and Stillman, 1936). Trypsin has also been shown to be the prototype agonist capable of cleaving PAR-2 (Bohm et al., 1996a; Bohm et al., 1996b; Nystedt et al., 1994; Nystedt et al., 1995b). The rodent PAR-2 activating peptide, SLIGRL, induces mitogenesis of human endothelial cells (Mirza et al., 1996), whereas it inhibits keratinocyte cell growth (Derian et al., 1997). There is currently no data on the effects of either human or rodent PAR-2 activating peptides on fibroblast proliferation. Therefore, the mitogenic effects of trypsin and the human and rodent PAR-2 activating peptides, SLIGKV and SLIGRL, respectively, were investigated on human foetal lung fibroblasts.
3.4.1 Effect of trypsin on human foetal lung fibroblast proliferation

Trypsin (1-20 nM) stimulated fibroblast proliferation (Figure 3.17). Concentrations above 20 nM caused disruption of the cell monolayer and the rounding of cells, a similar effect seen with high concentrations of tryptase (see Section 3.1.1).

![Graph showing effect of trypsin on human foetal lung fibroblast proliferation](image)

**Figure 3.17** Effect of trypsin on human foetal lung fibroblast proliferation. Human foetal lung fibroblasts were treated with trypsin at concentrations between 1 and 20 nM for 48 h. Growth was assessed spectrophotometrically as described in Section 2.4.1.2. Each point is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 2 further experiments.

3.4.2 Effects of PAR-2 activating peptides on human lung fibroblast proliferation

The non-aminated PAR-2 AP's corresponding to the human (SLIGKV) and the rat (SLIGRL) sequences caused significant increases in human foetal lung fibroblast proliferation (Figure 3.18). At a concentration of 1 mM, SLIGKV and SLIGRL caused stimulation of 22 ±2 and 37 ± 1.5% respectively above media control (P<0.01, n=5). This was confirmed by direct cell counts with SLIGKV (1mM) and SLIGRL (1mM), which increased cell numbers by ~25 and 50% respectively (control, 55,200 ± 4,900 cells; SLIGKV, 67,800 ± 900 cells, SLIGRL, 82,800 ± 5600 cells).
Figure 3.18  Effect of PAR-2 activating peptides SLIGKV and SLIGRL on human foetal lung fibroblast proliferation. Fibroblasts were incubated with activating peptides corresponding to human PAR-2 sequence SLIGKV (0.01 – 1mM) or rat PAR-2 sequence SLIGRL (0.01-1mM). Increases in cell proliferation were assessed after 48h spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 4 further experiments.

In addition to media controls in proliferation studies with PAR-2 AP, two positive controls for cell proliferation were included – 5% newborn calf serum (NCS) and tryptase (17.6 mU/ml) to ensure that the cells demonstrated consistent mitogenic responses. Both these controls produced either equal to or greater responses compared to the highest concentration of PAR-2 AP’s used (5% NCS, 64.7 ± 5.82; tryptase 17.6 mU/ml, 35.6 ± 2.88). Neither of the control peptide sequences at concentrations similar to those used with the PAR-2 AP affected fibroblast proliferation (LSIGKV, 1mM, 1 ± 1%; LSIGRL, 1 mM, 3 ± 1%). The mitogenic effects seen with these activating peptides, in particular the human derived peptide, SLIGKV, were lower than that seen with either tryptase (Figure 3.1 and 3.2) or trypsin (Figure 3.17).

3.4.3 Human lung parenchymal and airway fibroblast proliferation

Both SLIGKV and SLIGRL caused concentration-dependent stimulation of adult parenchymal (Figure 3.19A and B) and airway fibroblast proliferation (Figure 3.19C
and D). Stimulation was seen at concentrations of 10 μM and above. As shown for human foetal lung fibroblasts, SLIGRL generally caused a greater degree of stimulation than SLIGKV in both adult parenchymal and airway fibroblasts (Figure 3.19). The control peptides for the human and rat PAR-2 activating peptides had no significant effect on fibroblast proliferation (LSIGKV, Parenchymal fibroblasts 2.4 ± 0.88%, p>0.05, airway fibroblasts 3.4 ± 2.53%, p>0.05; LSIGRL, parenchymal fibroblasts 2.0 ± 1.79%, p>0.05, airway fibroblasts 1.8 ± 1.90%, p>0.05).

Figure 3.19 Effect of PAR-2 activating peptides, SLIGKV and SLIGRL, on human lung parenchymal and airway fibroblast proliferation. Human lung parenchymal fibroblasts were incubated with activating peptides corresponding to human (SLIGKV, panel A) or rat (SLIGRL, panel B) PAR-2 sequence. Human airway fibroblast were incubated with activating peptides corresponding to human (SLIGKV, panel C) or rat (SLIGRL, panel D) PAR-2 sequence. Increases in cell proliferation were assessed after 48h spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 2 further experiments.
3.4.4 Effect of neutral endopeptidase inhibitors on PAR-2 AP-induced fibroblast proliferation.

The concentration of the PAR-2 AP's that induces cell proliferation is approximately 100-fold greater than induced by either trypsin or rh-tryptase. In an attempt to explain this concentration difference fibroblasts were incubated with neutral endopeptidase inhibitors phosphoramidon (Phosp.), bestatin or thiorphan for 60 min prior to the addition of the PAR-2 AP. There were no significant difference in the PAR-2 AP responses in the presence of the neutral endopeptidase inhibitors (Figure 3.20).

![Figure 3.20 Effect of neutral endopeptidase inhibitors on PAR-2 activating peptide-induced human foetal lung fibroblast proliferation.](image)

Confluent fibroblast cell cultures were incubated with NEP inhibitors for 60 min at 37°C followed by co-incubation with PAR-2 AP. Cell proliferation was assessed after 48h incubation. Increases in cell proliferation were assessed after 48h spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 2 further experiments.
These data suggest that the PAR-2 activating peptides were not being degraded by neutral endopeptidases. The use of receptor agonists alone has limited value in the characterisation of receptors. However, agonists potencies alone have been used to classify receptor types and subtypes in the absence of selective antagonists (Coleman et al., 1994). Currently, with no selective small molecular weight antagonists for PAR-2, the use of the selective, potent PAR-2 agonist, trans-cinnamoyl-LIGRLO-NH₂ (tc-LIGRLO-NH₂) which is not subject to neutral endopeptidase degradation (Al Ani et al., 1999b; Roy et al., 1998) has proved to be useful in further characterising the receptor on the lung fibroblasts. This compound was made available to me as a kind gift from Professor Morley Hollenberg, University of Calgary, Canada.

3.4.5 Effect of highly selective PAR-2 AP, tc-LIGRLO-NH₂, on human lung airway fibroblast proliferation

In human airway fibroblasts tc-LIGRLO-NH₂ (0.01 – 0.1 mM) caused concentration-dependent increases in cell proliferation, whereas 1mM caused a smaller increase in cell proliferation than 0.1 mM (Figure 3.21). It is of note that tc-LIGRLO-NH₂, 0.1 mM, caused a similar increase in cell proliferation to that of tryptase 17.6 mU/ml in these studies. On comparison of the responses to 0.1mM of the three PAR-2 activating peptides used in this proliferation assay (Figure 3.18), tc-LIGRLO-NH₂ appears to be more potent than SLIGRL, which was slightly more potent than SLIGKV (tc-LIGRLO-NH₂ > SLIGRL ≥ SLIGKV).
Figure 3.21 Effect of tc-LIGRLO-NH$_2$ on human airway fibroblast proliferation. Fibroblasts were incubated with tc-LIGRLO-NH$_2$ (0.01-1 mM). Increases in cell proliferation were assessed after 48h spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 2 further experiments.

3.4.6 Effect of trypsin and PAR-2 activating peptides on human dermal fibroblasts

Tryptase has been shown to cause dermal fibroblast proliferation (Section 3.1.3 and (Abe et al., 1998)). However, it is reported that dermal fibroblasts do not express PAR-2 (Santulli et al., 1995), this is supported by data presented in Section 3.3.1 in which only a low level expression of PAR-2 mRNA was found. In addition, there was little or no immunohistochemical staining for PAR-2 on the cell surface of human adult dermal fibroblasts (Section 3.3.2.1). Therefore, the effects of the known activators of PAR-2, trypsin and the PAR-2 activating peptides, were investigated on dermal fibroblast proliferation.
3.4.7 Effect of trypsin and PAR-2 activating peptides on dermal fibroblast proliferation.

Trypsin (1-20 nM) was found to be only a weak agonist (Figure 3.22, \( P<0.01 \) compared to media alone). The lack of effect of trypsin on dermal fibroblast proliferation would be consistent with the previous data suggesting that these cells do not possess PAR-2. Further evidence of the lack of PAR-2 activated fibroblast mitogenesis is presented in Figure 3.22. Human dermal fibroblasts were incubated with PAR-2 activating peptides for 48h and cell proliferation assessed by spectrophotometric assay. Both the human and rat PAR-2 activating peptides failed to stimulate dermal fibroblast proliferation (SLIGKV, 1mM, 6 ± 2%; SLIGRL, 1mM, 4.0 ± 1.79%, Figure 3.22), as did the control PAR-2 activating peptides (LSIGKV, 2.56 ± 2.16%, \( P>0.05 \); LSIGRL, 1.73 ± 1.44%, \( P>0.05 \)).
Figure 3.22 Effects of trypsin and PAR-2 activating peptides on human dermal fibroblast proliferation. Confluent cell cultures of human dermal fibroblasts were treated with trypsin (4 – 20 nM, panel A), SLIGKV (0.01 – 1 mM, panel B) or SLIGRL (0.01 – 1 mM, panel C) for 48h. Increases in cell proliferation were assessed spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 2 further experiments.

Summarising the data presented in Sections 3.1 and 3.2, tryptase was found to induce lung fibroblast proliferation. These responses were dependent on its catalytic activity suggesting that it may be acting via a protease activated receptor. Tryptase has been postulated to activate PAR-2 (Corvera et al., 1997; Fox et al., 1997; Molino et al., 1997b). However, the literature suggested that fibroblasts, at least dermal fibroblasts, did not express PAR-2 (Belham et al., 1996; Santulli et al., 1995). Therefore in Section 3.3 I went on to determine if lung fibroblasts express PAR-2 using an antisera that cross-reacts with human PAR-2. I
was able to show that, similar to the published data, dermal fibroblasts express PAR-2 at very low levels, whereas, the receptor is highly expressed in lung fibroblasts. In Section 3.4 I demonstrate that activation of PAR-2 on lung fibroblasts by the known PAR-2 activators, trypsin and the PAR-2 activating peptides, induced cell proliferation. Therefore the circumstantial evidence would suggest that tryptase does activate PAR-2 in lung fibroblasts to induce mitogenesis. However, there are no antagonists of PAR-2 currently available to prove this, although an antibody is available (rabbit anti-rat PAR-2 antisera used in the localisation studies, Section 3.3) which has been generated against the tethered ligand of the rodent PAR-2. It is not known whether these are neutralising antibodies that bind to the peptide sequence and blocks the interaction of the tethered ligand with the PAR-2 activation site. Therefore, experiments were performed in which the interactions of tryptase and trypsin with the PAR-2 antibodies were studied.

3.5 Effect of rat PAR-2 antisera on tryptase- and trypsin-induced human foetal lung fibroblast proliferation.

In these studies, the mitogenic responses of human foetal lung fibroblasts, at various time points, following exposure to tryptase or trypsin, were evaluated in the absence or presence of the rabbit anti-rat PAR-2 polyclonal antisera.

The PAR-2 antisera caused concentration-dependent inhibition of both tryptase- and trypsin-induced cell proliferation (Figure 3.23). After a total agonist exposure time of 48h, a 1:2000 dilution of PAR-2 antisera partially inhibited the effects of tryptase (63.3 ± 4.1%, P<0.01), whereas it completely inhibited the effects of trypsin (100 ± 17.5%, P<0.01) (Figure 3.23). Partial inhibition was also observed when cells were incubated with tryptase for only 5 min and 24hr (44.9 ± 11.4%; 35.8 ± 9.9%, respectively, P<0.01).
Figure 3.23  Effect of PAR-2 antisera on tryptase- and trypsin-induced human foetal lung fibroblast proliferation. Cells were incubated with PAR-2 antisera for 60 min at 37°C prior to addition of single concentration of either tryptase (17.6 mU/ml) or trypsin (10nM). Increases in cell proliferation were assessed spectrophotometrically as described in Section 2.4.1.2. Data shown is a mean ± SEM of 6 observations from an individual experiment. Similar results were obtained in 3 further experiments.

As demonstrated in Figure 3.4, tryptase-induced fibroblast proliferation is time dependent. It is able to induce proliferation after being in contact with the cells after only 5 min exposure. To investigate whether these early responses were mediated by activation of PAR-2, confluent cells were incubated with 1:2000 dilution of PAR-2 antisera, for 60 min at 37°C, prior to the addition of trypase for various lengths of time. Figure 3.24 shows that the PAR-2 antisera can partially inhibit the effects of trypase at all time points studied. After
exposure times of 10 min, 8h, 24h and 48h, the PAR-2 antisera inhibited the tryptase responses by approximately 25, 35.0, 45 and 50% respectively (Figure 3.24).

These experiments demonstrate the ability of the PAR-2 antisera used in the localisation studies to act as a neutralising antiserum. The complete blockade of the trypsin-induced fibroblast proliferation by the 1:2000 dilution of the antisera suggests that these responses are solely mediated by activation of PAR-2. However, the partial inhibition of the tryptase-induced responses by the same concentration of antisera, suggests that these responses may be only partially mediated via PAR-2.

With the demonstration of PAR-2 on the cell surface of cultured lung fibroblasts, isolated from normal tissue, it is important to establish whether fibroblasts/myofibroblasts in situ
express PAR-2. Therefore, the following section investigates the localisation of PAR-2 in airway biopsy samples from normal, non-asthmatic patients who had died of non respiratory events, and attempts to co-localise this expression with a marker of fibroblast stress fibres, rhodamine phalloidin. This section also investigates the expression of PAR-2 in bronchial biopsy tissue from patients who have been diagnosed with asthma. Finally, PAR-2 expression is studied on cultured fibroblasts derived from both asthmatic and non-asthmatic bronchial airways.

### 3.6 Localisation of PAR-2 in airway biopsy samples from normal non-asthmatic patients

PAR-2 was localised in tissue samples obtained at autopsy from patients who had died of non-respiratory causes. PAR-2 in the airway tissue samples was localised with anti-rat PAR-2 antisera conjugated with FITC, which in some samples was co-localised with the F-actin microfilament stain, rhodamine phalloidin using confocal microscopy.

Frozen airway tissue was cut into 7 and 60 μm sections and fixed in ice-cold acetone to investigate which would be the best thickness to use to study the distribution of PAR-2 in this tissue. 60μm sections were found to be too thick as any antibody staining would be masked by the high degree of auto-fluorescence. Therefore all the subsequent studies were performed on 7μm sections.

Auto-fluorescence, by its nature, can been detected over a wide range of wavelengths, including those used to identify FITC (green channel, 488 nm) and rhodamine phalloidin (red, channel, 568 nm). Figure 3.25 shows a study in which images were obtained from tissue samples demonstrating auto-fluorescence at 488 nm (Figure 3.25A) and an image in which the auto-fluorescence at 568 nm was subtracted from that obtained at 488 nm (Figure
Thus demonstrating the auto-fluorescence seen at the two wave lengths is from the same source, the collagen fibrils.

**Figure 3.25**  **Auto-fluorescence of human airway tissue.** Human airway tissue (7µm), fixed in ice-cold acetone, was incubated in PBS overnight at 4°C and visualised using confocal microscopy. Images demonstrate the auto-fluorescence produced at a wavelength of 488nm by human airway tissue (A) and the ability to reduce it by subtraction of the auto-fluorescence at 568nm from that at 488nm (B).

3.6.1.1  *Localisation of PAR-2 in human bronchus*

PAR-2 was localised to the airway epithelium (Figure 3.26) and to sub-mucosal glands (Figure 3.27) as shown by the intense green (FITC) staining of the tissue. The anti-rat PAR-2 antiserum was co-localised with rhodamine phalloidin as shown by the orange or yellow staining in a number of sections (Figure 3.28). This staining was present below the epithelium (Figure 3.28A & C) and within extracellular matrix, identified as collagen (Figure 3.28E & F). Figures 3.28B & D highlight further the co-localisation. In these figures, the top left panel is the fluorescence at 488nm (green channel - FITC), the top right hand panel is the fluorescence at 568nm (red channel – rhodamine phalloidin), the bottom left panel is the combined green and red images. Finally, the bottom right image is a computer generated image, where both FITC and rhodamine phalloidin are co-localised. The scale bar on the right hand side of this last image is an arbitrary measure of intensity of co-localisation (yellow high intensity, blue low intensity).
Figure 3.26  Localisation of PAR-2 to bronchial epithelium. Tissue was cut into 7μm sections, fixed in ice-cold acetone and incubated with rabbit anti-rat PAR-2 antisera (1:500 dilution) overnight at 4°C. The slides were washed and then incubated with swine anti-rabbit antibody conjugated to FITC. The FITC labelled PAR-2 receptors (green) were visualised using confocal microscopy. Panel A shows the staining as visualised in the green channel (488nm), whereas, panel B shows the same image where the auto-fluorescence in the red channel (568nm) has been subtracted from the image.

Figure 3.27  Localisation of PAR-2 in bronchial sub-mucosal glands. Tissue was cut into 7μm sections, fixed in ice-cold acetone and incubated with rabbit anti-rat PAR-2 antisera (1:500 dilution) overnight at 4°C. The slides were washed and then incubated with swine anti-rabbit antibody conjugated to FITC. The FITC labelled PAR-2 receptors (green) were visualised using confocal microscopy.
Figure 3.28  Co-localisation of PAR-2 and fibroblast F-actin fibres in human airway. 7μm sections of human airway were fixed in ice-cold acetone, followed by incubation with anti-rat PAR-2 antisera overnight at 4°C. After washing, the sections were incubated with rhodamine phalloidin (1:100 dilution) for 60 min followed by swine anti rabbit IgG conjugated to FITC (1:40 dilution) for 60 min in a dark, humidified chamber. Images were taken at 1μm intervals through each section using a confocal microscope and re-constructed to give a pseudo-3D image. Panel A and C shows the co-localisation of PAR-2 and F-actin microfilament staining in cells below the epithelial basement membrane. The co-localisation in these sections is demonstrated in panels B & D. In these figures, the top left panel is the fluorescence at 488nm (green channel - FITC), the top right hand panel is the fluorescence at 568nm (red channel – rhodamine phalloidin), the bottom left panel is the combined green and red images. Finally, the bottom right image is a computer generated image, where both FITC and rhodamine phalloidin are co-localised. The scale bar on the right hand side of this last image is an arbitrary measure of intensity of co-localisation (yellow high intensity, blue low intensity). Panel E & F show co-localisation in cells present within the collagen fibrils.
The co-localisation of PAR-2 to cells that express F-actin microfilament and the location of these cells to an area below the epithelial basement membrane (lamina reticularis) suggests these cells to be myofibroblasts. These cells are also present within the extracellular matrix within the airway wall. Using the auto-fluorescent nature of this tissue, Figure 3.29 clearly shows a fibroblast between the epithelial layer and the deeper sub-mucosa and adventitia of the airway wall. This image was generated by the computer using Simulated Fluorescent Processing (SFP). This is where the incident laser light hits the tissue at an angle and the emitted light is captured at an angle to give the impression of shadowing of the tissue.

In control studies sections were incubated with rabbit IgG antibodies, as the isotype of IgG antibody within the rabbit antisera was unknown, and PBS prior to addition of the secondary FITC conjugated antibodies. No fluorescent staining was evident using confocal microscopy. In addition, staining controls were done using rabbit anti-rat PAR-2 antisera pre-absorbed for 60 min with the antigenic peptide used to generate the antibodies. Again no fluorescent staining was observed using confocal microscopy.
3.6.1.2  *Localisation of PAR-2 in bronchial biopsies from patients with asthma*

With the localisation of PAR-2 in epithelium, submucosal glands and fibroblasts in normal human bronchial tissue, it was of interest to study the localisation in bronchial tissue from patients with asthma. In particular, to investigate whether there was a change in PAR-2 expression on the epithelium and fibroblasts within the lamina propria compared to normal tissues. A pilot study was performed with biopsy material kindly supplied to me by Dr Tudor Toma (Spitalul Clinic Universitar de Pneumoftiziologic Iasi, Romania) which had been obtained from patients diagnosed with asthma. In the previous study (Section 3.6.1.1) the tissues obtained from post-mortem patients allowed transverse sections of the airway wall to be used. However, for this study the depth of these samples was approximately 1 mm, therefore, only the mucosa, the lamina reticularis and small areas of the sub-mucosa could be studied. In these sections, PAR-2 was localised using standard two-layer immunohistochemical techniques with antibody binding visualised with 3,3′-diaminobenzidine (DAB, brown) staining. PAR-2 was localised to the epithelium and to spindle-shaped cells beneath the epithelial basement membrane of the airway wall (Figure 3.30B & C).


Figure 3.30  Localisation of PAR-2 in human airway biopsies from asthmatic patients. Sections were initially incubated with anti-rat PAR-2 antisera for 16 overnight at 4°C. A second biotinylated antibody was applied for 60 min followed by incubation with streptavidin-alkaline phosphatase. The antibody binding was visualised using the phosphatase substrate, DAB, which gave a brown staining. Panel A is a non-specific rabbit IgG control showing no staining with DAB. Panels B and C show specific staining of PAR-2 in epithelial cells and spindle-shaped cells below the basement membrane, respectively, as denoted by the arrows. Final magnification x 1000.

The spindle-shaped cells were further characterised in parallel sections by staining with α-smooth muscle actin or vimentin, two antibodies used in the identification of fibroblasts. In Figure 3.31, panel A shows the lack of non-specific staining. Panel B shows the staining of the actin filaments in smooth muscle bundles by α-smooth actin antibodies (arrow heads), smooth muscle of arterioles (large open arrows) and spindle-shaped cells beneath the epithelial basement membrane (large closed arrow). Panel C shows the staining of spindle-shaped cells by vimentin (large closed arrow). From the staining with α-smooth muscle actin and vimentin antibodies and their location, the spindle-shaped cells can be classified as fibroblasts with α-smooth muscle actin filaments i.e. myofibroblasts.
Figure 3.31 Characterisation of spindle-shaped cells in human airway biopsy sections. Sections were initially incubated with non specific antibodies (A), α-smooth muscle actin antibodies (B) or vimentin antibodies (C) at room temperature for 60 min. A second biotinylated antibody was applied for 60 min followed by incubation with streptavidin-alkaline phosphatase. The antibody binding was visualised using the phosphatase substrate, DAB, which gave a brown staining. The final magnification for panel A and B were x400 and x1000 respectively. Panel C was visualised by interference contrast microscopy at a final magnification of x1000.

Following the localisation of PAR-2 to fibroblasts/myofibroblasts within biopsy section of patients with and without asthma, the expression of PAR-2 on fibroblasts which have been isolated from a similar group of patients was investigated. These studies used a series of well characterised fibroblast cell lines kindly given to me by Dr Jamila Chakir (Laval Hospital Research Centre, Quebec, Canada, (Tremblay et al., 1998b; Tremblay et al., 1998a).
3.6.1.3 *Localisation of PAR-2 in fibroblasts isolated from bronchial biopsy samples*

Fibroblasts isolated from bronchial biopsies obtained from asthmatic and non-asthmatic patients were cultured until approximately 60% confluent. The cells were fixed and stained as described in Section 2.5.2.1. PAR-2 was localised on the cell surface of the fibroblasts isolated from both asthmatic and non-asthmatic airway (Figure 3.32). There was no visual evidence of increased staining associated with either the asthmatic or non-asthmatic derived fibroblast. Similar results were obtained in a total of 5 cell lines derived from asthmatic airways and three cell lines derived from non-asthmatic airways.

Thus in summary, this section has shown that PAR-2 is expressed on the cell surface of fibroblasts isolated and cultured from normal human lung and airway. PAR-2 is also localised to fibroblasts within the lamina reticularis and extracellular matrix of normal human bronchus. In pilot studies, although PAR-2 was expressed on fibroblasts derived from bronchial biopsy samples from asthmatic patients, there appeared to be no difference in the expression when compared to fibroblasts isolated from non-asthmatic patients.
Figure 3.32  Localisation of PAR-2 in airway fibroblasts from asthmatic and non-asthmatic airways. Fibroblasts isolated from bronchial biopsies from asthmatic and non-asthmatic patients were cultured and stained with anti-rat PAR-2 antisera. PAR-2 localisation was demonstrated by FITC fluorescence and visualised using confocal microscopy in bronchial fibroblasts from asthmatic (A) and non-asthmatic (B) patients. Panel C shows the lack of fluorescent staining after incubation with non-specific rabbit IgG antibodies. Arrows denotes apparent enhanced staining of PAR-2 at the cell surface where a number of optical sections (8 x 1μm) through the cell have been overlaid.
3.7 The effects of tryptase and protease activated receptor activating peptides on extracellular matrix metabolism.

The effects of tryptase on extracellular matrix synthesis have not been extensively studied. In dermal fibroblasts, tryptase has been shown to induce type I procollagen mRNA and to increase the levels of collagen protein in cell culture supernatants, using an enzyme-linked immunosorbent assay (ELISA) for type I procollagen C-peptide (Abe et al., 1998; Gruber et al., 1997). In addition, tryptase has been demonstrated to increase collagen production measuring $^3$H-proline incorporation into the collagen molecule in a human lung cell line, MRC-5 (Cairns and Walls, 1997). To the best of my knowledge, to date, no studies have been reported on the effects of tryptase on proteoglycan production in human lung fibroblasts. In addition, the effects of the PAR-2 activating peptide on procollagen and proteoglycan metabolism in human fibroblasts have not been reported.

Procollagen gene expression was studied by measuring the effects of tryptase and the PAR-2 activating peptides on procollagen gene promoter activity in lung fibroblasts that had been transiently transfected with a human procollagen $\alpha_2$(I) gene promoter linked to a luciferase reporter gene and procollagen type I mRNA levels in non-transfected fibroblasts by Northern analysis.

Studies were also performed to investigate the effects of tryptase and a PAR-2 activating peptide on procollagen protein and proteoglycan metabolism in human foetal lung fibroblasts. Hydroxyproline is present in relatively few proteins, including procollagen. The level of hydroxyproline in intact procollagen molecules and free hydroxyproline, from low molecular weight procollagen degradation products, can be used as a biomarker of procollagen metabolism. The amounts of hydroxyproline synthesised and degraded by cells in culture are relatively small, therefore, to study the agonists effects on these levels, a highly sensitive assay method using High Pressure Liquid Chromatography (HPLC) was employed.
The methods used to evaluate the effects of tryptase on proteoglycan production in human foetal lung fibroblasts were similar to those reported by Chambers and colleagues (Chambers et al., 1994).

3.7.1 Effect of tryptase and PAR-2 activating peptide, SLIGKV on procollagen α2(I) gene promoter activity

The activation of the procollagen gene has previously been studied by using a procollagen α2(I) promoter linked to either a chloramphenicol acetyl transferase (CAT) or luciferase genes (Boast et al., 1990; Inagaki et al., 1994). Transforming growth factor-β causes a 5-23-fold increase in the reporter gene activity associated with this construct and a parallel increase in procollagen mRNA expression (Kahari et al., 1990). To investigate further the role of tryptase and the PAR-2 activating peptide, SLIGKV, on procollagen gene activation, the procollagen α2(I) promoter construct, linked to the luciferase gene, was transfected into human foetal lung fibroblasts and luciferase activity measured after 24h exposure to either agonist (See section 2.6.1).

Tryptase caused concentration-dependent increases in luciferase activity compared with media alone (Figure 3.33A). SLIGKV, 1 mM, also induced an 85.1 ± 9.17% increase in luciferase activity compared to media alone (Figure 3.33B), whereas the control peptide, LSIGKV (1mM), had no significant effect (Figure 3.33B). Trypsin (10nM) induced a 72.9% ± 4.63% increase in the luciferase activity (media alone: 756.1 ±47.95; trypsin 10nM: 1358.3 ± 36.41 RLU). In a separate experiment the known procollagen gene activator, TGFβ induced a 230% increase in luciferase activity compared with media alone (media: 1693 ± 261.5 RLU; TGFβ, 1ng/ml, 3892 ± 462.3 RLU). These data suggest that the serine proteases, tryptase and trypsin, and the PAR-2 activating peptide, SLIGKV, are able to induce procollagen gene promoter activity, possibly via the activation of PAR-2. However, the responses induced by these agonists are considerably lower than that seen with TGFβ.
The activation of a gene promoter does not ensure gene transcription. Therefore the effects on procollagen mRNA levels were studied using Northern analysis in the section 3.7.2.
Figure 3.33 Effect of tryptase and PAR-2 activating peptides on the human procollagen α2(I) gene promoter. Human foetal lung fibroblast were grown to 80% confluence and then transfected with the procollagen α2(I) gene promoter construct. The transfected cells were exposed to concentrations of tryptase (0.7 - 17.6 mU/ml, A) or SLIGKV (1 mM, B) for 24 h, prior to the evaluation of α2(I) gene promoter activity by assaying luciferase activity. Data presented is from a representative experiment and each data point is a mean of at least 3 individual wells. Data was repeated in a further study.
3.7.2 Effect of tryptase, SLIGKV, and TGFβ on steady state levels of α1(I) procollagen mRNA

In a preliminary experiment, total RNA was extracted from confluent human foetal lung fibroblasts exposed to tryptase (35 or 70 mU/ml), SLIGKV (1 mM), LSLGKV (1 mM) or TGFβ (1 ng/ml) for 24h. The RNA was subjected to agarose gel electrophoresis and hybridised with cDNA probe for α1(I) procollagen. The resulting Northern blot was visualised using a phosphoimager and the resulting image analysed by densitometry with each band normalised to 28 S rRNA (Figure 3.34 and 3.35). Tryptase, 35 or 70 mU/ml, did not cause a significant increase in α1(I) procollagen mRNA expression (Tryptase: 35 mU/ml, +6%, P=0.26; 70 mU/ml, +9%, P=0.19), whereas TGFβ (1 ng/ml) caused a significant 115% increase in levels compared to media alone (P<0.01). SLIGKV (1 mM) tended to increase α1(I) procollagen mRNA (control, 0.73 ±0.05 densitometry units, SLIGKV 1mM, 0.93 ±0.18 densitometry units) but this failed to reach significance (P=0.22). The levels after treatment with the control peptide, LSIGKV (1 mM), was not significantly different from media control (+8%, P=0.39). There was no significant difference in 28 S rRNA expression after any treatment (P>0.12). These data suggest that neither tryptase nor SLIGKV increase the steady state levels of α1(I) procollagen mRNA, whereas, TGFβ induced at least a doubling in the mRNA levels.
Figure 3.34 Effect of tryptase and TGFβ on α1(I) procollagen mRNA expression. Total RNA was extracted from confluent human foetal lung fibroblasts after exposure to tryptase (35 or 70 mU/ml) or TGFβ (1 ng/ml) for 24 h. The RNA was subjected to agarose gel electrophoresis and hybridised with a cDNA probe for α1(I) procollagen. The upper panels show representative Northern blots for human α1(I) procollagen, demonstrating two mRNA transcripts (4.8 and 5.8 kb) and 28 S rRNA (A and B, respectively). The lower panel shows the analysis of the blots by densitometry (C). The data for both α1(I) procollagen transcripts (4.8 and 5.8 kb) were combined before normalising for 28 S rRNA expression. These values are means ± SEM from three replicate cultures. * P <0.05 compared to untreated control.
Figure 3.35 Effect of SLIGKV on α1(I) procollagen mRNA expression. Total RNA was extracted from confluent human foetal lung fibroblasts after exposure to SLIGKV (1 mM) or LSIGKV (1 mM) for 24 h. The RNA was subjected to agarose gel electrophoresis and hybridised with a cDNA probe for α1(I) procollagen. The upper panels show representative Northern blots for human α1(I) procollagen and 28S rRNA (A and B, respectively). The lower panel shows the analysis of the blots by densitometry (C). The data for both α1(I) procollagen transcripts (4.8 and 5.8 kb) were combined before normalising for 28 S rRNA expression. The values are means ± SEM from three replicate cultures.
3.7.3 Effects of tryptase on collagen synthesis and degradation by human foetal lung fibroblasts

Human foetal lung fibroblasts were grown to visual confluence and treated with tryptase (17.6 and 35 mU/ml) for periods of 24h and 48h. As the cell monolayer contains a small amount of procollagen, the amount of hydroxyproline present in the combined culture medium and cell layer at the start of incubation was determined in both the ethanol-soluble and -insoluble fractions. This background level, which represented 0.57 ± 0.03 nmol hydroxyproline per 10^6 cells in the ethanol-insoluble fraction and 0.13 ± 0.06 nmol hydroxyproline per 10^6 cells in the ethanol soluble fraction, was subtracted from the sample values.

The hydroxyproline found in the ethanol-insoluble fraction was taken to represent hydroxyproline in procollagen and the hydroxyproline present in the soluble-fraction from each culture was taken to represent hydroxyproline derived from the degradation of procollagen during the culture period. Total procollagen production was estimated by the addition of the hydroxyproline content in the ethanol-insoluble fraction and the hydroxyproline content of the ethanol-soluble fraction.

As part of the assay, cell numbers were estimated by direct cell counting at the beginning of the incubation period and following 24h and 48h incubation with media alone and tryptase. Tables 3.3 and 3.4 demonstrate that tryptase induced cell proliferation, which was both concentration and time dependent. Therefore, the increases in cell numbers were taken into consideration when calculating the amounts of hydroxyproline measured.
Table 3.3  Effect of 24h treatment of tryptase on human foetal lung fibroblast proliferation. Cells were grown to visual confluence and then treated with media alone or tryptase. Cell numbers were assessed by visual counting using a haemocytometer after 24h. Data is also shown comparing the percentage changes in cell numbers after treatment, with those prior to addition of the agonist (T0), and after 24h treatment (T24). Data are expressed as mean and SEM of cell numbers from at least 4 repeats an individual experiment. Statistical significance is shown for data compared with media alone at T0 and T24. ns denotes not significant.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>P vs. T0</th>
<th>P vs. T24</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>111094</td>
<td>9414</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>106667</td>
<td>4024</td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Tryptase 17.6 mg/ml</td>
<td>147500</td>
<td>6250</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+32.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T24</td>
<td>+38.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptase 35mU/ml</td>
<td>151667</td>
<td>7129</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+36.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T24</td>
<td>+42.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4  Effect of 48h treatment with tryptase and TGFβ on human foetal lung fibroblast proliferation. Cells were grown to visual confluence and then treated with media alone, tryptase or TGFβ. Cell numbers were assessed by visual counting using a haemocytometer after 48h. Data is also shown comparing the percentage changes in cell numbers after treatment, with those prior to addition of the agonist (T0), and after 48h treatment (T48). Data are expressed as mean ± SEM of cell numbers from at least 3 repeats from an individual experiment. Similar results were found in 4 other experiments. Statistical significance is shown for data compared with media alone at T0 and T48. P<0.05 was considered significant. ns denotes not significant.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>P vs. T0</th>
<th>P vs. T48</th>
</tr>
</thead>
<tbody>
<tr>
<td>To</td>
<td>163457</td>
<td>17864</td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>T48</td>
<td>133568</td>
<td>13892</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptase 17.6 mU/ml</td>
<td>216250</td>
<td>10327</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+32.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T48</td>
<td>+61.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptase 35 mU/ml</td>
<td>246648</td>
<td>27610</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+50.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T48</td>
<td>+84.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ 1ng/ml</td>
<td>149464</td>
<td>11110</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>-8.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T48</td>
<td>+11.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There was a significant increase in the basal procollagen production in the presence of media after 24h and 48h incubation at 37°C in 10% CO₂ in air (hydroxyproline, nmol per 10^6 cells: 24h, 2.14 ± 0.08; 48h, 4.62 ± 0.13, P<0.01). Tryptase did not induce procollagen synthesis at the concentrations tested compared with media alone, after either 24h or 48h treatment, in fact, a significant decrease in the amount of procollagen was observed (Figure 3.36 and 3.37). The same concentrations of tryptase did not affect procollagen degradation as assessed by the hydroxyproline content (P>0.05, Figure 3.36). However, tryptase (17.6 and 35 mU/ml) did induce significant decreases in the amount of procollagen degradation per 10^6 cells following 48h treatment (Figure 3.37).
Figure 3.36 Effect of 24h treatment with tryptase on procollagen metabolism by human foetal lung fibroblasts. Cells were grown to visual confluence and then treated with tryptase (17.6 or 35 mU/ml) for 24 h. Panel A shows the amount of hydroxyproline, per \(10^6\) cells, in the ethanol-insoluble fraction (procollagen) of the culture supernatant. Panel B show the amount of hydroxyproline, per \(10^6\) cells, in the ethanol-soluble degraded procollagen). Whereas Panel C shows the total amount of procollagen produced by the cell culture over the 24h incubation period. Each value represents the mean ± SEM from 4-6 data points from an individual experiment. * P<0.05.
Figure 3.37 Effect of 48h treatment with tryptase on procollagen metabolism by human foetal lung fibroblasts. Cells were grown to visual confluence and then treated with tryptase (17.6 or 35 mU/ml) for 48 h. Panel A shows the amount of hydroxyproline, per $10^6$ cells, in the ethanol-insoluble fraction (procollagen) of the culture supernatant. Panel B shows the amount of hydroxyproline, per $10^6$ cells, in the ethanol-soluble degraded procollagen). Whereas Panel C shows the total amount of procollagen produced by the cell culture over the 48h incubation period. Each value represents the mean ± SEM from 4-6 data points from an individual experiment. * P< 0.05. Similar effects were observed in 4 further experiments.

To demonstrate these human lung fibroblasts could be stimulated to produce procollagen, cells were incubated with TGFβ (1 ng/ml) for 48h and the hydroxyproline production measured. TGFβ caused a 533% increase in procollagen production compared with media.
alone (basal levels: 1.36 ± 0.40 nmol hydroxyproline per 10^6 cells, TGFβ 1 ng/ml: 7.26 ± 0.36 nmol hydroxyproline per 10^6 cells).

3.7.4 Effect of PAR-2 activating peptide on procollagen metabolism in human foetal lung fibroblasts

After 24h treatment, the PAR-2 activating peptide, SLIGKV, and the control peptide, LSIGKV, had no affect on fibroblast cell numbers in this assay (Table 3.5).

Table 3.5 Effect of 24h treatment of SLIGKV on human foetal lung fibroblast proliferation. Cells were grown to visual confluence and then treated with media alone, SLIGKV or LSIGKV. Cell numbers were assessed by visual counting using a haemocytometer after 24h. Data is also shown comparing the percentage changes in cell numbers after treatment, with those prior to addition of the agonist (T0), and after 24h treatment (T24). Data are expressed as mean and SEM of cell numbers from at least 4 individual wells. Statistical significance is shown for data compared with media alone at T0 and T24. ns denotes not significant.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>P vs T0</th>
<th>P vs T24</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>106666</td>
<td>4024</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>111093</td>
<td>9414</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLIGKV 1mM</td>
<td>112500</td>
<td>1909</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+5.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T24</td>
<td>+1.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSIGKV 1mM</td>
<td>104583</td>
<td>4104</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>-1.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs.T24</td>
<td>-5.90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Neither SLIGKV (1 mM) or LSIGKV (1mM) had an effect on procollagen synthesis per 10^6 cells, following 24h treatment (Figure 3.36). Although, SLIGKV (1mM) did induce a decrease in the amount of hydroxyproline per 10^6 cells, no significant change in the total amount of hydroxyproline per 10^6 cells was observed (Figure 3.38).
Figure 3.38 Effect of 24h treatment with PAR-2 activating peptide, SLIGKV on procollagen metabolism by human foetal lung fibroblasts. Cells were grown to visual confluence and then treated with SLIGKV or LSIGKV (1mM) for 24 h. Panel A shows the amount of hydroxyproline, per 10^6 cells, in the ethanol-insoluble fraction (procollagen) of the culture supernatant. Panel B shows the amount of hydroxyproline, per 10^6 cells, in the ethanol-soluble degraded procollagen. Whereas Panel C shows the total amount of procollagen produced by the cell culture over the 24h incubation period. Each value represents the mean ± SEM from 4-6 data points from an individual experiment. * P< 0.05. Similar effects were observed in 3 further experiments.

Following 48h treatment SLIGKV, 1 mM, caused a significant increase in cell numbers, whereas LSIGKV had no affect (Table 3.6). Similar to the 24h data, SLIGKV had no affect on the hydroxyproline content of the ethanol-insoluble fraction, or the total procollagen.
synthesis from the cell culture (Figure 3.39), but induced a significant decrease in the hydroxyproline content of the ethanol-soluble fraction of the culture media (Figure 3.39).

Figure 3.39 Effect of 48h treatment with PAR-2 activating peptide, SLIGKV on procollagen metabolism by human foetal lung fibroblasts. Cells were grown to visual confluence and then treated with SLIGKV or LSIGKV (1mM) for 48h. Panel A shows the amount of hydroxyproline, per 10⁶ cells, in the ethanol-insoluble fraction (procollagen) of the culture supernatant. Panel B show the amount of hydroxyproline, per 10⁶ cells, in the ethanol-soluble degraded procollagen). Whereas Panel C shows the total amount of procollagen produced by the cell culture over the 48h incubation period. Each value represents the mean ± SEM from 4-6 data points from an individual experiment. * P< 0.05. Similar effects were observed in 3 further experiments.
Table 3.6  Effect of 48h treatment of SLIGKV on human foetal lung fibroblast proliferation. Cells were grown to visual confluence and then treated with media alone, SLIGKV or LSIGKV. Cell numbers were assessed by visual counting using a haemocytometer after 48h. Data is also shown comparing the percentage changes in cell numbers after treatment, with those prior to addition of the agonist (T0), and after 48h treatment (T48). Data are expressed as mean and SEM of cell numbers from at least 4 individual wells. Statistical significance is shown for data compared media alone at T0 and T48.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>P vs T0</th>
<th>P vs T48</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>106666</td>
<td>4024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T48</td>
<td>96875</td>
<td>7212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLIGKV 1mM</td>
<td>112500</td>
<td>1909</td>
<td>ns</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>+5.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T48</td>
<td>+16.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSIGKV 1mM</td>
<td>104583</td>
<td>4103</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>% change vs. T0</td>
<td>-1.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change vs. T48</td>
<td>+7.95</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To summarise this section, tryptase failed to induce an increase in procollagen production after either 24 or 48h incubation. However, it was able to induce a concentration dependent increase in cell number during the culture period. After 24h treatment tryptase had no significant effect on the degradation of newly synthesised procollagen, whereas, it induced a concentration-dependent decrease in degradation after 48h. Finally, tryptase had no significant effects on total procollagen synthesis at 24h, but induced significant decreases after 48h pretreatment. These data suggest that tryptase does not induce increases in procollagen synthesis in human foetal lung fibroblasts. The PAR-2 activating peptide, SLIGKV, had no significant effect on procollagen production at either 24 or 48h. However, it did induce significant decreases in procollagen degradation at both time points, but not enough to cause significant changes in total procollagen synthesis. It is interesting to note that the control peptide, LSIGKV, caused significant decreases in procollagen production, degradation and total synthesis. These data suggest that the PAR-2 activating peptide can influence the degradation of procollagen but this may not to be via the activation of PAR-2.
Chapter 4

4 Discussion

4.1 Proliferation studies

4.1.1 Tryptase-induced fibroblast proliferation

Human mast cell tryptase was shown to be a potent mitogen for lung fibroblasts. It caused concentration-dependent increases in proliferation of human foetal lung fibroblasts (Figure 3.1) and adult parenchymal and airway fibroblasts (Figure 3.5) as measured by both indirect (spectrophotometric assay) and direct (visual cell counts) methods.

However, there was a discrepancy between the spectrophotometric data and the visual counts, such that the spectrophotometric data underestimated the number of cells. Methylene blue is a basic dye that is positively charged at pH8.5. It binds electrostatically to negatively charged groups within cells, in particularly the phosphate groups of nucleic acids and some of the charged groups of proteins.

Possible reasons for the underestimation of the cell numbers in this assay may be due to practical considerations. The assay can only be used with cells that are strongly adherent, any cells that are weakly bound to the culture plates would be lost during the washing phases of the assay. In addition, tryptase has trypsin-like effects, inducing cells to round and be lost from culture plate. From examinations of the cultures this only appeared to occur at the highest concentrations of tryptase used. These effects may offer an explanation for the maximal effects of tryptase seen in the spectrophotometric assay in which a maximal response appeared to have been reached, since at this concentration there may have been some cell loss. Within these studies, all the proliferation assays were performed on confluent cultures of cells that do not exhibit contact inhibition. The addition of tryptase stimulates cell proliferation, inducing the formation a multi-layer culture. This may restrict
the access of methylene blue to the cell within the cell layer, thus reducing the estimation of
the cell number, or the cells on the upper layers may be weakly adherent and are lost.

Another possibility is that if a large percentage of the cells are proliferating, the absorbance
may be reduced due to the cells rounding up during metaphase of mitosis. However, this is
not thought to be a significant problem, since in rapidly proliferating cells, only 1% of the
cells would be in metaphase at any one point (Oliver et al., 1989). Also, as previously
mentioned, tryptase, like trypsin, can induce the rounding up of cells within its own right.
All these practical issues may lead to an underestimation of the cell number by the
spectrophotometric assay. Although the spectrophotometric assay underestimated cell
numbers, the trends were the same in both assays and therefore unlikely to affect the
conclusions of the study.

Previous studies have shown that tryptase can induce lung fibroblast mitogenesis in
confluent cultures (Ruoss et al., 1991, Hartmann et al., 1992, Cairns and Walls, 1997).
Ruoss and colleagues demonstrated that dog tryptase was able to induce $^3$H-thymidine
incorporation in Chinese hamster lung fibroblasts and Rat-1 lung fibroblasts that
corresponded to increased cell numbers (Ruoss et al., 1991). Tryptase was able to induce
mitogenesis in human primary lung fibroblasts and a lung fibroblast cell line, MRC-5, over
the concentration range 0.3 – 30 nM (Cairns and Walls, 1997; Hartmann et al., 1992). The
concentrations of tryptase at which activity was observed in the present study were
comparable to those previously reported, expressed in either units of enzymatic activity or
molar concentrations (Cairns and Walls, 1997; Hartmann et al., 1992; Ruoss et al., 1991).
They are also comparable to reported levels of tryptase in plasma and BAL fluid. In patients
who exhibited allergic reactions plasma tryptase levels ranged from 4 - 75 ng/ml, whereas, in
patients with mastocytosis tryptase levels were found to be as high as 88 ng/ml (Schwartz et
al., 1987a). Using an assumed molecular weight for tryptase of 134K kDa, and an average
specific activity of the commercially available tryptase of 10,000mU/mg, these
concentrations of tryptase, in terms of activity units used in this thesis, would be 0.03 – 0.6mU/ml. Similar concentrations have been found in BAL from symptomatic asthmatic patients and in atopic asthmatics following antigen challenge (13 ng/ml and 10 ng/ml, respectively, (Broide et al., 1991; Wenzel et al., 1988). These concentrations are 10-30 fold lower than the calculated concentrations used within the proliferation assays used in this present study. However, they represent greatly diluted concentrations of tryptase in plasma and BAL, whereas, the local concentration at the site of release would be expected to be many times greater due to the initially high amounts released and the smaller fluid volume surrounding the tissue. The concentration at the cell surface may therefore be similar to those used in these in vitro proliferation assays.

This concept has been demonstrated following nasal challenge with cold air or mannitol in atopic patients, in which high levels of tryptase (900ng/ml) could be induced in low volume nasal washings (Proud et al., 1992) and in a skin chamber model, in which an immediate cutaneous reaction was induced and the levels of tryptase (in excess of 300ng/ml) measured in a restricted fluid volume within the chamber (Schwartz et al., 1987b).

The response to tryptase has been further characterised and for the first time I have shown that tryptase was able to stimulate proliferation after only being in contact with the fibroblasts for 5 min (Figure 3.4) suggesting that tryptase is capable of directly stimulating the cells. However, these studies do not rule out the possibilities of tryptase releasing growth factors bound to the cell surface or the extracellular matrix deposited by the growing fibroblasts. Fibroblasts have been shown to produce a range of growth factors including TGFβ, IGF-1, PDGF-AA and PDGF-BB (see Akers et al., 2000; Powell et al., 1999 for reviews). Therefore to confirm any direct effects of tryptase on these cells, future studies need to be performed in the presence of inhibitors of these mediators e.g. neutralising antibodies, receptor antagonists, soluble receptors. In addition, the levels of these mediators
could be measured in the presence of tryptase, before and following treatment with highly selective tryptase inhibitors. This would establish whether tryptase was able to release such mediators from binding sites on the extracellular matrix.

The time course data shown in Figure 3.4 demonstrates a further increase in the mitogenic effects of tryptase after 24 h. It is tempting to suggest that this secondary increase was attributable to the release of a secondary growth factor stimulated by tryptase, however, there is no current evidence to suggest that tryptase can induce or stimulate the release of other growth factors. Although tryptase has been implicated in the activation of urinary plasminogen activator (uPA, Stack and Johnson, 1994).

The activation of uPA could initiate a sequence of events that would have the potential to induce fibroblast proliferation. Activated uPA converts plasminogen to plasmin, which in turn has been shown to activate TGFβ by cleaving the latency-associated peptide (LAP) from latent-TGFβ (Khalil et al., 1996). At low concentrations TGFβ can induce fibroblast proliferation, although at higher concentrations it has been shown to inhibit proliferation, which can be blocked by indomethacin suggesting a modulating role for PGE2 (McAnulty et al., 1997). The activation of the plasminogen-plasmin pathway has been shown in human lung fibroblasts and thus forms a plausible explanation (Idell et al., 1992; Knox and Crooks, 1988). However, there is no direct evidence for the release of plasminogen from cultured fibroblasts. Thus further studies will be required to investigate the role, if any, of this pathway in the activation of human fibroblast proliferation.

In pilot studies, the PAR-1 activator, thrombin, was shown to only stimulate fibroblast proliferation after being in contact with the cells for at least 24h (data not shown). This data is consistent with the findings that smooth muscle cell mitogenesis via the activation of
PAR-1 by thrombin induces the release of autocrine growth factors e.g. PDGF, FGF-2, EGF and TGFβ (see Molloy et al., 1996; Stouffer and Runge, 1998 for reviews). A similar pathway may be responsible for the further increase in proliferation observed in response to tryptase between 24 and 48h.

4.1.1.1 Inhibition of tryptase-induced fibroblast proliferation

The disruption of the enzyme structure, by heating the enzyme solution to 65°C for 60 min, induced a substantial reduction in the enzyme’s ability to induce fibroblast proliferation (Figure 3.6). However, at the highest enzyme concentration used (17.6 mU/ml), the effects were not totally abolished. Upon heating, the tryptase protein structure would be disrupted, destabilising its tetrameric structure, with the possible dissociation into dimers and monomers. The reason for this lack of total inhibition is unclear. However, it is possible that at this concentration of tryptase, the temperature and length of time exposed to the heat was not sufficient to totally inactivate the enzyme. In addition, during the 48h incubation period at 37°C, the tetrameric structure of tryptase may be able to reform, thus allowing the induction of a mitogenic response. Heat inactivation has been shown to inhibit tryptase-induced neutrophil and eosinophil chemotaxis in mouse peritoneum and epithelial mitogenesis (Cairns and Walls, 1996; He et al., 1997). However, it only partially inhibited tryptase-induced ³H-thymidine incorporation and IL-8 release in human umbilical vein endothelial cells (HUVEC, Compton et al., 1998).

Using the non-selective serine protease inhibitors, antipain and benzamidine, it was possible, to reduce the mitogenic effects of tryptase by 60-70%. This is consistent with the partial inhibition of tryptase-induced cleavage of the synthetic peptide substrate, tosyl-gly-pro-arg-p-nitroanilide demonstrated in this thesis. Benzamidine has also been shown to partially inhibit the proliferative responses to tryptase in a human lung fibroblast cell line, MRC-5
Similarly, it only partially inhibits tryptase-stimulated IL-8 release from HUVECs and a human epithelial cell line, H292 (Cairns and Walls, 1996; Compton et al., 1998). This could be interpreted as the remaining responses being due to a catalytic-independent mechanism or more likely reflects the concentration of the inhibitor used, as the concentration used in these assays (10μM) was lower than the published Ki from benzamidine against tryptase (12-25μM) (Burgess et al., 1999; Caughey et al., 1993; Sturzebecher et al., 1992). A 10-fold higher concentration of benzamidine (100μM) was tested in the proliferation assays but it induced a significant degree of cell loss (data not shown) and was therefore excluded from the data analysis.

Using the selective tryptase inhibitor, BABIM, a 5000-fold more potent inhibitor of tryptase than benzamidine (Caughey et al., 1993; Katz et al., 1998), both the mitogenic effects and the tryptase-induced cleavage of the synthetic peptide were totally inhibited (Figures 2.7 and 3.8). These data clearly demonstrate the mitogenic effects of tryptase are dependent on an active catalytic site. This conclusion was further strengthened by the use of the specifically designed tryptase inhibitor, APC366 (N-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride (Clark et al., 1995; Clark et al., 1996)). This compound totally inhibited the mitogenic effects of the enzyme. It is worth mentioning at this point that the slow inhibitory kinetics of this compound (Figure 3.9) compared with the effects of antipain, benzamidine and BABIM, is similar to that demonstrated in animals models (Clark et al., 1995; Clark et al., 1996) and within the clinic (Seife, 1997; Zhang and Timmerman, 1997). It is postulated this compound inhibits tryptase by the formation of a chemically reactive form of the inhibitor that binds slowly and irreversibly to the catalytic sites of tryptase (Clark et al., 1996).
A possible reason for the growth factor activity of the purified tryptase is an impurity present in the commercially prepared tryptase preparation. However, there are several lines of evidence that dispute this as a possible explanation. Firstly the inter-batch variation of the tryptase preparation with respect to the mitogenic effects were not significant, even though there was a variable total protein concentration within the samples. Secondly, recombinant human tryptase induced concentration-dependent increases in fibroblast proliferation, albeit at lower concentrations than the purified tryptase used (Figure 3.3). Recombinant tryptase has been shown to have similar properties to purified tryptase but to be more potent at cleaving a range of tryptase-specific synthetic substrates (Chan et al., 1999; Niles et al., 1998). Thirdly, the blocking of the proliferative responses and the potent inhibition of the cleavage of synthetic peptide substrate, tosyl-gly-pro-arg-p-nitroanilide, by the protease inhibitors, strongly suggests this not to be the case, and that tryptase, not a contaminant, mediated the mitogenic effect.

In contrast to the effects of the serine protease inhibitors, antipain, benzamidine, BABIM and APC366, the proposed endogenous inhibitors of tryptase, lactoferrin (Elrod et al., 1997) and secretory leukocyte proteinase inhibitor (SLPI, Wright et al., 1999) did not significantly inhibit the mitogenic effects of tryptase. Lactoferrin is a potent and selective inhibitor of tryptase-induced cleavage of a synthetic substrate with a Ki of 24nM. It is a member of the transferrin class of proteins, associated with neutrophil granules and is secreted from serous cells in the sub-mucosal glands (Jeffery, 1987; Marriott, 1989). It possesses high affinity binding sites for heparin and therefore may compete with other molecules for endogenous heparin. It has been suggested that the mechanism of tryptase inhibition by lactoferrin is by disruption of the enzyme’s tetrameric complex, due to its higher binding affinity for heparin. This competition for the endogenous heparin would cause the dissociation of the enzyme into it’s inactivate monomeric form. This inhibitory effect of lactoferrin can be reversed by the addition of excess heparin. The commercially supplied stock solution of tryptase used in
these studies included 20μM heparin. The concentration of tryptase used for these proliferation studies was a 1:20 dilution of this stock solution, giving a final heparin concentration of 1μM. The concentration of heparin shown to completely reverse the inhibition of tryptase by lactoferrin was 5μM (Elrod et al., 1997). It is therefore conceivable that in these studies, the excess heparin present in the media could bind to the high affinity sites on the lactoferrin molecule, reducing its effectiveness in binding to the tryptase-heparin complex. Further studies are required to confirm these findings. A recent publication has demonstrated that another neutrophil-associated protein, myeloperoxidase, can inhibit tryptase via a similar mechanism (Cregar et al., 1999).

Secretory leukocyte proteinase inhibitor tended to reduce the mitogenic effects of tryptase, however the responses did not reach statistical significance. At the concentrations used (1-100μM) SLPI would have been expected to totally inhibit the catalytic site of tryptase, since in an in-vitro enzyme assay, using vasoactive intestinal peptide (VIP) as the substrate, SLPI inhibited the catalytic activity of tryptase with a Ki of 0.34 nM. SLPI has a high affinity for heparin (Faller et al., 1992; Fath et al., 1998), which like lactoferrin and myeloperoxidase could compete for the heparin in the tryptase-heparin complex. In contrast to the effects of heparin on lactoferrin, the binding of heparin to SLPI increases it’s potency to inhibit serine proteases by inducing a favourable conformational change in the molecular structure (Faller et al., 1992; Fath et al., 1998). Thus, the reason for the limited inhibitory activity is unclear. It could be explained if SLPI, due to it’s size and structural conformation, could partially inhibit the catalytic activity of tryptase by blocking one catalytic site within the tetramer, leaving the remaining sites free. It is also possible that unlike lactoferrin, which is a non-competitive inhibitor, SLPI may be reversible and therefore over the 48 h incubation period tryptase may be able to reassert its enzyme activity. There is currently no mechanistic data available on how SLPI inhibits tryptase, therefore further studies are required to investigate this interaction.
These studies did not include positive controls for the proposed actions of lactoferrin or SLPI. A suitable assay would have to be employed to confirm the bioactivity of lactoferrin, such as inhibition of bacterial growth (Brock, 1995, Bellamy et al., 1992). However, as the concentration used in this assay were 400-fold greater than the Km for inhibition of enzymatic activity, it would have been expected to inhibit the tryptase-induced proliferation. Although there was a non-significant inhibition of tryptase-induced proliferation by SLPI, the data suggests that there may have been a degree of enzyme inhibition. Again to confirm that SPLI was active a suitable assay should be performed, for example an enzyme inhibition assay using trypsin. The data sheet supplied with the compound by the manufacturer claimed that SLPI was active at inhibiting trypsin at a concentration ration of 1:1 (Calbiochem, UK).

If it was confirmed that both lactoferrin and SLPI can inhibit tryptase, they could have a role in modulating the activity of the enzyme released from activated mast cells in the asthmatic airway. There are increased circulating levels of lactoferrin and myeloperoxidase in mild asthmatics, and in BAL of smokers and chronic bronchitis patients, which have been used to suggest an increase in the numbers of activated neutrophils present in these respiratory conditions (Kallenbach et al., 1992; Thompson et al., 1990). The concentration of lactoferrin in these patients is similar to its Ki (24nM) for tryptase, suggesting that, in atopic asthmatics, lactoferrin may be able to modulate the effects of tryptase. There is no comparable data for levels of SLPI in asthma, however, both molecules have been shown to reduce the late phase allergic reactions in animal models, which are generally believed to be mediated by mast cell degranulation (Elrod et al., 1997; Wright et al., 1999). The data from this current study does not support the inhibition of tryptase by lactoferrin or SLPI. However, the studies were complicated by the excess amounts of heparin present in the commercially acquired tryptase. Therefore, it would be necessary to redesign these experiments to take into consideration the presence of the heparin and to repeat the
proliferation assays along side inhibition studies using a synthetic peptide substrate. The interaction of tryptase with lactoferrin and SLPI may be important during the chronic phases of asthma, in which fibroblasts play a crucial role in the deposition of extracellular matrix during airway remodelling.

4.1.2 Tryptase-induced proliferation of dermal fibroblasts

In addition to the stimulation of lung fibroblast proliferation, tryptase was found to induce dermal fibroblast proliferation. This is consistent with previous work (Abe et al., 1998; Gruber et al., 1997; Pohlig et al., 1996). Tryptase is able to induce proliferation in epithelial cells (Blair et al., 1997; Cairns and Walls, 1996), endothelial cells (Mirza et al., 1996), and smooth muscle cell (Brown et al., 1995). However, using rat vascular smooth muscle cells, it does not induce an increase in $^3$H-thymidine incorporation or an increase in cell numbers (Hartmann et al., 1992, Hartmann et al., 1994). In a recent report, tryptase was shown to induce $^3$H-thymidine incorporation in human umbilical vascular endothelial cells (HUVECS), but this did not correspond to an increase in cell number, in fact a decrease was observed (Compton et al., 1998). The reasons for the difference in the cellular response to tryptase is unclear. It could be due to the type of cell or the culture conditions, however the use of $^3$H-thymidine as a surrogate for cell proliferation has been questioned (Murat et al., 1990). In the proliferation assays performed as part of this thesis, the increase in the uptake of methylene blue as a surrogate marker for cell proliferation did corresponded with increases in cell number.

Tryptase was found to be equally potent at inducing proliferation of human foetal lung, airway and dermal fibroblasts, but was less so for human lung parenchymal fibroblasts. This was not the case for trypsin, which was a potent mitogen for lung derived fibroblasts (Figure
3.17) but was a weak mitogen for dermal fibroblasts (Figure 3.22). This suggests that the mitogenic effects of tryptase and trypsin on human lung and dermal fibroblasts may be mediated by different mechanisms.

In summary, tryptase-induced increases in proliferation were inhibited by the serine protease inhibitors antipain, benzamidine and BABIM at concentrations which inhibit its enzyme activity, and which have been used previously to inhibit its cellular effects. The effects of tryptase are not mediated by a contaminant of the commercially available tryptase. Together, these data demonstrate that the tryptase-induced fibroblast proliferation is dependent on the catalytic activity of the enzyme suggesting that one of the protease-activated receptor (PAR) family of receptors may be involved in the response (Dery et al., 1998; Ishihara et al., 1997; Nystedt et al., 1994; Vu et al., 1991a; Xu et al., 1998).

Tryptase induces phosphoinositol hydrolysis and calcium mobilisation in endothelial cells, colonic myocytes and keratinocytes (Corvera et al., 1997; Molino et al., 1997b; Schechter et al., 1998) and these effects are mimicked by the PAR-2 activating peptides (Al Ani et al., 1995; Hwa et al., 1996; Magazine et al., 1996; Mirza et al., 1996; Molino et al., 1997a; Nystedt et al., 1996; Saifeddine et al., 1996; Schechter et al., 1998; Storck et al., 1996). In addition, tryptase and trypsin have been shown to mediate cell activation by cleavage of protease activated receptors (Corvera et al., 1997; Molino et al., 1997b; Nystedt et al., 1994; Nystedt et al., 1995b; Nystedt et al., 1995a). Therefore with this literature evidence and the data from this current study that the mitogenic effects of tryptase are inhibited by serine protease inhibitors, I hypothesised that tryptase and trypsin mediate their proliferative effects on lung fibroblasts by activation of PAR-2.
4.2 Localisation and function of Protease Activated Receptor-2

4.2.1 PAR-2 expression by RT-PCR

To demonstrate the presence of PAR-2 in lung fibroblasts the total mRNA from human foetal, adult airway fibroblasts, and human adult dermal fibroblasts were subjected to RT-PCR using specific primers to PAR-2. Figure 3.12 demonstrates PAR-2 mRNA is expressed in foetal lung and adult airway fibroblasts, whereas the bands were less prominent in dermal fibroblasts. PAR-2 has previously been identified in human lung by Northern analysis (Bohm et al., 1996a; Nystedt et al., 1994), however these studies used whole lung extracts and therefore could not identify expression in any particular cell type. Using immunohistochemical localisation, D'Andrea and colleagues were able to localise PAR-2 to the epithelial layer, bronchial smooth muscle in the lung and to myofibroblasts in the ovary and prostate gland (D'Andrea et al., 1998).

The limited PAR-2 mRNA expression in dermal fibroblasts supports the work of Santulli and co-workers who also failed to show PAR-2 mRNA in a dermal fibroblast cell line, although they were able to demonstrate expression in human keratinocytes (Santulli et al., 1995). Similarly, Hou and colleagues were unable to demonstrate PAR-2 mRNA in human gingival fibroblasts (Hou et al., 1998).

Even though PAR-2 mRNA is expressed by a cell it does not necessarily mean that the receptor protein is expressed on the cell surface. Therefore, it was also important to demonstrate expression of the protein.
4.2.2 Localisation of PAR-2 by immunohistochemistry

4.2.2.1 Fibroblast cell cultures

To localise PAR-2 to the cell surface of fibroblasts, a rabbit anti-rat PAR-2 antisera (B5) was used. This antisera has previously been used to demonstrate PAR-2 on the cell surface of rat enterocytes, colonic myocytes and a sarcoma virus transformed rat kidney epithelial (KNRK) cell line transfected with human cDNA for PAR-2 (Bohm et al., 1996a; Bohm et al., 1996b; Kong et al., 1997).

In this current study PAR-2 was demonstrated on the cell surface of human foetal, adult parenchymal and airway fibroblasts. This is the first time that the rabbit anti-rat antisera had been used to identify PAR-2 on isolated human cells. This result was not surprising given the similarity of this region of the receptor sequence between human and rodent PAR-2 (Bohm et al., 1996a; Nystedt et al., 1994; Nystedt et al., 1995b; Saifeddine et al., 1996) and given that an antibody to a similar region of the human PAR-2 sequence also cross reacts with rat PAR-2 (Corvera et al., 1997; Kong et al., 1997).

It was possible to analyse 1 μm optical sections through the cell body of the fibroblasts using confocal microscopy and to identify antibody staining on the cell membrane (Figure 3.15). Human foetal and adult airway fibroblasts appeared to have a higher level of PAR-2 expression at the cell surface than the human parenchymal fibroblasts. Using the same antibody, there was very little receptor expression on the cell surface of dermal fibroblasts, although a low expression of PAR-2 was seen in the cytoplasm in this cell type. In comparison, only weak staining was found in cells which were incubated with non-specific rabbit IgG antibodies in place of the primary antisera or when the antisera was pre-absorbed with the specific PAR-2 peptide sequence used to generate the antisera (Figure 3.16). Further evidence of the presence of PAR-2 in these cells was obtained by the use of specific...
rabbit polyclonal antibodies to human PAR-2 that has been used to localise the receptor in human skin biopsies (Hou et al., 1998). Using this antibody, a similar pattern of PAR-2 expression in the lung fibroblasts was observed to that seen with the rabbit anti-rat antisera (Figure 3.16). Finally, it was possible to demonstrate that incubation of the cells with tryptase or trypsin reduced the cell surface localisation of PAR-2 and caused an apparent increase in the diffuse intracellular (cytoplasmic) staining compared to untreated cells (Figure 3.16). These data therefore suggest that both tryptase and trypsin are able to cleave and activate PAR-2 on the cell surface of human lung fibroblasts.

The decrease in the cell surface staining induced by tryptase and trypsin may be due to the inactivation process of PAR-2 following stimulation. PAR-2 is a G-protein coupled receptor, and is therefore liable to inactivation by a number of different mechanisms that have been postulated for other G-protein coupled receptors, including Neurokinin-1 (NK1) and β2-adrenergic receptors (Bohm et al., 1997). The termination of this signal is mediated by the uncoupling and internalisation of the receptor. The phosphorylation of the receptor-G-protein complex is by G-protein receptor kinases (GRK) and the receptor uncoupling is mediated by the interaction of the phosphorylated receptor with β-arrestin.

In the case of receptors that have ligands attached (NK1 and β2 adrenergic receptors), the ligand and receptor are dissociated by acid hydrolysis and the receptor is secreted into the cytoplasm along with β-arrestin to be recycled back to the cell surface membrane. In the case of PAR-2, where no independent ligand is attached to the receptor, a similar process has been postulated, in which the β-arrestin is secreted to the cytoplasm although the receptor is not re-cycled, but sorted to lysosomes for degradation (DeFea et al., 2000; Dery et al., 1999).
The data presented in figure 3.16 suggests a similar process. Tryptase and trypsin were in contact with the cells for 30 min prior to fixation, in which time they may have cleaved PAR-2 and stimulated receptor internalisation. However, to gain a better understanding of the inactivation of PAR-2 in this cell type, further studies are required, in which the receptors are co-localised with antibodies to the various components in the suspected inactivation pathway for example: mannosidase II antibody – golgi apparatus, transferrin receptor – early endosomes and LAMP-1 antibody – lysosomes.

4.2.2.2 Location of PAR-2 in normal human airways

Since I have been able to demonstrate PAR-2 on the cell surface of isolated cultured human fibroblasts, it was of interest to investigate the localisation of PAR-2 in biopsies from normal human bronchus. For consistency, the primary antibody used for these studies was the rabbit anti-rat antisera and the secondary antibody was conjugated to FITC.

An initial problem was the level of auto-fluorescence. In pilot experiments, the sections were fixed using paraformaldehyde. Following discussions with histopathologists, it was thought that this fixative might be enhancing the auto-fluorescence. A discernible reduction in auto-fluorescence was obtained when using ice-cold acetone as the fixative and was therefore, used as the fixative of choice in all subsequent studies. In addition, initial studies used thick section (60μM) in an attempt to obtain enough histological data that would allow a computer reconstruction of the airway tissue, to try and locate fibroblasts in-situ. However, the degree of auto-fluorescence seen in these sections restricted any further histological analysis. Therefore I decided to revert to using semi-thin sections (7μM) to minimise the amounts of auto-fluorescence.
With the nature of auto-fluorescence, being defined as emission of light across a wide spectrum, a further reduction in auto-fluorescence was obtained by visualising the image at two different wavelengths (488 nm and 568 nm). By electronically subtracting the image obtained at the higher wavelength (568 nm) from the image obtained at the lower wavelength (488 nm) it was possible to eliminate most of the auto-fluorescence (Figure 3.25). Using this technique, PAR-2 expression was demonstrated on epithelial cells (Figure 3.26) and in bronchial sub-mucosal glands (Figure 3.27).

In addition, PAR-2 was co-localised to cells containing F-actin filaments or stress fibres. These cells were identified as fibroblasts/myofibroblasts, due to their localisation directly beneath the basement membrane (Figure 3.28 A-D & 3.29) in close association with collagen fibres (Figure 3.28 E & F), and the expression of the F-actin filaments (stress fibres), a characteristic of myofibroblasts (Powell et al., 1999).

Fibroblasts and myofibroblasts have been localised to the lamina reticularis of normal rat airways (Evans et al., 1993; Evans et al., 1999). The authors described a fragmented layer of fibroblasts around the airway. They also reported similar structures in tracheas from sheep, monkey, cat, dog, rabbit, mouse and hamster (Evans et al., 1989; Evans et al., 1990). Within human airways, although a fibroblast sheath has not been demonstrated, fibroblasts have been identified by means of fibroblast markers (PR2 D3, vimentin, monoclonal antibody for the β-subunit of prolyl-4-hydroxylase, α-smooth muscle actin) and electron microscopy (Brewster et al., 1990; Chakir et al., 1996; Gabrielli et al., 1994; Hoshino et al., 1998c; Hoshino et al., 1998a; Hoshino et al., 1998b).

During the course of this present study bronchial biopsy specimens were made available from patients who were diagnosed with asthma. The samples were obtained from patients
while undergoing investigative diagnostic procedures for other pulmonary disorders. In these biopsy samples it was possible to localise PAR-2 to epithelial cells and to spindle-shaped cells in the lamina reticularis. In parallel sections, these spindle-shaped cells were identified as fibroblast-like, due to their staining with vimentin and α-smooth muscle actin. (Figure 3.30 & 3.31). The position of the fibroblasts in these biopsy sections in this current study are similar to that found in airways of mild atopic asthmatic patients (Figure 4.1, Gizycki et al., 1997).
**Figure 4.1** Electronmicrograph of subepithelial collagen and fibroblast (arrowed) from airway of mild asthmatic patient. B - basal cells, BM - basement membrane, LR- lamina reticularis. Reprinted with permission from Wilson JW & Li X (1997) Inflammation and cytokines in airway wall remodelling In: Airway wall remodelling in asthma. Eds. Stewart AG Publ. CRC Press, Inc. Copyright CRC Press, Boca Raton, Florida.

### 4.2.2.3 PAR-2 expression in human airway fibroblasts

An increase in the mRNA level and protein expression of PAR-2 has been noted in HUVEC's after exposure to TNFα, IL-1β or bacterial lipopolysaccharide (Nystedt et al., 1996). It was therefore of interest to investigate if PAR-2 expression was increased in fibroblasts cultured from the airways of asthmatic and non-asthmatic patients. Fibroblasts from such patients were kindly made available by Dr Jamilla Chakir (Université Laval, Quebec, Canada). As shown in figure 3.32, PAR-2 expression was similar in both cell types. This was an unexpected result, since the pro-inflammatory cytokines in the asthmatic airway would have been expected to induce an increase in the expression of PAR-2, similar to that seen in the endothelial cells following stimulation with pro-inflammatory mediators. This difference could be due to the different cell type studied, as has already been shown within
this study and within the published literature, the expression of PAR-2 is different between cell types. Also, the endothelial cells were exposed to pro-inflammatory mediators during culture, whereas the fibroblasts used in this study were obtained from airway biopsy material and cultured in the absence of pro-inflammatory mediators. In addition, any increase in receptor level could be down regulated during the culture period.

The biopsy material was obtained from patients diagnosed as having mild asthma and who had not recently had an exacerbation of their disease and were only using β2-adrenergic receptor agonists on demand. At this stage of their disease it is possible that the levels of the pro-inflammatory mediators are low and therefore PAR-2 expression would be correspondingly low. It is possible that PAR-2 on the cell surface of the fibroblasts obtained from the asthmatic group of patients may have already been activated, potentially contributing to the airway remodelling. This activation would lead to receptor down-regulation, back to basal levels, giving the impression of no change in receptor expression.

On the other hand, the expression of PAR-2 on cell surface of fibroblasts obtained from the normal group of patients may have been induced during the isolation and culture period, again leading to the conclusion that the expression of the receptor was similar in both groups.

These data do not support the findings of a recent publication at the American Thoracic Society Conference in Toronto, that showed, in a more severe asthmatic group of patients, requiring maintenance steroid therapy, an increase in the expression of PAR-2 on epithelial cells compared with normal controls. Although, there were no significant differences in the expression of the receptor on bronchial smooth muscle in severe asthmatic airways compared with controls (Knight et al., 2000).
As an interim summary, it has been possible to demonstrate PAR-2 on the cell surface of isolated human lung fibroblasts, on fibroblast-like cells within the wall of normal human bronchus, and expression of PAR-2 on fibroblast-like cells within airway biopsy samples from patients with mild asthma. In addition, PAR-2 was expressed on fibroblasts isolated and cultured from airway biopsy material from a separate group of mild asthmatic patients. Therefore, the evidence so far suggests that tryptase can induce lung fibroblast mitogenesis and it may be via the activation of PAR-2 which are located on the fibroblast cell surface. However, further evidence was required to show that the activation of PAR-2 receptors can lead to fibroblast mitogenesis and that blocking the PAR-2 receptor can inhibit the effects of tryptase.

### 4.2.3 Mitogenic effects of PAR-2 activating peptides

The human and rat PAR-2 activating peptides, SLIGKV and SLIGRL, induced concentration-dependent increases in lung fibroblast proliferation, whereas they had no effect on dermal fibroblast proliferation. In all three lung fibroblast cell types used, SLIGRL was slightly more potent than SLIGKV. This was particularly prominent in airway fibroblasts. This difference in potency has been seen in other assay systems in which both SLIGKV and SLIGRL have been used (Al Ani et al., 1999b; Corvera et al., 1999; Hollenberg et al., 1997). To ensure that these differences were not due to the selective breakdown by known endopeptidase enzymes the proliferation assays were repeated in the presence of endopeptidase inhibitors (phosphoramidon, bestatin or thiophan). These inhibitors had no significant effect on the response to either SLIGKV or SLIGRL suggesting that there was no substantial degradation that would affect the responses to the agonists.

To further characterise the receptor, the highly selective PAR-2 peptide-based agonist, tc-LIGRLO-NH₂ was used to induce concentration-dependent increases in fibroblast
proliferation. When the effects of tc-LIGRLO-NH$_2$ at 100µM are compared with similar concentrations of SLIGKV and SLIGRL, it was a more potent mitogen, in keeping with previous reports in other model systems (Al Ani et al., 1999b; Vergnolle et al., 1998). Unlike SLIGKV and SLIGRL, tc-LIGRLO-NH$_2$, 1000µM failed to induce a greater proliferative response than 100µM. The reason for the lack of increase is unclear. However, in previous reports in which the ability of this compound to induce intracellular calcium release was studied, the highest concentration investigated was 100µM, as it was shown that the responses had reached a maximum at this concentration (Al Ani et al., 1999a; Al Ani et al., 1999b; Vergnolle et al., 1998). In addition, using a ligand binding assay, maximum binding was seen at 100µM (Al Ani et al., 1999b).

The concentrations of the SLIGKV and SLIGRL required for maximal stimulation were between 3-10-fold higher compared with previous reported studies. A possible reason for this difference could be due to the activating peptides used in this study not having a C-terminal amino group. It is unclear why this modification of the peptide would increase the potency of the peptide, although it is thought to protect the peptide from endopeptidase degradation. This did not appear to be the case in this study, as the inhibition of endopeptidases by specific inhibitors did not increase the potency of the peptides. It is possible that the aminated peptides may have improved binding to the receptor. A recent publication has identified specific amino acid residues within PAR-1 that have high affinity sites for thrombin and its activating peptides, of which some are essential for the induction of an intracellular signal (Blackhart et al., 2000). If such binding mechanisms could be identified for PAR-2, this may offer a further explanation for the apparent difference in potency of the activating peptides in different assays.
The receptor has very specific structural requirements for activation (Al Ani et al., 1999a). For example, if the isoleucine in position 3 of the activating peptide is substituted with an alanine, or the serine in position 1 is substituted with a threonine residue, there is a dramatic reduction in potency (Hollenberg et al., 1997). It is interesting to note that if the glycine in position 4 of SLIGRL-NH$_2$ is changed for an alanine, to give SLIARL-NH$_2$, there is only a modest loss in potency. In fact, this peptide has a similar potency to SLIGKV-NH$_2$ (Hollenberg et al., 1997). There is also a small reduction in potency when the amino acid in position 6 of SLIGRL-NH$_2$ is lost, leaving the peptide SLIGR-NH$_2$ (Al Ani et al., 1999b). It is therefore conceivable that the addition of a C-terminal amino group to the activating peptides may have important effect on the structural conformation of the peptides, allowing them to bind to the receptor with higher affinity.

Following cleavage of the N-terminal sequence of the receptor, the resulting ‘tethered ligand’ is presented to the binding site on the 2nd extracellular loop in the correct structural conformation. A further possibility is that due to the limited structural features of the soluble activating peptides, they are not being presented correctly and therefore will only interact with the binding site due to the law of mass action. Thus, with the complex structural requirements of the cell surface receptor and the peptide nature of the agonists, higher concentrations of the activating peptides are required to obtain measurable responses.

4.2.4 The effect of anti-rat PAR-2 antisera (B5) on the mitogenic responses to trypsin and tryptase

To enable further characterisation of the receptor pharmacologically, a selective receptor antagonist is required. With the evidence that the anti-rat PAR-2 antisera can cross-react with human PAR-2, it was used as a potential neutralising antisera. Pre-incubation of the cells with the antisera completely inhibited the mitogenic response of trypsin. However, it
only partially inhibited the effects of tryptase. The mechanisms of these inhibitions are unclear and require further investigation. It could be speculated that the antibody selectively binds to the new N-terminal sequence of the cleaved receptor, since the antisera was generated against a peptide sequence homologous to this region in the human receptor (Table 1.9). It would therefore block the binding of the tethered ligand to the receptor by steric hindrance. It is also possible that the antibody could block the interaction of tryptase or trypsin with their cleavage sites, reducing the ability of the enzyme to cleave the N-terminus of the receptor. Other antibodies are available that have been generated against different regions of the receptor (Table 1.9) and therefore could be used to clarify the mechanism of action.

The PAR-2 antisera did not completely inhibit tryptase-induced fibroblast mitogenesis and the reason for this is unknown. However, coupled together with the evidence of the biphasic nature of the proliferative response in human foetal lung fibroblasts and the potent mitogenic activity on dermal fibroblasts, despite the low levels of PAR-2 expression and the lack of response to the PAR-2 activating peptides, suggests that tryptase can induce fibroblast proliferation via PAR-2-dependent and independent mechanisms.

However, the effects can be totally inhibited by serine protease inhibitors, suggesting that the remaining activity still requires an intact catalytic site and thus involves another protease activated receptor or as previously discussed could also be the cleavage of growth factors from the extracellular matrix. Using structure activity relationships to characterise PAR-2 in different tissues, Hollenberg and co-workers have postulated that there are PAR’s distinct from PAR-2 (Al Ani et al., 1999b; Roy et al., 1998; Saifeddine et al., 1998; Vergnolle et al., 1998). In a similar approach, SLIGRL-NH₂ was reported to induce smooth muscle relaxation in guinea pig taenia coli, whereas trypsin was without effect, implying that the activating peptide was not stimulating a classical PAR-2 receptor (Cocks et al., 1999b). It is
therefore conceivable that there are sub-types of PAR’s that are unable to be activated by
known enzymes, but can be stimulated by binding of selective peptide sequences. In support
of this a polymorphism in the PAR-2 gene has been identified which causes a single amino
acid change in the 2nd extracellular loop of the receptor. This change would be enough to
inhibit the activation by a tethered ligand, but the receptor may still be available to activation
by soluble activating peptides (Compton et al., 2000).

The mitogenic effects of the PAR-2 activating peptides, SLIGKV and SLIGRL, and tryptase
on a primary human lung fibroblast cell line have recently been published in abstract form
(Freeman et al., 1999). The assay studied ³H-thymidine incorporation as a surrogate for cell
proliferation. They were able to show that tryptase induced a large increase in ³H-thymidine
incorporation, SLIGKV induced a much lower rate of incorporation, whereas SLIGRL failed
to induce incorporation. PAR-2 mRNA was demonstrated within the cells. Due to the lack
of effect with SLIGRL and the relatively low levels of mitogenesis induced by SLIGKV
compared with tryptase, these authors conclude that in lung fibroblasts mitogenesis may not
be induced via activation of PAR-2 but via a different protease activated receptor. In
contrast, the evidence from this current study would support the conclusion that tryptase can
induce human lung fibroblast proliferation partly via the activation of PAR-2 and a PAR-2
independent mechanism, possibly another PAR.

In addition to these hypotheses, recent studies have shown that cleaved PAR-1 can activate
PAR-2 by intermolecular transactivation (O’Brien et al., 2000). This could be a possible
mechanism in these cell types. Protease activated receptor-1 mRNA and the cell surface
protein are present in lung fibroblasts (Figure 3.14, Chambers et al., 1998). Also tryptase
has been shown to cleave a peptide homologous to the N-terminal sequence PAR-1 (Molino
et al., 1997b). This may form another possible explanation for the biphasic response seen
when tryptase was incubated with human foetal lung fibroblasts for various lengths of time
(Figure 3.4). The initial short incubation periods may activate PAR-2 to induce mitogenesis, whereas the extended incubation times may allow the cleavage of PAR-1, inducing mitogenesis, and/or subsequent transactivation of PAR-2. In a series of pilot studies when thrombin was incubated with human foetal lung fibroblasts, mitogenesis did not occur until after 24h incubation. Thus the combined activation of PAR-2 and PAR-1 would lead to an enhancement of cell proliferation. A similar interaction has been postulated for PAR-3 and PAR-4, in which PAR-3 acts as a co-factor for the intracellular activation of PAR-4 (Nakanishi-Matsui et al., 2000). If a similar mechanism were operating in the current studies, in addition to direct activation of PAR-2, tryptase may also activate an unknown PAR (s) or a PAR-2 subtype, which can interact with PAR-2 either by extracellular transactivation or by receptor dimerisation leading to intracellular signalling. Further studies are required to clarify the effects of tryptase, in which it would be important to identify small molecular antagonists to allow full and complete characterisation of these receptors.

4.3 Effect of tryptase and PAR-2 activating peptides on extracellular matrix production

4.3.1 The effects of tryptase, trypsin, and PAR-2 activating peptides on procollagen metabolism

It is worth reviewing at this point previously published data on the effects of tryptase on procollagen mRNA expression and procollagen protein synthesis. Three publications have investigate the effects of tryptase on human adult dermal fibroblasts in which tryptase was shown to increase α1(I) procollagen mRNA expression, and increase procollagen protein production (Abe et al., 1998; Abe et al., 2000; Gruber et al., 1997). The concentration used to induce an increase in procollagen protein was approximately 147mU/mL, at least 4 times higher than the concentrations used in my studies on human foetal lung fibroblasts. They also reported significant proliferative responses to tryptase, similar to the mitogenic effects
seen with the dermal fibroblasts cell line presented in this current study. Tryptase has also been shown to induce proliferation and collagen production in a human foetal lung fibroblast cell line, MRC5, using concentrations similar to those I used to demonstrate tryptase-induced proliferation of human foetal lung, adult parenchymal and airway fibroblasts (Cairns and Walls, 1997). The technique used to analyse collagen in this study was to measure the total amount of $^3$H-proline incorporation (total protein), subtracting the non-collagenous protein counts (i.e. collagenase-resistant proteins), leaving an estimate of intact collagen protein production. They confirmed these findings by demonstrating increases in the levels of hydroxyproline using a spectrophotometric assay.

Tryptase induced concentration-dependent increases in the activation of procollagen $\alpha_2(1)$ gene promoter activity (Figure 3.33). At the highest concentration used, tryptase induced a 55% increase in activity that was approximately 20% of the activity of the known procollagen gene activator, TGF$\beta$. Therefore, tryptase is a relatively weak inducer of procollagen $\alpha_2(1)$ promoter activity. Similar responses were obtained with single concentrations of trypsin and SLIGKV, whereas, the control peptide LSIGKV was without effect (Figure 3.33). This data, for the first time, suggests that both tryptase and PAR-2 may have a role in $\alpha_2(1)$ procollagen gene activation.

However, no significant increase in procollagen $\alpha_1(1)$ mRNA levels was obtained after 24h treatment with tryptase or SLIGKV (Figures 3.34 & 3.35). Although there was a trend towards an increase with SLIGKV, this may be a reflection of the slight decrease in the 28S rRNA signal. In these cells, TGF$\beta$ induced a 115% increase (Figure 3.34). Previous studies have reported that tryptase can induce procollagen $\alpha_1(1)$ mRNA in dermal fibroblasts which was inhibited by BABIM, suggesting that the catalytic activity of the enzyme is required for this response (Gruber et al., 1997).
One explanation for the lack of a correlation between the gene promoter assay and mRNA levels could be the use of the promoter for procollagen α2(I) gene, whereas the probe used in the Northern assay was for procollagen α1(I) mRNA. It is conceivable that the promoter region of the procollagen α2(I) gene has a tryptase and/or PAR-2 sensitive regions, whereas, the procollagen α1(I) gene does not, which would lead to a preferential activation of the procollagen α2(I) gene compared to procollagen α1(I) gene. However, this is unlikely since these genes are co-ordinately regulated (Bateman et al., 1996). In fact, similar gene regulatory sites control transcriptional regulation of both α1(I) and α2(I) procollagen genes (Chambers and Laurent, 1997). A further possible explanation for these results is that there may be controlling elements upstream of the promotor regions that are sensitive to tryptase or PAR-2 which modulate the transcription of the procollagen mRNA. Since the sequences of the construct used for the α2(I) procollagen promotor contained the nucleotides +58 to −3500 relative to the transcription start site, it is possible that in this assay the promotor activation by tryptase and SLIGKV was allowed to proceed unhindered (Inagaki et al., 1994). Another possible explanation is that tryptase and SLIGKV could induce procollagen mRNA instability, in contrast to the actions of TGFβ, in which part of its actions are known to be via an increase in procollagen mRNA stability (Penttinen et al., 1988; Raghow et al., 1987).

It is also possible that the procollagen promotor assay is more sensitive compared with the Northern Assay. The comparison of TGFβ (1ng/ml) in both assays would suggest that this could be the case. In the promotor assay TGFβ caused a 230% increase compared with control, whereas, in the Northern assay it only achieved a 100% increase. The increases in the promotor activity induced by tryptase and SLIGKV were approximately one fifth of that seen with TGFβ. Therefore, if this reduced effect were paralleled in the Northern assay, it
would be difficult to discern changes in mRNA levels using the densitometry techniques used in this assay, leading to the conclusion that tryptase and SLIGKV had no effect. Therefore, future studies require the use of more sensitive measures of mRNA levels e.g. quantitative and semi-quantitative PCR (TaqMan or Gene Chip technology).

It is interesting to note that following 24h treatment with tryptase, there was a concentration-dependent decrease in procollagen production, and a similar trend in total synthesis but this failed to achieve statistical significance (Figure 3.36). Following 48h treatment with tryptase, there were concentration-dependent decreases in procollagen production, degradation and total synthesis (Figure 3.37).

With the decrease in the production of procollagen following exposure to tryptase, it may reasonably have been expected to see an increase in the levels of degraded procollagen. Tryptase has been demonstrated to stimulate the release of matrix metalloproteinases (MMP) from fibroblasts and activate pro-stromelysin (pro-MMP-3) to stromelysin (MMP-3) which, in turn, activates pro-collagenase (pro-MMP-1) to collagenase (MMP-1) leading to the degradation of procollagen molecules (Cairns and Walls, 1997; Lees et al., 1994). However, this would only be true if there were no increases in total synthesis. In this current study there were significant decreases in total synthesis, leading to the conclusion that there were no significant change in the levels of collagen degradation (Figure 3.37). With the procollagen promotor data and given similar steady state levels of mRNA following treatment with tryptase, it suggests that the enzyme acts at a post transcriptional/translational level to reduce procollagen production and total synthesis. However, due to the lack of an increase in procollagen degradation, it does not appear to affect post-translational mechanisms.
An alternative explanation for the effect of tryptase on procollagen synthesis by human lung fibroblasts is that they are unable to metabolise procollagen protein while the cells are proliferating. Although tryptase failed to increase procollagen total synthesis, it did cause significant increases in cell numbers during the incubation period, whereas TGFβ, which did not cause a change in cell numbers, caused a significant increase in the synthesis of procollagen. However, it is thought that rapidly proliferating cells usually make more collagen and degrade a greater proportion, thus with tryptase, it would have been expected to see an increase in hydroxyproline levels in both the ethanol-insoluble and -soluble fractions of the assay, but as there was a decrease in both, this is unlikely to be an explanation.

Although, it may be possible that mediators could independently control lung fibroblast proliferation and collagen production.

Previously published data has demonstrated that although PDGF and FGF-2 were able to stimulate cardiac fibroblast proliferation, whereas TGFβ and IGF-1 did not, although all four mediators were able to stimulate collagen production (Butt et al., 1995b). In contrast to TGFβ and IGF-1, tryptase may be able to induce proliferation but not procollagen synthesis. In contrast to this hypothesis, tryptase has been shown to induce both proliferation and collagen production in dermal fibroblasts (Abe et al., 1998; Abe et al., 2000), and in a human lung fibroblast cell line (Cairns and Walls, 1997) albeit using separate collagen and mitogenesis assays.

This is the first time that this methodology has been used to try and investigate procollagen synthesis induced by agonists that also induce cell proliferation. Previous reports have studied the effects of TGFβ and thrombin on procollagen synthesis at concentrations that did not to induce cell proliferation (Chambers et al., 1998; McAnulty et al., 1995). In this current study the results have been expressed related to the cell number at the end of the
assay. This may have caused an over estimation in the reduction of procollagen synthesis induced by tryptase, since the number of cells present over the time course of the assay was constantly increasing. To illustrate this, Table 4.1 shows data for procollagen production either uncorrected for cell number or corrected for the mean cell counts at the end of the 48h

Table 4.1  Procollagen production data corrected for cell counts

<table>
<thead>
<tr>
<th></th>
<th>Media (nmole hydroxyproline)</th>
<th>Tryptase 17.5 mU/ml (nmole hydroxyproline)</th>
<th>Tryptase 35 mU/ml (nmole hydroxyproline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncorrected data</td>
<td>4.48 ± 0.124</td>
<td>4.84 ± 1.042</td>
<td>2.43 ± 0.123</td>
</tr>
<tr>
<td>Corrected using mean cell number following 48h incubation</td>
<td>4.95 ± 0.139</td>
<td>2.56 ± 0.551</td>
<td>1.12 ± 0.05</td>
</tr>
</tbody>
</table>

The data in table 4.1 shows that the analysis is clearly dependent on cell numbers. Therefore this methodology may not be appropriate to study procollagen synthesis induced by mediators that can induce cell proliferation. In previously published data, the effects of tryptase on the levels of procollagen secreted from human lung fibroblasts into the cell culture supernatant has been investigated (Cairns and Walls, 1997). Tryptase was shown to have a greater response at lower, compared with higher concentrations, suggesting that tryptase can induce an increase in collagen synthesis which is dependent on the concentration used, however these responses appeared to be independent of cell number.

Therefore, the effects of tryptase on procollagen synthesis in human foetal lung fibroblasts I have obtained are contrary to the published literature. The reason for this is unclear and requires further work. Currently, most of the literature relates to the effects of tryptase on human dermal fibroblasts, with one other published study on human lung fibroblasts albeit using different assay systems. Therefore, there are questions regarding the types of assay
used to measure procollagen synthesis, the effects of tryptase on procollagen mRNA stability and post-transcriptional/translational regulation that require further investigation.

Following the incubation of human lung fibroblasts with the PAR-2 activating peptides, SLIGKV, there was no effect of procollagen production, whereas there was a decrease in procollagen degradation after 24 and 48h treatment (Figure 3.39). After 48h treatment, there were significant increase in cell numbers induced by SLIGKV, which may add further support to the hypothesis, that fibroblast proliferation and procollagen production may be controlled independently (Table 3.6). The decrease in procollagen production, degradation and total synthesis by the control peptide, LSIGKV, was unexpected. However, one interpretation of this data is that the effects seen with SLIGKV (decrease in procollagen degradation) were not mediated through PAR-2, although, this does not rule out these effects being mediated through different PAR.

In summary, using the HPLC methodology and corrected the data for cell number, it can be concluded that neither tryptase nor the activating peptide, SLIGKV induce procollagen production in human foetal lung fibroblasts. However, this data should be viewed with caution, as it was clear that this increase in cell number has profound effects on the measurement of procollagen. This data is contrary to the data obtained with human dermal fibroblasts and a different human lung-derived fibroblast cell line. Possible reasons for this could be a change in procollagen mRNA stability, the effects of tryptase on post-transcriptional/translational mechanisms or that tryptase, and the PAR-2 activating peptides, are able to stimulate mitogenesis independently from procollagen synthesis. It is possible however to speculate that PAR-2 activation may not induce procollagen production.
In summary therefore, the effects of tryptase and PAR-2 activating peptides on extracellular matrix metabolism is complex. The effects of tryptase and PAR-2 activating peptides on procollagen synthesis have not be defined in this limited study, although it is clear that the effects of these agonists on fibroblast proliferation is an important contributing factor associated with these responses. Therefore more extensive studies are required to be able to draw firmer conclusions about the activity of tryptase and the activation of PAR-2 on extracellular matrix molecules metabolism. Thus, more work is needed in this area, but these preliminary findings suggest that tryptase may be important in disease pathology and therefore will be discussed in the following section.

4.4 Implications of these findings for disease

4.4.1 The potential role of mast cells and mast cell tryptase in lung disease pathology

Activated mast cells have been associated with a wide range of lung pathologies including asthma and fibrosis. It has been well established that mast cell numbers are increased in asthma and that these cells are activated (see Table 1.4). In adult patients diagnosed with asthma, who had not received anti-inflammatory therapy, mast cells are associated with airway epithelium, in addition mast cell degranulation within the airway wall and lung parenchyma has been demonstrated in patients who had died of an asthmatic attack (Heard et al., 1989; Laitinen et al., 1993). In contrast, there was no increase in the levels of tryptase in BAL from children with asthma, demonstrating that in early stages of the disease, mast cells may not be activated (Ennis et al., 1999; Jacobi et al., 1998). This could suggest that mast cells might not play a predominant role in these early stages of the disease.

In a recent study of mild asthmatics, short term treatment with fluticasone propionate decreased the numbers of mast cells in the lamina reticularis within bronchial biopsies and reduced the levels of tryptase in BAL. In addition they reported a decrease in the
subepithelial fibrosis seen in the airways of these patients (Olivieri et al., 1997a). Although no association was made between the numbers of mast cells or the levels of tryptase and the degree of airway remodelling in this study, it could be speculated that the reduction of inflammatory cells and mediators, including mast cells and tryptase, led to the reduction in airway remodelling. Similar findings were observed in patients with occupational asthma who were sensitised to toluene diisocyanate (TDI, Saetta et al., 1995). After cessation of the TDI exposure, the numbers of mast cells and fibroblasts were significantly reduced, with a concomitant reduction of the subepithelial fibrotic lesion in the bronchial biopsies. Although mast cells do not appear to be activated in the early stages of asthma, the reduction in the number of these cells and the mediators derived from them during the later stages of this disease may contribute to a reduction in airway remodelling. These data would suggest that mediators released from activated mast cells, possibly tryptase, might be important in the development of the fibrotic lesion seen in the lamina reticularis of the asthmatic airways. However, it may also be speculated that mast cells may not be the primary cells associated with the onset of airways remodelling in such diseases as asthma, but may be secondary to the effects of other inflammatory cells and mediators.

Increased numbers of mast cells have also been demonstrated in the lungs of sarcoidosis patients (Chanez et al., 1993; Eklund et al., 1993; Ohrn et al., 1995). This disease is characterised by granulomatous and fibrotic lesions in many organs of the body, including liver, skin, brain and lung. Mast cells have been shown to be activated based on the presence of increased levels of the mast cell mediators, tryptase and histamine in BAL and sputum of these patients (Chanez et al., 1993; Eklund et al., 1993; Ohrn et al., 1995; Walls et al., 1991). Mast cell numbers are also increased in interstitial pulmonary fibrosis (or cryptogenic fibrosing alveolitis, CFA, (Agius et al., 1985; Walls et al., 1990) with accompanying increased levels of tryptase found in the BAL (Walls et al., 1991). In patients with fibrotic lung disease, Kawanami and colleagues demonstrated significant increases in
the numbers of mast cells within the parenchymal tissue of the lung, the majority of which were closely associated with fibroblasts, providing evidence for the concept of fibroblasts activation by mast cell mediators in these conditions (Heard et al., 1992; Kawanami et al., 1979). In the fibrotic lung, mast cells undergo a chronic process of partial degranulation, which is different to the total degranulation seen in an anaphylactic reaction (Kawanami et al., 1979). This partial degranulation may explain the continuous nature of the fibrotic response in the lung, as the mast cell mediators released would constantly stimulate the surrounding resident and inflammatory cells in the tissue including fibroblasts.

In a rat model of fibrosis induced by over-expression of TGFβ, the early stages of the fibrotic response were associated with an initial increase in inflammatory cells, which were followed by increases in the numbers of fibroblasts and extracellular matrix deposition (Sime et al., 1997). Using the same model, increases in the numbers of mast cells were only seen during the later stages of the fibrotic response, with the conclusion that a role for these cells in the initiation of fibrosis was unlikely, however, they may be involved with the persistence nature of the fibrotic response. These data would be consistent with the data presented in this thesis of tryptase-induced fibroblast proliferation, since during the time period of increased mast cells numbers in the rat lungs there were also large increases in the numbers of fibroblasts associated with the fibrotic parenchyma and pleura.

In addition to the proposed involvement of mast cells in fibroblasts proliferation and fibrotic lesions, they have also been shown to be increased in bronchial cancer (Jordana et al., 1988; Marquez Perez et al., 1998; Walls et al., 1990). The levels of tryptase are increased in the BAL of these patients and could therefore stimulate fibroblast proliferation and subsequent extracellular matrix deposition within the carcinoma (fibrotic component of the lesion). In addition, tryptase could activate matrix metalloproteinases to degrade the surrounding extracellular matrix, allowing cell migration and metastasis.
4.4.2 Fibroblast association with sub-epithelial thickening

In 1960, Glynn and Michaels demonstrated the presence of fibroblasts within the airway wall and their association with an increase in the thickness of the lamina reticularis (Glynn and Michaels, 1960). However, Brewster and colleagues were the first group to report an increase in the numbers of myofibroblasts in the lamina reticularis of the asthmatic compared with the non-asthmatic airway (Brewster et al., 1990).

In a series of papers, Hoshino and co-workers were able to show the presence of fibroblasts in the lamina reticularis of the airways from asthmatics and correlated their numbers with the thickness of the lamina reticularis (Hoshino et al., 1998c; Hoshino et al., 1998a; Hoshino et al., 1998b). These same authors related this increase in thickness to an increase in the number of asthmatic attacks and bronchial hyperreactivity. In contrast, Roche and colleagues, although they found an increase in the thickness of the lamina reticularis in asthmatic patients compared with control, they could not correlate this with disease severity (Roche et al., 1989). This data was supported by that of Chu and co-workers who were unable to correlate sub-epithelial thickness with disease severity, however, in their study they were not able to show a difference between normal control and asthmatic subjects (Chu et al., 1998).

The variation in the measurement of sub-epithelial thickness is highlighted in Table 4.2. There is a wide range of values in control (2.52 to 8.4 μm) and asthmatic airways (5.5 to 23.13 μm) which can depend on a number of factors. These include the biopsy technique which may lead to mechanical damage (Soderberg et al., 1990), the angle of cut through the airway, the type of analysis performed on the sample including the number of measurements.
taken and the size of interval between samples (Sullivan et al., 1998; Wilson and Li, 1997),
the severity of the disease (Minshall et al., 1997), the age of the patients and the calibre of
the airway (Watanabe et al., 1997). There is also a on-going debate as to whether the
thickness of this layer has any effect on lung function, as only 3 out of 7 studies investigating
these effects, showed some correlation with function (Table 4.2). The use of steroids, and in
one case a β-adrenoceptor agonist, decreased sub-epithelial thickening, however, this effect
appeared to be both dose- and time-depandant (Table 4.3).
Table 4.2 Measurements of thickness of lamina reticularis in normal control and asthmatic subjects

<table>
<thead>
<tr>
<th>Disease State</th>
<th>Thickness of lamina reticularis (µm)</th>
<th>Associated with change in lung function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>8.0 ± 2.82</td>
<td>FEV1 NT</td>
<td>(Soderberg et al., 1990)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.17 ± 0.59</td>
<td>FEV1 No</td>
<td>(Roche et al., 1989)</td>
</tr>
<tr>
<td>Asthma</td>
<td>7.95 ± 1.17</td>
<td>FEV1 No</td>
<td>(Chu et al., 1998)</td>
</tr>
<tr>
<td>Normal control</td>
<td>5.2 (4.0 - 7.1)</td>
<td>FEV1 No</td>
<td>(Roche et al., 1989)</td>
</tr>
<tr>
<td>Asthma mild</td>
<td>5.5 (4.5 - 9.5)</td>
<td>FEV1 No</td>
<td>(Chu et al., 1998)</td>
</tr>
<tr>
<td>moderate</td>
<td>6.8 (4.6 - 9.0)</td>
<td>FEV1 No</td>
<td>(Chu et al., 1998)</td>
</tr>
<tr>
<td>severe</td>
<td>6.5 (5.2 - 9.3)</td>
<td>FEV1 No</td>
<td>(Chu et al., 1998)</td>
</tr>
<tr>
<td>Asthma</td>
<td>6 - 30</td>
<td>FEV1 NT</td>
<td>(Molina et al., 1977)</td>
</tr>
<tr>
<td>Normal control</td>
<td>~ 7.5 *</td>
<td>FEV1 Yes</td>
<td>(Jeffery et al., 1989)</td>
</tr>
<tr>
<td>Asthma mild</td>
<td>~ 11.75*</td>
<td>FEV1 No</td>
<td>(Jeffery et al., 1992)</td>
</tr>
<tr>
<td>Normal control</td>
<td>8.2 ±0.5</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>Asthma mild</td>
<td>11.0 ±0.6</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>moderate</td>
<td>11.0 ±0.7</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>Normal control</td>
<td>~ 1.5*</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>~ 6.1*</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>mild to severe</td>
<td>~ 8.5*</td>
<td>FEV1 NT</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.68 (2.26 - 6.26)</td>
<td>FEV1 No</td>
<td>(Brewster et al., 1990)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>7.55 (3.75 - 11.1)</td>
<td>FEV1 No</td>
<td>(Ollerenshaw and Woolcock, 1992)</td>
</tr>
<tr>
<td>Asthma</td>
<td>12 ± 2</td>
<td>FEV1 NT</td>
<td>(Lundgren et al., 1988)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.8 (2.9 - 6.7)</td>
<td>FEV1 NT</td>
<td>(Trigg et al., 1994)</td>
</tr>
<tr>
<td>Asthma – severe</td>
<td>7.0 (3.8 - 9.6)</td>
<td>FEV1 NT</td>
<td>(Trigg et al., 1994)</td>
</tr>
<tr>
<td>Asthma - mild</td>
<td>23.13 ± 3.44</td>
<td>FEV1 NT</td>
<td>(Trigg et al., 1994)</td>
</tr>
<tr>
<td>Normal control</td>
<td>2.52 ± 1.04</td>
<td>FEV1 NT</td>
<td>(Wilson and Li, 1997)</td>
</tr>
<tr>
<td>Asthma - mild</td>
<td>5.68 ± 2.42</td>
<td>FEV1 NT</td>
<td>(Wilson and Li, 1997)</td>
</tr>
<tr>
<td>moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>5.15 ± 0.23</td>
<td>FEV1 NT</td>
<td>(Watanabe et al., 1997)</td>
</tr>
<tr>
<td>Asthma</td>
<td>8.19 ± 1.36</td>
<td>FEV1 NT</td>
<td>(Sullivan et al., 1998)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.5 *</td>
<td>FEV1 NT</td>
<td>(Watanabe et al., 1997)</td>
</tr>
<tr>
<td>Asthma</td>
<td>5.5 *</td>
<td>FEV1 NT</td>
<td>(Sullivan et al., 1998)</td>
</tr>
<tr>
<td>COPD</td>
<td>7.0 *</td>
<td>FEV1 NT</td>
<td>(Watanabe et al., 1997)</td>
</tr>
<tr>
<td>Normal control</td>
<td></td>
<td></td>
<td>(Watanabe et al., 1997)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>8.31 (7.02 - 10.04)</td>
<td>FEV1 NT</td>
<td>(Hoshino et al., 1998b)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.02 (3.7 - 4.6)</td>
<td>FEV1 No</td>
<td>(Hoshino et al., 1998a)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>8.09 (6.4 - 11.8)</td>
<td>FEV1 No</td>
<td>(Hoshino et al., 1998a)</td>
</tr>
<tr>
<td>Normal control</td>
<td>4.5 (3.7 - 5.7)</td>
<td>FEV1 Yes</td>
<td>(Hoshino et al., 1998a)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>10.3 (5.3 - 15.2)</td>
<td>FEV1 Yes</td>
<td>(Hoshino et al., 1998a)</td>
</tr>
<tr>
<td>Normal control</td>
<td>8.4 ± 3.4</td>
<td>FEV1 No</td>
<td>(Chakir et al., 1996)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>12.8 ± 4.6</td>
<td>FEV1 No</td>
<td>(Chakir et al., 1996)</td>
</tr>
<tr>
<td>Asthma – mild</td>
<td>10.8 ± 0.5</td>
<td>FEV1 NT</td>
<td>(Olivieri et al., 1997b)</td>
</tr>
</tbody>
</table>

* denotes an estimation from the original graphical data. NT the correlations were not tested.
Table 4.3  The effects of asthma treatment on subepithelial thickening in airways of asthmatic patients

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µg)</th>
<th>Route</th>
<th>Dosing</th>
<th>Treatment time</th>
<th>Effect on subepithelial thickening</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDP</td>
<td>500</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>4 months</td>
<td>↓</td>
<td>(Trigg et al., 1994)</td>
</tr>
<tr>
<td>FP</td>
<td>250</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>6 weeks</td>
<td>↓</td>
<td>(Olivieri et al., 1997b)</td>
</tr>
<tr>
<td>Bud</td>
<td>400</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>4-6 weeks</td>
<td>↓</td>
<td>(Laitinen et al., 1997)</td>
</tr>
<tr>
<td>BDP</td>
<td>400</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>6 months</td>
<td>↓</td>
<td>(Hoshino et al., 1998b)</td>
</tr>
<tr>
<td>Albuterol</td>
<td>200</td>
<td>inhaled</td>
<td>q.i.d</td>
<td>12 weeks</td>
<td>↓ tenascin NSE Collagen</td>
<td>(Altraja et al., 1999)</td>
</tr>
<tr>
<td>Nedocromil Sodium Bud</td>
<td>200</td>
<td>inhaled</td>
<td>q.i.d</td>
<td>12 weeks</td>
<td>↓ tenascin NSE Collagen</td>
<td>(Altraja et al., 1999)</td>
</tr>
<tr>
<td>Bud</td>
<td>800</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>4 weeks</td>
<td>↓ tenascin NSE Collagen</td>
<td>(Laitinen et al., 1994)</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>500</td>
<td>inhaled</td>
<td>q.i.d</td>
<td>4 weeks</td>
<td>NSE</td>
<td>(Jeffery et al., 1992)</td>
</tr>
<tr>
<td>Bud</td>
<td>800</td>
<td>inhaled</td>
<td>b.i.d</td>
<td>7-15 months</td>
<td>NSE</td>
<td>(Laursen et al., 1988)</td>
</tr>
</tbody>
</table>

BDP - beclomethasone dipropionate  FP - fluticasone propionate  Bud - Budesonide.  b.i.d - twice daily treatment.  q.i.d - four times daily treatment.  ↓ denotes steroid-dependant asthmatic.  NSE - no significant effect

In a recent study, increased numbers of myofibroblasts were demonstrated in the lamina reticularis of mild asthmatic patients after antigen challenge, which were suggested to be associated with the deposition of extracellular matrix (Gizycki et al., 1997). Although, in this study, allergen challenge induced both early and late phase responses (demonstrated by a decreases in FEV₁) there were no differences in mast cell numbers or tissue morphology during the late phase response. As highlighted by the authors, this was only an acute challenge in which the mediators released induced an immediate bronchoconstriction and an inflammatory cell infiltration that led to myofibroblast chemotaxis and/or proliferation.

From the data presented in this thesis, the tryptase released from the activated mast cells may contribute to fibroblast/myofibroblast proliferation, which may be partly mediated via
activation of PAR-2. It is therefore conceivable that with the increased numbers of fibroblasts/myofibroblasts, the activation of these cells by other pro-fibrotic mediators released from inflammatory cells may increase the secretion and deposition of extracellular matrix in the lamina reticularis. However, extracellular matrix secretions from other cell types within the airway wall i.e. smooth muscle cells, epithelial and endothelial cells cannot be ruled out (reviewed in Bienkowski, 1991).

Hyaluronan, versican, biglycan and decorin were shown to be increased in the airways of post-mortem patients whose deaths were primarily associated with severe asthma and a recent report demonstrated evidence for an increase in proteoglycan deposition in the lamina reticularis of atopic mild asthmatics (Huang et al., 1999; Roberts, 1995). Increased proteoglycan production has been shown from bronchial fibroblast cell lines cultured from asthmatic patients (Tremblay et al., 1998a). Thus, with the potential increase in the number of fibroblasts within the atopic asthmatic airway, partly due to the mitogenic effects of tryptase, the activation of these cells by pro-fibrotic mediators may lead to an increase in the levels of proteoglycan deposition within the airway wall. In addition, due to the hydrophilic nature of these molecules, it can be speculated that an increased fluid content within the submucosa may further thicken the airway wall.

It is of note that I used the same human asthmatic fibroblast cell lines as Drs Chakir and Tremblay in their proteoglycan experiments for my studies of PAR-2 localisation. The preliminary evidence from my studies suggests that, in human foetal lung fibroblasts, the activation of PAR-2 may not have a direct role in procollagen synthesis or proteoglycan metabolism. In contrast, the direct and indirect effects of tryptase on procollagen and proteoglycan synthesis may be mediated independently of PAR-2 via another PAR or the release of other pro-fibrotic mediators. This potential increase in extracellular matrix deposition may lead to an increase in the sub-epithelial fibrosis seen in these patients. This
theory would also apply to other fibrotic conditions in which mast cells, and tryptase levels, are increased. However, these limited studies can not rule out a role for PAR-2 in this condition, but further studies would be required to confirm these speculations. In addition to a role for tryptase and possible activation of PAR-2 in fibroblast proliferation and extracellular matrix deposition, their effects on other aspects of airway pathology have been investigated, in particular, their effects on airway tone, airway inflammation and airway secretions. These aspects will be discussed in the following three sections and related to the histological findings within this thesis.

4.4.3 A role for tryptase and PAR-2 activation in bronchoconstriction

Tryptase can enhance histamine-induced constriction of human isolated bronchi with similar findings being reported in isolated guinea pig and dog airways, in which increased sensitivity induced by tryptase, was inhibited by SLPI and APC366, demonstrating that the catalytic activity of the enzyme was required for this response (Barrios et al., 1998; Berger et al., 1999; Johnson et al., 1997; Sekizawa et al., 1989). This effect of tryptase may be an indirect effect via the proteolytic release or activation of other mediators or possibly a direct effect via protease activated receptors located on human airway smooth muscle (D'Andrea et al., 1998). Therefore the increased levels of tryptase in BAL fluid and airway tissue may activate PAR-2, on the smooth muscle leading to an increase in bronchial sensitivity or reactivity to other agents.

Tryptase can also induce airway smooth muscle proliferation (Brown et al., 1995). These direct effects of tryptase on smooth muscle cells have implications for changes in the function of the airways in atopic, allergic asthmatic patients. The increase in the sensitivity of smooth muscle will allow the airways to constrict to lower concentrations of inciting
agents, making the airways more sensitive or hyperreactive than in normal patients (Figure 1.5). In addition, the increase in the amount of smooth muscle present in the airway wall would add to the sub-mucosal thickness, reducing the resting lumenal diameter, thus allowing a greater degree of bronchoconstriction.

The role of PAR-2 receptors in modulating airway tone has been investigated. Messenger RNA and the protein for PAR-2 have been demonstrated in guinea pig airway tissue, in particular in the epithelium and smooth muscle (Ricciardolo et al., 2000). In anaesthetised guinea pig, bronchoconstriction induced by trypsin or SLIGRL-NH$_2$ was blocked by the inhibition of NK-1 and NK-2 neurokinin receptors suggesting that these agents induce bronchoconstriction indirectly via the release of neurokinins. The incubation of guinea pig airway slices with SLIGKV-NH$_2$ induced the release of Substance P, which was inhibited by exposure to capsaicin. It was therefore concluded that the activation of PAR-2 induced the release of substance P, and possibly neurokinin A, from sensory nerve terminals inducing bronchoconstriction and a possible contribution to airway hyperreactivity in asthma.

Another interesting finding, was the activation of PAR-2 on bronchial smooth muscle in guinea pig induced relaxation in the upper airways (tracheal and bronchi), whereas it caused constriction of the lower, intrapulmonary airways. The inhibition of cyclo-oxygenase and nitric oxide synthase, and the removal of the epithelium could block this relaxation indicating the effects are mediated by substances released from the epithelium, which could include prostanoids (PGE$_2$) and/or nitric oxide (NO, Ricciardolo et al., 2000). The inhibition of cyclo-oxygenase by indomethacin was also able to inhibit PAR-2 mediated contraction of rat gastric longitudinal smooth muscle (Al-Ani et al., 1995).
The contractions of the intrapulmonary airways appeared to be via a direct effect on the smooth muscle as they were not blocked by inhibitors of cyclo-oxygenase and NO synthase or antagonists of muscarinic, histamine H$_1$, bradykinin B$_2$, neurokinin NK$_1$ or NK$_2$ receptors (Ricciardolo et al., 2000). Other groups have shown that PAR-2 activating peptide-induced contractions of other tissues - rat duodenal muscle strips, rat colon and mouse ureter were not blocked by a range of inhibitors or antagonists, again suggesting a direct effect of PAR-2 on smooth muscle (Corvera et al., 1997; Kawabata et al., 1999; Moffatt and Cocks, 1999).

In the case of the contraction of endothelium-denuded rat pulmonary arteries, the potency of PAR-2 activating peptides suggested that low concentrations induce responses via activation of PAR-2, whereas high concentrations were via an unknown receptor (Roy et al., 1998). To further demonstrate the complex nature of these responses, PAR-2 activating peptides induced contractions of endothelium-intact human umbilical vein smooth muscle (HUVEC) via the release of an endothelium-derived contracting factor (EDCF, (Saifeddine et al., 1998).

Therefore, tryptase and PAR-2 activating peptides induce complex response in many different cell types associated with the airway and lung parenchymal tissue, demonstrating how difficult it would be to investigate the effects of these agonists within an in-vivo model. In this thesis although the studies were performed under highly controlled conditions in-vitro, they lacked the potential influence of extracellular matrix interactions, adhesion molecules and mediators released from surrounding resident and/or inflammatory cells that undoubtedly have a role in modulating airway fibroblast activation in vivo. Thus, although these current studies add to the understanding of the effects of tryptase and PAR-2 activating peptides on the activation of isolated fibroblasts, there are many other potential mediators that would enhance or decrease these effects in-vivo.
In isolated mouse bronchial rings, activation of PAR-2 by SLIGRL-NH₂ induced concentration-dependent smooth muscle relaxation (Cocks et al., 1999a). This response was blocked by the cyclo-oxygenase inhibitor, indomethacin, and mimicked by PGE₂. This bronchoprotective response to SLIGRL-NH₂ was confirmed in the anaesthetised rat following bronchoconstriction to 5-hydroxytryptamine. The localisation of PAR-2 in the epithelium of human airways, led Cocks and Colleagues to speculate that the activation of PAR-2 by trypsin or tryptase released from activated mast cells within the epithelium, led to the release of PGE₂, which may have induced cytoprotection and also induced bronchial smooth muscle relaxation, protecting the airway from excessive bronchoconstriction. The cytoprotection of the epithelium would maintain the airways protective barrier against noxious chemicals and particles, in addition to maintaining a local source of PGE₂. It has been shown that in-vitro, the activation of fibroblasts by TGFβ induces the release of PGE₂ that can suppress fibroblast proliferation (McAnulty et al., 1997). Therefore, PGE₂ released from the intact epithelium would potentially reduce the degree of fibroblast proliferation induced by other inflammatory mediators, including tryptase and trypsin, known to be present in the asthmatic airway.

PAR-2 has been localised to cells associated with the smooth muscle layer within the mouse bronchi, which were identified as fibroblasts (Cocks et al., 1999a). This data would be consistent with the finding of PAR-2 on fibroblasts from normal and asthmatic airways within this current study. Cocks and co-workers postulated that the activation of these receptors would be detrimental, in that they would induce fibroproliferation and may lead to excessive extracellular matrix production. Therefore, if PGE₂ production was reduced due to a damaged epithelium, within the asthmatic airway, extracellular matrix deposition would occur unhindered, leading to the remodelling of the airways.
Within airway remodelling, inflammation plays a major role. This may be seen as cellular infiltration, oedema and plasma protein extravasation. Although the effects of tryptase and the PAR-2 activating peptides have not been investigated on inflammation within the airways, their effects have been studied in other organs and tissues.

4.4.4 The potential role of tryptase and PAR-2 in inflammation

Tryptase plays an important role in inflammation. It is a chemoattractant for inflammatory cells including eosinophils and neutrophils in guinea pig skin (He et al., 1997; He and Walls, 1997), neutrophils in mouse peritoneum (He et al., 1997; Louis et al., 1997) and fibroblasts (Gruber et al., 1997). If tryptase is incubated with purified eosinophils or neutrophils it induces cellular activation shown by the induction of cell shape changes (Walls et al., 1995). It is also able to activate epithelial and endothelial cells to secrete the granulocyte chemotactic agent, interleukin-8 (IL-8) and up-regulate ICAM-1 (Cairns and Walls, 1996; Compton et al., 1998) which both depend on the catalytic activity of the enzyme. With the localisation of PAR-2 to epithelial cells within the airways of normal and asthmatic airways in this present study, confirming previously published data, it is possible to speculate a role for this receptor in these tryptase-induced responses (Cocks et al., 1999a; D'Andrea et al., 1998).

The localisation and activation of PAR-2 on endothelial cells and neutrophils induces leukocyte rolling and adherence, and subsequent migration (Howells et al., 1997; Molino et al., 1997a; Nystedt et al., 1996; Vergnolle, 1999). With the upregulation of PAR-2 mRNA on endothelial cells following stimulation with IL-1β, TNFα or LPS (Nystedt et al., 1996), PAR-2, and its activators, may play an important role in the inflammatory responses.
(leukocyte migration) associated with lung diseases such as asthma, ARDS, fibrosis and sarcoidosis.

In addition to the proposed role of PAR-2 in cellular influx in the airways, its activation has been implicated in vascular permeability in the rat hindpaw oedema model (Kawabata et al., 1998; Vergnolle et al., 1999). PAR-2 was located on endothelial cells within the rat paw as well as on the infiltrating cells and their activation induced oedema and granulocyte infiltration. Similar to the contractions of smooth muscle, these responses were not inhibited by indomethacin or NO synthase inhibitors, however, they were reduced by mast cell stabilisation. This was not a consistent finding however, as in a separate study, SLIGRL did not activate rat peritoneal mast cells, whereas, the control peptide, LSIGRL, induced histamine release which was thought to be a non-specific effect on mast cell degranulation (Nishikawa et al., 2000).

PAR-2 has been located on dorsal root ganglia (DRG) neurones and co-localised with both Substance P and calcitonin gene related peptide (CGRP, Steinhoff et al., 2000). The authors were able to demonstrate that in isolated DRG neurones in culture, SLIGRL-NH$_2$ induced intracellular calcium mobilisation, and in tissue slices, the PAR-2 activating peptide induced the release of both Substance P and CGRP. Importantly, they were also able to show that both trypsin and tryptase were able to release these agents. Similar to previously published data, PAR-2 activating peptides induced inflammation (neutrophil influx) and oedema in rat hindpaw, and were inhibited by pretreatment with capsaicin, an NK1-receptor antagonist (RP67580) or CGRP$_1$ receptor antagonist (CGRP$_8$-37). The control peptide, LRGILS-NH$_2$, induced a degree of oedema which was less than that seen with SLIGRL-NH$_2$, but greater than saline control. This response was reduced by pretreatment with compound 48/80, confirming that this compound was inducing a non-specific degranulation of mast cells.
Although these findings are not directly relevant to the findings of this thesis, they do indicate that the activation of PAR-2 by tryptase and trypsin may play important roles in all aspects of airway neurogenic and cellular inflammation and remodelling. However, depending on the location of the receptor, its activation can have a cytoprotective role, proinflammatory or profibrotic effect. This complex interplay activities is further exemplified by the identification of a role for PAR-2 in increasing glandular secretions.

4.4.5 PAR-2 and airway secretions

Within this current study I was able to demonstrate that in the normal human bronchi, PAR-2 was co-located to cells within the submucosal glands expressing F-actin stress fibres. These cells have features similar to epithelial cells, but contain contractile elements and have been termed myoepithelial cells (Bensch et al., 1965; Meyrick and Reid, 1970). PAR-2 has been localised to glands in the intestine and stomach (D'Andrea et al., 1998), however, this has not been previously shown in the lung. PAR-2 mRNA has been demonstrated in the rat salivary glands (Kawabata et al., 2000a) and the activating peptide SLIGRL-NH$_2$ was able to induce mucin secretion from rat and mouse sublingual glands, amylase secretion from rat parotid glands and pancreatic secretions in the rat (Kawabata et al., 2000a, Kawabata et al., 2000b). It is tempting to speculate that the activation of the PAR-2 by tryptase and other PAR-2 activators, on the myoepithelial cells would induce secretion of airway mucus and serosal fluid from sub-mucosal glands, adding to the pathology of the asthmatic and chronic bronchitic airway. Further work is required in this area, in particular the co-localisation of PAR-2 to cells that contain specific mucin types found in the airway - MUC2, MUC5B and MUC5AC.
4.5 Summary

In summary, the data presented in this thesis demonstrates that human lung fibroblasts can express PAR-2 mRNA, which is translated and expressed as receptor protein on the cell surface. The proliferation studies show that the known PAR-2 activating enzymes, tryptase and trypsin, and PAR-2 activating peptides can induce human lung fibroblast proliferation suggesting that the activation of PAR-2 on these cells induces cell proliferation. Using neutralising antibodies to PAR-2 I totally inhibited the mitogenic effects of trypsin demonstrating that these effects are mediated solely through activation of PAR-2. Whereas, the mitogenic effects of tryptase were only partly mediated via PAR-2 due to the partial inhibition of the responses with the neutralising antibody. This has led to the conclusion that the remaining mitogenic effects of tryptase may be mediated directly through the activation of a subtype of PAR-2 or a different PAR, which are not blocked by the neutralising antibodies, or indirectly through the release of other mitogenic agents.

In biopsies obtained from normal and mild asthmatic airways, PAR-2 was localised to epithelial and sub-mucosal tissue. It was also co-localised with phalloidin, and associated with α-smooth muscle actin or vimentin staining, demonstrating the expression of PAR-2 on fibroblasts/myofibroblasts within these airways. In addition PAR-2 was localised to the cell surface of airway fibroblasts isolated from biopsy specimens from mild asthmatic patients. However, the role of PAR-2 in these tissues has not been clarified, although the potential modulation of epithelial cells, smooth muscle cells, fibroblasts and myofibroblasts mitogenesis, following activation by trypsin, or tryptase released from mast cells, has led to the speculation that activation of these receptors may contribute to the structural remodelling of diseased airways and the lung parenchyma.
In pilot studies, both tryptase and the PAR-2 activating peptide, SLIGKV, were able to induced α2 (I) procollagen promoter activity, but were not able to induced either increases in α1 (I) procollagen mRNA or procollagen protein synthesis. In fact, both agents induced decreases in procollagen production, degradation and total synthesis. The mechanisms of these responses are unclear, although tryptase and SLIGKV may have effects on procollagen gene regulation, type I procollagen mRNA stability, activate or have effects on post-transcriptional/translational regulation. Within these extracellular matrix studies, both tryptase and SLIGKV induce fibroblast proliferation which had profound effects on extracellular matrix production. It was hypothesised that these agents may modulate fibroblast mitogenesis and extracellular matrix production independently.

Therefore, in answering the specific aims of this thesis posed at the beginning of these studies, a cell proliferation assay was developed using confluent cultures of lung and airway fibroblasts in which it was demonstrated that both tryptase and trypsin were able to induce mitogenesis. Secondly it was found that both human lung and airway fibroblasts from normal and asthmatic tissue expressed PAR-2. It was shown that the mitogenic responses to tryptase were at least partly mediated by PAR-2. Finally, tryptase may have a limited role in the control of extracellular matrix production by human lung fibroblasts.

4.6 Future studies

Although these studies addressed a number of aspects of tryptase activity as regards to fibroblast mitogenesis and extracellular matrix production, and proposed a role for PAR-2 in fibroblast activation, there are a number of new questions that require further investigation e.g. the PAR-2 independent induction of fibroblast proliferation by tryptase. Although both direct and indirect pathways were postulated for these effects, further studies using specific
growth factor inhibitors, receptor antagonists, solublised receptors or other neutralising antibodies are needed. In addition, the novel findings with the neutralising antibody to PAR-2 needs further characterisation using PAR-2 agonists and selectivity studies on other known PAR’s. It is tempting from these studies to suggest the existence of an unknown PAR’s, therefore further studies identifying and cloning PAR receptor polymorphism’s and new PAR proteins, will help in the understanding of the responses to tryptase in lung, airway and dermal fibroblasts. To help characterise and gain a better understanding of these PAR-2-independent responses, the study of intracellular Ca$^{2+}$ release in these cells will give an insight into the activation mechanisms of this receptor and may be useful in the characterisation of the intracellular pathways involved, allowing a comparison with other published studies of known protease activated receptors.

The studies on the effects of tryptase and PAR-2 activating peptide on extracellular matrix metabolism in lung fibroblasts were only pilot studies and therefore need to be extended to confirm these initial findings. In particular it would be interesting to gain a better understanding of the effects of these agonists on procollagen metabolism in fibroblasts. These studies would include the quantification of procollagen mRNA (TaqMan, Gene Chip technologies) and the measurement of the mRNA stability (nuclease protection assay). Finally, since the findings in lung fibroblasts are contrary to the published data in dermal fibroblasts, it would be interesting to study the effects of tryptase on these cells and identify any role for PAR-2, or other PAR’s, in its response.
5 References


Ref Type: Abstract


266


Ref Type: Report


Ref Type: Journal (Full)


Ref Type: Abstract


Ref Type: Journal (Full)


6 Appendix

6.1 Patient Details

6.1.1 Adult human lung and airway fibroblasts

Patient 1

Name: AM  
Sex: Female  
DOB: 5-9-39  
Personal History: 
Notes: Mass in upper left lung. Underwent lung resection. Fibroblast cells grown from explant of lung parenchyma and airway tissue from macroscopically normal tissue.

Patient 2

Name: DN  
Sex: Female  
Age: 60  
Personal History: 
Notes: Mass in left lung. Underwent lung resection. Fibroblast cells grown from explant of lung parenchyma and airway tissue from macroscopically normal tissue.

Patient 3

Name: Unknown  
Sex: Male (Caucasian)  
Age: 23  
Personal History: Possible tobacco or cannabis smoker  
Notes: Tissue obtained following road traffic accident. Fibroblast cells grown from explant of lung parenchyma and airway tissue from macroscopically normal tissue.
6.1.2 Lung tissue for PAR-2 and fibroblast localisation

Patient biopsies were obtained from patients who attended the clinic of Dr Tudor Toma, Spitalul Clinic, Universitar de Pneumoftiziologic, Isai, Romania.

**Patient 1 (Control)**

Name: BS  
Sex: Male  
Age: 72  
Personal History: Former TB in left upper lobe.  
Notes: The biopsies were collected from the bifurcation of the lower lobe subsegmental bronchi.

**Patient 2 (Control)**

Name: SN  
Sex: Male  
Age: 59  
Personal History: Screened for endobronchitic tumour, found to be macroscopically normal.  
Notes: The biopsies were collected from the bifurcation of the lower lobe subsegmental bronchi.

**Patient 3 (Asthmatic)**

Name: CC  
Sex: Female  
Age: 50  
Personal History: Moderate asthma, treated with inhaled corticosteroids and long acting beta-mimetics.  
Notes: The biopsies were collected from the bifurcation of the lower lobe subsegmental bronchi.

**Patient 4 (Control)**

Name: OE  
Sex: Male  
Age:  
Personal history: TB upper right lobe  
Notes: Acute bronchitis with heamoptosis. Under went biospy for differential diagnosis
**Patient 5 (Control)**

Name: RC  
Sex: Male  
Age: 62  
Personal history: Smoker  
Notes: ?upper left apical nodule (TB?). Biopsy taken for diagnosis

**Patient 6 (Asthma)**

Name: GM  
Sex: Female  
Age: 64  
Personal history: Asthma diagnosed for more than 20 years. Recently hospitalised for an asthmatic attack of moderate severity during an acute lower respiratory tract infection with haemophilus influenza  
Drug treatments: Salbutamol 200μg (MDI) 2 puffs x 6/day. Prednisolone 30mg/day. Theophylline (acute i.v.). Amoxicillin 3g/day  
Notes: Biopsy taken for evaluation of inflammation at the bronchial level.

**Patient 7 (Control)**

Name: RG  
Sex: Male  
Age: 67  
Personal history: Smoker (~30 pack years)  
Notes: ?upper left apical nodule on chest x-ray. Biopsy taken for diagnosis. Biopsy found to be normal.

**Patient 8 (Asthmatic)**

Name: AN  
Sex: Female  
Age: 41  
Personal history: Diagnosed with asthma in 1991. Repetitive asthma attacks in the last 2 weeks.  
Drug Treatment: Theophylline (acute i.v.)  
Notes: Biopsy taken for evaluation of inflammation in the mucosa. As recent aggravation of asthma with no obvious reason, bronchoscopy performed for differential diagnosis. Bronchoscopy found to be normal.
6.2 Publications associated with this work


Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2

IAN A. AKERS,1 MADDY PARSONS,1 MICHAEL R. HILL,1 MORLEY D. HOLLENBERG,2 SHAHIN SANJAR,3 GEOFFREY J. LAURENT,1 AND ROBIN J. MCAULI

1Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, The Royal Free and University College Medical School, The Rayne Institute, London WC1E 6JJ; 2Respiratory Diseases Unit, Glaxo Wellcome Research and Development Limited, Medicines Research Centre, Stevenage SG1 2NY, United Kingdom; and 3Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Akers, Ian A., Maddy Parsons, Michael R. Hill, Morley D. Hollenberg, Shahin Sanjar, Geoffrey J. Laurent, and Robin J. McAnulty. Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L193–L201, 2000.—Mast cells play a potentially important role in fibrogenic diseases, releasing mediators including tryptase that are capable of stimulating fibroblast proliferation and procollagen synthesis. The mechanism by which tryptase stimulates fibroblast proliferation is unclear, although recent studies suggest it can activate protease-activated receptor (PAR)-2. We therefore investigated the role of PAR-2 in tryptase-induced proliferation of human fetal lung and adult lung parenchymal and airway fibroblasts and, for comparative purposes, adult dermal fibroblasts. Tryptase (0.7–70 mU/ml) induced concentration-dependent increases in proliferation of all fibroblasts studied. Antipain, bis(5-amidino-2-benzimidazolyl)methane, and benzamidine inhibited tryptase-induced fibroblast proliferation, demonstrating that proteolytic activity is required for the proliferative effects of tryptase. RT-PCR demonstrated the presence of PAR-2 mRNA, and immunohistochemical staining localized PAR-2 to the cell surface of lung fibroblasts. In addition, specific PAR-2 activating peptides, SLIGKV and SLIGRL, mimicked the proliferative effects of tryptase. In contrast, human dermal fibroblasts only weakly stained with the PAR-2 antibody. PAR-2 mRNA was almost undetectable, and fibroblasts did not respond to PAR-2 activating peptides. These results suggest that tryptase induces lung, but not dermal, fibroblast proliferation via activation of PAR-2 and are consistent with the hypothesis that the release of tryptase from activated mast cells may play an important role in the fibrogenic response observed in asthma, chronic obstructive pulmonary disease, and patients with pulmonary fibrosis.

asthma; airway remodeling; interstitial fibrosis; serine proteinase

Many diseases of the lung are associated with a fibroproliferative response, with increased numbers of fibroblasts/myofibroblasts and excessive deposition of extracellular matrix proteins. These include interstitial lung diseases such as cryptogenic fibrosing alveolitis, sarcoidosis, bronchopulmonary dysplasia, farmer’s lung, bronchiolitis obliterans organizing pneumonia, pulmonary fibrosis associated with systemic sclerosis, and rheumatoid arthritis (38) as well as diseases such as asthma and chronic obstructive pulmonary disease where there is an increase in fibroblast number and extracellular matrix deposited around the airways (7, 15, 53). However, the mechanism by which fibroblast proliferation and excessive extracellular matrix deposition occurs is unclear.

Lung tissue from patients with these diseases has been shown to contain increased numbers of mast cells (13, 24, 30, 39, 49, 50, 52), and these are often found in close apposition to fibroblasts (28, 34). The increase in mediators derived from mast cells, such as histamine and tryptase, in bronchoalveolar lavage fluid from these patients (1, 11, 13, 21, 39, 63, 66) and their state of degranulation (34) suggest that they are in a state of activation. In addition, bronchoalveolar lavage fluid from asthmatic patients contains an increased number of mast cells (60, 65), and the number of mast cells in bronchial biopsies has been related to the degree of subepithelial thickening within the airway wall of asthmatic patients (19, 20, 22). Together, these data suggest that the mediators released from mast cells may play an important role in stimulating fibroblast proliferation and collagen synthesis.

One mediator that is released in a high concentration from degranulating mast cells is tryptase. It is a 130-kDa serine protease stored in the granule as an active tetrameric enzyme bound to heparin (48). Tryptase is a potent stimulant of fibroblast, epithelial, and smooth muscle cell proliferation (8–10, 27) and is capable of stimulating synthesis of type I collagen by human fibroblasts (9, 26). The mechanisms by which tryptase exerts these cellular effects is unclear, although recent evidence suggests that tryptase may activate one member of the protease-activated receptor (PAR) family, PAR-2 (18).

PAR-2 is a member of the PAR family that includes PAR-1 (62), PAR-3 (32), and PAR-4 (33, 67). Activation of these receptors requires the cleavage of the extracellular NH2-terminal domain, revealing a new peptide
sequence (tethered ligand) that is able to bind to sites within the second extracellular loop of the seven-transmembrane receptor (36). It has been suggested that trypsin (5, 6, 44, 45) and, more recently, tryp-
tase (14, 25, 40, 42) may activate PAR-2. In addition, PAR-2 can be selectively activated by the peptides serine-leucine-isoleucine-glycine-lysine-valine (SLIGKV) and serine-leucine-isoleucine-glycine-arginine-leucine (SLIGRL), which correspond to the first six amino acids of the new NH$_2$-terminal domain exposed after cleavage of human and rat PAR-2, respectively (5, 6, 35, 43). Tryptase-induced calcium mobilization in various cells has been shown to be mediated via PAR-2 (14, 42, 57). The role of PAR-2 in cell proliferation is controversial, with activation of PAR-2 in human vascular endothelial cells and keratinocytes causing proliferative and anti-
proliferative effects, respectively (17, 41). The role of PAR-2 in tryptase-induced fibroblast proliferation is currently unknown, although it has been suggested that dermal and arterial fibroblasts do not express PAR-2 mRNA (3, 56, 57).

In this study, we investigated the role of PAR-2 in mediating the effects of tryptase on lung fibroblast proliferation. To address this, we examined the expres-
sion and localization of PAR-2 in human lung fibro-
basts with RT-PCR and immunohistochemical stain-
ing. In addition, we compared the mitogenic effects of tryptase with those of trypsin and the specific PAR-2 activating peptides.

**METHODS**

**Materials.** Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin-streptomycin, Fugnizone, and trypsin-
EDTA solutions were all obtained from Life Technologies (Paisley, UK); newborn calf serum (NCS) was obtained from Imperial Laboratories (Andover, UK); phosphate-buffered saline (PBS) was from Oxoid (Basingstoke, UK); antipain, benzamidine, BSA, holo-transferrin, methylene blue, and cell dissociation solution were obtained from Sigma (Poole, UK); human fetal lung fibroblasts (HFL1) were purchased from American Type Culture Collection (Manassas, VA); and human mast cell tryptase was purchased from Bioprocessing (Scarborough, ME). Tryptase activity is expressed in milli-
units and was defined as the amount of enzyme that gave a change of 1 absorbance unit/min at 410 nm with N-benzyl-DL-
arginine p-nitroanilide (pNA) as the substrate. The batches of tryptase used in these studies had specific activities ranging
from 5,275 to 6,089 mU/mg of total protein. Rat PAR-2 activating peptide SLIGRL was purchased from Neo
systems Laboratoire (Strasbourg, France). Human PAR-2 activating peptide SLIGKV and the control peptides leucine-serine-
isoleucine-glycine-lysine-valine (LSIGKV) and leucine-serine-
isoleucine-glycine-arginine-leucine (LSIGRL) were kindly syn-
thesized by Dr. Robert Mecham (Washington University School of Medicine, St. Louis, MO). All peptides were in the
free carboxylate form. The synthetic peptides were prepared by conventional solid-phase synthesis on an Applied Bio
systems model 431A synthesizer with FastMoc chemistry. The sequence and purity of the peptides were confirmed by electrospray mass spectrometry. All synthetic peptides were soluble in the tissue culture medium at the concentrations tested. Bis(3-amidino-2-benzimidazolyl)methane (BABIM) was a kind gift from Dr. David Andrews (Glaxo Wellcome Research and Development, Stevenage, UK); rabbit anti-rat PAR-2 serum (B5) was raised in rabbits against a peptide that spans the rat PAR-2 trypsin cleavage/activation site: 3GPNNSKR(trypsin cleavage site)/SLIGRLDT 3P-TGGC coupled to keyhole limpet hemocyanin (YGGC for coupling). The role of the B5 antibody in immunohistochemical staining has been previously described (14, 35). Goat anti-rabbit IgG conjugated to FITC was obtained from DAKO (High Wy
come, UK). TRIZol Reagent and Superscript Kit were ob-
tained from Life Technologies; agarose, ethidium bromide, and mineral oil were obtained from Sigma.

**Tissue explant.** Fibroblast cell lines were established from lung tissue obtained from patients undergoing resection of localized lung tumors. For parenchymal lung fibroblasts, tissue was carefully cut into pieces of <1 mm$^3$ that were placed into 10-cm-diameter petri dishes (Corning Costar, Cambridge, MA) with 2 ml of DMEM containing penicillin (100 U/ml), streptomycin (100 μg/ml), Fugnizone (amphoterin B; 2.5 μg/ml), and 10% NCS and incubated at 37°C in a humidified atmosphere of air containing 10% CO$_2$. When the tissue had firmly attached to the plastic surface, a further 8 ml of culture medium were slowly added to the petri dish and incubated as before. The medium was changed every 7 days. For airway fibroblasts, the airways were dissected free of parenchymal connective tissue, and <1-mm$^3$ pieces of tissue were cultured as for the parenchymal tissue. Samples of skin obtained from patients undergoing breast reduction surgery were dissected free of fatty tissue, and 1-mm$^3$ pieces were placed, dermal side down, into petri dishes containing 2 ml of DMEM as described above and cultured. After ~3 wk of culture, fibroblasts were seen growing out from the explanted tissue. These were cultured for at least 2 wk until confluent islands of cells were observed. The cultures were then passaged with a split ratio of 1:2 to 75-cm$^2$ flasks (Falcon, Marathon, London, UK) containing culture medium without Fugnizone. Thereafter, confluent cultures were pas-
saged with a split ratio of 1:4. Experiments were performed with cells between passages 2 and 8. Human fetal lung fibroblasts (HFL1) were grown to confluence in DMEM containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% NCS in 75-cm$^2$ flasks. For the experiments, cells were used between passages 15 and 23. Fibroblast lines were characterized immunohistochemically to confirm their purity. Staining with antibodies to cytokeratin, von Willebrand factor, and desmin was negative, indicating that the cultures did not contain significant numbers of epithelial or mesothe-
lial cells, endothelial cells, or smooth muscle cells. Greater than 95% of the cells stained positively for vim entin, and between 20 and 30% of the cells were also positive for α-smooth muscle actin, confirming the fibroblast/myofibro-
blast phenotype of the cells lines. All cells were tested for mycoplasma infection.

**Cell proliferation.** In pilot studies, tryptase-induced cell proliferation was assessed in subconfluent and confluent cultures. In subconfluent cultures, trypase at concentra-
tions ≤ 3.5 μM/ml caused a small mitogenic response; however, at concentrations > 3.5 μM/ml, trypase caused cell detachment (data not shown). In confluent cultures, cell detachment was not seen until concentrations > 80 μM/ml were used. In parallel control plates, cell numbers were assessed at the time of agonist addition and at the end of the incubation 48 h later. There was an ~10% decrease in cell number (76,328 ± 3,841 cells at time of addition compared with 67,109 ± 5,258 cells after 48-h incubation); however, this was not significant (P = 0.18). Therefore, all subsequent studies were performed on confluent cultures with the follow-
ing protocol. Cells were trypsinized [trypsin (0.05% wt/vol)-
EDTA (0.02% wt/vol)] and seeded onto 96-well microtiter
ACTIVATION OF PAR-2 BY HUMAN MAST CELL TRYPTASE

L195

plates (Nunc, Life Technologies) at 10^4 cells/well in 100 μl of DMEM containing penicillin, streptomycin, and NCS for 5 days as described in Tissue explant. When the cells were visually confluent, the medium was replaced with 100 μl of serum-free medium (incubation medium) containing BSA (1 mg/ml) and holo-transferrin (1 μg/ml) and incubated for a further 24 h. The medium was replaced with 100 μl of fresh incubation medium containing growth factors (tryptase, 0.7–70 μM; trypsin, 1–20 nM; activating peptides SLIGKV and SLIGRL, 0.01–1 mM; control peptides LSIGKV and LSIGRL, 1 mM; and NCS, 5%, or trypatase, 17 μM/ml) that had been preincubated with various protease inhibitors (anti-pain, benzamidine, or BABIM at concentrations of 1–100 μM) for 60 min at 37°C. Cells were then incubated for a further 48 h.

Cell number was assessed by the use of a spectrophotometric assay previously described (47). Briefly, the medium was removed, and the plates were washed in PBS. The cells were fixed in Formalin-saline (10% vol/vol Formalin in 0.15 M NaCl) for at least 30 min. The Formalin-saline was removed, and the plates were blotted dry. The cells were stained with 100 μl/well of methylene blue (1% wt/vol in 0.01 M borate buffer) for 30 min. The excess dye was removed by washing in 0.1 M borate buffer (pH 8.5) with a plate washer (Denley Instruments Cellwash, Life Science International, Basingstoke, UK). The bound dye was then eluted from the cells by the addition of 100 μl of acidified alcohol (0.01 M HCl-ethanol, 1:1 vol/vol), and the absorbance was measured at 650 nm with a microplate spectrophotometer (Titretek Multispec MCC/340 MK II, Flow Laboratories, Rickmansworth, UK). Results are expressed as a percent change in the mean absorbance compared with that in cells exposed to incubation medium alone. Changes in fibroblast cell number were confirmed by direct cell counting with a hemacytometer (British Drug House/Merck, Lutterworth, UK).

PAR-2 localization. Confluent cultures of fibroblasts were treated with nonenzymatic cell dissociation fluid to obtain single-cell suspensions. Cells were seeded onto eight-well chamber slides (Lab-Tek Permanox, Nunc, Life Technologies) at a density of 10^5 cells/well in 400 μl of DMEM containing 5% NCS. The cells were incubated at 37°C in a humidified atmosphere of air containing 10% CO₂ for 48 h. The cells were fixed with 4% paraformaldehyde for 3 min at room temperature. The fixed cells were washed with PBS and treated with normal swine serum for 20 min followed by three 5-min washes with PBS. The cells were incubated with rabbit anti-rat PAR-2 antisera (1:1,000 dilution) for 16 h at 4°C. Normal rabbit serum (1:1,000) or rabbit IgG (20 mg/ml) was used instead of the primary antiserum as a negative control. The cells were thoroughly washed and then incubated with a secondary swine anti-rabbit IgG conjugated to FITC for 60 min at room temperature. After three 5-min washes with PBS, the chambers were removed, and coverslips were mounted with an antifade glycerol-PBS-based mountant (AP1, Citiflour Products, Canterbury, UK). The cells were visualized by confocal microscopy (Leica TCS NT, Leica UK, Milton Keynes, UK). Digital images were obtained with a Polaroid Digital Palette HR6000 (Leica UK), and photomicrographs were obtained with a Polaroid Presentation Chrome 35-mm (ASA100) film.

RT-PCR for PAR-2 in lung and dermal fibroblasts. Human fetal lung, adult airway, and adult dermal fibroblasts were grown to confluence as described in Tissue explant. Total RNA was extracted from the cells with TRIzol Reagent (GIBCO BRL, Life Technologies) according to the manufacturer’s instructions. First-strand cDNA was synthesized by reverse transcription of the RNA with the Superscript Preamplification System for First Strand cDNA Synthesis (GIBCO BRL, Life Technologies) according to the manufacturer’s instructions. PCR amplification was performed with 2.5 U of Taq DNA polymerase on 5 μg of cDNA with the following oligonucleotide primers for human PAR-2: forward, 5'-GGTGATGG-CCACATCCCAAGTC-3', and reverse, 5'-GTACAGGGCATAGACATGC-3'. The reaction was allowed to proceed for 35 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min. The quality of the PCR product was checked by 1.5% agarose gel electrophoresis at 90 V for 45 min and visualized by ethidium bromide. Its identity was confirmed by sequencing with AmpliTaq DNA polymerase FS (Perkin-Elmer) fluorescently labeled dye-terminator chemistry and the use of an Applied Biosystems 377 PRISM automated sequencer. The signal obtained in the PCR amplification of PAR-2 cDNA was compared with the product generated from a glyceraldehyde-3-phosphate dehydrogenase PCR control with the following primer pair: forward, 5'-ACCAGATGTCATGGCATCAC-3', and reverse, 5'-TCCACACCTCTTGTGCTGTA-3', giving an expected product size of 452 bp.

Assay for inhibition of tryptase activity. Tryptase activity and its inhibition by serine protease inhibitors were assayed with the synthetic peptide substrate tosyl-Gly-Pro-Arg pNA. Stock solutions of substrate and inhibitors were dissolved in DMSO. Twenty microliters of tryptase (17 μM/ml final concentration) were added to the reaction mixture containing 50 μl of 10 mM Tris and 120 mM NaCl at pH 7.8, 20 μl of substrate (stock solution, 2 mM), and 10 μl of antipain, BABIM, or benzamidine to give a final concentration of 100 μM. The reaction volume was 100 μl, with a final DMSO concentration of 1.8%. Absorbance was measured at 405 nm with a 630/650-nm reference filter. The results are expressed as a percent inhibition of tryptase-induced cleavage of the synthetic peptide.

Statistics. In cell proliferation studies, means ± SE were calculated. The variation between data sets was tested with ANOVA, and the significance was tested with unpaired t-tests, with a Bonferroni modification for multicomparison of data (23). Differences were considered significant when P was <0.05.

RESULTS

Effect of tryptase on fibroblast proliferation. Figure 1 shows the effect of tryptase on human fetal lung fibroblast proliferation. Tryptase stimulated proliferation of fibroblasts in a concentration-dependent manner. Proliferation was induced at concentrations of 0.7 μM/ml and above, with a maximal stimulation of ~50% obtained at a concentration of 35 μM/ml. The effect of tryptase was reproducible; in eight separate experiments, 17.6 μM/ml of tryptase stimulated fibroblast proliferation by 38 ± 2%. The effect of tryptase on fibroblast proliferation was confirmed by direct cell counting. Values were generally higher than for the spectrophotometric assay. Tryptase at concentrations of 3.5 and 17.6 μM/ml increased cell numbers above those seen in parallel control plates by ~25 and 70%, respectively (control, 124,200 ± 12,800 cells; 3.5 μU/ml, 156,700 ± 32,500 cells; 17.6 μU/ml, 210,000 ± 11,100 cells).

Tryptase also caused concentration-dependent increases in the proliferation of fibroblasts derived from adult human lung parenchyma and airways over a similar concentration range to that seen with fetal lung
ACTIVATION OF PAR-2 BY HUMAN MAST CELL TRYPTASE

Fig. 1. Effect of tryptase on human fetal lung fibroblast proliferation. Cells were incubated with tryptase at concentrations between 0.7 and 70 mU/ml for 48 h. Each point is mean ± SE of 6 observations from a representative experiment. Similar results were obtained in 5 separate experiments. Where error bars are not shown, they are within the point. *P < 0.01 compared with medium control.

Tryptase also caused reproducible, concentration-dependent increases in human dermal fibroblast proliferation over a similar concentration range (0.7–17.6 mU/ml) to that seen with the lung fibroblasts (Fig. 2C).

Effect of proteolytic inhibitors on tryptase-induced fibroblast proliferation. The effect of proteolytic inhibitors on tryptase-induced fibroblast proliferation is shown in Fig. 3. The nonselective serine protease inhibitor antipain and the relative selective tryptase inhibitors BABIM and benzamidine caused concentration-dependent inhibition of tryptase-induced human fetal lung fibroblast proliferation (Fig. 3). At the highest concentration used (100 μM), antipain, BABIM, and benzamidine inhibited the response to tryptase by 68 ± 17.7, 100 ± 6.8, and 70 ± 5.8%, respectively. The addition of the inhibitors alone to the cells had no effect on basal cell proliferation (Fig. 3).

Tryptase-induced cleavage of the synthetic peptide substrate tosyl-Gly-Pro-Arg pNA was inhibited by all three serine protease inhibitors tested. Antipain and BABIM at a concentration of 100 μM caused inhibition of 87.8 ± 3.3 and 93.4 ± 2.5%, respectively, similar to

Fig. 2. Effect of tryptase on fibroblasts isolated from adult human lung and dermal biopsies. Confluent cell cultures of human lung parenchymal (A), airway (B), and dermal (C) fibroblasts were treated with tryptase (0.7–17.6 mU/ml) for 48 h. Values are means ± SE of 6 observations from a representative experiment. Data were reproduced in at least 4 independent experiments. *P < 0.01 compared with medium control.

Fig. 3. Effect of serine protease inhibitors on tryptase-induced proliferation of human fetal lung fibroblasts. Tryptase (17.6 mU/ml) was preincubated with antipain (1–100 μM; A), bis(5-amidino-2-benzimidazolyl)methane (1–100 μM; B), or benzamidine (1–100 μM; C) for 60 min at 37°C before addition to cells. Controls included tryptase preincubated with medium in absence of inhibitors and with inhibitors in absence of tryptase. Cell proliferation was assessed after a subsequent 48-h incubation. Values are means ± SE of 6 observations from a representative experiment. Similar results were obtained in at least 4 further experiments. Significant difference compared with medium control: *P < 0.05; **P < 0.01.
their degree of inhibition of tryptase-induced fibroblast proliferation. However, benzamidine (100 μM) appeared less effective in the biochemical assay, inhibiting tryptase-induced cleavage by 24 ± 13.0%.

Effect of trypsin on fibroblast proliferation. Trypsin, a known activator of PAR-2, also induced concentration-dependent increases in lung fibroblast proliferation. Proliferation was induced at concentrations of 5 nM and above, with a maximal achievable stimulation at 20 nM (Fig. 4). At higher concentrations, the cells detached from the plate. Trypsin also induced proliferation of dermal fibroblasts but to a lesser extent (Fig. 4).

Effect of PAR-2 activating peptides on fibroblast proliferation. The PAR-2 activating peptides corresponding to the human (SLIGKV) and rat (SLIGRL) sequences also caused increases in fetal lung fibroblast proliferation (Fig. 5). At a concentration of 1 mM, SLIGKV and SLIGRL caused stimulation of 22 ± 2 and 37 ± 1.5%, respectively, above medium control values (P < 0.01). This was confirmed by direct cell counts with SLIGKV (1 mM) and SLIGRL (1 mM), which increased cell number by —25 and 50%, respectively (control, 55,200 ± 4,900 cells; SLIGKV, 67,800 ± 900 cells; SLIGRL, 82,800 ± 5,600 cells). Coincubation with the peptidase inhibitor phosphoramidon (10 μM), bestatin (10 μM), or thiorphan (10 μM) did not increase the activity of either peptide agonist (data not shown). Neither of the control peptide sequences at concentrations similar to those used with the activating peptides affected fibroblast proliferation (1 mM LSIGKV, 1 ± 1%; 1 mM LSIGRL, 3 ± 1%).

Both SLIGKV and SLIGRL caused a concentration-dependent stimulation of adult parenchymal and airway fibroblast proliferation. Stimulation was seen at concentrations of 10 μM and above. Figure 6 shows the effects of the activating and control peptides at a concentration of 1 mM. As shown for human fetal lung fibroblasts, SLIGRL generally caused a greater degree of stimulation than SLIGKV in both adult parenchymal and airway fibroblasts. The control peptides did not affect fibroblast proliferation (Fig. 6). In contrast to the lung fibroblasts, both the human and rat PAR-2 activating peptides failed to stimulate human dermal fibroblast proliferation (Fig. 6).

Immunolocalization of PAR-2. Figure 7 shows the localization of PAR-2 in human fetal lung and adult parenchymal and airway fibroblasts after fixation for 3 min at room temperature. PAR-2 was clearly localized to the cell surface as demonstrated by a greater intensity of staining around the perimeter of the cells in the confocal images (Fig. 7, A–C). Weak staining was also observed in two different human adult dermal fibroblasts.

![Fig. 4. Effect of trypsin on human fetal lung and dermal fibroblast proliferation. Human fetal lung (■) and dermal (○) fibroblasts were treated with trypsin at concentrations between 1 and 20 nM for 48 h. Each point is mean ± SE from 6 observations from a representative experiment. Similar results were obtained in 2 further experiments. *P < 0.01 compared with medium control.](image)

![Fig. 5. Effect of protease-activated receptor (PAR)-2 activating peptides SLIGKV and SLIGRL on human fetal lung fibroblast proliferation. Fibroblasts were incubated with activating peptides corresponding to human PAR-2 sequence SLIGKV (0.01–1 mM; open bars) or rat PAR-2 sequence SLIGRL (0.01–1 mM; solid bars). Increases in cell proliferation were assessed after 48 h. Each value is mean ± SE of 6 observations from a representative experiment. Similar results were obtained in 4 further experiments *P < 0.01 compared with medium control.](image)

![Fig. 6. Effect of PAR-2 activating peptides SLIGKV and SLIGRL on human lung parenchymal, airway, and dermal fibroblast proliferation. Cells were incubated with activating peptides corresponding to human SLIGKV (hatched bars) or rat SLIGRL (crosshatched bars) PAR-2 sequence as well as control peptides LSIGKV (solid bars) and LSIGRL (open bars) at a concentration of 1 mM. Increases in cell proliferation were assessed after 48 h. Each value is mean ± SE of 6 observations from a representative experiment. Similar results were obtained in at least 2 further experiments *P < 0.01 compared with medium control.](image)
activation of PAR-2 by human mast cell tryptase

Fig. 7. Localization of PAR-2 immunoreactivity in human fetal lung fibroblasts (A), adult lung parenchymal (B) and airway (C) fibroblasts, and adult dermal fibroblasts (D). Cells were fixed for 3 min in 4% paraformaldehyde. Arrows, localization of PAR-2 to cell surface. Final magnification, x630.

blast cell lines (Fig. 7D). Fluorescent staining was absent in control preparations with normal rabbit serum (1:1,000 dilution) or rabbit IgG (20 ng/ml; data not shown).

Detection of PAR-2 mRNA by RT-PCR. RT-PCR with primers targeted to human PAR-2 amplified a product corresponding to the predicted size (813 bp) and sequence, indicating the presence of PAR-2 mRNA in human fetal lung and adult airway fibroblasts. A similar PCR product was also detected in human dermal fibroblasts but at much lower intensity relative to the signal observed in the lung and airway fibroblasts. RT-PCR with primers to glyceraldehyde-3-phosphate dehydrogenase gave a product of similar intensity in all cell types studied (Fig. 8). Similar results were obtained with another airway and dermal fibroblast line (data not shown).

DISCUSSION

In this study, we have shown that human mast cell tryptase causes proliferation of fibroblasts derived from fetal and adult human lung parenchyma and adult airways. Tryptase-induced fibroblast proliferation was inhibited by proteolytic inhibitors, demonstrating that the effect was dependent on its catalytic activity. In addition, as far as we are aware, we have demonstrated for the first time the presence of PAR-2 on the surface of lung fibroblasts and that PAR-2 activating peptides corresponding to either the human or rat sequence mimicked the proliferative effect of tryptase. These data suggest that tryptase induces lung fibroblast proliferation via cleavage and activation of PAR-2 located on the fibroblast cell surface. In contrast, although tryptase stimulated proliferation of human adult dermal fibroblasts, the PAR-2 activating peptides had no effect, suggesting that in this cell line tryptase was acting via other mechanisms.

Tryptase has previously been shown to be a potent mitogen for fibroblasts derived from the lung (9, 27, 54) and dermis (51). The concentrations of tryptase at which activity was observed in the present study were comparable to those previously reported, expressed in either units of enzymatic activity or molar concentration. The present study confirms previous data and extends these observations to show that tryptase can stimulate proliferation of fibroblasts derived from both the lung parenchyma and airway.

Tryptase-induced increases in proliferation were inhibited by the serine protease inhibitors antipain, BABIM, and benzamidine at concentrations that inhibit its enzyme activity and that have been used in previous studies to inhibit the effects of tryptase (9, 10, 12, 59, 64). Together, these data demonstrate that the tryptase-induced fibroblast proliferation is dependent on the catalytic activity of the enzyme.

The evidence that tryptase-induced proliferation requires its proteolytic activity suggests that a member of the PAR family may be involved in the response (18,
32, 45, 62, 67). It has recently been shown that trypsin induces phosphoinositid hydrolysis and calcium mobilization in endothelial cells, colon myocytes, and keratinocytes (14, 42, 57) and that these effects are mimicked by the PAR-2 activating peptides (2, 31, 37, 41, 43, 46, 55, 57, 58). In this study, we demonstrated that PAR-2 activating peptides also mimic the effects of trypsin on lung fibroblast proliferation. In contrast, the control peptides, in which the first two amino acids were reversed (LSIGKV and LSIGRL), were without activity, demonstrating the specificity of the responses to the activating peptides. Because PAR-2 activating peptides do not cross-react with other known PARs (4, 29, 36), this suggests that the proliferative effects of trypsin on lung fibroblasts may be mediated via PAR-2.

Further evidence for the presence of PAR-2 on the cell surface of fibroblasts was obtained by immunohistochemical staining. We used a rabbit anti-rat PAR-2 antiserum, which has previously been used to demonstrate PAR-2 on the cell surface of both rat enterocytes and colon myocytes and a rat kidney epithelial cell line transfected with human cDNA for PAR-2 (14, 35). We have demonstrated the presence of PAR-2 on the cell surface of both human fetal and adult parenchymal and airway fibroblasts. As far as we are aware, this is the first time that the B5 antiserum has been used to identify PAR-2 on human cells, although its cross-reactivity has previously been confirmed by the expression of human PAR-2 in rat cells (35). This result was not surprising given the similarity of this region of the receptor sequence between human and rodent PAR-2 (6, 44, 45, 55) and given that an antibody to a similar region of the human PAR-2 sequence also cross-reacts with rat PAR-2 (14, 35). In addition, we have demonstrated the expression of PAR-2 mRNA by RT-PCR in human fetal lung and adult airway fibroblasts. Further evidence demonstrating the presence of PAR-2 in the human lung has been provided by Northern analysis (6, 44) and immunolocalization (16), although in this study, PAR-2 was only identified within the epithelial layer and bronchial smooth muscle. Thus the evidence suggests that PAR-2 is present in the human lung, and we have shown in this study with RT-PCR, immunohistochemistry, and the use of PAR-2 activating peptides that PAR-2 is present on human lung fibroblasts and may be activated by trypsin to induce fibroblast proliferation.

In contrast to our observations in human lung fibroblasts, previous studies (16, 56) have suggested that human dermal fibroblasts do not express PAR-2 mRNA. From our data with human dermal fibroblasts, we observed very weak mRNA expression and immunostaining for PAR-2. In addition, we were unable to obtain responses to the PAR-2 activating peptides, suggesting there may be insufficient receptors to elicit a proliferative response. Furthermore, the response to trypsin, another known activator of PAR-2, was limited. Interestingly, we obtained responses to trypsin that were similar to those seen on lung fibroblasts. This result suggests that trypsin may cause its proliferative effects either 1) via a subtype of PAR-2 that is not activated by the PAR-2 activating peptides; 2) via a novel protease-activated receptor; 3) via PAR-4, which can be activated equally well by trypsin and thrombin (33, 67); or 4) via a non-PAR mechanism. Furthermore, a recent study (61) suggests the presence of a non-PAR-2, trypsin-sensitive receptor in the rat jejunum, providing further evidence for the existence of multiple PAR-2-like receptors. If it can be confirmed that there are subtypes of PAR-2 or multiple trypsin-sensitive PARs, this would have important implications for the pharmacological regulation of trypsin-mediated cellular effects. The discovery of distinct PAR-2 subtypes would raise the possibility of developing tissue-specific PAR-2 inhibitors.

In summary, we have confirmed that trypsin is mitogenic for both fetal and adult lung fibroblasts derived from either the parenchyma or airway. Proteolytic cleavage is required for the proliferative activity of trypsin. We also demonstrated the expression and localization of PAR-2 to the cell surface of the lung fibroblasts and that activation of these receptors with PAR-2 activating peptides induced proliferation. These data are consistent with the hypothesis that trypsin-induced lung fibroblast proliferation occurs via the
activation of PAR-2 and that release of tryptase from activated mast cells may play an important role in fibroblast proliferation and the extracellular matrix protein deposition observed in the airways of patients with asthma and chronic obstructive pulmonary disease and in the lungs of patients with pulmonary fibrosis.

This work was funded by Glaxo Wellcome Research and Development Limited.

Address for reprint requests and other correspondence: R. J. McNulty, Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, The Royal Free and University College Medical School, The Rayne Institute, 5 University St., London WC1E 6JJ, UK (E-mail: r.mcnulty@ucl.ac.uk).

Received 19 March 1999; accepted in final form 25 August 1999.

REFERENCES


Fibroblasts and myofibroblasts

Ian A. Akers, Robin J. McAnulty and Geoffrey J. Laurent

The Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, Royal Free and University College London Medical School, The Rayne Institute, 5, University Street, London WC1E 6JJ, UK

Introduction

In this chapter we will briefly review the structure of the normal airway and the extracellular matrix components from which it is composed. The role of the fibroblast/myofibroblast in maintaining this structure will be discussed, together with the evidence for their role in remodelling the airway wall in asthma and COPD. Finally we discuss the potential for pharmacological modulation of fibroblast function as a potential means of reversing the changes in airway structure in these diseases.

Structure of the normal airway

The trachea divides into two main bronchi, which further subdivide into segmental bronchi that enter the left and right lungs. The trachea and bronchi are supported by surrounding cartilage, however, as the bronchi subdivide further into bronchioles the cartilage is lost. The terminal bronchioles give rise to respiratory bronchioles and finally the alveoli. As bronchioles normally control airflow in the lung we focus on their structure in the next section.

Structure of the bronchiole and its connective tissue components

The bronchiole wall consists of a mucosal lining (epithelium), a basement membrane, sub-epithelial connective tissue (lamina reticularis), a sub-mucosal layer (smooth muscle) and adventitia (Fig. 1a). The epithelium is mainly of pseudo-stratified, ciliated, columnar epithelial cells, clara cells, goblet (mucus), serous and basal cells. The structure and function of these cells within the mucosal layer are discussed in an accompanying chapter in this book (see Devalia et al.). In this chapter we focus on the extracellular matrix components within the basement membrane, lamina reticularis, sub-mucosal and adventitial layers (Fig. 1b).
Fibroblasts and myofibroblasts

The basement membrane is separated into two regions – lamina rara and lamina densa (Fig. 1c) which contain matrix molecules, including collagen types IV, VII and XV, the glycoproteins, laminin, nidogen (entactin), fibronectin, SPARC (BM-40) and proteoglycans. These structures provide a scaffold for attachment of cells and regulate their surrounding milieu by controlling the flux of small and large molecules [1]. It is the distribution and density of these molecules which gives rise to the characteristic features of the lamina rara and lamina densa.

Collagen IV is the most abundant protein in the basement membrane. This molecule is composed of triple helical domains, interrupted by short non-helical regions, providing flexibility. It forms an open-meshed, three-dimensional network (Fig. 2) in close association with laminin, nidogen and the heparan sulphate-linked proteoglycan, perlecan. Collagen type IV in basement membrane is probably produced by epithelial cells [2, 3], although type IV collagen mRNA expression has also been reported in mesenchymal cells [4]. Type V collagen is also found in small amounts within the basement membrane [5].

Collagen XV has recently been demonstrated in basement membrane and it has been suggested this molecule is responsible for the attachment of the basement membrane to the underlying interstitium (Fig. 1c) [6]. Similar roles have been postulated for collagen types V and VII [7, 8].

Laminins are the most abundant non-collagenous protein within the basement membrane. They have a cross-like structure (Fig. 2) and have been reported to mediate cellular attachment to extracellular matrix [9], cell migration, growth and differentiation [10]. A recent study has shown that laminin subtype expression changes during the development of the lung, suggesting that these molecules may play a role in the regulation of airway morphogenesis [11].

The normal basement membrane also contains nidogen, a single polypeptide, composed of three globular domains connected by a flexible link (Fig. 2). It binds to both collagen IV and laminin, via one of the globular regions, possibly linking these two molecules [12]. It also binds to cell surface integrins, via an Arg-Gly-Asp (RGD) domain [13].

Fibronectin is a high molecular weight glycoprotein present in basement membrane and on cell surfaces. It exists as a disulphide-linked dimer (Fig. 2) which forms
high molecular weight insoluble polymers. It contains a variety of binding sites for other extracellular matrix molecules including collagen, heparin/heparan sulphate, hyaluronan and integrins. It has been implicated in a number of processes including cell attachment, migration, proliferation, opsonisation and wound healing (for extensive reviews see [14, 15]).

Another basement membrane component, SPARC (secreted protein, acidic and rich in cysteine) (Fig. 2) is able to bind cell surfaces, collagen types I, II and V and the glycoprotein, thrombospondin. Its exact role is unknown but is associated with morphogenesis, tissue remodelling and inhibits the spreading of a variety of cells, including fibroblasts [16–19].

Heparan sulphate-linked proteoglycans are ubiquitous components of basement membranes [20]. One such proteoglycan is perlecan which has a core protein with 5–7 globular domains and two or three heparan sulphate side chains attached to one
Fibroblasts and myofibroblasts end (Fig. 2). It has been associated with cell attachment [21], immobilisation of growth factors, such as FGF-2 [22] and serine proteases [23]. Therefore, in addition to its structural role it may also act as a reservoir for growth factors and metabolic enzymes.

Syndecan is a proteoglycan containing both heparan sulphate and chondroitin sulphate glycosaminoglycan side chains. It is able to bind to extracellular matrix molecules as well as the intracellular actin cytoskeleton. It is mainly associated with epithelial cells and is proposed to regulate their shape and organisation. It is localised to the basolateral surface of epithelial cells, binding these structures to the basement membrane.

In summary, extracellular matrix components of the basement membrane provide a supporting structure for epithelial and basal cells within the airway wall, links to the lamina reticularis and acts as a selective molecular sieve. These components are continuously synthesised and degraded by epithelial and mesenchymal cells. They also influence the behaviour of cells by binding to integrins on cell surfaces, regulating their migration, proliferation and metabolism.

The main components of the lamina reticularis are collagen types I and III, elastin, proteoglycans and glycoproteins, which contribute to the structural and mechanical properties of the airways. The structure and function of these are described below.

Collagens

There are at least 19 different collagens, of which, 11 have been shown to be present in the lung. These collagens are classified into three groups – the fibril-forming types (I, II, III, V, XI), the non-fibril-forming types (IV, VI, VII, VIII) and the FACIT (fibril-associated collagens with interrupted triple helices, XII, XIV). Table 1 shows the collagen types known to be associated with the airways.

Collagen types I and III are produced by fibroblasts [24, 25]. It has been proposed that type I collagen provides the tensile strength to all flexible surfaces of the lung, whilst type III contributes to the tissue compliance [26]. They also provide a structural scaffold to which resident cells attach via specific integrins. The α-chains of these collagens have a single triple helical domain which forms 95% of the molecule (Fig. 2). Once secreted into the interstitial space, they form long, thin, cable-like structures covalently bonded to homologous regions of neighbouring fibrils. These fibrils exist as copolymers with common associations between collagens I–V, I–VI, III–VI and III–VII (for review see [27, 28].

Type VI collagen consists of alternating filamentous and beaded regions [29]. The α1 (VI) and α2 (VI) chains contain a number of collagen binding sites and RGD sequences suggesting that collagen type VI participates in matrix-matrix interactions and cell-matrix interactions [29].
<table>
<thead>
<tr>
<th>Type</th>
<th>Supramolecular structure</th>
<th>Function</th>
<th>Distribution in the airway wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibril</td>
<td>Structural component, tensile strength</td>
<td>Lamina reticularis, sub-mucosa, adventitia</td>
</tr>
<tr>
<td>II</td>
<td>Fibril</td>
<td>Structural component, tensile strength</td>
<td>Bronchial and tracheal cartilage</td>
</tr>
<tr>
<td>III</td>
<td>Fibril</td>
<td>Structural component, compliance</td>
<td>Lamina reticularis, sub-mucosa, adventitia</td>
</tr>
<tr>
<td>IV</td>
<td>Non-fibrillar 3-dimen­sional network</td>
<td>Molecular sieving, cell support</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>V</td>
<td>Fibril</td>
<td>Regulation of type I collagen fibrillogenesis</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
<tr>
<td>VI</td>
<td>Beaded filament</td>
<td>Cell adhesion to matrix</td>
<td>Associated with type I and III collagen, interstitium</td>
</tr>
<tr>
<td>VII</td>
<td>Fibril</td>
<td>Anchors basement membrane to matrix</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
<tr>
<td>VIII</td>
<td>Filamentous lattice (short chain)</td>
<td>Mechanical strength</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>IX</td>
<td>FACIT</td>
<td>Regulation of type II collagen fibrillogenesis</td>
<td>Cartilage, associated with type II collagen</td>
</tr>
<tr>
<td>XI</td>
<td>Fibril</td>
<td>Regulation of type II collagen fibrillogenesis</td>
<td>Cartilage, associated with type II collagen</td>
</tr>
<tr>
<td>XV</td>
<td>Multiplexin</td>
<td>Anchors basement membrane to matrix</td>
<td>Basement membrane, lamina reticularis</td>
</tr>
</tbody>
</table>

FACIT, fibril-associated collagens with interrupted triple helix; Multiplexin, multiple triple-helix domains and interruptions

Elastin

Mature elastin is composed of two chemically and morphometrically distinct components – amorphous elastin and a highly structured microfibrillar component. Elastin is formed by the cross-linking of lysine residues in secreted tropoelastin by lysyl oxidase, followed by spontaneous formation of desmosines and isodesmosines, the stable elastin cross-links. The microfibril component acts as a scaffold or template for the development of amorphous elastin which consists of fibrillin, microfibril associated glycoprotein and associated microfibril protein (for a full review see [30]).

Elastin is the second most abundant matrix molecule in the airway representing about 5% of the total protein content [31]. It is produced by fibroblasts [32–34]
Fibroblasts and myofibroblasts

forming parallel and longitudinal fibres localised to the lamina reticularis and submucosa [35–37].

During both the inflation and deflation cycles of the lung, airway patency is maintained by the radial tension induced by elastin within the wall restricting excessive smooth muscle contraction or relaxation [38].

Proteoglycans and glycoproteins

The core proteins of eighteen different proteoglycan molecules have been reported. Figure 2 shows the structure of four proteoglycan molecules found in airway tissue, perlecan, syndecan, decorin and versican. Each core protein is linked to a variety of glycosaminoglycan side chains which consist of alternating galactosamine and glucuronic/iduronic acid units (chondroitin/dermatan sulphate), alternating glucosamine and glucuronic/iduronic acid units (heparin and heparan sulphate) or alternating glucosamine and galactose units (keratan sulphate). Their functions include matrix hydration, modulation of collagen fibre formation, cell-matrix and cell-cell interactions and the binding of growth factors [39].

Proteoglycans are synthesised by a variety of cell types including fibroblasts [40], type II epithelial cells [41] and pulmonary arterial endothelial cells [42]. Platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) are capable of stimulating proteoglycan production by fibroblasts [43, 44].

Decorin and biglycan are two small chondroitin/dermatan sulphated proteoglycans, which are found associated with type I and type VI collagens, the lamina reticularis and smooth muscle bundles of the sub-mucosa within airways [45–47]. These molecules can bind polypeptide growth factors e.g. TGFβ [48] protecting them from denaturation and proteolytic degradation [49, 50]. It has therefore been suggested that these proteoglycans can act as a reservoir for growth factors, storing them until their release by proteases during tissue remodelling.

Versican, a large aggregating chondroitin sulphate proteoglycan is localised around the smooth muscle bundles in the sub-mucosa of the airway wall, in association with hyaluronan [51]. It is thought to change the mechanical properties of the airways in a similar manner to aggrecan in cartilage, by regulating the fluid (osmotic) balance within the airway tissue (see [46]).

Glypican, a glycosyl phosphatidylinositol linked heparan sulphate proteoglycan is expressed on the surface of human lung fibroblasts and bronchiolar epithelial cells. It binds fibronectin, collagen type I and anti-thrombin III via the heparan sulphate side chains. The function of glypican is not known, but its binding characteristics suggests a role in cell attachment and control of coagulation. A second heparan sulphate-linked proteoglycan is perlecan. This molecule is found in all basement membranes and gives the structure a fixed negative charge, which is important for the filtration properties of the membrane. It is also able to bind to
other extracellular matrix molecules such as laminin and collagen type IV, and is an attachment substrate for cells.

Tenascin

Tenascin is a large disulphide-linked glycoprotein of six identical subunits. In the normal lung it has been localised to both basement membrane and lamina reticularis of proximal airways [52, 53]. It inhibits cell adhesion, migration and causes cell shape changes [54]. Its distribution suggests involvement with tissue morphogenesis and wound healing. These functions and evidence that inflammatory cytokines can increase the secretion of this molecule from cultured epithelial cells [55], suggest a potential role for tenascin in airway remodelling.

Fibroblasts in the normal airways

Fibroblast which possess the greatest capacity to produce extracellular matrix molecules (see [56]), appear flattened and have a stellate appearance, with an average diameter of 28 µm and a thickness of 0.55 µm [57]. They are localised to the lamina reticularis, forming a sheath around the airways (Fig. 1b) [57]. The cells contain prominent rough endoplasmic reticulum, ribosomes and Golgi apparatus, reflecting their high metabolic activity.

A cell of similar phenotype to fibroblasts, but morphologically larger and containing a larger proportion of cytoplasm, is the myofibroblast [58-60]. These cells have been identified within the lamina reticularis of normal airways [61, 62] and can be distinguished from fibroblasts by the prominent amounts of contractile filaments containing α-smooth muscle actin [61, 63]. They are found in multicellular strands and in close apposition with other cells [64]. Myofibroblasts appear in increased numbers subsequent to tissue injury [65, 66].

The source of myofibroblasts at sites of tissue injury is uncertain, both differentiation from fibroblasts and smooth muscle cells have been proposed [67, 68]. TGFβ can induce smooth muscle-actin expression in fibroblasts, thus giving the cell a myofibroblast-like phenotype [69]. Myofibroblasts cultured from granulating wounds are capable of reverting back to a fibroblast phenotype [70].

The role of fibroblasts and myofibroblasts in airway morphogenesis and homeostasis

The fibroblast has been postulated to play an important role in airway morphogenesis and development. The co-culture of human epithelial cells with human foetal
Fibroblasts and myofibroblasts in collagen gels causes epithelial cell invasion of extracellular matrix, which then develop into groups of cells that resemble primordial sub-mucosal glands, forming tubular structures, which undergo dichotomous branching [71].

The development of the airways occurs during the pseudoglandular phase of lung development, between weeks 5–17 of gestation (for review see [72, 73]). The pattern of airway branching is dependent on the interactions between the developing mesenchyme, epithelial cells, cellular adhesion molecules and the extracellular matrix, in particular, proteoglycans and glycosaminoglycans [74]. In the developing lungs of rats, the differentiation of mesenchymal stem cells into fibroblasts-like and myofibroblast-like cells is associated with the production and deposition of extracellular matrix molecules [75]. The deposition of collagen, syndecan, laminin and fibronectin from these cells is essential for airway morphogenesis, in particular tubule formation and terminal branching [65, 73, 76].

In the mouse, syndecan expression also influences the development of alveolar sacs during the canalicular stages of lung development [77]. The formation of the alveoli is also associated with the synthesis and secretion of elastin, and possibly other extracellular matrix molecules by myofibroblasts at the leading edge of new alveolar buds [33]. Three growth factors produced by mesenchymal and epithelial cells, EGF, IGF-1 and TGFβ, also have a role in airway branching and alveolar development [73, 74].

The fibroblast/myofibroblast plays a key role in the maintenance of the airway extracellular matrix. Collagens are continually synthesised and degraded in the normal lung, with average turnover rates estimated to be 10% per day in lungs of young adult rats and rabbits [78–80]. These rates decrease with age, but proceed at relatively rapid rates throughout life [81]. Furthermore, the balance of intracellular and extracellular breakdown pathways also change with age. In young animals about 30% of newly synthesised collagen is degraded intracellularly within minutes of its synthesis [79, 82] but this proportion increases with age to about 80% [81]. In addition, the rates of these processes change when the lung is injured. For example, in experimental models of pulmonary fibrosis the proportion of newly synthesised collagen degraded intercellularly decreases, contributing to the increased collagen deposition in the injured lung [82]. The modulation of these processes could also play an important role in airway remodelling.

The rate of extracellular degradation is thought to depend on the degree of cross-linking and in older animals the mature collagen fibrils may be protected from degradation. Nevertheless, extracellular collagens are susceptible to breakdown and are degraded rapidly both in growing and diseased tissue. This is accomplished by a family of metalloproteinases, which are produced by both resident cells (fibroblasts, epithelial and endothelial cells) and inflammatory cells (activated macrophages and neutrophils) [83].

There are at least fifteen zinc- and calcium-dependent metalloproteinases which have the capacity to breakdown a wide range of extracellular matrix proteins [84–
They are important in embryonic tissue development, cell migration, inflammation and wound healing. The actions of the metalloproteinases is tightly regulated by a diverse group of anti-proteinases including the tissue inhibitors of metalloproteinases (TIMPs), which are synthesised and secreted by activated mesenchymal cells, and circulating anti-proteinases such as α2-macroglobulin, α1-antitrypsin, α2-antiplasmin and secretory leukocyte protease inhibitor. The recent development of synthetic metalloproteinase inhibitors is enabling the characterisation of the role of metalloproteinases in human disease [86].

In summary, fibroblasts and myofibroblast are involved in the morphogenesis and development of the airways. They respond to cytokines and growth factors producing a variety of extracellular matrix molecules, the degradation of which is tightly regulated by both intracellular and extracellular proteinases and their inhibitors. When this tight regulation breaks down, as seen in the collagen diseases (e.g. osteogenous imperfecta) or in inflammatory diseases (e.g. rheumatoid arthritis, asthma, COPD or lung fibrosis) compromised tissue function ensues.

**Regulation of fibroblast and myofibroblast extracellular matrix production in normal and diseased airways**

As mentioned in the previous section there are several mediators able to influence extracellular matrix production by fibroblasts and myofibroblasts during development, in particular, IGF-1, TGFβ1 and PDGF. However, mediators regulating extracellular matrix turnover during development or maintenance of the airway wall has not been extensively studied. A variety of cytokines, polypeptide growth factors, lipid mediators and coagulation cascade products are known to activate mesenchymal cells [28, 87]. Many of these mediators are released by both resident and inflammatory cells in the lungs of asthmatics (for review see [88, 89]. We will focus on the effects of the mediators that are currently known to be increased in this group of patients and which have been shown to modulate fibroblast function (Tab. 2).

**Cytokines and growth factors**

A large number of cytokines and polypeptide mediators released in the inflamed airway can cause fibroblast chemotaxis, proliferation and induce extracellular matrix molecule production (Tab. 2). TGFβ increases procollagen [90–92], fibronectin [91] and proteoglycan [44] production. It is able to regulate procollagen production by direct and indirect mechanisms. In fibroblasts, it increases the synthesis of procollagen, decreases its degradation intracellularly and extracellularly by down-regulation of metalloproteinases and up-regulation of tissue inhibitors of metalloproteinases, thus maximising its effect on collagen deposition [93].
### Table 2 - Mediators reported to be increased in asthma which have been shown to promote fibroblast migration, proliferation and matrix production.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Likely source</th>
<th>Increased directed migration</th>
<th>Enhanced proliferation</th>
<th>Increased matrix production</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Mφ, TC, BC, MC</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>94, 95</td>
</tr>
<tr>
<td></td>
<td>Fb, Ep, En, Neut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>TC, MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>96-99</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mo, Mφ, MC, Fb, Ep, En</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>100, 101</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Mφ, TC MC, Fb, Ep, Eos</td>
<td>↑</td>
<td></td>
<td></td>
<td>102</td>
</tr>
<tr>
<td>TNFα</td>
<td>Mφ, MC, Eos</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>103-106</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Mφ, Fb, Ep</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>107-109</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Mo, Mφ, Fb, MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>44, 90, 92</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fb, En, SMC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>117, 118</td>
</tr>
<tr>
<td>ECP</td>
<td>Eos</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>119-122</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>Mo, Mφ, Bo, MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>123-125</td>
</tr>
<tr>
<td></td>
<td>Eos, Neut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>Mφ, Fb, Ep, En</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>126-128</td>
</tr>
<tr>
<td>Tryptase</td>
<td>MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>129-132</td>
</tr>
<tr>
<td>Thrombin</td>
<td>plasma</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>133-136</td>
</tr>
<tr>
<td>Fibrinogen &amp; fibrinopeptides</td>
<td>plasma</td>
<td>↑</td>
<td></td>
<td></td>
<td>134</td>
</tr>
<tr>
<td>Neuropeptides</td>
<td>Sensory nerves</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>137</td>
</tr>
<tr>
<td>Histamine</td>
<td>Bo, MC</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>138, 139</td>
</tr>
</tbody>
</table>

Mo, monocyte; Mφ, macrophage; TC, T cell; BC, B cell; Bo, basophil; MC, mast cell; Fb, fibroblast; Ep, epithelial cell; En, endothelial cell; Eos, eosinophil; Neut, neutrophil; SMC, smooth muscle cell; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; INF α, interferon alpha; IL-1, interleukin-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNFα, tumour necrosis factor α; ECP, eosinophil cationic protein; ET-1, endothelin-1, FGF-2, fibroblast growth factor 2, ↑ increased function

TGFβ has been detected in the sputum of patients with asthma [140], in eosinophils infiltrating the epithelium of asthmatic airway [141], in nasal polyps [142] and in eosinophils, macrophages and fibroblasts within the lamina reticularis of airways from asthmatic patients [143, 144]. The latter finding is associated with
an increase in the sub-epithelial thickness within the airway and disease severity [143, 144]. In addition, TGFβ has been shown to cause a change in the phenotype of fibroblasts to myofibroblasts [69] and may therefore contribute to the increased numbers of myofibroblasts in asthmatic airways [61, 145]. These data, coupled with the known activity of TGFβ as a potent promoter of procollagen production, suggest that it may have a pivotal role in tissue remodelling within the diseased airway.

Granulocyte macrophage-colony stimulating factor (GM-CSF) secreted by inflammatory cells including lymphocytes, macrophages and fibroblasts [146, 147] prolongs the survival and activation of eosinophils potentiating the deleterious effects of these cells in the airways of asthmatics [146, 148]. Consistent with this, recent studies have demonstrated that transient over-expression of the GM-CSF gene leads to peri-bronchial accumulation of eosinophils, an increased number of macrophages in the lung parenchyma, increases in the levels of TGFβ and irreversible fibrotic lesions [149, 150]. GM-CSF is chemotactic for myofibroblasts [102], suggesting another mechanism by which these cells could accumulate in the lamina reticularis of asthmatic airways [61, 145] with subsequent activation by TGFβ.

Fibroblasts are able to produce inflammatory cytokines such as IL-1, IL-6, chemokines and GM-CSF in response to activation by the acute phase proteins IL-1 and TNFα [56]. These mediators have paracrine effects leading to enhanced inflammatory cell accumulation (Fig. 3). In this way the fibroblast may both activate and prolong the inflammatory response.

A close apposition of eosinophils and fibroblasts was noted by Glyn and Michaels [151] in the airways of asthmatics. Furthermore, activated eosinophils degranulate and release mediators, which include TGFβ, and granule products, eosinophil cationic proteins (ECP) and major basic protein (MBP), into the surrounding tissue or airway lumen. ECP stimulates the proliferation of fibroblasts and increases the synthesis of collagen and proteoglycans [119, 120, 122, 152]. Whereas, MBP, acts synergistically with TGFβ and IL-1 to enhance IL-6 production by fibroblasts, which stimulates fibroblast proliferation and extracellular matrix protein production by both autocrine and paracrine mechanisms [100, 101, 153].

The mast cell has been shown to play a pivotal role in asthma, both in the transient bronchoconstriction and the chronic inflammatory phase of the disease [154]. As shown in Table 2 many of the mediators released by mast cells are able to modulate fibroblast function.

One such mediator is tryptase, a trypsin-like serine protease [155] which is elevated in bronchoalveolar lavage fluid from patients with allergic inflammation [156] and asthma [157]. Tryptase is chemotactic and mitogenic for fibroblasts [129–132, 158], stimulates type I collagen production in human lung fibroblasts [132] and increases procollagen mRNA levels in dermal fibroblasts [129]. With the established role of mast cells and their mediators in allergy and asthma, it is likely
that they also play an important role in the activation of fibroblasts and myofibroblasts in the diseased airway.

Other peptides present in the airways of asthmatic patients also have the capacity to activate fibroblasts. Endothelin-1 is found in high concentrations within the airways of asthmatic patients [159–161] and has been demonstrated to be a chemoattractant and mitogen for fibroblasts and also stimulates collagen synthesis [126–128].

Neuropeptide-containing sensory nerves have been localised in human airways and apposed to fibroblasts (Fig. 1b) [162, 163]. The activation of these sensory nerves, with the subsequent release of the neuropeptides, substance P and neurokinin A, in the asthmatic airways may lead to bronchoconstriction and neurogenic inflammation [164]. In addition to these recognised roles both substance P and neurokinin A are capable of stimulating fibroblast proliferation and chemotaxis [137].

**Coagulation cascade products**

Fibrin has been shown to be deposited within the airway wall [165, 166] demonstrating the activation of the blood coagulation cascade. Several products of this cascade are known to have pro-fibrotic effects.

Thrombin is mitogenic and chemotactic for lung fibroblasts [133, 134] and increases collagen synthesis by these cells at least partly by protease-activated receptor-1 [136]. Thrombin may also activate fibroblasts indirectly by the release of mediators from resident cells, platelets and the extracellular matrix. In addition, recent studies from our laboratory demonstrate Factor Xa stimulation of fibroblast proliferation and collagen synthesis [167].

The generation of fibrin by the actions of thrombin on fibrinogen generates fibrinogen-cleavage products, fibrinopeptides A and B which are mitogenic for fibroblasts [134]. Also, the isolated &A and Bβ chains from the fibrinogen molecule possess mitogenic activity [168]. Thus products of the coagulation cascade derived from plasma protein exudation observed in the inflamed airway can activate fibroblasts and have the potential to contribute to airway remodelling.

**Mechanical load**

Mechanical load is now recognised as an important regulator of cell function. The cyclic mechanical stretch of fibroblasts has been shown to cause an increase in cell proliferation [169] and procollagen production [170]. A clinical feature of asthma is repeated, transient bronchoconstriction. This raises the possibility that changes in contractile properties of the airways may influence the activation of the mesenchymal cells to proliferate and secrete increased amounts of matrix proteins.
Hypoxia

Hypoxemia occurs in asthmatic patients which is associated with the degree of airway obstruction [171]. As the bronchioles receive their blood supply via the bronchial artery, which is derived from the aorta, this suggests that the airways may be exposed to transient hypoxia. Hypoxia is known to have marked effects on fibroblast function increasing both proliferation and collagen synthesis in-vitro [172], which is mediated, in part, by TGFβ [173]. In addition hypoxic endothelial and epithelial cells release increased amounts of profibrotic cytokines such as PDGF and endothelin-1 [174].

In summary a number of cytokines and structurally diverse polypeptide and lipid mediators are able to cause fibroblast chemotaxis, proliferation and stimulation of extracellular matrix protein production. In addition mechanical load and hypoxia also capable of activating fibroblasts. Current research implicates several cytokines such as TGFβ and endothelin-1 as important agents promoting matrix deposition but many other mediators could be important (see Tab. 2) including proteases generated by the coagulation cascade and released by activated mast cells.

Fibroblasts as antigen presenting and effector cells in the immune response

It has been suggested that fibroblasts can act as antigen presenting cells based on the expression of thymocyte-1 (Thy-1) antigen on their cell surface (Fig. 3) [175–177]. Murine lung fibroblasts not expressing the Thy-1 antigen up-regulate the expression of MHC class II antigen and therefore have the ability to present antigen to T cells (Fig. 3). In contrast, human lung fibroblasts expressing Thy-1 were able to up-regulate MHC class II antigen, but only after stimulation with IFNγ. A subset of these cells demonstrated the ability to cause T-cell proliferation [177].

Fibroblasts could therefore act as accessory antigen presenting cells, in conjunction with dendritic cells and macrophages, to enhance the local immune and inflammatory response (Fig. 3). More studies are needed to establish the extent to which fibroblasts contribute to the overall immune response.

Recent observations have also shown that a soluble mediator secreted by fibroblasts can prevent lymphocyte apoptosis by maintaining or increasing the expression of the Bcl-XL gene [178, 179]. Thus, there would be a persistence of lymphocytes adding to the chronicity of the inflammatory response.

Extracellular matrix molecule deposition within the lamina reticularis

A change in the structure of airways from patients who had died from asthma was first noted by Huber and Koessler [180]. These findings were confirmed in mor-
Fibroblasts as antigen presenting and inflammatory cells. Antigen presentation by fibroblasts leads to an enhancement of the immune response, and stimulation of cytokine and extracellular matrix production. The cytokines produced by the fibroblast enhance the inflammatory response by both autocrine and paracrine mechanisms.

Figure 3

Phological studies in which the airway wall was found to be significantly thicker in asthmatic compared with non-asthmatic patients [181]. Carroll and colleagues [182] demonstrated increased wall thickness in both the large and small airways in fatal asthma, but only in the small airways in non-fatal asthma.

This increase has also been noted in other airways diseases such as chronic obstructive pulmonary disease [183–185], bronchiectasis, bronchitis, and tuberculosis [183]. The thickening results from increase in the thickness of the mucosa [181, 186], lamina reticularis [5, 166, 187, 188] and sub-mucosal layers of the airway [180, 181, 187, 189, 190]. Changes in the sub-mucosa are likely to involve both smooth muscle hyperplasia [191] and hypertrophy [192].

It was initially thought that the changes in the lamina reticularis were a consequence of the thickening of the epithelial basement membrane, due to increased collagen deposition [151, 180, 189, 193]. However, using ultrastructural techniques, the thickening was localised to the lamina reticularis, with the lamina rara and the lamina densa of the basement membrane remaining unchanged [5, 166, 187]. These changes appear to be an early event in the pathology of asthma, as increases in the
sub-epithelial layer are seen in children with asthma [190] and in patients with mild disease [166].

It was postulated that the increased thickness of the lamina reticularis was due to the activation of the epithelial cells [166], which have been shown to secrete collagen in vitro [194, 195]. However, the composition of this layer was shown to consist predominantly of collagen types I, III and V, fibronectin [5, 196] and elastin [145], molecules that are generally thought to be produced by fibroblasts, rather than epithelial cells. Also, no laminin was identified within the lamina reticularis, but was found within the “true” basement membrane, again, suggesting that the matrix deposition in the sub-epithelial layer was not associated with epithelium [5].

The increased thickness of the lamina reticularis has been correlated with increased numbers of fibroblasts/myofibroblasts [61, 145, 151]. Furthermore, in a recent study, increased numbers of myofibroblasts were observed in the lamina reticularis of mild asthmatic patients after antigen challenge [62]. From these studies it was suggested that myofibroblasts were responsible for deposition of the increased extracellular matrix. However, other cell types within the airway wall are capable of producing extracellular matrix molecules and are likely to be involved in remodelling. For example, smooth muscle cells are capable of producing similar amounts of procollagen to fibroblasts [197].

In addition to the increase in collagen, amounts of fibronectin, elastin and other extracellular matrix components have been shown to be increased within the lamina reticularis of asthmatic airways. Hyaluronan, versican, biglycan and decorin were increased in the airways of patients with severe asthma [46]. Due to the hydrophilic nature of these molecules, it can be speculated that the airway wall may be further thickened by an increased fluid content within the sub-mucosa. Increased proteoglycans production has been shown from bronchial fibroblast cell lines cultured from asthmatic patients [198] and, increased levels have been demonstrated in the sputum from asthmatic subjects [199]. Currently no data is available for the content of these matrix molecules in bronchial biopsies from mild asthmatics. Fibroblasts may also be involved in the regulation of fluid content of tissues via integrin mediated cell-matrix interaction [200–202].

Functional effects of a thickened airway wall

Both the airways and the parenchyma contribute to the elastic recoil of lung tissue. Mead hypothesised that the parenchyma is able to stabilise the alveoli and terminal bronchioles, by generating a recoil force (interdependence), thus preventing airway collapse (Fig. 4a) [203]. A decrease in elastic recoil may have profound effects on the airway contractility. This hypothesis was extended to suggest that the adventitial tissue around the airway is an important determinant of the magnitude of bronchoconstriction (Fig. 4a and b) [204–206]. Several groups have hypothesised that
the load exerted by the lung parenchyma in the normal airways must be overcome by the smooth muscle to enable the airway to contract (Fig. 4c) [205–207]. Excessive extracellular matrix deposition outside the smooth muscle layer (peri-bronchial fibrosis) would uncouple these opposing forces allowing the smooth muscle to contract more easily (Fig. 4d) [208].

The increase in the deposition of extracellular matrix within the mucosal layer and lamina reticularis would also have consequences for airway constriction. A thickened mucosal layer, compared to normal, causes an increase in the narrowing of the lumen for any given degree of smooth muscle contraction (Fig. 4c) [209]. An important normal physiological response to smooth muscle contraction is the folding of the mucosa which restricts lumenal narrowing. However, in the inflamed airway, the deposition of the extracellular matrix within the lamina reticularis would limit the number of folds generated, thus allowing a further reduction in lumenal diameter (Fig. 4b and d) [210].

In early stages of disease, the initial deposition of extracellular matrix proteins by fibroblasts and other mesenchymal cells may be beneficial. Extending the hypothesis of Mead and others, the initial deposition of extracellular matrix around the airways, within the lamina reticularis, adventitia and the alveolar connections, may increase the parenchymal load on the airways, increasing interdependence, thus restricting the degree of airway closure.

However, as the disease progresses, excessive deposition of extracellular matrix, along with the thickening of the airway wall due to increased smooth muscle, oedema and epithelial cell hyperplasia, becomes detrimental to airway function. There is now anecdotal evidence that there is a reduction in the number of alveolar connections to the airways of asthmatics, which would reduce interdependence and increase the degree of basal airway tone. Also this reduction in interdependence will allow an increase in airways contractility (airway hyperresponsiveness) to inciting agents (e.g. antigen, cold air, exercise, stress).

In addition to the increase in extracellular matrix deposition, a reduction in the amount of elastin is seen in some patients, while disorganisation of the elastin fibrils is seen in others [35, 211]. This data would support the theory of interdependence, as the force required to overcome the elastic nature of the parenchymal tissue would be less thus increasing the magnitude of the bronchoconstriction. The change in elastin content and structure may partly explain the reduction in lung elasticity seen in asthmatic patients [212, 213]. In contrast, no change in the elastin content of the airways was found by Godfrey and colleagues [214]. Thus no consensus of opinion has been reached regarding the effects on elastin metabolism in the airways during chronic inflammation. However, this is an important feature of remodelling in the inflamed airway and needs further investigation.

A limited number of studies have been performed specifically to examine the interaction of sub-epithelial thickening and airway function. Roche and colleagues found no correlation between sub-epithelial thickness and disease severity or air-
A schematic diagram showing the effect of structural changes on the airway lumen. In the normal airway, constriction and relaxation within this airway is a reversible process. (a) shows the forces exerted on the normal dilated and constricted airway wall which keeps the lumen open (interdependence). During bronchoconstriction smooth muscle contraction must overcome the external forces before the airway is able to constrict. At this point the epithelium folds to compensate for the reduction of the airway lumen, however the external forces restrict the degree of constriction. On smooth muscle relaxation the external elastic forces will allow the airway to relax back to its resting state.

(b) In the inflamed airway constriction is greatly enhanced. The increase in wall thickness due to a thicker epithelium and smooth muscle hypertrophy and hyperplasia, reduces the resting lumen diameter, thus, in the inflamed airway a similar degree of airway constriction to that seen in the normal airways, would result in a greatly reduced lumen diameter. Thus having profound effects of airway function.

(c) In addition to the wall thickening, the increase in matrix deposition within the lamina reticularis reduces the ability of the mucosa to fold. Therefore, in combination with the increased extracellular matrix within the peri-bronchial adventitia, which reduces the load exerted by the parenchyma on the airway wall, this would allow the airway to constrict to a greater extent than in either a) or b). Subsequently, the combination of the thickened airway wall and extracellular matrix deposition also restricts the degree to which the airway can return to its normal resting diameter, potentially leading to a chronic reduction in airflow.
Fibroblasts and myofibroblasts

ways hyperresponsiveness [5]. However, the thickness of the sub-epithelial layer or bronchial wall has been correlated to airway hyperresponsiveness by others [166, 215, 216] and in a recent study, Minshall and colleagues were able to demonstrate a correlation between sub-epithelial thickness and lung function (FEV₁) in mild, moderate and severe asthmatic patients [144].

In summary, changes in the structure of the airways in inflammatory diseases, due to changes in key extracellular matrix components such as elastin, collagen and proteoglycans, will have marked effects on the mechanical properties of the lung. This suggests pharmacological agents directed at controlling extracellular matrix deposition may have a place in the treatment of asthma. A reduction of the fibrotic lesions, may lead to the re-establishment of the interdependence between the airway wall and the lung parenchyma, thus returning lung function towards normal. The effects of selected agents on collagen production and degradation will be reviewed in the next section.

Pharmacological modulation of the fibroblast

The current treatment for asthma involves the combination of symptomatic relief with bronchodilators (β-adrenoceptor agonist, theophylline, ipratropium bromide) and anti-inflammatory agents (beclomethasone, budesonide, fluticasone propionate). The steroids are currently the most effective treatment for inflammation in asthma [217]. However, there is a group of patients who are refractive to these agents.

Steroids may inhibit fibroblast function, in vitro, either by inhibiting procollagen gene expression [218] or by inhibiting the release of pro-fibrotic cytokines from inflammatory cells. In vivo studies are more controversial but there is evidence that steroids can inhibit collagen production and block fibrosis [219]. The effects of inhaled budesonide on extracellular matrix deposition in a number of studies in asthmatic patients, have been unable to show a reduction in the thickening of the sub-epithelial layer [188, 220, 221] or a reduction in the levels of extracellular matrix molecules within this layer [222]. In contrast, in two studies of short-term treatment with either beclomethasone or fluticasone propionate a reduction in sub-epithelial thickening was noted [223, 224]. A similar decrease in sub-epithelial thickening was seen after the removal of inciting agents in occupational asthma [225, 226] suggesting that it is possible to reduce extracellular matrix deposition in chronically inflamed tissue.

The conflicting results obtained with these steroids may be due to the type and dose of steroid used, patient compliance or even a reflection of disease severity. Alternatively, these discrepancies could be accounted for by differing techniques associated with sampling, cutting and analysis of the bronchial biopsies. Further studies are required to determine the effects of steroids on extracellular matrix deposition.
Table 3 - Agents which modulate collagen metabolism

<table>
<thead>
<tr>
<th>Likely mechanisms of action</th>
<th>Agent</th>
<th>Evaluation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in procollagen mRNA levels</td>
<td>Tranilast</td>
<td>Clinical</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>Minoxidil; pirfenidone</td>
<td></td>
<td>231, 232</td>
</tr>
<tr>
<td>Decrease transcription or procollagen mRNA stability</td>
<td>Glucocorticoids</td>
<td>Animal models</td>
<td>233-241</td>
</tr>
<tr>
<td></td>
<td>INFγ; PGE₁; PGE₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Translation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incorporated to form unstable collagen chains</td>
<td>Proline analogues</td>
<td><em>in vitro</em></td>
<td>242-250</td>
</tr>
<tr>
<td></td>
<td>Procollagen peptides</td>
<td>Animal models</td>
<td></td>
</tr>
<tr>
<td>Feedback inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiaproline; D-α-methylproline</td>
<td></td>
<td>251</td>
</tr>
<tr>
<td>Blocks chain elongation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Removal of cofactors for hydroxylation</td>
<td>Bivalent cations, e.g. Zn²⁺; ascorbate analogues</td>
<td><em>in vitro</em>; animal models, clinical</td>
<td>252-259</td>
</tr>
<tr>
<td>Inhibition of prolyl 4-hydroxylase</td>
<td>Proline analogues; Safironil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of lysyl hydroxylase</td>
<td>Minoxidil</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microtubular systems/Secretion</strong></td>
<td>Colchicine; Cytochalasin B; Taxol; Nocodazole; Vincristine; Vinblastine; Brefeldin A</td>
<td><em>in vitro</em></td>
<td>260-262</td>
</tr>
<tr>
<td>Disruption of microtubules, Golgi and endoplasmic reticulum system</td>
<td>Animal models</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of procollagen peptides</td>
<td>Amino acids and polyamines</td>
<td><em>in vitro</em></td>
<td>263</td>
</tr>
<tr>
<td><strong>Polymerisation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prevention of cross-linking</td>
<td>D-Penicillamine; β-Aminopropionitrile</td>
<td><em>in vitro</em>; animal studies; clinical</td>
<td>264-266</td>
</tr>
<tr>
<td>Inhibition of lysyl oxidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Extracellular degradation</strong></td>
<td>Colchicine; Relaxin; Pentoxifylline</td>
<td><em>in vitro</em>; animal models; clinical</td>
<td>267-269</td>
</tr>
<tr>
<td>Increases production of collagenase</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4 - Agents which modulate fibroblast activation and proliferation mechanisms

<table>
<thead>
<tr>
<th>Likely mechanisms of action</th>
<th>Agent</th>
<th>Evaluation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth factor mediators</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of mediator release</td>
<td>Tranilast (MK341)</td>
<td>Clinical</td>
<td>230</td>
</tr>
<tr>
<td>Inhibition of TGFβ, PDGF, FGF</td>
<td>Pirfenidone</td>
<td>In vitro</td>
<td>270-274</td>
</tr>
<tr>
<td>Binds and inactivates TGFβ</td>
<td>Decorin</td>
<td>Animal studies</td>
<td></td>
</tr>
<tr>
<td><strong>Growth factor receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of TGFβ activation</td>
<td>Mannose-6-phosphate</td>
<td>Animal models</td>
<td>275-278</td>
</tr>
<tr>
<td>Inactivation of growth factors</td>
<td>TGFβ antisense; TGFβ and TNFα antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of both proliferation and procollagen synthesis</td>
<td>Receptor antagonists, e.g. endothelin; angiotensin II</td>
<td>In vitro</td>
<td>279-281</td>
</tr>
<tr>
<td><strong>Fibroblast proliferation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of proliferation and matrix synthesis</td>
<td>Retinoids</td>
<td>In vitro</td>
<td>282, 283</td>
</tr>
<tr>
<td>Inhibits fibroblast proliferation</td>
<td>5-fluorouracil; mitomycin-C, Clinical</td>
<td>Animal models</td>
<td></td>
</tr>
<tr>
<td>Decrease in matrix production</td>
<td>Minoxidil; Taxol</td>
<td>Clinical</td>
<td>261, 262</td>
</tr>
</tbody>
</table>

As steroids have a number of side effects [227] alternative approaches have been investigated, including inhibition of procollagen DNA transcription, mRNA translation or post-translational packaging of the procollagen molecules [87, 228, 229]. These, and other strategies for inhibiting collagen deposition and fibroblast proliferation are shown in Tables 3 and 4 along with other agents which are currently under investigation for a number of fibrotic conditions, including lung fibrosis. These new compounds are now beginning to be evaluated in experimental models and man.

### Summary

There is now strong evidence that fibroblasts and myofibroblasts play key roles in airway morphogenesis, maintenance of airway inflammation and tissue repair. They are metabolically active cells, continuously producing extracellular matrix compo-
nents and are responsible for the maintenance of the structural framework of the airway. Through cell-matrix interactions they can also regulate the three-dimensional structure and mechanical properties of the airways. In asthmatic airways the number of fibroblasts/myofibroblasts and the amount of extracellular matrix increases profoundly affecting airway mechanics and cell function. The mechanisms for this increase in extracellular matrix deposition are uncertain. We propose that structurally diverse mediators from resident cells, inflammatory cells and blood derived proteins modulate the function of fibroblasts and myofibroblasts, increasing mesenchymal cell numbers locally within the airway tissue and enhancing extracellular matrix protein deposition. In addition, with the recently ascribed role of fibroblasts in antigen presentation, the production of pro-inflammatory cytokines and enhancement of inflammatory cell survival, suggests that these cells could also enhance and perpetuate the inflammatory response in the airway. A better understanding of the mechanisms by which fibroblast and myofibroblast functions are modulated, will identify new targets for the development of therapeutic agents for use in diseases associated with inflammation and airway remodelling such as asthma, chronic obstructive pulmonary disease (COPD) and bronchitis.

References

Fibroblasts and myofibroblasts


181
Fibroblasts and myofibroblasts


183


67 Phan SE (1996) Role of the myofibroblast in pulmonary fibrosis. *Kid Int* 49 (suppl 54) S46-S48


Fibroblasts and myofibroblasts for rapid rates of collagen turnover with extensive degradation of newly synthesised collagen in tissues of the adult rat. Collagen Rel Res 7: 93–104


96 Postlethwaite AE, Holness MA, Katai H, Raghow R (1992) Human fibroblasts synthet-


109 Goldstein RH, Polgar P (1982) The effect and interaction of bradykinin and prosta-
glandins on protein and collagen production by lung fibroblasts. *J Biol Chem* 257: 8630–8633


mRNA for matrix proteins both in the presence and in the absence of changes in mRNA
stability. Proc Natl Acad Sci USA 85: 1105–1108

112 Khalil N, Greenberg AH (1991) The role of TGFβ in pulmonary fibrosis. In: GR Bock,
211

113 Lyons RM, Moses HL (1990) Transforming growth factor and the regulation of cell pro-

114 Ignotz RA, Massague J (1986) Transforming growth factor-β stimulates the expression
of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol
Chem* 261: 4337–4345

enhances the production of hyaluronan in human lung but not in skin fibroblasts. *Exp
Cell Res* 186: 192–195

A (1993) Altered expression of small proteoglycan, collagen and transforming growth
factor-β₁ in developing bleomycin-induced pulmonary fibrosis in rats. *J Clin Invest* 92:
632–637

fibroblasts into a denuded area in a cell monolayer is mediated by basic fibroblast

stimulate cardiac fibroblast activity. *Ann NY Acad Sci* 752: 387–393

119 Pincus SH, Ramesh KS, Wyler DJ (1987) Eosinophils stimulate fibroblast DNA synthe-
sis. *Blood* 70: 572–574

120 Birkland TP, Cheavens MD, Pincus SH (1994) Human eosinophils stimulate DNA syn-
thesis and matrix production in dermal fibroblasts. *Arch Dermatol Res* 286: 312–318

(1991) Eosinophils adhere to and stimulate replication of lung fibroblasts “in vitro”.
*Clin Exp Immunol* 86: 185–190

Eosinophil cationic protein alters proteoglycan metabolism in human lung fibroblast


tions. Granule major basic protein interacts with IL-1 and transforming growth factor-
\( \beta \) in the stimulation of lung fibroblast IL-6 type cytokine production. *J Immunol* 156: 4449–4456


169 Bishop JE, Mitchell JJ, Absher PM, Baldor L, Geller HA, Woodcock-Mitchell J, Ham-
Fibroblasts and myofibroblasts


180 Huber HL, Koessler KK (1922) The pathology of bronchial asthma. Arch Intern Med 30: 689–760


and morphology of airways in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 143: 1189–1193


199 Shute JK, Criss J, Hockey P, Howarth PH (1998) Increased proteoglycans in sputum in asthma and cystic fibrosis (CF) and increased GAG levels in CF. Am J Respir Crit Care Med 157: A607


202 Berg A, Ekwall AK, Rubin K, Stjernschantz J, Reed RK (1998) Effect of PGE1, PGJ2 and
PGF$_2$ alpha analogs on collagen gel compaction \textit{in vitro} and interstitial pressure \textit{in vivo}.  
\textit{Am J Physiol} 274: H663–H671


bronchoscopy and bronchial mucosal biopsies in asthmatics undergoing long-term high-dose budesonide aerosol treatment. *Allergy* 43: 284–288


Fibroblasts and myofibroblasts
ticosteroids decrease the synthesis of type I procollagen mRNAs. Biochemistry 25:
3202–3209
fibroblast collagen synthesis by interferons. J Clin Invest 74: 1112–1116
236 Baum BJ, Moss J, Breul SD, Crystal RG (1978) Association in normal human fibrob­
lasts of elevated levels of adenosine 3′: 5′-monophosphate with a selective decrease in
gen types I and III. Effects of PGE_1 and isoproterenol. Fed Proc 40: 1813
238 Baum BJ, Moss J, Breul SD, Berg RA, Crystal RG (1980) Effect of cyclic AMP on the
239 Varga J, Diaz-Perez A, Rosenbloom J, Jimenez SA (1987) PGE_2 causes a coordinate
decrease in the steady state levels of fibronectin and types I and III procollagen mRNAs
in normal human dermal fibroblasts. Biochem Biophys Res Commun 147: 1282–1288
ferential effects of gamma-interferon on collagen and fibronectin gene expression. J Biol
Chem 262: 13348–13351
methylprednisolone aceponate and momethasone furoate on collagen synthesis in
human skin in vivo. Skin Pharmacol 10: 261–264
242 Uitto J, Prockop DJ (1975) Inhibition of collagen accumulation by proline analogues: the
mechanism of their action. In: H Poppu, K Becker (eds): Collagen metabolism in the
liver, vol. 1. Stratton Intercontinental, New York, 139–148
vention of bleomycin-induced pulmonary fibrosis in the hamster by cis-4-hydroxyl-L-
proline Am Rev Respir Dis 123: 388–393
244 Tan EM, Ryhänen L, Uitto J (1983) Proline analogues inhibit human skin fibroblast
245 Westner M, Krieg T, Hörlein D, Glanville RW, Fietzek P, Müller PK (1979) Inhibiting
effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. J Biol
Chem 254: 7016 7023
bition of collagen and fibronectin synthesis by a synthetic homolog of a portion of the
expression: a trojan horse approach Proc Natl Acad Sci USA 88: 10158–10162
of proline analogue with sustained antifibrotic activity in lung fibrosis. Am J Respir Crit
Care Med 155: 1391–1397
synthesis in IMR-90 human lung fibroblast cell cultures. Evidence for transcriptional con­
trol. J Biol Chem 266: 2983–2987

195
Fibroblasts and myofibroblasts


279 Mutsaers SE, Foster ML, Chambers RC, Laurent GJ, McAnulty RJ (1997) Increased


