ROLE OF TRANSFORMING GROWTH FACTOR β ISOFORMS

IN THE PATHOGENESIS OF PULMONARY FIBROSIS

by

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A thesis submitted to the University of London for the degree of PhD

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ABSTRACT

Pulmonary fibrosis is a disease of the lung interstitium characterised by excessive deposition of extracellular matrix proteins including collagen. The aetiology is frequently unclear, but the last decade has generated significant advances in our understanding of the pathogenesis. One current hypothesis is that polypeptide mediators, released by resident lung cells and recruited inflammatory cells, stimulate fibroblast replication and increased collagen synthesis. Interstitial collagen deposition then impedes gas exchange. Of the cytokines studied so far, current evidence strongly implicates transforming growth factor β1 (TGFβ1). However, it is now known that there are at least five TGFβ isoforms, of which TGFβ1,3 are found in mammals. The role of TGFβ2 and TGFβ3 in the pathogenesis of pulmonary fibrosis is currently unclear. The overall aim of this thesis was to examine the role of the three different TGFβ isoforms in the pathogenesis of pulmonary fibrosis. In so doing, I addressed the hypothesis that TGFβ1, TGFβ2 and TGFβ3 play distinct but overlapping roles in the pathogenesis of this disease. To address this hypothesis, the effect of TGFβ2 and TGFβ3 on human lung fibroblast procollagen metabolism was examined in vitro. TGFβ1,3 all stimulated fibroblast procollagen production. TGFβ3 was the most potent and also reduced intracellular procollagen degradation. Secondly, a non-isotopic in situ hybridisation technique was developed for use in lung tissue. This enabled the localisation of TGFβ isoform gene expression in normal and fibrotic murine and human lung. TGFβ1 and TGFβ3 mRNA transcripts were demonstrated in a wide variety of lung cells not hitherto recognised to express these genes, and TGFβ3 gene expression was demonstrated in human lung for the first time. TGFβ1 but not TGFβ3 gene expression was enhanced during bleomycin-induced lung injury in mice, and TGFβ1 gene expression was more consistently enhanced in human lung fibrosis than was that of TGFβ3. Taken together, these data suggest that TGFβ1 is the predominant isoform implicated in the pathogenesis of this disease. Finally, results with the TGFβ2 riboprobes yielded positive hybridisation signal using the sense probe, but little or no signal using the antisense probe. These results and further studies involving characterisation of the TGFβ2 probes and Northern analysis of rat lung and murine lung cells suggested that a natural TGFβ2 antisense transcript is present in mammalian lung.
I should particularly like to thank the following people without whom this thesis would not have been written.

Dr Robin McAnulty, who supervised these studies on a day to day basis. I am especially grateful for his unfailing enthusiasm, expertise and patience.

Professor Geoffrey Laurent, my supervisor and head of department, whose support and advice have been invaluable.

Dr Kim Harrison, who provided the initial inspiration and has been a continued source of encouragement along the way.

Dr Ron du Bois, Dr Peter Jeffery and Dr Carol Black, for supplying me with open lung biopsy samples from their patients for in situ hybridisation studies.

Dr Shahzeidi, who provided the murine lung samples from the bleomycin studies.

All my colleagues who have contributed, with lively discussions and numerous cups of tea, to making my stay in this department an extremely valuable and enjoyable one.

My parents, for their unfailing support throughout my undergraduate and postgraduate studies.
# INDEX OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>3</td>
</tr>
<tr>
<td>INDEX OF CONTENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>14</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>18</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>19</td>
</tr>
<tr>
<td>CHAPTER ONE: INTRODUCTION</td>
<td>22</td>
</tr>
<tr>
<td>1.1 Preamble</td>
<td>23</td>
</tr>
<tr>
<td>1.2 Defining pulmonary fibrosis</td>
<td>23</td>
</tr>
<tr>
<td>1.2.1 Historical overview</td>
<td>26</td>
</tr>
<tr>
<td>1.2.2 Clinical features</td>
<td>27</td>
</tr>
<tr>
<td>1.2.3 Investigations</td>
<td>27</td>
</tr>
<tr>
<td>1.2.3.1 Radiology</td>
<td>28</td>
</tr>
<tr>
<td>1.2.3.2 Pulmonary function testing</td>
<td>30</td>
</tr>
<tr>
<td>1.2.3.3 Bronchoalveolar lavage</td>
<td>30</td>
</tr>
<tr>
<td>1.2.3.4 Histology</td>
<td>31</td>
</tr>
<tr>
<td>1.3 The nature of the problem</td>
<td>35</td>
</tr>
<tr>
<td>1.3.1 Pathogenesis</td>
<td>35</td>
</tr>
<tr>
<td>1.3.2 Epidemiology</td>
<td>35</td>
</tr>
<tr>
<td>1.3.3 Prognosis</td>
<td>39</td>
</tr>
<tr>
<td>1.3.4 Current treatments</td>
<td>39</td>
</tr>
<tr>
<td>1.4 Collagen</td>
<td>40</td>
</tr>
<tr>
<td>1.4.1 Collagen in normal lung</td>
<td>40</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.4.1.1</td>
<td>Collagen types and distribution</td>
</tr>
<tr>
<td>1.4.1.2</td>
<td>Collagen turnover</td>
</tr>
<tr>
<td>1.4.1.3</td>
<td>Collagen synthesis</td>
</tr>
<tr>
<td>1.4.1.4</td>
<td>Collagen degradation</td>
</tr>
<tr>
<td>1.4.1.5</td>
<td>Mechanisms regulating collagen deposition</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Collagen regulation in pulmonary fibrosis</td>
</tr>
<tr>
<td>1.4.2.1</td>
<td>Animal models</td>
</tr>
<tr>
<td>1.4.2.2</td>
<td>Human pulmonary fibrosis</td>
</tr>
<tr>
<td>1.5</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>1.5.1</td>
<td>The TGFβ superfamily</td>
</tr>
<tr>
<td>1.5.2</td>
<td>The TGFβ isoforms</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Chemistry</td>
</tr>
<tr>
<td>1.5.3.1</td>
<td>Structure</td>
</tr>
<tr>
<td>1.5.3.2</td>
<td>Activation</td>
</tr>
<tr>
<td>1.5.3.3</td>
<td>Receptors</td>
</tr>
<tr>
<td>1.5.4</td>
<td>Biological activity</td>
</tr>
<tr>
<td>1.5.4.1</td>
<td>Immunoregulation</td>
</tr>
<tr>
<td>1.5.4.2</td>
<td>Regulation of cell proliferation and differentiation</td>
</tr>
<tr>
<td>1.5.4.3</td>
<td>Regulation of extracellular matrix protein turnover</td>
</tr>
<tr>
<td>1.6</td>
<td>TGFβ in the pathogenesis of fibrosis</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Role of TGFβ₁ in extra-pulmonary fibrosis</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Role of TGFβ₁ in pulmonary fibrosis</td>
</tr>
<tr>
<td>1.6.2.1</td>
<td>Animal studies</td>
</tr>
<tr>
<td>1.6.2.2</td>
<td>Human studies</td>
</tr>
<tr>
<td>1.7</td>
<td>Current in situ hybridisation techniques</td>
</tr>
<tr>
<td>1.8</td>
<td>Aims of this thesis</td>
</tr>
<tr>
<td>1.8.1</td>
<td>Hypothesis</td>
</tr>
<tr>
<td>1.8.2</td>
<td>Specific aims</td>
</tr>
</tbody>
</table>
CHAPTER TWO: METHODS

2.1 Materials (*in vitro* studies) 74

2.2 Fibroblast cell culture 74
2.2.1 Culture conditions 74
2.2.2 Cell passage 76

2.3 Determination of procollagen metabolism 76
2.3.1 Cell culture conditions 76
2.3.1.1 TGFβ₁,₃ dose response relationships 77
2.3.1.2 Comparison of TGFβ isoforms 77
2.3.2 Sample processing 77
2.3.2.1 Cell harvesting 77
2.3.2.2 Separation of ethanol-insoluble and ethanol-soluble fractions 77
2.3.3 Measurement of hydroxyproline by reverse-phase HPLC 78
2.3.3.1 Pre-column derivatisation 80
2.3.3.2 Instrumentation and chromatographic conditions 80
2.3.3.3 Quantitation of hydroxyproline content 82
2.3.3.4 Calculation of procollagen synthesis, production and degradation rates 85
2.3.3.5 Calculation of the proportion of newly-synthesised procollagen degraded 85

2.4 Determination of DNA content of cell monolayers 85
2.4.1 Culture conditions 85
2.4.2 Assay conditions 86
2.4.3 Calculation of DNA content 87

2.5 Determination of TGFβ₁ production 89
2.5.1 Mesothelioma cell culture conditions 89
2.5.2 TGFβ₁ antisense oligonucleotide synthesis 89
2.5.3 Experimental conditions 89
2.5.4 TGFβ₁ enzyme-linked immunoabsorbent assay 90
2.5.4.1 Plate coating 90
2.5.4.2 Sample preparation 90
2.5.4.3 Assay 91

2.6 Bleomycin model of lung fibrosis 92
2.6.1 Animals, bleomycin instillation and sacrifice 92
2.6.2 Murine lung tissue preparation for *in situ* hybridisation 92
2.6.3 Rat lung tissue preparation for RNA extraction 93

2.7 Selection of patient samples 94
2.7.1 Patient evaluation 94
2.7.1.1 Systemic sclerosis 94
2.7.1.2 Cryptogenic fibrosing alveolitis 94
2.7.2 Open lung biopsies 94

2.8 Materials (*in vivo* studies) 95

2.9 General molecular biology procedures 95
2.9.1 Spectrophotometric evaluation of nucleic acids 95
2.9.2 Nucleic acid precipitation and recovery 95
2.9.3 Agarose gel electrophoresis of nucleic acids 96
2.9.4 Phenol-chloroform purification of DNA samples 96

2.10 Plasmid preparation 97
2.10.1 TGFβ₁-3 probes and plasmid vectors 97
2.10.2 Preparation of bacterial plates 101
2.10.3 Bacterial transformation 101
2.10.4 Freezing transformed bacteria 102
2.10.5 Small-scale plasmid DNA preparation 102
2.10.5.1 Detergent lysis 102
2.10.5.2 Alkaline lysis 103
2.10.6 Large-scale plasmid DNA preparation 104

2.11 Digoxigenin-labelled riboprobe preparation 105
2.11.1 Plasmid linearisation 105
2.11.2 In vitro transcription 108
2.11.3 Assessment of digoxigenin incorporation into riboprobes 108
2.11.4 Reduction of riboprobe length by alkaline hydrolysis 111

2.12 32P labelled cDNA probe preparation 111
2.12.1 Preparation of template DNA 111
2.12.2 Random prime labelling 112
2.12.3 Calculation of labelling efficiency 113
2.12.4 Interpretation of results 113

2.13 In situ hybridisation 114
2.13.1 Prehybridisation 114
2.13.1.1 Slide and coverslip preparation and tissue sectioning 114
2.13.1.2 Prehybridisation tissue treatments 114
2.13.2 Hybridisation 115
2.13.2.1 Hybridisation buffer preparation 115
2.13.2.2 Hybridisation of tissue sections 115
2.13.3 Post hybridisation treatments 116
2.13.4 Histological analysis 116
2.13.4.1 Counterstaining and mounting 116
2.13.4.2 Microscopy and photography 116

2.14 Characterisation of TGFβ2 riboprobes 117
2.14.1 Asymmetric restriction enzyme digestion 117
2.14.2 Dideoxynucleotide sequencing 118
2.14.2.1 Template preparation 118
2.14.2.2 Primer annealing, labelling and termination reactions 118
2.14.2.3 DNA electrophoresis 120
2.14.2.4 Autoradiography and sequence analysis 121
2.14.3 Assessment of digoxigenin labelling 121

2.15 RNA extraction 121
2.15.1 Total RNA extraction 121
2.15.2 Polyadenylated RNA extraction 122
2.15.3 Concentration of polyadenylated RNA 123

2.16 Northern analysis 125
2.16.1 RNA electrophoresis 125
2.16.2 Northern transfer 127
2.16.3 Prehybridisation 127
2.16.4 Hybridisation and stringency washes 128
2.16.5 Imaging 128

CHAPTER THREE: RESULTS 130

3.1 The effect of TGFβ1,3 on fibroblast proliferation 131

3.2 The effect of TGFβ1,3 on procollagen metabolism 133
3.2.1 Dose-response relationships for TGFβ1,3 133
3.2.2 The effect of TGFβ1,3 on procollagen production 137
3.2.3 The effect of TGFβ1,3 on procollagen degradation 137
3.2.4 The effect of TGFβ1,3 on procollagen synthesis 137
3.2.5 Summary 138

3.3 Validation of methods 142
3.3.1 Plasmid preparation 142
3.3.1.1 Yield and purity of purified plasmid DNA 142
3.3.1.2 Electrophoresis of purified plasmids 143
3.3.2 Digoxigenin labelling of riboprobes 146
3.3.2.1 Electrophoresis of digoxigenin-labelled riboprobes 146
3.3.2.2 Assessment of digoxigenin incorporation into riboprobes 146
3.3.2.3 Reduction of riboprobe length by alkaline hydrolysis 146
3.3.2.4 Effect of riboprobe length on detection of mRNA transcripts by 
  in situ hybridisation 150
3.3.3 Assessment of $^{32}$P incorporation into cDNA probes 150
3.3.4 RNA isolation 150
3.3.4.1 Yield and purity of total lung and cellular RNA 150
3.3.4.2 Electrophoresis of purified total RNA 152
3.3.5 Specificity of TGFβ1,3 probes 152
3.3.6 Summary 152

3.4 TGFβ1 and TGFβ3 gene expression in normal murine lung 155
  3.4.1 Sense and antisense controls for TGFβ1 and TGFβ3 155
  3.4.2 Localisation of TGFβ1 mRNA transcripts 155
  3.4.3 Localisation of TGFβ3 mRNA transcripts 155
  3.4.4 Summary 161

3.5 TGFβ1 and TGFβ3 gene expression 
  in bleomycin-induced lung fibrosis 161
  3.5.1 Characteristics of murine lung tissue following bleomycin 161
  3.5.2 TGFβ1 gene expression 162
  3.5.3 TGFβ3 gene expression 170
  3.5.4 Summary 170

3.6 TGFβ1 and TGFβ3 gene expression in normal human lung 174
  3.6.1 Characteristics of control patients 174
  3.6.2 Sense and antisense controls for TGFβ1 and TGFβ3 174
  3.6.3 Localisation of TGFβ1 mRNA transcripts 174
  3.6.4 Localisation of TGFβ3 mRNA transcripts 174
  3.6.5 Summary 175
3.7 TGF\(\beta_1\) and TGF\(\beta_3\) gene expression in fibrotic human lung

3.7.1 Patient characteristics

3.7.2 TGF\(\beta_1\) gene expression

3.7.3 TGF\(\beta_3\) gene expression

3.7.4 Summary

3.8 \textit{In situ} hybridisation with TGF\(\beta_2\) riboprobes

3.8.1 Normal murine lung

3.8.2 Bleomycin-induced murine lung fibrosis

3.8.3 Normal rat lung

3.8.4 Normal human lung

3.8.5 Summary

3.9 Characterisation of TGF\(\beta_2\) probes

3.9.1 Determination of TGF\(\beta_2\) probe orientation by asymmetric restriction enzyme mapping

3.9.2 Determination of TGF\(\beta_2\) sense riboprobe sequence

3.9.3 Assessment of digoxigenin incorporation into TGF\(\beta_2\) riboprobes

3.9.4 Summary

3.10 Identification of transcripts detected by the TGF\(\beta_2\) sense riboprobe

3.10.1 Identification of a transcript in rat lung

3.10.2 Identification of a transcript in murine mesothelioma cells

3.10.3 Summary

3.11 Preliminary studies: effect of TGF\(\beta_1\) antisense on mesothelial cell growth and TGF\(\beta_1\) secretion

3.11.1 Murine mesothelioma cell morphology and cell number

3.11.2 Murine mesothelioma cell TGF\(\beta_1\) secretion

3.11.3 Summary
CHAPTER FOUR: DISCUSSION

4.1 The effect of TGFβ isoforms on procollagen metabolism

4.2 Use of digoxigenin-labelled riboprobes
   4.2.1 Previous in situ hybridisation studies
   4.2.2 Advantages over isotopic probes
   4.2.3 Advantages over biotinylated probes
   4.2.4 Probe hydrolysis
   4.2.5 Use in Northern analysis
   4.2.6 Summary

4.3 In situ hybridisation in murine lung
   4.3.1 TGFβ1 and TGFβ3 expression in normal lung
   4.3.2 Functions of TGFβ1 and TGFβ3 in normal lung
   4.3.3 TGFβ1 and TGFβ3 expression during development of bleomycin-induced pulmonary fibrosis
      4.3.3.1 Altered localisation of TGFβ1 expression
      4.3.3.2 Mesenchymal cell TGFβ1 expression
      4.3.3.3 Mesothelial cell TGFβ1 expression
      4.3.3.4 Type II cell TGFβ1 expression
      4.3.3.5 Time course of TGFβ1 expression
      4.3.3.6 TGFβ3 expression
      4.3.4 TGFβ1 and TGFβ3 expression in wound healing
      4.3.5 Summary

4.4 In situ hybridisation in human lung
   4.4.1 TGFβ1 and TGFβ3 expression in normal lung
   4.4.2 TGFβ1 and TGFβ3 expression in fibrotic lung
      4.4.2.1 Differential TGFβ1 and TGFβ3 expression
      4.4.2.2 Localisation of gene expression
      4.4.2.3 Gene expression in dense fibrosis
   4.4.3 Summary
4.4.3 Summary 233

4.5 TGFB2 expression 234
   4.5.1 In situ hybridisation 234
   4.5.2 Characterisation of TGFB2 riboprobes 234
   4.5.3 Northern analysis 234
   4.5.4 Endogenous antisense transcripts 235
      4.5.4.1 Antisense transcripts in prokaryotes 235
      4.5.4.2 Antisense transcripts in eukaryotes 235
      4.5.4.3 Cytokine antisense transcripts 236
      4.5.4.4 TGFB antisense transcripts 236
      4.5.4.5 Future studies and potential applications 237
   4.5.5 Summary 238

4.6 Future perspectives 238
   4.6.1 The effect of TGFB1 antisense in vitro 238
   4.6.2 TGFB1 antisense as a therapeutic strategy in pulmonary fibrosis 240
   4.6.3 Summary 240

4.7 Summary and conclusions 241

APPENDIX Manufacturers and suppliers 244
BIBLIOGRAPHY 247
PUBLICATIONS ARISING FROM THIS THESIS 300
LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

1.1 High resolution CT scan findings in pulmonary fibrosis 29
1.2 Macroscopic appearances of fibrotic lung 34
1.3 Post 1990 mortality from CFA in the UK 38
1.4 Steps involved in collagen synthesis and degradation 44
1.5 Electron micrographs of normal and fibrotic lung 47
1.6 The high molecular weight latent TGFβ complex 55
1.7 Possible mechanisms regulating TGFβ activation 59

CHAPTER TWO: METHODS

2.1 Morphology of human foetal lung fibroblasts 75
2.2 Hydroxyproline derivatisation with NBD-Cl 79
2.3 Separation of hydroxyproline by reverse-phase HPLC 83
2.4 Standard curve for DNA 88
2.5 Vector map for TGFβ1 probe 98
2.6 Vector map for TGFβ2 probe 99
2.7 Vector map for TGFβ3 probe 100
2.8 Digoxigenin-labelled uridine triphosphate (DIG-11-UTP) 107
2.9 Detection of mRNA transcripts using digoxigenin-labelled riboprobes 109
2.10 Cla I restriction enzyme mapping of pm TGFβ2-9A 119
2.11 Polyadenylated RNA isolation 124
2.12 Size calibration curve for electrophoretically separated RNA 126

CHAPTER THREE: RESULTS

3.1 The effect of TGFβ1,3 on DNA content of fibroblast cultures 132
3.2 Dose-response relationship for TGFβ1 134
3.3 Dose-response relationship for TGFβ2

3.4 Dose-response relationship for TGFβ3

3.5 The effect of TGFβ1,3 on HFL-1 procollagen production

3.6 The effect of TGFβ1,3 on HFL-1 procollagen degradation

3.7 The effect of TGFβ1,3 on HFL-1 procollagen synthesis

3.8 Agarose gel electrophoresis of plasmid DNA prepared using the Promega Wizard Maxipreps DNA Purification System

3.9 Agarose gel electrophoresis of digoxigenin-labelled TGFβ1 antisense riboprobe

3.10 Assessment of digoxigenin incorporation into riboprobes

3.11 Reduction of riboprobe length by alkaline hydrolysis

3.12 Effect of riboprobe length on detection of mRNA transcripts by in situ hybridisation

3.13 Electrophoresis of purified total RNA

3.14 Northern analysis using TGFβ1,3 cDNA probes

3.15 In situ hybridisation in normal murine lung: sense and antisense controls for TGFβ1

3.16 In situ hybridisation in normal murine lung: sense and antisense controls for TGFβ3

3.17 Cellular localisation of TGFβ1 gene expression in normal murine lung (I)

3.18 Cellular localisation of TGFβ1 gene expression in normal murine lung (II)

3.19 Cellular localisation of TGFβ1 gene expression in normal murine lung

3.20 Changes in murine lung following bleomycin

3.21 Sense and antisense controls for TGFβ1 in murine lung ten days after bleomycin

3.22 Altered pattern of TGFβ1 gene expression in murine lung ten days after bleomycin

3.23 Enhanced TGFβ1 gene expression in mesenchymal and mesothelial cells ten days after bleomycin
3.24 Enhanced TGFβ₁ gene expression in pulmonary endothelial and alveolar type II cells ten days after bleomycin

3.25 TGFβ₁ gene expression in murine lung 21 and 35 days after bleomycin

3.26 Sense and antisense controls for TGFβ₃ in murine lung ten days after bleomycin

3.27 TGFβ₃ gene expression in murine lung ten days after bleomycin

3.28 In situ hybridisation in normal human lung: sense and antisense controls for TGFβ₁

3.29 In situ hybridisation in normal human lung: sense and antisense controls for TGFβ₃

3.30 Cellular localisation of TGFβ₁ gene expression in normal human lung

3.31 Cellular localisation of TGFβ₃ gene expression in normal human lung

3.32 In situ hybridisation in fibrotic human lung: sense and antisense controls for TGFβ₁

3.33 TGFβ₁ gene expression in bronchial epithelium and alveolar macrophages in fibrotic human lung

3.34 TGFβ₁ gene expression in alveolar epithelial and mesothelial cells in fibrotic human lung

3.35 Absence of hybridisation signal for TGFβ₁ in a patient with CFA and advanced fibrosis

3.36 In situ hybridisation in fibrotic human lung: sense and antisense controls for TGFβ₃

3.37 TGFβ₃ gene expression in bronchial epithelium and mesothelial cells in fibrotic human lung

3.38 TGFβ₃ gene expression in alveolar macrophages and alveolar epithelial cells in fibrotic human lung

3.39 Absence of hybridisation signal for TGFβ₃ in a patient with CFA and advanced fibrosis
3.40 In situ hybridisation in normal murine lung:
sense and antisense controls for TGFβ₂ 195

3.41 In situ hybridisation with TGFβ₂ probes in murine lung
ten days after bleomycin 196

3.42 In situ hybridisation with TGFβ₂ probes in murine lung
21 days after bleomycin 197

3.43 In situ hybridisation with TGFβ₂ probes in normal rat lung 199

3.44 In situ hybridisation with TGFβ₂ probes in normal human lung 200

3.45 Clal restriction enzyme digest of pm TGFβ₂-9A 203

3.46 Dideoxynucleotide sequencing of pm TGFβ₂-9A 204

3.47 Electrophoresis of TGFβ₂ riboprobes 205

3.48 Digoxigenin incorporation into TGFβ₂ riboprobes 206

3.49 Northern analysis in rat lung
with digoxigenin-labelled TGFβ₂ riboprobes 209

3.50 Northern analysis in murine mesothelioma cells
with digoxigenin-labelled TGFβ₂ riboprobes 210

3.51 Morphology of murine mesothelioma (AC29) cells 212

3.52 Effect of TGFβ₂ antisense on AC29 cell number 213

3.53 Effect of TGFβ₂ antisense on TGFβ₁ production 214

CHAPTER FOUR: DISCUSSION

4.1 The mammalian TGFβ promoters 226
LIST OF TABLES

CHAPTER ONE: INTRODUCTION

1.1 Pulmonary fibrosis: clinical associations 25

CHAPTER TWO: METHODS

2.1 Chromatographic conditions for separation of hydroxyproline
by reverse-phase HPLC 81
2.2 Reproducibility of HPLC measurements 84
2.3 Restriction enzymes and RNA polymerases
used to prepare TGFβ₁,₃ riboprobes 106

CHAPTER THREE: RESULTS

3.1 Yield and purity of plasmid DNA obtained
following small and large-scale preparation 144
3.2 Time course of changes in lung collagen content during the
development of bleomycin-induced pulmonary fibrosis 163
3.3 Cells expressing TGFβ₁ and TGFβ₃
in normal and fibrotic murine lung 173
3.4 Cells expressing TGFβ₁ and TGFβ₃
in normal and fibrotic human lung 192
3.5 Quantitative changes in TGFβ₁ and TGFβ₃
gene expression in fibrotic human lung 193
LIST OF ABBREVIATIONS

aa    amino acid
A-a   alveolar-arterial
AC29  murine mesothelioma cell line
Arg   arginine
ATP   adenosine triphosphate
BAL   bronchoalveolar lavage
BMPs  bone morphogenetic proteins
C     carboxy
cDNA  complementary DNA
CFA   cryptogenic fibrosing alveolitis
CO₂   carbon dioxide
CRE   cyclic AMP responsive element
CTP   cytosine triphosphate
CXR   chest radiograph
Cys   cysteine
dATP  deoxyadenosine triphosphate
dCTP  deoxycytosine triphosphate
ddATP dideoxyadenosine triphosphate
ddCTP dideoxycytosine triphosphate
ddGTP dideoxyguanine triphosphate
ddTTP dideoxythymidine triphosphate
dGTP  deoxyguanine triphosphate
DIG   digoxigenin
DMEM  Dulbecco’s modified Eagle’s medium
DNA   deoxyribonucleic acid
dTTP  deoxythymidine triphosphate
EBV   Epstein-Barr virus
EDTA  ethylenediaminetetraacetic acid
EGF   epidermal growth factor
ELISA enzyme-linked immunoabsorbent assay
ET-1  endothelin-1
FCS  foetal calf serum
Gly  glycine
Grp  glucose-regulated protein
GTP  guanine triphosphate
HCl  hydrochloric acid
HFL-1  human foetal lung fibroblast cell line
H2O  water
H2O2  hydrogen peroxide
HPLC  high pressure liquid chromatography
HRCT  high resolution computed tomography
Hsp  heat-shock protein
HYP  hydroxyproline
Ig  immunoglobulin
IGF-1  insulin-like growth factor 1
IL-1  interleukin 1
iNOS  inducible nitric oxide synthase
IPF  idiopathic pulmonary fibrosis
KCO  carbon monoxide transfer coefficient
kd  kilodalton
LAP  latency associated peptide
LTBP  latent TGFβ binding protein
Mad  Mothers against dpp
MgCl2  magnesium chloride
MHC  major histocompatibility complex
MOPS  3-(morpholinopropanesulphonic acid)
M-6-P  mannose-6-phosphate
mRNA  messenger ribonucleic acid
N  amino
NaCl  sodium chloride
NaOH  sodium hydroxide
NBD-Cl  7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
NCS newborn calf serum
NMR nuclear magnetic resonance
NO nitric oxide
PAI-1 plasminogen activator inhibitor 1
PBS phosphate-buffered saline
PCA perchloric acid
pCO₂ partial pressure of carbon dioxide
PCR polymerase chain reaction
PDGF platelet-derived growth factor
PGE₂ prostaglandin E₂
poly A⁺ RNA polyadenylated ribonucleic acid
RNA ribonucleic acid
RNase ribonuclease
RT room temperature
SA-PMPs streptavidin-paramagnetic particles
SDS sodium dodecyl sulphate
SEM standard error of the mean
SSC saline sodium citrate
SSc systemic sclerosis
SSPE saline sodium phosphate
TEMED tetramethylethylenediamine
TGFβ transforming growth factor β
TIMP tissue inhibitor of metalloproteinase
TNFα tumour necrosis factor α
Tris tris (hydroxymethyl) aminomethane
tRNA transfer ribonucleic acid
uPA urokinase
UTP uridine triphosphate
UV ultraviolet
CHAPTER ONE

INTRODUCTION
1.1 PREAMBLE

Pulmonary fibrosis is a disease characterised predominantly by interstitial lung pathology. It is a final common sequel to various insults to the lung and therefore arises in diverse clinical settings. Treatment is generally inadequate and the prognosis poor. Although the pathogenesis remains incompletely understood, pulmonary injury appears to cause an influx of inflammatory cells. These cells, together with resident lung cells, release polypeptide mediators, or cytokines. Cytokines then stimulate target cells such as fibroblasts to replicate and synthesise increased amounts of extracellular matrix proteins including collagen. Various cytokines have been implicated in the fibrotic process and considerable evidence currently implicates transforming growth factor [TGF]β1 in the pathogenesis of this disease. However, little is known about the role played by the other two mammalian TGFβ isoforms, TGFβ2 and TGFβ3. This thesis attempts to address the question of the role played by the three mammalian TGFβ isoforms in the pathogenesis of pulmonary fibrosis.

The specific questions I have addressed are detailed at the end of this introduction (section 1.8). The work described in this thesis focuses on examining the effects of the three TGFβ isoforms on lung fibroblast procollagen metabolism in vitro, and localising TGFβ1,3 gene expression in normal and fibrotic murine and human lung. Current radiolabelled techniques for localising gene expression in tissue are limited in terms of cellular resolution, and a non-radioactive method was therefore developed for in situ hybridisation in lung tissue. The introduction reviews clinical aspects of pulmonary fibrosis and summarises current concepts of the pathogenesis. Regulation of lung collagen metabolism and the biology of transforming growth factor β (TGFβ) are then described. Evidence is presented that TGFβ is implicated in the pathogenesis of fibrotic disorders, and current in situ hybridisation techniques briefly outlined.

1.2 DEFINING PULMONARY FIBROSIS

This section introduces the clinical entity of pulmonary fibrosis. The major clinical settings in which pulmonary fibrosis may arise are summarised in table 1.1. It may
follow environmental exposure to a variety of dusts, both inorganic such as coal, asbestos or silica, or organic such as mycelial spores or avian serum proteins, giving rise to the diseases known as farmer's and pigeon fancier's lung respectively. Fibrosis may follow infections, such as pulmonary tuberculosis, where the fibrosis is characteristically localised to the upper lobes, or *Mycoplasma* pneumonia, when it is typically diffuse. Pulmonary fibrosis may follow exposure to certain drugs, such as practolol and amiodarone. It may arise in association with granulomatous diseases such as sarcoidosis, or connective tissue diseases such as systemic sclerosis, polymyositis, Sjögren's syndrome or ankylosing spondylitis. Rarer associations include neurofibromatosis and tuberose sclerosis. Alternatively, pulmonary fibrosis may occur with no known associated disease or aetiology; in this case the disease is known as cryptogenic fibrosing alveolitis (CFA), or idiopathic pulmonary fibrosis (IPF) in the USA.
Table 1.1 Pulmonary fibrosis: clinical associations
Table shows the major clinical settings in which pulmonary fibrosis can arise. For convenience they are grouped into two main categories. The first comprises instances where the cause is to some extent defined; the second those cases where the cause remains obscure.

<table>
<thead>
<tr>
<th>KNOWN CAUSES</th>
<th>UNKNOWN CAUSES</th>
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<tbody>
<tr>
<td>◆ Inorganic dusts</td>
<td>◆ Granulomatous diseases</td>
</tr>
<tr>
<td>◆ Organic dusts</td>
<td>◆ Inherited disorders</td>
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<td>◆ Infections</td>
<td>◆ Vasculitic disorders</td>
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<td>◆ Drugs</td>
<td>◆ Connective tissue disorders</td>
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<tr>
<td>◆ Neoplasia</td>
<td>◆ Cryptogenic fibrosing alveolitis</td>
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<td>◆ Radiation</td>
<td>(CFA, IPF)</td>
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<td>◆ Acute lung injury</td>
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Where human lung fibrosis is considered, emphasis in this thesis is placed on CFA and on pulmonary fibrosis arising in association with systemic sclerosis (SSc). This section gives a brief historical overview before summarising the clinical and pathological features. Pulmonary fibrosis in both CFA and SSc shares many common characteristics and these two entities are therefore described together except where there are distinguishing aspects.

1.2.1 Historical overview

The first reports of acute diffuse pulmonary fibrosis of unknown cause are attributed to Hamman and Rich in the first half of this century (Hamman, Rich, 1935; Hamman, Rich, 1944). These authors described four patients who died of a rapidly progressive disease characterised by the presence of lung inflammation and fibrosis. A fifth patient presented with chronic disease, but all patients died within six months of the onset of symptoms. No aetiological agent was identified; in one patient mycobacteria were isolated from the sputum but autopsy revealed only a small fresh tuberculous cavity in the left lung which was unrelated to the diffuse fibrosis.

However, subacute diffuse lung fibrosis of unknown cause was probably recognised before 1944. In 1803 an illustration of a longitudinal section of whole lung was published in Baillie's Morbid Anatomy apparently depicting diffuse fibrosis with honeycombing (Baillie, 1803). In 1890 two case reports of fibrosis subsequent to 'subacute indurative pneumonia' were published in The Lancet (Kidd, 1890). Both patients were male, aged 44 and 51. Both died of the disease, the first six years after the onset of respiratory symptoms including a 'slight morning cough', the second four months after the onset of illness. No infective agent was identified in either case at autopsy. The author concludes, "we affirm the existence of a subacute ... pneumonia distinct from the acute classical type, characterised by a tendency to fibrous ... changes in the lung. The indurative process may be mainly or exclusively interstitial, it may be represented wholly or in part by organisation of an alveolar exudation, or it may comprise both of these lesions". In the 19th century Harley, Dinkler and Osler also recognised lung fibrosis arising in association with scleroderma (Harley, 1877; Dinkler,
1891; Osler, 1892). Radiological changes of pulmonary fibrosis associated with scleroderma were first described in 1941 (Murphy et al 1941; Linenthal, Talkov, 1941).

Several reports of chronic lung fibrosis of unknown cause followed (Grant et al 1956; Rubin, Lubliner, 1957; Scadding, 1960; Livingstone et al 1964; Stack et al 1965). Many of these cases were initially referred to as 'Hamman-Rich' syndrome, but this term is no longer used. In 1964 Scadding proposed a definition of the disease, which he called fibrosing alveolitis, as a condition characterised by inflammation and fibrosis of the pulmonary interstitium and peripheral airspaces (Scadding, 1964). He added the prefix 'cryptogenic' to indicate that no cause could be demonstrated. CFA is synonymous with the term idiopathic pulmonary fibrosis (IPF) used to describe the condition in the United States (Crystal et al 1976), but the British name emphasises the inflammatory component of the disease.

1.2.2 Clinical features

The commonest presenting symptom is breathlessness on exertion associated with an unproductive or minimally productive cough. Non-specific symptoms such as malaise and weight loss are also recognised and particularly marked in patients with progressive disease. Pulmonary symptoms usually progress gradually and up to 20% of patients remain stable for many years (Rubin, Lubliner, 1957; Scadding, 1970; Carrington et al 1978; Winterbauer, 1991). Finger clubbing has been reported in between 66 and 85% of patients (Scadding, 1960; Livingstone et al 1964; Stack et al 1965; Turner-Warwick et al 1980b) and fine basal crackles are almost universally heard on auscultation (Turner-Warwick et al 1980b). Central cyanosis may follow exertion.

1.2.3 Investigations

General laboratory investigations may show the presence of circulating immune complexes (Dreisin et al 1978; Martinet et al 1984), a positive rheumatoid factor or antinuclear factor, and an elevated erythrocyte sedimentation rate (Turner-Warwick, Doniach, 1965; Turner-Warwick et al 1980b).
1.2.3.1 Radiology

Chest radiograph (CXR) typically shows small lungs with round or irregular nodular or reticulonodular shadows usually most marked in the lower zones. Honeycombing is seen in severe cases. Less commonly the CXR may be normal or show an amorphous increase in lung density known as a 'ground glass' pattern (Liebow et al 1965). The radiological features of pulmonary fibrosis associated with collagen vascular diseases such SSc are indistinguishable from those seen in CFA (Bergin, Müller, 1987).

High resolution computed scanning (HRCT) permits detection of subtle interstitial changes and is now more accurate than CXR in predicting histological diagnosis in a variety of diffuse interstitial lung diseases (Mathieson et al 1989; Padley et al 1991). Honeycombing not recognised on CXR may be pronounced on HRCT (Staples et al 1987). Typical findings include predominant involvement of the lower lung zones and subpleural regions.

Reticular abnormalities correspond histologically with areas of fibrosis on open lung biopsy in both CFA (Müller et al 1986; Nishimura et al 1992) and SSc (Wells et al 1992) while areas of so-called 'ground-glass' attenuation represent regions of increased cellularity and inflammation (Müller et al 1987; Vedal et al 1988; Remy-Jardin et al 1993). Irregular lines of attenuation, mainly of the lower zones, and subpleural lower zone honeycombing may also be seen; these features follow the ground-glass changes (Terriff et al 1992). Serial HRCT is therefore of value in following the course of the disease. Figure 1.1 illustrates the HRCT findings of ground-glass and reticular shadowing, seen in two different patients.
Figure 1.1 High resolution CT scan findings in pulmonary fibrosis.
Figure illustrates HRCT features of ground-glass (a) and reticular (b) shadowing, in scans taken from two different patients. These changes correspond to distinct histological patterns (section 1.2.3.1).
1.2.3.2 **Pulmonary function testing**

Pulmonary function testing reveals a restrictive ventilatory defect, with reduced vital capacity and total lung capacity, reduced compliance and impaired carbon monoxide transfer factor (diffusing capacity). The latter is in part due to reduction in lung volumes, but the transfer coefficient (KCO) is also usually reduced, probably reflecting a reduced pulmonary capillary volume (Denison et al 1984). Residual volume typically remains normal. Some patients have evidence of airflow obstruction. Where this occurs in non-smokers there is evidence that the disease process involves the small airways (Fulmer et al 1977). Arterial blood gas sampling usually reveals hypoxaemia in symptomatic patients, a widened alveolar-arterial (A-a) gradient variably increased on exercise (Carrington et al 1978) and a low or normal pCO₂.

In SSc, impaired gas transfer and a restrictive ventilatory pattern are frequently found even when the patient has no respiratory symptoms and the CXR normal. A review of 19 series of 512 patients with SSc revealed an average of 81% patients with physiological impairment, but review of 30 series of 1620 patients by the same authors showed the prevalence of radiological evidence of lung involvement to be only 36% (Alton, Turner-Warwick, 1988). This figure included patients with vascular disease causing pulmonary arterial hypertension as well as those with interstitial involvement causing lung fibrosis. Lung function testing is therefore a vital element in the pulmonary assessment of patients with SSc.

1.2.3.3 **Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) is increasingly used in the evaluation of patients with CFA (Daniele et al 1985). It typically yields an increased cell count of 3-6 x 10⁶/ml (Turner-Warwick, Haslam, 1987), with 10-20% neutrophils. In some cases these authors and others (Watters et al 1987) also noted a lymphocyte excess. Furthermore, mast cell numbers are also frequently increased in patients with lung fibrosis (Kawanami et al 1979). These findings are not diagnostic of CFA since a granulocyte excess is found in a variety of interstitial lung diseases where a degree of fibrosis is present. However, lavage differential cell counts are of some value as a guide to prognosis and treatment. Response to corticosteroid treatment is associated with higher
numbers of lymphocytes (Haslam et al. 1990) and reduced numbers of eosinophils (Turner-Warwick, Haslam, 1987). Furthermore, clinical, radiological and physiological response to corticosteroid treatment is usually accompanied by a reduction in neutrophils on repeat BAL.

In patients with SSc, a neutrophilia has been associated with an accelerated decline in pulmonary function (Silver et al. 1990). Correlation of BAL findings with HRCT appearances in patients with SSc suggests that a neutrophilia is associated with extensive fibrosis, while eosinophilia is often observed in less advanced disease, particularly when HRCT appearances suggest lung inflammation (Wells et al. 1994b).

1.2.3.4 Histology

The fibrosing alveolitis seen in association with SSc is histologically identical to CFA (Harrison et al. 1991b). Autopsy reveals that the disease is most severe in the lung periphery. Early pathological features described were cellular thickening of the alveolar wall usually associated with fibrosis, and infiltration of alveolar spaces with large mononuclear cells (Scadding, Hinson, 1967). The interstitial changes are frequently patchy. The histological features now recognised and summarised below comprise alveolar epithelial cell damage and regeneration, endothelial cell damage, inflammatory cell infiltration, fibrosis, peripheral airway involvement and vascular changes.

A constant histological feature is damage to and loss of type I alveolar epithelial cells (Crouch, 1990), with consequent proliferation and hypertrophy of alveolar type II cells (Brody, Craighead, 1976; Coalson, 1982; Hammar et al. 1985). The earliest changes are those of epithelial and endothelial cell injury (Harrison et al. 1991b), which is thought to lead to inflammatory cell infiltration. The degree of infiltration can vary considerably within a section of the lung, and may be predominantly interstitial or intralveolar. When the cellular pattern predominates, the findings are of infiltration with macrophages and monocytes, together with smaller numbers of neutrophils, eosinophils and lymphocytes. Mast cell numbers are also increased (Hunt et al. 1992; Pesci et al. 1993). The populations of inflammatory cells in the alveoli commonly differ from those in the interstitium, with macrophages and monocytes predominating in the air.
spaces and lymphocytes and plasma cells predominating in the interstitium (Davis et al 1978).

Blood-borne mediators and cytokines released by inflammatory and resident lung cells then stimulate enhanced fibroblast collagen synthesis and fibrosis. The degree of fibrosis in alveolar walls is variable, but when the fibrotic pattern predominates relatively few inflammatory cells are seen. The alveolar walls are thickened and electron microscopy reveals that they not only contain increased amounts of collagen, but that the fibrils are deposited in a disorganised pattern (Laurent et al 1988). Interstitial and intra-alveolar deposition of connective tissue leads to loss of normal alveolar architecture. Air spaces may collapse, or become enlarged as a consequence of damage to alveolar walls. The term 'honeycombing' is used to describe the phenomenon of enlarged distal air spaces lined with bronchiolar epithelial-like or proliferating type II alveolar cells. Fig 1.2 shows the autopsy appearances of a fibrotic lung with honeycombing.

The disease can involve peripheral bronchioles as well as the alveoli, with peribronchial inflammation and/or fibrosis a common feature, frequently accompanied by a reduction in small airway diameter (Fulmer et al 1977). In some patients there is also mucous gland hyperplasia, increased luminal mucus and increased smooth muscle content in the large airways (Edwards, Carlile, 1982; Andoh et al 1992). Vascular changes are also observed in pulmonary fibrosis, affecting both pre-acinar and intra-acinar vessels. Muscular pulmonary arteries exhibit medial hypertrophy, intimal smooth muscle hyperplasia and connective tissue deposition, while distal arterioles develop a smooth muscle coat. There is evidence of injury to the endothelium of alveolar capillaries (Coalson, 1982; Corrin et al 1985).

In summary, pulmonary fibrosis is a disease predominantly involving the lung interstitium and was recognised over 100 years ago. Patients typically present with a dry cough and gradually worsening breathlessness. Lung function tests show a restrictive defect and radiology typically reveals bilateral basal reticulonodular opacities. The pathology is characterised by alveolar epithelial and endothelial damage,
inflammatory cell infiltration of alveolar spaces and fibrotic thickening of alveolar walls. Typically these changes lead to loss of alveolar architecture. The next section outlines the nature of the problem in terms of its pathogenesis, epidemiology, response to currently available treatments and the prognosis.
Figure 1.2 Macroscopic appearances of fibrotic lung
Figure shows a sagittal section of an autopsy specimen of fibrotic lung. Peripheral honeycombing is seen, with relative sparing of central areas. Reproduced with permission from Respiratory Medicine, eds Brewis RAL, Corrin B, Geddes DM, Gibson GJ. WB Saunders Company Ltd, second edition, London 1995.
1.3 THE NATURE OF THE PROBLEM

1.3.1 Pathogenesis

Although the pathogenesis of pulmonary fibrosis remains incompletely understood, the last decade has generated significant advances in our understanding. Current concepts in the pathogenesis of pulmonary fibrosis are as follows. Morphological and biochemical studies suggest that alveolar epithelial and/or endothelial cell injury are early events in the disease process (Harrison et al 1991b). Injury leads to an influx of inflammatory cells recruited from the circulation, including monocytes and alveolar macrophages. These cells, together with resident lung cells including epithelial (Khalil et al 1991) and endothelial cells (Phan et al 1991a), are then stimulated to release polypeptide mediators or cytokines (Martinet et al 1987; Jordana et al 1988; Khalil et al 1989). These growth factors stimulate fibroblasts, the major producers of lung collagen, to replicate and to increase production of extracellular matrix proteins including collagen. Various well-characterised cytokines such as platelet-derived growth factor (PDGF), interleukin 1 (IL-1), tumour necrosis factor α (TNFα), insulin-like growth factor 1 (IGF-1), endothelin 1 (ET-1) and transforming growth factor β (TGFβ) derived from resident and inflammatory lung cells can modify fibroblast function (Martinet et al 1987; Jordana et al 1988; Piguet et al 1989; Cambrey et al 1995; Harrison et al 1994; Dawes et al 1994; Cambrey et al 1994; Khalil et al 1989). More recently, blood-derived proteins such as thrombin have also been implicated in the fibrotic process (Hernandez-Rodriguez et al 1995). The result is excessive extracellular matrix deposition in alveolar walls, leading to loss of alveolar architecture, a restrictive lung function defect and impaired gas exchange.

1.3.2 Epidemiology

Current prevalence of CFA in the UK is estimated to be three per 100,000 population (Grant, 1987). It most frequently presents in individuals aged between 40 and 70 years of age and the male:female ratio is 1:1. Patients with SSc are predominantly female and usually present at an earlier age. Autopsy evidence of pulmonary fibrosis is
virtually universal in patients dying of SSc (Weaver et al 1968; D’Angelo et al 1969). A retrospective study has shown that mortality from CFA in England and Wales is rising steadily (Johnston et al 1990), is higher in males than in females, and is greatest in the industrialised regions of England and Wales. These authors also proposed that the number of deaths officially recorded as due to the disease may underestimate the real number of patients dying with the disease by up to half.

More recently published data show that the crude mortality rate, which was 0.7/100,000 in 1979, rose to over 1.6/100,000 in 1992 (Hubbard et al 1996a). Recent data from the Office of Population of Censuses and Surveys (Anonymous 1995; Anonymous 1993a; Anonymous 1993b) suggest that this upward trend was maintained in 1993 (see fig 1.3). Changes in automated cause coding necessitate caution in interpreting later figures, but these changes are estimated to reduce the 1994 figures by no more than 7% (Anonymous 1996), suggesting that the 1993 figures were maintained in 1994. If Johnston and colleagues are correct that official mortality figures are an underestimate, annual mortality in 1994 may therefore have exceeded 2000. For comparison, mortality from asthma, over which there is currently much concern, was 1516 in the same year (Anonymous 1996) and has fallen from 1858 in 1990. Mortality from SSc alone was 103 in 1994 and has not significantly changed since 1990 when it was 95, illustrating that different aetiological factors are implicated in SSc and CFA.

The aetiology of pulmonary fibrosis is unknown, but there is evidence that both genetic and environmental factors may be implicated. Several studies have documented a familial form of CFA, arising in two or more first degree relatives, including pairs of twins (for a recent review see Sulavik 1995 (Sulavik, 1995). The familial form of pulmonary fibrosis is identical to non-familial CFA. No specific genetic defect has yet been defined, but one study has suggested that genes located on chromosome 14 may play a role in the fibrotic process in CFA (Musk et al 1986). There is also evidence for immunogenetic factors being important in the development of pulmonary fibrosis in patients with SSc (Briggs et al 1991). This study of MHC class II genes and autoantibodies in 75 patients revealed that 57% with pulmonary fibrosis, but only 6% without, had either HLA DR3/DRw53a or anti-Scl-70. For a recent review of existing

Regional variation in mortality in England and Wales, highest in the East Midlands and lowest in the South West, suggests that industrial environmental exposure may have an aetiological role. Two case-control studies have indicated that exposure to metal or wood dust may be an important factor in the aetiology of CFA (Scott et al 1990; Hubbard et al 1996b). The latter study of 218 patients with CFA estimated that the combined aetiological fraction attributable to exposure to metal or wood dust was approximately 20%.
Figure 1.3 Post 1990 mortality from CFA in the UK
Figure shows annual deaths plotted against year (data from the Office of Population Censuses and Surveys for 1991 to 1994).
A viral aetiology for CFA has also been sought. One candidate is the Epstein-Barr virus (EBV). Raised serum antibodies to EBV and IgA against viral-capsid antigen have been documented in 13 patients with CFA (Vergnon et al 1984). More recently, antigen specific for replicating EBV has been identified in alveolar type II cells in lung biopsies from patients with CFA (Egan et al 1995). Further studies are required to determine the significance of these findings.

1.3.3 Prognosis

The prognosis of CFA is poor. Several studies have demonstrated that the five year survival rate is approximately 50% (Stack et al 1972; Turner-Warwick et al 1980b; Turner-Warwick et al 1980a). In addition to death from respiratory failure and pulmonary infections, patients with CFA exhibit excess mortality from ischaemic heart disease, cerebrovascular accidents, thromboembolism and bronchial neoplasms (Turner-Warwick et al 1980b). In contrast, the rate of progress of pulmonary fibrosis in association with SSc is often slow when compared with that of CFA (Alton, Turner-Warwick, 1988; Wells et al 1994a). Figures are variable, but lung involvement in SSc has been reported to reduce five year survival from approximately 90% to around 70% (Steen et al 1985). Treatment of pulmonary fibrosis in either setting has not improved the prognosis.

1.3.4 Current treatments

Three therapeutic agents are currently used, comprising corticosteroids (oral or parenteral), and the immunosuppressants cyclophosphamide and azathioprine. Treatment is usually initiated with a high dose of prednisolone (60-80 mg daily) alone, or with a combination of prednisolone at a lower dose (20 mg on alternate days) and either cyclophosphamide 2mg/kg up to 120 mg daily or azathioprine 2.5 mg/kg up to 150 mg daily. Several studies have used one or more of these agents. An early response to treatment is associated with a more favourable long-term prognosis (Stack et al 1972; Turner-Warwick et al 1980b) and in some patients a combination of prednisolone and cyclophosphamide may offer advantages over prednisolone alone.
(Johnson et al 1989). Finally, there is no difference in survival between patients treated with corticosteroids and those patients which receive no treatment (Carrington et al 1978).

Lung transplantation remains the only treatment option for patients who deteriorate despite medical therapy, and the first successful single lung transplantation was performed in 1983 for pulmonary fibrosis. Although limited by organ availability and the generally more advanced age of patients with CFA, experience so far indicates that single or double lung transplantation can provide a good quality of life in patients free from infection and rejection. However, chronic rejection resulting in obliterative bronchiolitis remains a problem in long-term survivors. Transplantation for pulmonary fibrosis in association with SSc is at present rarely a viable option, given the poor wound healing and the high incidence of renal impairment exhibited by these patients.

In summary, pulmonary fibrosis is a disease of unknown aetiology from which mortality is rising. Evidence exists that both genetic and environmental factors are important in aetiology but there are as yet no conclusive data. The prognosis is poor and current treatments are inadequate. For these reasons the study of the pathogenesis of this disease is both fascinating and vital if better treatments are to be made available for patients suffering from this disease. The remainder of this introduction is devoted to discussing the possible role of altered extracellular matrix protein regulation, particularly collagen, and the transforming growth factor β family of cytokines, in the pathogenesis of pulmonary fibrosis.

1.4 COLLAGEN

1.4.1 Collagen in normal lung

1.4.1.1 Collagen types and distribution

Extracellular matrix protein accumulation in alveolar walls is a key feature of the pathogenesis of pulmonary fibrosis as well as a potential therapeutic target. In order to appreciate the ways in which altered collagen metabolism may lead to the
development of lung fibrosis, our existing knowledge of the role played by collagen in normal lung and of the mechanisms underlying collagen biosynthesis and degradation is summarised below.

Collagens comprise the largest group of proteins in the lung and account for approximately 20% of its dry weight in the adult human lung (Bradley et al 1975; Hurst et al 1977). Together with other extracellular matrix proteins such as elastin, proteoglycans and fibronectin, collagens provide a three-dimensional structural framework which also plays roles in regulating cellular function including adhesion, migration and cell-matrix interactions (for a review see Schnaper and Kleinman 1993 (Schnaper, Kleinman, 1993).

The collagens consist of a family of at least 19 closely related proteins, denoted by Roman numerals, which share common structural properties. All are composed of three polypeptide α-chains, which may be identical or different, and which are linked by hydrogen bonding to form a right handed triple helix. At least 30 α-chains are now recognised, denoted by Arabic numerals and encoded by separate genes (for recent reviews see Kielty et al 1993; Mays, Laurent, 1994; Chambers, Laurent, 1996). The α-chains have a high glycine content and contain repeated triplets of the structure Gly-X-Y, where approximately 30% of the X residues are proline and 30% of the Y residues are hydroxyproline (Miller, 1985).

Collagen types I, II, III, V and XI are the fibril-forming interstitial collagens. Types I and III constitute approximately 90% of collagen in adult human lung, in a ratio of 2:1 (Rennard, Crystal, 1982; Kirk et al 1984). Type II collagen is found only in cartilage and is therefore restricted to the trachea and larger airways. Type V collagen is found in small amounts in basement membranes and in association with type I in the lung interstitium (Madri, Furthmayr, 1979) and type XI collagen is thought to be associated with type II collagen in the airways (Laurent, 1986).

1.4.1.2 Collagen turnover
Mammalian lung collagen content increases rapidly around and after birth (Bradley et
al 1974), and continues to increase during growth to adulthood, with a five to ten fold increase in lung collagen concentration (Mays et al 1988). Moreover, collagens are not inert proteins, but are continuously synthesised and degraded throughout life. Synthesis and degradation rates are quite rapid, with a daily turnover rate of the order of 10% in adult rats and rabbits (Laurent, 1986). In vitro studies have shown that a proportion of collagen produced by lung fibroblasts is degraded intracellularly within minutes of synthesis (Bienkowski et al 1978b; Bienkowski et al 1978a). In vivo, the process occurs within 15 mins (McAnulty, Laurent, 1987). Since collagen deposition in the lung is determined by the balance between synthesis and degradation, changes in either or both of these processes may lead to net collagen deposition and fibrosis.

1.4.1.3 Collagen synthesis

The fibroblast is an important source of pulmonary collagen (Hance et al 1976) but other cell types including endothelial, alveolar epithelial and mesothelial cells can also produce collagen (see review Bienkowski 1991 (Bienkowski, 1996). The pathways involved in collagen biosynthesis are now well-described (see reviews by Nimni, Harkness, 1988 and Kielty et al 1993) and the principal steps, illustrated in figure 1.3, are outlined below.

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 peptide is cleaved in the rough endoplasmic reticulum. There is growing 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 (Nakai et al 1992; Ferreira et al 1994; Freyria et al 1995; Lamande et al 1995). These proteins play important roles in binding malfolded procollagen molecules, preventing their secretion and promoting correct folding.

Further post-translational modification occurs in the Golgi apparatus. This includes hydroxylation of proline and lysine residues in the X position by prolyl-4-hydroxylase
and 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 the 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.

Interchain and intrachain disulphide bond formation then 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 triple helices spontaneously assemble to form fibrils, four molecules are required to initiate fibril formation. Electron microscopy studies have shown that collagen fibrils have highly tapered and symmetrical pointed tips, and grow by addition of further molecules in the C- to N-terminal direction (Kadler et al 1990). Electrostatic interactions initially hold the molecules together, but as they mature, lysyl oxidase catalyses the formation of aldehyde derivatives from some of the lysine and hydroxylysine residues, generating covalent bonds between the chains of neighbouring molecules and stabilising the fibrils (Ricard-Blum, Ville, 1988).
Figure 1.4 Steps involved in collagen synthesis and degradation

Figure shows the principal pathways involved in collagen biosynthesis. Reprinted from Laurent 1986, with permission from *Thorax*, London, UK.
1.4.1.4 Collagen degradation

Collagen degradation may occur at both extracellular and intracellular sites. Mature collagen molecules are relatively resistant to proteolysis. However, a group of metalloproteinases (MMPs) are capable of degrading collagen outside the cell (see Murphy and Reynolds 1993 (Murphy, Reynolds, 1993) for a review). This family of enzymes is dependent on calcium and zinc and includes the interstitial collagenases, the gelatinases and the stromelysins. They are produced mainly by mesenchymal cells, neutrophils and macrophages. They are generally secreted as inactive precursors and are activated by proteolytic cleavage of the propeptides. Under normal circumstances, extracellular collagen degradation is regulated by a balance between proteinases and a variety of antiproteinases, the major group being the tissue inhibitors of metalloproteinases (TIMPs).

Intracellular procollagen degradation occurs rapidly during biosynthesis. Degradation of defective molecules (the 'enhanced' pathway) is thought to take place in lysosomes (Berg, 1986), while 'basal' degradation has been proposed to occur between the endoplasmic reticulum and Golgi apparatus (Barile et al 1990). Early studies in rats, measuring urinary excretion of isotopically labelled hydroxyproline, indicated that recently synthesised collagen was subject to rapid degradation with a half-life of less than 24 hrs (Lindstedt, Prockop, 1961). This rapid breakdown was subsequently confirmed in various tissues including the lung (Bienkowski et al 1978b; McAnulty, Laurent, 1987), and in vitro studies have shown that lung fibroblasts degrade 10 to 40% of newly synthesised procollagen (for a review see Rennard et al 1982). Degradation in vivo is rapid, occurring within 15 mins of synthesis (McAnulty, Laurent, 1987) before secretion has had time to occur. The proportion of newly synthesised lung procollagen degraded increases with age, from 30% in young rats to 80% in old animals (Mays et al 1989).

1.4.1.5 Mechanisms regulating collagen deposition

Collagen deposition can therefore be regulated at various stages. These include modulation of gene transcription, RNA processing, RNA transport into the cytoplasm.
and subsequent translation, intracellular and extracellular degradation and fibrillogenesis. Alterations in the number of collagen-synthesising cells will also play a role. Numerous mediators including cytokines, hormones and vitamins, together with alterations in physical and mechanical environments, can interact to modulate procollagen mRNA levels \textit{in vitro} by altering gene transcription or mRNA processing and stability. These mediators include TGF\(\beta\), IL-1, TNF\(\alpha\), glucocorticoids, interferon gamma, ascorbic acid and prostaglandin (PG) \(E_2\) (for a recent review see Chambers, Laurent, 1996).

1.4.2 Collagen regulation in pulmonary fibrosis

It is clear that changes in either procollagen synthesis or degradation could potentially lead to collagen accumulation in the lung and consequent fibrosis. Figure 1.5 shows the electron micrograph appearances of collagen in normal and fibrotic lung, and the current evidence for increased collagen deposition in pulmonary fibrosis is summarised below.
Figure 1.5 Electron micrographs of normal and fibrotic lung.
Figure shows the electron microscopy appearances of normal (upper panel) and fibrotic (lower panel) lung. Of note are the thickened alveolar wall and increased numbers of collagen fibrils seen in the fibrotic specimen. BM = basement membrane, Ep = epithelium, En = endothelium, Rbc = red blood cell, Co = collagen.
1.4.2.1 Animal models

A variety of animal models of pulmonary fibrosis have been developed. One of the most widely used is the bleomycin model, in which animals receive a single intratracheal instillation of the chemotherapeutic agent bleomycin. Bleomycins are a family of compounds produced by *Streptomyces verticillis*. They have potent antineoplastic properties but pulmonary fibrosis is a major adverse effect (see review Hay et al 1991) exploited in this model. The histological changes, similar to those seen in fibrosing alveolitis, have been well characterised and include oedema, necrosis of type I epithelial cells and inflammatory cell influx, followed by proliferation and metaplasia of type II epithelial cells together with fibrosis (Adamson, Bowden, 1974b; Adamson, Bowden, 1979; Thrall et al 1979).

Several studies have reported increased lung collagen content following bleomycin (Laurent et al 1981; Phan et al 1981; Shahzeidi et al 1991). Collagen synthesis rates also increase (Phan et al 1981; Laurent, McAnulty, 1983), as does collagen gene expression assessed by Northern analysis (Kelley et al 1985a; Raghow et al 1985; Hoyt, Lazo, 1988) or by *in situ* hybridisation (Shahzeidi et al 1993; Shahzeidi et al 1994). Collagen degradation rates following bleomycin were decreased (Phan et al 1981; Laurent, McAnulty, 1983).

Another well-characterised animal model is that of fibrosis induced by internal or external irradiation. Fibrosis under these circumstances is slowly progressive, in contrast to the acute fibrosis produced by bleomycin. In radiation models of pulmonary fibrosis increased rates of both collagen synthesis and degradation have been reported (Pickrell et al 1975; Pickrell et al 1976; Murray, Parkins, 1987; Walklin et al 1987; McAnulty et al 1991b) suggesting that distinct mechanisms may underlie the pathogenesis of pulmonary fibrosis induced by different agents.

1.4.2.2 Human pulmonary fibrosis

Immunohistochemistry suggests that collagen I is increased in areas of established lung fibrosis, whereas type III is increased in areas of early fibrosis (Bateman et al 1981). This is borne out by biochemical studies (Seyer et al 1976; Kirk et al 1984). Early
studies differed as to whether the lungs of patients with pulmonary fibrosis contained increased amounts of collagen (Fulmer et al 1980; Madri, Furthmayr, 1980), probably owing to the patchy nature of the disease. However, increased collagen content in CFA has now been established (Kirk et al 1986; Selman et al 1986).

Indirect evidence for increased collagen synthesis in patients with lung fibrosis is provided by studies demonstrating increased levels of procollagen peptides in BAL fluid from patients with CFA (Low et al 1983; Cantin et al 1988; Bjermer et al 1989) and SSc (Harrison et al 1990). Elevated serum levels of glucosyl transferase, another marker of collagen synthesis, have been found in patients with pulmonary fibrosis (Anttinen et al 1985). Enhanced collagen gene expression has been demonstrated in primary fibroblast lines derived from human fibrotic lung tissue (McSharry et al 1987) and in fibrotic foci from the lungs of patients with pulmonary fibrosis (Broekelmann et al 1991). However, increased collagen production by primary fibroblast lines from human fibrotic lung has not been demonstrated (Raghu et al 1989).

Alterations in collagen degradation are less well studied. Reduced collagenolytic activity in lung tissue has been reported in CFA (Selman et al 1986) while increased collagenase levels have been reported in BAL fluid from patients with CFA (Gadek et al 1979). The latter result may reflect the products of inflammatory cells present in BAL fluid rather than events occurring within the interstitium. A study of primary fibroblast lines derived from normal and fibrotic lung showed that fibrotic lung fibroblasts secreted half the amount of collagenase compared with control cells, suggesting that a predominance of low collagenase-producing fibroblast subpopulations could contribute to the development of fibrosis (Pardo et al 1992).

Increased collagen content in pulmonary fibrosis could also result from increased numbers of interstitial fibroblasts. There is evidence suggesting that this may be an important factor. A primary fibroblast line derived from a patient with familial CFA showed a peak thymidine incorporation double that of control lines (Gauldie et al 1987) and fibroblasts from patients with early lung fibrosis showed increased proliferative potential compared with controls (Raghu et al 1988).
Most biochemical studies have concentrated on collagen, but the lung content of other extracellular matrix proteins is also altered in lung fibrosis. The relevance of this to the pathogenesis of the disease is at present unclear. Increased elastin content (McCullough et al 1978; Laurent et al 1981; Goldstein et al 1979) and gene expression (Lucey et al 1996) have been demonstrated in animal models of pulmonary fibrosis. Pulmonary fibronectin gene (Kelley et al 1985b; Raghow et al 1985; Hoyt, Lazo, 1988) and protein (Hernnäs et al 1992) expression increases following bleomycin as do mRNA and protein content of the proteoglycan biglycan (Westergren-Thorsson et al 1993), while lung decorin gene and protein expression fall. Histological evidence of enhanced elastin deposition in fibrotic lesions in human lung is also reported (Eskenasy, 1982). Our understanding of the roles played by non-collagenous extracellular matrix protein components in the pathogenesis of pulmonary fibrosis is likely to increase in the future.

In summary, collagen is an important component of normal lung, providing a structural framework for tissue components as well as participating in the regulation of cellular function. Significant collagen turnover occurs throughout life. The pathways regulating biosynthesis and degradation are now fairly well understood and there are a number of different points at which excess synthesis or reduced degradation, or both, could arise and lead to collagen accumulation. Excess collagen deposition is now established as a feature of experimental and human pulmonary fibrosis. The next section discusses how the TGFβ family of cytokines might be involved in regulating collagen deposition in pulmonary fibrosis.

1.5 TRANSFORMING GROWTH FACTOR β

Although it is unlikely that a single cytokine is the sole mediator of the fibrotic response, one cytokine which is strongly implicated in the pathogenesis of pulmonary fibrosis is TGFβ₁. This section summarises the nature and functions of the TGFβ family of cytokines. Since the subject of this thesis is the role of the TGFβ family of cytokines in the pathogenesis of pulmonary fibrosis, this section will conclude by reviewing the evidence that TGFβ₁ is a key mediator in the pathogenesis of this disease.
1.5.1 The TGFβ superfamily

The TGFβ superfamily comprises at least 25 distinct but related molecules which have in common a number of structural features (for recent reviews see Lyons et al 1991, Laiho, Keski-Oja, 1992, Kingsley, 1994). All family members are initially synthesised as larger precursor molecules with an N-terminal signal sequence and a pro-domain of varying size. The precursor protein is usually cleaved to release a mature C-terminal segment of 110-140 amino acids, and the mature peptide consists of hetero- or homodimers of this C-terminal segment. Seven cysteine residues in the mature peptide are an almost invariant feature.

Some of the members can be grouped into distinct sub-families. These include the mammalian activins and inhibins, the Mullerian inhibitory substances, the DVR group comprising the decapentaplegic (dpp) gene complex and 60A subfamily, the bone morphogenetic proteins (BMPs) and growth differentiation factor 1 and 3. Other members include dorsalin, nodal, glial-derived neurotrophic growth factor and more recently described genes such as fugacin (Echochard et al 1995) and lefty (Meno et al 1996). Key unifying aspects of the biology of these peptides are their involvement in the regulation of developmental processes, and the multiple functions of each individual family member in a given organism.

1.5.2 The TGFβ isoforms

TGFβ₁ is the prototype of the TGFβ subfamily which consists of at least five different TGFβ isoforms. TGFβ₂ was isolated from bone as a cartilage-inducing factor and named CIF-B before its identity as TGFβ₂ was recognised (Seyedin et al 1985). It was also independently identified as a renal epithelial cell growth inhibitor (Holley et al 1980) and T cell suppressor factor (Wrann et al 1987) before being cloned (Arrick et al 1994; Madisen et al 1988; Hanks et al 1988; Miller et al 1989). Human TGFβ₃ was initially identified by cDNA characterisation (ten Dijke et al 1988b; Derynck et al 1988) and was subsequently expressed in recombinant form (Graycar et al 1989; ten Dijke et al 1990b). To date only TGFβ₁,₃ have been described in mammals.
Two further TGFβ isoforms have been described, designated as TGFβ₄ and TGFβ₅, present in chicken (Jakowlew et al. 1988c) and the frog Xenopus laevis (Kondaiah et al. 1990) respectively. Heterodimers between different TGFβ gene products have also been reported. TGFβ₁₂ heterodimers have been identified in porcine platelets (Cheifetz et al. 1987) and both TGFβ₁₂ and TGFβ₂₃ heterodimers have been isolated from bovine bone (Ogawa et al. 1992). The degree of identity between the five isoforms ranges from 64% (TGFβ₂ compared with TGFβ₄) to 82% (TGFβ₁ compared with TGFβ₄) (Kondaiah et al. 1990). TGFβ₁,₅ are all encoded as larger precursors (varying from 304 to 412 amino acids in length) and are processed to 112 amino acid chains, with the exception of TGFβ₄ which contains 114 amino acids. All mature peptides contain nine cysteine residues.

Cloning of individual TGFβ isoforms reveals that they are extremely well conserved between species. The sequences of the mature murine (Derynck et al. 1986), bovine (Van Obberghen-Schilling et al. 1987), porcine (Derynck, Rhee, 1987; Kondaiah et al. 1988), simian (Sharples et al. 1987) and chicken (Jakowlew et al. 1988a) TGFβ₁ peptides are identical to that of human TGFβ₁, with the exception of murine TGFβ₁ which has serine substituted for alanine at position 75. The sequences of the mature human (de Martin et al. 1987; Madisen et al. 1988) and simian (Hanks et al. 1988) TGFβ₂ are identical, as are the partial sequences of porcine and bovine TGFβ₂ (Cheifetz et al. 1987; Seyedin et al. 1987). Human (ten Dijke et al. 1988b; Derynck et al. 1988) and chicken TGFβ₃ (Jakowlew et al. 1988b) differ only in the substitution of a tyrosine for a phenylalanine, and human and porcine TGFβ₃ differ only in two amino acids (Derynck et al. 1988).

Comparison of human and murine TGFβ₁, and human and porcine TGFβ₃, N-terminal precursor regions reveals a high degree of polypeptide sequence conservation between species consistent with its important biological functions (Derynck et al. 1986; Derynck et al. 1988). In contrast, comparison of all three human TGFβ precursor sequences reveals striking dissimilarity (Derynck et al. 1988) with only three potential N-glycosylation sites preserved in all three isoforms and the RGD sequence preserved in the TGFβ₁ and TGFβ₃ precursors only.
Isoform sequence homology is apparent at genomic level. The TGFβ, gene in various mammalian species has a seven-exon structure (Derynck et al 1987; Van Obberghen-Schilling et al 1987) largely conserved in genes encoding the other TGFβ isoforms (Derynck et al 1988). Comparison of the sequences of the TGFβ, and TGFβ3 genes shows that all intron-exon junctions are localised at exactly corresponding nucleotide positions, the one exception being that of the first intron (Derynck et al 1988). The high conservation of the splice junctions has led to the suggestion that the TGFβ genes arose from early gene duplication events, with the structural similarities between the TGFβ isoforms at polypeptide level preserved by functional requirements. Nevertheless, chromosomal mapping studies show that in mouse and man the TGFβ1,3 genes are located on separate chromosomes. In the mouse they are located on chromosomes 7,1 and 12; in man they map to chromosomes 19q13, 1q412 and 14q24 respectively (Fujii et al 1986; Barton et al 1988; ten Dijke et al 1988a).

1.5.3 Chemistry

1.5.3.1 Structure
TGFB1 was first described as a soluble factor present in conditioned medium of murine sarcoma virus and chemically transformed cells (de Larco, Todaro, 1978). It was subsequently shown to be identical to the growth inhibitor of BSC-1 African green monkey kidney cells described by Holley and colleagues (Holley et al 1978). TGFB1 was later purified and characterised from human platelets (Assoian et al 1983), human placenta (Frolik et al 1983), and bovine kidney (Roberts et al 1983). The peptide was named for its ability to induce normal rat kidney fibroblasts to grow and form colonies of cells in soft agar in the presence of epidermal growth factor (EGF) (Roberts et al 1981). TGFB1 is a 25 kd homodimer, each chain containing 112 amino acids including nine cysteine residues. Crystallography of TGFB2 reveals that eight of the cysteine residues are grouped together to produce a series of intrachain disulphide bridges, forming a rigid structure called a cysteine knot, while the remaining cysteine residue forms an interchain disulphide bond linking the monomers (Daopin et al 1992; Schluengger, Grütter, 1992). NMR analysis of TGFB1 suggests that the structures of TGFB1 and TGFB2 are virtually identical (Archer et al 1993).
Cloning of TGFβ₁ (Derynck et al 1985) revealed that the TGFβ₁ monomer is initially synthesised in a latent form as the C-terminal segment of a 390 amino acid (aa) precursor polypeptide. The precursor has a short N-terminal signal peptide designating the molecule for secretion. The high molecular weight latent TGFβ₁ complex released by platelets consists of three subunits (see figure 1.6) whose structure has been elucidated in some detail (Wakefield et al 1988; Miyazono et al 1988). The precursor sequence is termed the latency associated peptide (LAP). It is glycosylated at three N-linked sites (Brunner et al 1988) and itself forms a disulphide-bonded dimer of approximately 75 kd. LAP is further covalently linked by a disulphide bond to a 120-205 kd glycoprotein, latent TGFβ binding protein (LTBP), which is transcribed from a separate gene (Kanzaki et al 1990). The third component is the mature, processed homodimeric TGFβ₁.

The TGFβ₁ precursor is cleaved intracellularly at an Arg-Arg site between residues 278 and 279. LAP is a homodimer representing two 249 aa remnants of the N terminals. Mature TGFβ₁ and LAP remain associated through noncovalent interactions and are secreted as latent TGFβ₁ (Pircher et al 1986). LTBP is synthesised separately but becomes associated with latent TGFβ₁ prior to secretion. This probably promotes correct folding and secretion of the TGFβ₁ molecule and is mediated by covalent bonding between an eight cysteine repeat of LTBP and Cys33 of LAP (Miyazono et al 1991; Saharinen et al 1996).
Figure 1.6 The high molecular weight latent TGFβ complex

Figure shows the three subunits of the high molecular weight latent TGFβ complex. These comprise the latency associated peptide (LAP), the latent TGFβ binding protein (LTBP) and the mature, processed homodimeric TGFβ. LAP is covalently linked by a disulphide bond to LTBP. Adapted from Flaumenhaft et al 1993b.
The role of LAP in regulating TGFβ₁ activity is being elucidated. At least two of the LAP carbohydrate side chains contain phosphorylated mannose-6-phosphate residues (Purchio et al 1988). Since agents that inhibit glycosylation block secretion (Sha et al 1989), the carbohydrate side chains are thought to play a role in secretion of the latent complex. This is supported by studies showing that if the cDNA encoding the precursor is engineered to produce the propeptide region of the precursor only, LAP is still secreted effectively (Gentry, Nash, 1990). LAP purified from cells transfected with a TGFβ₁ precursor cDNA containing a stop codon prior to the sequence encoding the mature TGFβ₁ can associate with and inactivate TGFβ₁. Furthermore, in transgenic mice with elevated hepatic levels of LAP, activity of hepatic TGFβ₁ is inhibited (Bottinger et al 1996). Association with LAP after secretion therefore probably restricts TGFβ₁ activity to locations where the latent complex can be activated. TGFβ₁ LAP also binds TGFβ₂ and TGFβ₃ tightly (Miller et al 1992) and recombinant TGFβ₁ LAP is a potent inhibitor of the activities of TGFβ₁,₂,₃ in vitro (Bottinger et al 1996).

The role of LTBP is still not entirely clear since it does not associate with mature TGFβ₁ (Kanzaki et al 1990) and is not required for latency. However, amino acid sequencing by these authors revealed that it contains various motifs involved in protein-protein interactions, including 16 EGF-like domains (for a review on EGF-like repeats see Appella et al 1988), an Arg-Gly-Asp (RGD) sequence which may mediate interactions with integrins (Ruoslaiti, Pierschbacher, 1987) and a sequence of eight amino acids identical to a sequence in the B2 chain of laminin (Sasaki, Yamada, 1987). Both free LTBP and LTBP complexed to TGFβ₁ are efficiently incorporated into fibroblast extracellular matrix, and proteolytic cleavage of LTBP by plasmin releases latent TGFβ₁ from the matrix (Taipale et al 1994). Recently it has been shown that the N terminal of LTBP associates covalently with extracellular matrix (Saharinen et al 1996). LTBP may thus play a role in binding of the high molecular weight latent TGFβ₁ complex to cell surface and matrix proteins.

Two further LTBP molecules have recently been identified, termed LTBP-2 and LTBP-3 and having beween 38 and 41% homology with LTBP-1 (Moren et al 1994; Yin et al 1995). All three proteins show structural similarities to the microfibril protein.
fibrillin and form a high molecular weight complex with the TGFB1 precursor. The different associated proteins may serve to direct the complexes to specific sites. The binding of TGFB2 and TGFB3 to individual LTBP isoforms has not been characterised.

In some cell types, including CHO cells transfected with the TGFB1 precursor cDNA (Gentry et al 1987) and cultured osteoblasts (Bonewald et al 1991), TGFB1 is produced as a low molecular weight (100 kd) latent complex consisting only of the mature TGFB1, noncovalently associated with LAP. In serum, a latent form of TGFB1, consisting of the mature dimeric form of TGFB1 complexed to α-macroglobulin, has been described (O'Connor-McCourt, Wakefield, 1987). This has been proposed to act as a clearance mechanism for excess TGFB1 following its release. However, TGFB1 bound to α-macroglobulin can be released by heparin, suggesting that α-macroglobulin may act as a carrier protein to deliver TGFB to cells. The growth factor may be released on encountering the cell surface and an environment rich in heparan sulphate proteoglycans (McCaffrey et al 1989).

1.5.3.2 Activation

The observation that conditioned cell culture medium treated with acid contained TGFB activity (Lawrence et al 1984) was the first indication that TGFB might be secreted in latent form. It is now known that all three TGFB isoforms are secreted as latent complexes and activated extracellularly by release of the mature cytokines from their noncovalently associated LAPs. TGFB1 has been shown to be released from degranulating platelets and secreted from most cells in inactive form. In vitro the peptide can be activated by extremes of pH, exposure to urea or more than 0.02% sodium dodecyl sulphate (Lawrence et al 1985; Miyazono et al 1988), suggesting that latency is conferred by electrostatic interactions between TGFB1 and LAP.

Mechanisms which activate latent TGFB1, in vivo are still being elucidated (see figure 1.7). Proteases such as plasmin can activate latent TGFB1, in vitro (Lyons et al 1988), possibly by cleaving a peptide bond in LAP and causing an alteration in its conformation which releases mature TGFB1 (Lyons et al 1990). The concentrations of plasmin required (0.5 U/ml) are such that they are unlikely to exist in solution in vivo.
However, they could occur at the cell surface where TGFβ₁ activation is postulated to take place (Dennis, Rifkin, 1991). The requirement for TGFβ₁ activation may allow TGFβ₁ to regulate its own production. In co-cultures of endothelial and smooth muscle cells active TGFβ increases plasminogen activator inhibitor 1 (PAI-1) expression. This inhibits conversion of plasminogen to plasmin by urokinase (uPA) and prevents further TGFβ₁ activation (Sato et al 1990). TGFβ also down-regulates uPA directly (Nunes et al 1995).

Two recent studies support the view that plasmin activates TGFβ₁ in vivo. Transgenic mice expressing human apolipoprotein a (apo a) fail to exhibit TGFβ activation in aortic wall and serum, possibly reflecting apo a inhibition of plasminogen activation (Grainger et al 1994). Following in vivo lung injury in rats, a rise in active TGFβ₁ secretion by BAL fluid-derived alveolar macrophages is paralleled by a rise in plasmin release by the same cells, and α₂ antiplasmin added to the cultures prevents TGFβ₁ activation (Khalil et al 1996a).

Binding of latent TGFβ₁ to the mannose-6-phosphate/insulin like growth factor II (IGF-II) receptor is required for activation (Dennis, Rifkin, 1991), and probably occurs via interaction with mannose-6-phosphate residues on LAP. LTBP antibody inhibits TGFβ₁ activation (Flaumenhaft et al 1993a), suggesting that LTBP directs TGFβ₁ to activation sites on the cell surface. Recent studies have also implicated thrombospondin 1, a platelet alpha-granule and extracellular matrix glycoprotein, in TGFβ₁ activation (Schultz-Cherry, Murphy-Ullrich, 1993; Schultz-Cherry et al 1995). This pathway of activation is protease-independent and does not require interaction with cell surface molecules. Since thrombospondin can co-localise with TGFβ in extracellular matrix (Slater et al 1995), thrombospondin may regulate activation of matrix-bound TGFβ.
Figure 1.7 Possible mechanisms regulating TGFβ activation
Figure shows the mechanisms which may regulate TGFβ activation in vivo. Latent TGFβ bound to the cell surface is probably activated by plasmin. Active TGFβ then upregulates PAI-1 and downregulates the plasminogen activator uPA, thus reducing subsequent activation of latent TGFβ. An alternative, protease-independent activation pathway has also been described. This does not require interaction with cell surface proteins but utilises thrombospondin, an extracellular matrix glycoprotein. M-6-P = mannose-6-phosphate.
1.5.3.3 Receptors

The actions of the TGFβ family of cytokines are mediated through binding to specific cell membrane receptors, and almost all cell types bind TGFβ, with affinities in the picomolar concentration range (Frolik et al 1984; Tucker et al 1984; Massagué, Like, 1985; Wakefield et al 1987). In most cell types, three different types of TGFβ receptor, type I (53 kd), type II (70-80 kd) and type III (250-350 kd) have been identified (Cheifetz et al 1988a). The type III receptor is a proteoglycan (Segarini, Seyedin, 1988; Cheifetz et al 1988a) named betaglycan because of its ability to bind TGFβ (Andres et al 1989).

Cells lacking the type III receptor, including haematopoietic progenitor cells (Ohta et al 1987) and endothelial or epithelial cells in primary or early passage cultures (Segarini et al 1989) are still responsive to TGFβ, indicating that the type III receptor is not required for signalling. Cloning of the type III receptor demonstrated that the betaglycan structure contained a short cytoplasmic domain with no signalling motif (Wang et al 1991). Betaglycan exists in soluble as well as membrane-bound form (Andres et al 1989). The membrane-bound form of betaglycan presents TGFβ to the type II signalling receptor (Lopez-Casillas et al 1993) while the soluble form binds TGFβ and inhibits binding to membrane receptors. Conversion of membrane-bound betaglycan to the soluble form may thus provide a mechanism for TGFβ inhibition (Lopez-Casillas et al 1994).

The type I and II receptors are distantly related transmembrane serine/threonine kinases. Studies of mutant cells resistant to TGFβ action have demonstrated that TGFβ binding to the type I receptor requires the presence of the type II receptor, and that both type I and type II receptors are required for TGFβ signalling (Attisano et al 1994; Franzen et al 1993; Bassing et al 1994; Laiho et al 1991). Studies in mink lung epithelial cells (Wrana et al 1994) suggest that TGFβ binds directly to the type II receptor, which is a constitutively active kinase. Bound TGFβ is then recognised by the type I receptor, which is recruited into the complex and becomes phosphorylated by the type II receptor. Phosphorylation subsequently enables the type I receptor to propagate the signal to downstream substrates. TGFβ signalling is therefore mediated by a heteromeric
complex. Isoforms of the type I and type II receptors with differing affinities for TGFβ have also been reported (Kalter, Brody, 1991; Zhou et al 1995; Tsang et al 1995).

In addition to the three well-characterised TGFβ receptors, several other membrane-bound proteins have been shown to bind TGFβ. The type IV receptor is a 60 kd protein so far only isolated from a pituitary tumour cell line (Cheifetz et al 1988b). The type V receptor is a 400 kd non-proteoglycan glycoprotein originally isolated from bovine liver but since recognised in various cells (O'Grady et al 1991). The type VI receptor is a 180 kd receptor also found on a wide variety of cell types (Segarini et al 1992). The functional importance of these receptors is at present unclear.

Components of the signalling pathways that lie downstream of the serine/threonine kinase receptors are as yet poorly defined, but recent evidence implicates a family of genes homologous to the Drosophila gene called Mothers against dpp (Mad). The different proteins encoded by these genes appear to be localised to the cytoplasm and to transmit specific information to the nucleus, resulting in different biological responses (Riggins et al 1996; Graff et al 1996).

1.5.4 Biological activity

TGFβ is the prototype of a multifunctional growth factor and its actions on any particular target cell are highly dependent on cell phenotype, culture conditions and the presence of other growth factors. For example, TGFβ stimulates the growth of fibroblasts from very early fetuses but inhibits the growth of fibroblasts derived from older fetuses (Hill et al 1986). Both TGFβ and TGFβ stimulate primitive mesenchymal cells to differentiate and express cartilage, but treatment of chondrocytes with TGFβ suppresses synthesis of cartilage constituents including type II collagen (Rosen et al 1988). The diversity of the effects of TGFβ, coupled with almost universal cellular expression of TGFβ receptors, place it in a unique position to regulate normal and pathological processes. The biological actions of TGFβ can be subdivided into three main categories. These comprise its regulatory effects on immune function, cell
differentiation and growth, and extracellular matrix protein turnover. The next section discusses these in turn, placing greatest emphasis on the last, since it is the effects of TGFβ on matrix turnover which have led to its implication in the pathogenesis of pulmonary fibrosis.

Most studies have examined TGFβ1. The biological activities of the TGFβ isoforms are similar, but differences in potency exist dependent on cell type and assay (Graycar et al 1989). This may partly reflect differential affinities of the receptors for the TGFβ isoforms (Segarini et al 1987; Cheifetz et al 1990; Lyons et al 1991; Mitchell et al 1992). The majority of type I and II receptors bind TGFβ1 and TGFβ3 with greater affinity than TGFβ2 (Massagué, 1992) but receptor subsets exist which bind all three isoforms with equal affinity (Segarini et al 1987). Where functional differences are known to exist between the isoforms they will be highlighted.

1.5.4.1 Immunoregulation

Both TGFβ1 and TGFβ2 have potent immunoregulatory actions. They inhibit T and B lymphocyte proliferation in femtomolar concentrations (Kehrl et al 1986a; Kehrl et al 1986b; Kehrl et al 1989). TGFβ1 inhibits thymocyte proliferation (Ristow, 1986), suppresses natural killer cell activity (Rook et al 1986) and inhibits production of lymphokine-activated killer cells and cytotoxic T lymphocytes (Mulé et al 1988; Espevik et al 1988). TGFβ1 also inhibits B cell IgG and IgM secretion.

TGFβ1 antagonises some actions of the interleukins IL-1, IL-2 and IL-3 (Kehrl et al 1986a; Kehrl et al 1986b; Ohta et al 1987; Wahl et al 1988) interferon-α (Rook et al 1986), interferon gamma (Czarniecki et al 1988) and TNFα (Ranges et al 1987). TGFβ1 directs isotype switching in B lymphocytes, upregulating IgA production by splenic B cells (Coffman et al 1989) or alternatively suppressing IgA secretion by activated IgA-secreting lymphocytes (van Vlasselaer et al 1992).

TGFβ1 and TGFβ2 can also suppress immune cell function in vivo. TGFβ2 was independently characterised as an immunosuppressive factor produced by human glioblastomas (de Martin et al 1987) which can cause impaired cell-mediated immune
responses in patients (Wrann et al 1987). Further evidence that TGFβ₁ plays an essential role in immunoregulation *in vivo* comes from studies of TGFβ₁ null mice (Shull et al 1992; Kulkarni, Karlsson, 1993). These mice are born healthy but within two to three weeks develop a rapidly progressive and fatal multifocal inflammatory disease characterised by severe wasting. Lymphocytes are required for this inflammatory response, indicating that TGFβ₁ normally plays a vital role in regulating lymphocyte proliferation and activation (Diebold et al 1995).

In contrast to its effects on lymphocytes, TGFβ₁ can activate macrophages and monocytes. It stimulates monocyte chemotaxis in picomolar concentrations and at higher concentrations enhances monocyte gene expression of various fibroblast growth factors including IL-1 (Wahl et al 1987). However, TGFβ₁, and to a lesser extent TGFβ₂, also selectively inactivate macrophages by suppressing the release of H₂O₂ (Tsunawaki et al 1988). TGFβ₁ also inhibits cytokine-induced macrophage nitric oxide (NO) release *in vitro* (Nelson et al 1991) by reducing translation of inducible nitric oxide synthase (iNOS) mRNA and increasing iNOS degradation (Vodovotz et al 1993). TGFβ₁ null mice exhibit elevated serum levels of NO reaction products and increased iNOS gene and protein expression, confirming that TGFβ₁ downregulates iNOS expression *in vivo* (Vodovotz et al 1996). It has therefore been suggested that TGFβ may participate in repair following injury by upregulating macrophage growth factor production and downregulating cytotoxicity.

1.5.4.2 Regulation of cell proliferation and differentiation

TGFβ is a potent growth inhibitor for many normal cell lines (for a review see Fynan, Reiss, 1993). It almost invariably inhibits epithelial cell replication in vitro, including that of keratinocytes (Moses et al 1985; Coffey et al 1988), tracheal (Jetten et al 1986) and bronchial epithelial cells (Masui et al 1986), intestinal epithelial cells (Kurokowa et al 1987) and renal proximal tubular cells (Fine et al 1985). Although differences in potency exist between the three TGFβ isoforms, TGFβ2 and TGFβ3 share the ability of TGFβ1 to inhibit proliferation of human keratinocytes and that of the mink lung epithelial cell line CCL64 (Graycar et al 1989). The latter biological effect is frequently used as a bioassay for TGFβ. TGFβ1 also inhibits haematopoietic cell proliferation but TGFβ2 is 100 times less potent (Ohta et al 1987).

TGFβ1 is also a potent inhibitor of endothelial cell growth (Baird, Durkin, 1986; Frater-Schröder et al 1986; Müller et al 1987). TGFβ2 is 100 times less active in inhibiting endothelial cell replication (Jennings et al 1988). Inhibition of cell growth by TGFβ1 is frequently associated with terminal differentiation (Jetten et al 1986; Masui et al 1986; Kurokowa et al 1987). TGFβ1 arrests the cell cycle in late G1 phase (Nakamura et al 1985; Laiho et al 1990), probably by blocking several steps involved in cyclin-dependent kinase activation and thereby preventing subsequent phosphorylation of retinoblastoma protein (Satterwhite, Moses, 1994).

In contrast, mesenchymal cells appear less susceptible to growth inhibition by TGFβ1 (see review Fynan, Reiss, 1993), with both inhibitory and stimulatory effects reported. TGFβ1,3 stimulate proliferation of embryonic mouse fibroblasts, with TGFβ2 and TGFβ3 equipotent and significantly more active than TGFβ1 (Graycar et al 1989). TGFβ3 stimulates proliferation of human fibroblasts isolated from fetuses weighing up to 50 g (Hill et al 1986) and rat mesenteric fibroblasts cultured in intact connective tissue (Franzen, Dahlquist, 1994). TGFβ1 also stimulates human mesothelial cell replication (Gabrielson et al 1988). TGFβ1 and TGFβ3 stimulate proliferation of osteoblast-enriched bone cell cultures, with TGFβ3 being three to five times more potent than TGFβ1 (ten Dijke et al 1990a).

However, a biphasic effect was noted in this study and has also been reported in
fibroblasts and smooth muscle cells, with stimulation at low concentrations and inhibition at higher concentrations (Kimura et al 1989; Battegay et al 1990). The proliferative response appears to be mediated in part by induction of platelet derived growth factor (PDGF) expression (Seifert et al 1994). At higher concentrations TGFβ₁ downregulates PDGF receptor expression (Battegay et al 1990) but may also inhibit proliferation directly.

Comparison of TGFβ₁,₂,₃ has revealed that all three isoforms stimulate human foetal lung fibroblast proliferation at 0.2 pM (McAnulty et al 1997) but inhibit proliferation at concentrations of 1.6 pM and above. TGFβ₂ was the most and TGFβ₁ the least potent in this study. Indomethacin overcame the inhibitory effects of higher concentrations of TGFβ₁,₂,₃, suggesting that prostaglandin E₂ (PGE₂) plays a role in regulating in mediating the antiproliferative effects of TGFβ. This is consistent with the observation that both the antiproliferative effects of TGFβ on epithelial cells and TGFβ₁-induced PGE₂ synthesis are mediated via pertussis toxin sensitive G proteins (Howe et al 1990; Fukami et al 1995; McAnulty et al 1995).

1.5.4.3 Regulation of extracellular matrix protein turnover
TGFβ₁ is the most potent stimulator of extracellular matrix protein deposition, particularly that of collagen, so far described. It does this by stimulating procollagen gene and protein expression, and inhibiting collagen degradation.

TGFβ₁ stimulates type I collagen and fibronectin synthesis in vitro by all fibroblast lines examined including mouse embryonic, rat kidney, rat lung, human skin, human gingival, human embryonic lung fibroblasts, adult normal and fibrotic lung fibroblasts (Ignotz, Massagué, 1986; Roberts et al 1986; Raghu et al 1989; Wrana et al 1986; McAnulty et al 1991a; McAnulty et al 1995). TGFβ₁ also increases collagen synthesis in vivo (Roberts et al 1986; Pierce et al 1989; Nabel et al 1993). TGFβ₁ augments synthesis of collagen types I and III by human embryonic lung fibroblasts without altering the proportion of collagen types (Fine, Goldstein, 1987) and this effect is selective for collagen as compared with total protein production.
TGFβ1 also stimulates collagen synthesis by myoblasts (Ignotz et al. 1987), rat embryo mesenchymal cells (Seyedin et al. 1985), osteoblasts (Centrella et al. 1987; ten Dijke et al. 1990a), hepatic lipocytes (Matsuoka, Tsukamoto, 1990) and foetal rat lung epithelial cells (DiMari et al. 1991). Little is known about the effects of the other two TGFβ isoforms on collagen synthesis. The only study to address this question showed that TGFβ3 was three to five times more potent than TGFβ1 at stimulating collagen synthesis by osteoblast-enriched bone cultures (ten Dijke et al. 1990a). TGFβ2 is less potent than TGFβ1 in stimulating type II collagen production by embryonic rat muscle cells, but more potent in stimulating proteoglycan synthesis by these same cells (Seyedin et al. 1985).

The pathways by which TGFβ1 enhances extracellular matrix protein synthesis, including that of collagen, are partly known. TGFβ1 stimulates increased collagen I and III and fibronectin mRNA levels (Ignotz et al. 1987; Raghow et al. 1987; Varga et al. 1987; Dean et al. 1988; Penttinen et al. 1988; Raghu et al. 1989). The mechanism involves both increased matrix protein transcription rates and increased mRNA stability (Raghow et al. 1987; Penttinen et al. 1988). TGFβ1 increases gene transcription by activation of the α2(I) (Rossi et al. 1988) and α1(I) collagen (Ritzenhaler et al. 1991) promoters at different regions (Ritzenhaler et al. 1991). In the case of the α2(I) collagen gene, nuclear factor-1 (NF-1)-like and AP-1 binding sites are thought to be partly responsible for mediating the effects of TGFβ1 (Rossi et al. 1988; Chung et al. 1996). An NF-1-like binding site is located in the α1(I) collagen promoter but apparently does not mediate TGFβ1 activation of the α1(I) collagen gene (Jimenez et al. 1994), and the activation mechanism remains unclear. TGFβ1 also increases elastin synthesis by smooth muscle cells (Liu, Davidson, 1988) and lung fibroblasts (McGowan, McNamer, 1990), probably by activation of the elastin promoter (Katchman et al. 1994).

TGFβ1 regulates the enzymes controlling degradation of newly synthesised extracellular matrix proteins. It reduces collagenase gene transcription (Edwards et al. 1987; Overall et al. 1991) and upregulates TIMP gene and protein expression (Edwards et al. 1987; Overall et al. 1991; Wright et al. 1991). TGFβ1 also increases PAI-1 synthesis, probably
by increasing transcription (Lund et al 1987). TGFβ₁ also reduces the proportion of newly synthesised procollagen degraded intracellularly prior to secretion (McAnulty et al 1991a) although the mechanism by which it does this is not known. Recent data suggest that TGFβ₁-mediated induction of lung fibroblast procollagen synthesis is negatively regulated by PGE₂ production in response to TGFβ₁ (McAnulty et al 1995).

In summary, the TGFβ isoforms are a family of highly homologous multifunctional peptides with potent effects including control of immune function, cell growth and differentiation and extracellular matrix metabolism. TGFβ₂ and TGFβ₃ appear to have distinct but in some cases overlapping roles to TGFβ₁. Their activities have led to their being implicated in a variety of normal biological processes including organ and tissue development and wound healing. The TGFβ family has also been implicated in the pathogenesis of a number of diseases characterised by inflammation, fibrosis and malignant transformation. The following section reviews the evidence that TGFβ is implicated in the pathogenesis of fibrotic disease.

1.6 TGFβ IN THE PATHOGENESIS OF FIBROSIS

Given the profibrotic potential of the TGFβ isoforms, it is not surprising that this family of cytokines has also been implicated in the pathogenesis of diseases characterised by chronic inflammation and fibrosis. The following section reviews the evidence that the TGFβ family is implicated in the pathogenesis of both extra-pulmonary and pulmonary fibrosis. Almost all studies have focused on TGFβ₁ and it is to the prototype of the family that this section will therefore refer.

1.6.1 Role of TGFβ₁ in extra-pulmonary fibrosis

Various studies have shown that TGFβ₁ gene expression is enhanced in situations where increased extracellular matrix protein deposition follows injury. These include experimental glomerulonephritis (Yamamoto et al 1994), liver regeneration (Jakowlew et al 1991) and experimental and clinical ureteropelvic junction obstruction (Seremetis, Maizels, 1996). In vivo transfection of pig arteries with the TGFβ₁ gene results in
substantially increased extracellular matrix production (Nabel et al 1993). The first conclusive evidence that TGFβ₁ plays a role in the pathogenesis of fibrosis in vivo came from studies in an animal model of renal fibrosis. In rats, administration of anti-TGFβ₁ serum coincident with the induction of acute mesangial proliferative glomerulonephritis suppressed the increased production of extracellular matrix usually seen in this disease (Border et al 1990). This group subsequently demonstrated that the proteoglycan decorin, which can bind and neutralise TGFβ₁, had the same effect (Border et al 1992).

An important development in elucidating the role of TGFβ₁ further has been the generation of transgenic mice overexpressing TGFβ₁. This has provided several lines of evidence suggesting that TGFβ₁ is profibrotic in vivo. Transgenic mice overexpressing TGFβ₁ in the central nervous system, pancreas and liver develop accumulation of extracellular matrix proteins in the tissues where the transgene is expressed (Wyss-Coray et al 1995; Lee et al 1995; Sanderson et al 1995). Finally, transgenic mice with high plasma TGFβ₁ levels develop progressive renal fibrosis (Kopp et al 1996).

The available patient data also suggests that TGFβ₁ is important in the development of fibrosis. Raised plasma TGFβ levels predict the risk of developing liver or lung fibrosis following autologous bone marrow transplant for advanced breast cancer (Anscher et al 1993), and persistently elevated plasma TGFβ levels correlate with the development of pneumonitis in patients with lung cancer who receive radiotherapy (Anscher et al 1994).

1.6.2 Role of TGFβ₁ in pulmonary fibrosis

TGFβ₁ is constitutively expressed in normal (Yamauchi et al 1988; Pelton et al 1991a; Khalil et al 1993; Magnan et al 1994) and fibrotic lung (Moreland et al 1992). Evidence that TGFβ is an important mediator in the pathogenesis of lung fibrosis will be reviewed by considering data obtained from animal models followed by that available from human studies.
1.6.2.1 Animal studies

Total lung TGFβ₁ mRNA levels increase prior to enhanced collagen type I and III gene expression following intratracheal bleomycin in mice (Hoyt, Lazo, 1988; Raghow et al 1989; Westergren-Thorsson et al 1993). The increase in TGFβ gene expression is confined to bleomycin-sensitive strains of mice (Hoyt, Lazo, 1988; Phan, Kunkel, 1992). Following bleomycin in rats, total lung TGFβ levels rise 30 fold, and the peak in TGFβ levels precedes maximal collagen synthesis (Khalil et al 1989). Immunohistochemistry shows that at seven days when TGFβ production is maximal, TGFβ₁ is almost exclusively localised to alveolar macrophages, whereas at 14 days when collagen synthesis peaks, TGFβ₁ is localised extracellularly in association with areas of developing fibrosis (Khalil et al 1989). Following bleomycin, much of the TGFβ released by alveolar macrophages is in active form (Khalil et al 1993).

*In vitro* studies show that bleomycin stimulates TGFβ release by endothelial cells (Phan et al 1991b), and stimulates TGFβ gene expression by rat lung fibroblasts (Breen et al 1992) suggesting that alveolar macrophages are not the only cells producing TGFβ during the course of bleomycin-induced lung fibrosis. The role of TGFβ₂ and TGFβ₃ in the pathogenesis of bleomycin-induced pulmonary fibrosis is not yet clear. However, antibodies to TGFβ₂ alone, or to TGFβ₂ and TGFβ₁ together, reduce total lung content in mice following bleomycin (Giri et al 1993).

1.6.2.2 Human studies

Although enhanced collagen production by fibrotic lung fibroblasts has not been demonstrated, there is evidence that primary lung fibroblast lines from patients with SSc and pulmonary fibrosis are more sensitive to TGFβ₁ than control cells as judged by their procollagen synthesis when stimulated by exogenous TGFβ₁ (Harrison et al 1991a). The results of this study contrast with that by Raghu and colleagues, who failed to demonstrate any difference in TGFβ₁-stimulated procollagen production between fibrotic and normal human lung fibroblasts (Raghu et al 1989). This could reflect differences in the stage of disease, since patients with SSc and pulmonary fibrosis often present earlier than do those with CFA. Fibroblasts at an earlier stage of disease may exhibit an enhanced response to stimulatory cytokines which is subsequently lost.
TGFB levels in BAL fluid are raised in infants who develop chronic lung disease of prematurity, in whom pulmonary fibrosis is a prominent feature (Kotecha et al 1996).

Immunohistochemistry for TGFB in lung biopsy tissue from patients with idiopathic pulmonary fibrosis has revealed prominent deposition in epithelial cells, macrophages and extracellular matrix (Khalil et al 1991). TGFB was not present in epithelial cells in normal lung tissue. A study of patients with pulmonary fibrosis including those with CFA and SSc showed TGFB protein localised to alveolar macrophages, airway epithelial cells, smooth muscle cells and hyperplastic type II pneumocytes (Corrin et al 1994). Control sections showed weak staining of alveolar macrophages, variable staining in airway epithelium and no staining in alveolar epithelial cells. TGFB gene expression is also increased in human pulmonary fibrosis (Broekelmann et al 1991), with predominant gene expression in alveolar macrophages and TGFB protein localised to foci containing activated fibroblasts.

In summary, TGFB gene and protein expression are enhanced in animal models of lung fibrosis and in the lungs of patients with the disease. Animal models reveal a temporal relationship between the rise in TGFB levels and increased collagen gene and protein expression. There is also a spatial association between TGFB deposition and extracellular matrix accumulation. Taken together these data strongly suggest an important role for TGFB in the pathogenesis of pulmonary fibrosis.

A number of questions need to be addressed. Firstly, it is not known whether TGFB2 and TGFB3 share the ability of TGFB1 to stimulate human lung fibroblast procollagen production and reduce intracellular degradation. Secondly it is not known which cells are the primary source of TGFB1 gene expression in the lung, and whether these alter during the course of the development of pulmonary fibrosis. A greater understanding of this would raise the possibility of cell-targeted therapy. It is also not known whether TGFB2 and TGFB3 play a role in the pathogenesis of pulmonary fibrosis in vivo. This is a particularly important question to address given the potential for specific anticytokine agents as therapeutic strategies in the treatment of this disease.
In order to address the question of localisation of TGFβ isoform gene expression in normal and fibrotic lung, *in situ* hybridisation techniques provide a powerful tool. However, current techniques are limited in a number of ways, and one of the aims of this thesis was to develop a technique which would adequately resolve these obstacles. The next section reviews briefly the current methods available and their limitations.

### 1.7 Current *in situ* hybridisation techniques

*In situ* hybridisation techniques permit the detection of specific nucleic acid sequences in morphologically preserved chromosomes, cells or tissue sections. The technique was first developed independently by two groups nearly thirty years ago (John et al. 1969; Pardue, Gall, 1969). At this time radioisotopes were the only labels available for nucleic acids, and autoradiography was the only means of detecting hybridised sequences. Furthermore, as molecular cloning was not possible, *in situ* hybridisation was restricted to those sequences which could be isolated and purified by conventional biochemical means, such as viral DNA and ribosomal RNAs.

Molecular cloning techniques and improved radiolabelling methods have generated significant improvements in the efficiency of *in situ* hybridisation. DNA sequences of a few hundred base pairs in length can be detected in metaphase chromosomes, and radioactive *in situ* detection of low copy number mRNA transcripts is now possible in individual cells. However, radioactive methods still confer significant limitations in terms of cellular resolution and the length of time required for autoradiography. In particular, the degree of cellular resolution afforded by the use of the $^{35}$S label frequently does not permit accurate identification of cell types expressing the gene product when whole tissue sections are examined. This becomes a significant disadvantage when examining lung tissue, which contains multiple cell types.

The localisation of TGFβ isoform gene expression was a key part of this thesis (see section 1.8.2). It was therefore important to develop a method for *in situ* hybridisation in lung tissue which would permit a high degree of cellular resolution. Two non-isotopic labels currently exist, biotin and digoxigenin. Digoxigenin was chosen for this
work, the reasons for which are discussed in section 4.2. The following section sets out the hypothesis and aims of this thesis.

## 1.8 AIMS OF THIS THESIS

### 1.8.1 Hypothesis

The overall aim of this thesis was to examine the role of the three different TGFβ isoforms in the pathogenesis of pulmonary fibrosis. In so doing I will address the hypothesis that TGFβ1, TGFβ2 and TGFβ3 play distinct but overlapping roles in the pathogenesis of pulmonary fibrosis.

### 1.8.2 Specific aims

The specific aims were fourfold and outlined below.

Firstly, to investigate whether TGFβ2 and TGFβ3 share the ability of TGFβ1 to stimulate human lung fibroblast procollagen production and elucidate the mechanisms in terms of their effects on procollagen synthesis and intracellular degradation.

Secondly, to localise TGFβ1, TGFβ2 and TGFβ3 gene expression in normal murine and human lung.

Thirdly, to localise TGFβ1, TGFβ2 and TGFβ3 gene expression during the course of murine bleomycin-induced lung fibrosis.

Fourthly, to localise TGFβ1, TGFβ2 and TGFβ3 gene expression in human pulmonary fibrosis arising in the context of CFA or SSc.

The following chapter describes the methods used to approach these questions. Chapter three gives an account of the results, which are then discussed in chapter four.
CHAPTER TWO

METHODS
IN VITRO STUDIES

2.1 MATERIALS

Chemicals, of analytical grade or above, were obtained from BDH/Merck unless otherwise indicated. Water for buffer preparation was distilled and deionised. Solvents used to prepare HPLC buffers and solutions were of HPLC grade and obtained from BDH/Merck. Sterile tissue culture plates, polypropylene centrifuge tubes and pipettes were purchased from Costar. Other disposable plasticware was obtained from Sterilin. Dulbecco’s modified Eagle’s medium, newborn calf and foetal bovine serum were purchased from Imperial laboratories. The same batch of serum was used throughout. Phosphate buffered saline, trypsin, streptomycin, penicillin and glutamine were obtained from the Institute of Cancer Research. Natural porcine TGFβ1, natural porcine TGFβ2 and recombinant chicken TGFβ3 were obtained from R&D Systems. Human foetal lung fibroblasts (HFL-1) were purchased from the American Type Culture Collection.

2.2 FIBROBLAST CELL CULTURE

2.2.1 Culture conditions

HFL-1 cells were cultured in 10 cm diameter tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 200 units/ml of penicillin, 200 units/ml of streptomycin, 4 mM glutamine and 10% newborn calf serum (NCS). Plates were incubated at 37°C in a humidified atmosphere of air containing 10% CO₂. Cells were passaged regularly and tested routinely for Mycoplasma infection using the Mycoplasma Gen-Probe Rapid Detection System kit (Laboratory Impex) according to the manufacturer’s instructions. Figure 2.1 shows the characteristically spindle-shaped HFL-1 cells in subconfluent culture, stained with 1% methylene blue and photographed using an inverted phase-contrast light microscope.
Figure 2.1 Morphology of human foetal lung fibroblasts
Figure shows HFL-1 cells in subconfluent culture, stained with 1% methylene blue and photographed with an inverted phase-contrast light microscope. Original magnification: x 100
2.2.2 Cell passage

HFL-1 cells exhibited contact inhibition and were passaged into fresh culture dishes on reaching visual confluence (approximately once every six days). The medium was aspirated and discarded, and the cell layer washed with phosphate buffered saline (PBS). Fibroblasts were resuspended with 1 ml of trypsin (0.05% w/v) followed by incubation at 37°C for 1 min. The cells were examined with an inverted phase contrast light microscope (Olympus TCK-2, Olympus Optical Company) to confirm that they had become rounded. Trypsin was neutralised by addition of 9 ml of DMEM supplemented with 10% NCS. The cell suspension was then split into six fresh cell culture plates. Cells remained viable for up to 15 passages after receipt from the supplier. Cells used for experiments were between passages 14 and 20. When plating cells for experiments, the trypsinised cells were resuspended in DMEM supplemented with 5% NCS prior to seeding in 12-well tissue culture plates at a density of $1 \times 10^5$ cells/well.

2.3 DETERMINATION OF PROCOLLAGEN METABOLISM

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

2.3.1 Cell culture conditions

HFL-1 cells cultured to visual confluence were resuspended in DMEM with 5% NCS as described (section 2.2.2) and seeded into 12 well plates. When they had reached visual confluence the DMEM was removed and replaced with 1 ml of pre-incubation medium containing 4mM glutamine, 50µg/ml ascorbic acid, 0.2mM proline and 2% NCS. After 24 hrs the medium was replaced with 1 ml of fresh pre-incubation medium.
containing TGFβ₁, TGFβ₂ or TGFβ₃. The cells were then incubated for a further 24 hrs before harvesting. Identical parallel cultures were set up to estimate cell number by cell counting or measurement of DNA content of the cell layer.

2.3.1.1 TGFβ₁,₃ dose response relationships
Lyophilised TGFβ isoforms were reconstituted in a sterile solution of 1 mg/ml of bovine serum albumin in 4 mM hydrochloric acid (HCl) to give a final concentration of 1µg/ml. Stock solutions were aliquoted and stored at -40°C. To determine dose response relationships for each TGFβ isoform, cells were incubated with incubation medium containing TGFβ₁, TGFβ₂ or TGFβ₃ at concentrations ranging from 0.05 to 5 ng/ml (2 to 200 pM). Control cells were incubated with medium without addition of TGFβ.

2.3.1.2 Comparison of TGFβ isoforms
Cells were incubated with incubation medium containing TGFβ₁, TGFβ₂ or TGFβ₃ at a concentration of 1 ng/ml (40 pM). Control cells were incubated with medium alone.

2.3.2 Sample processing

2.3.2.1 Cell harvesting
After 24 hrs the cell layer was scraped into the medium and aspirated. Each well was washed with 1 ml of PBS and the washings combined with the initial aspirate in glass tubes. Samples were immediately stored at -40°C to prevent proteolysis.

2.3.2.2 Separation of ethanol-insoluble and ethanol-soluble fractions
Samples were thawed on ice and proteins precipitated by addition of ethanol to a final concentration of 67% (v/v) at 4°C overnight. Precipitated proteins were subsequently separated from low molecular weight moieties by filtration through an acid-resistant 0.45 µm pore filter (type HV, Millipore) using a vacuum filtration unit (Millipore). Supernatants were collected into glass hydrolysis tubes and the protein pellets adherent to the filters washed twice with 1.5 ml ethanol (67% v/v in H₂O). The filters with adherent proteins were then transferred to Pyrex hydrolysis tubes.
The ethanol-insoluble fraction was hydrolysed in 6 M HCl for 16 h at 110°C. Hydrolysates were subsequently decolorised by mixing with 30 mg of activated charcoal and filtration through an acid-resistant 0.65 μm pore size filter (Millipore) prior to chromatography. The ethanol-soluble fraction was evaporated to dryness on a Dri-Block Sample Concentrator (DB-3 SC-3, Techne). These samples were then hydrolysed, mixed with activated charcoal and filtered as above.

2.3.3 Measurement of hydroxyproline by reverse-phase HPLC

Hydroxyproline content was determined by reverse-phase HPLC after derivatisation with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, Sigma). The chemical reaction between hydroxyproline and NBD-Cl is shown in figure 2.2. Secondary amino acids react with NBD-Cl to yield a chromophore with maximum absorbance at 495 nm. Interference from primary amino acids is minimised by restricting derivatisation time to 20 mins.
Figure 2.2 Hydroxyproline derivatisation with NBD-Cl
2.3.3.1 Pre-column derivatisation

100 μl of decolorised hydrolysate was transferred to a 1.5 ml microfuge tube (Marathon), covered with perforated parafilm and evaporated to dryness under vacuum with a sample concentrator (Savant Speedvac Plus AR SC110 AR, Life Science). Each sample was redissolved in 100 μl of water, buffered with 100 μl of 0.4 M potassium tetraborate, pH 9.5 (Sigma) and mixed with 100 μl of 36 mM NBD-Cl in methanol to a final concentration of 12 mM NBD-Cl. Samples were protected from light by wrapping the tubes in foil and incubated at 37°C for 20 mins. Derivatisation was terminated by acidification with 50 μl of 1.5 M HCl. 150 μl of a concentrated solution of HPLC running buffer A (167 mM sodium acetate in 26% aqueous acetonitrile, pH 6.4) was added and samples filtered with an HPLC 0.22 μm pore size filter (Millipore). A 100 μl aliquot was then injected onto the HPLC column and eluted with an acetonitrile gradient as described below.

2.3.3.2 Instrumentation and chromatographic conditions

Derivatised samples were separated on an LKB single pump HPLC system (LKB/Pharmacia) with a reverse-phase cartridge column (LiChroCART LiChrosopher 250 nm length x 4mm diameter, 5 μm particle size, 100 RP-18, BDH/Merck). The column was maintained at 40°C in a heated column oven (Jones Chromatography). At the start of each day of HPLC analysis, running buffers (A and B, defined in table 2.1) were degassed with helium and the HPLC system equilibrated in buffer A for 40 mins. The first HPLC run consisted of a blank gradient and was followed by analysis of three derivatised standard hydroxyproline solutions containing 50 pmol.

NBD-Cl derivatives in samples and standards were eluted with an acetonitrile gradient. This was generated by changing the relative proportions of each running buffer with time, thereby increasing acetonitrile concentration. The chromatographic conditions are shown in table 2.1. Post-column detection was achieved by monitoring absorbance at 495 nm using a flow-through variable wavelength monitor (LKB/Pharmacia). The signal was processed on an on-line chromatography computing integrator (Trio, Trivector) for quantitative analysis. The column eluent was collected and discarded. Total running and column regeneration time per sample was 25 mins.
<table>
<thead>
<tr>
<th>Column</th>
<th>LiChrosopher 100 RP-18, 250 x 4 mm, 5 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>Buffer A: 8% aqueous acetonitrile (v/v) and 50 mM sodium acetate pH 6.4</td>
</tr>
<tr>
<td></td>
<td>Buffer B: 75% aqueous acetonitrile (v/v)</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 (mins)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2.1 Chromatographic conditions for separation of hydroxyproline by reverse-phase HPLC
2.3.3.3  **Quantitation of hydroxyproline content**

The hydroxyproline content of each sample was determined by comparing the peak area of the chromatogram obtained to that of three separate aliquots of 50 pmol of hydroxyproline generated from standard conditions, derivatised and run at the beginning of each day. Since the cell monolayer contains a small amount of procollagen and the serum contains hydroxyproline, the amount of hydroxyproline present in the combined culture medium and cell layer at the start of the 24 hour incubation was determined in both ethanol-soluble and insoluble fractions. This background level ($I_0$) was subtracted from all the sample values.

Figure 2.3 shows typical chromatograms obtained for ethanol-insoluble (upper panel) and ethanol-soluble sample fractions following reverse-phase HPLC. Hydroxyproline eluted from the column after approximately 6 mins, between glutamine and serine. The peaks from both fractions were well-defined and predictable.

Table 2.2 shows that the coefficient of variation for HPLC measurements of hydroxyproline did not exceed 8%, indicating that the results were reproducible. Six replicate cultures for each experimental condition were therefore considered to be sufficient to demonstrate statistical significance.
Figure 2.3 Separation of hydroxyproline by reverse-phase HPLC

Figure shows typical chromatograms obtained for ethanol-insoluble (upper panel) and ethanol-soluble (lower panel) sample fractions following reverse-phase HPLC. The hydroxyproline peak was a separate and well-defined peak with an average column retention time of 5.73 minutes.


<table>
<thead>
<tr>
<th>Day</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.746</td>
<td>4.812</td>
</tr>
<tr>
<td>2</td>
<td>13.381</td>
<td>4.859</td>
</tr>
<tr>
<td>3</td>
<td>12.706</td>
<td>4.615</td>
</tr>
<tr>
<td>4</td>
<td>12.741</td>
<td>4.638</td>
</tr>
<tr>
<td>Mean</td>
<td>13.143</td>
<td>4.731</td>
</tr>
<tr>
<td></td>
<td>0.507</td>
<td>0.122</td>
</tr>
</tbody>
</table>

Coefficient of variation: 8% 5%

Table 2.2. Reproducibility of HPLC measurements
Table shows the coefficient of variation for HPLC measurements made on the same sample on different days. Values are given in nmol hydroxyproline. Aliquots of two samples were analysed on a total of eight different days. Sample 1 was from an ethanol-insoluble fraction and sample 2 was from an ethanol-soluble fraction.
2.3.3.4 Calculation of procollagen synthesis, production and degradation rates

Procollagen production was calculated from the quantity of hydroxyproline present in the ethanol-insoluble fraction. The proportion of newly-synthesised procollagen degraded intracellularly (expressed as a percentage) was calculated from the quantity of hydroxyproline in the ethanol-soluble fraction (the free amino acid pool) compared with that in both protein and the free pool. Total procollagen synthesis was represented by the sum of hydroxyproline in protein and the free pool. All rates were corrected for DNA, measured in identical parallel cell cultures, and results expressed as pmol hydroxyproline/μg DNA/hour.

2.3.3.5 Calculation of the proportion of newly-synthesised procollagen degraded

The proportion of newly synthesised procollagen degraded was calculated as follows and expressed as a percentage of total synthesis:

\[ \frac{\text{hyp in ethanol-soluble fraction}}{\text{hyp in ethanol-soluble + ethanol-insoluble fractions}} \times 100\% \]

where hyp = hydroxyproline

2.4 Determination of DNA content of cell monolayers

This assay was based on previously published methods (Burton, 1955; Burton, 1956) with modifications permitting the measurement of small amounts of DNA (McAnulty et al 1991a). RNA is extracted and DNA precipitated using 0.5 M perchloric acid (PCA) at 4°C. The RNA is then discarded and DNA extracted with 0.8 M PCA at 70°C. The colorimetric reaction between DNA phosphates and diphenylamine is then measured at 595 nm.

2.4.1 Culture conditions

Parallel cultures of HFL-1 fibroblasts were set up as described above (section 2.3.1)
and cells harvested after the 24 hr incubation period. The culture medium was discarded and cell layers washed with 1 ml of PBS which was then aspirated and discarded. Cell layers were scraped into 1 ml of fresh PBS and aspirated into a 1.5 ml microfuge tube which was centrifuged (GS-15R, Beckman) at 11,600 x g for 5 mins at 4°C. Supernatants were discarded, each well was washed with a further 1 ml of PBS which was used to resuspend the cell pellet, and samples then spun at 11,600 x g for 5 mins at 4°C. Supernatants were discarded and pellets frozen at -40°C until the assay.

2.4.2 Assay conditions

The assay was performed in microfuge tubes on ice. Samples were thawed, pellets resuspended in 1 ml of 0.5 M PCA and incubated for 10 mins. Samples were centrifuged at 11,600 x g for 5 min at 4°C, supernatants discarded and the pellets washed twice more, each time with 1 ml of 0.5 M PCA. After the third wash the supernatants were discarded, pellets resuspended in 1.2 ml of 0.8 M PCA and incubated at 70°C for 45 mins with occasional shaking.

A standard curve of calf thymus DNA (Sigma) was prepared in 1 ml of 0.8 M PCA, using glass tubes previously rinsed in acetone and dried. Concentrations ranged from 0 to 20 μg/ml. Standards were then heated to 70°C for 45 mins.

Samples and standards were incubated on ice for 15 mins, samples spun at 11,600 x g for 10 mins at 4°C and 1 ml of the supernatant transferred to a clean glass tube. To samples and standards were added 100 μl of an aqueous solution (1.6 mg/ml) of acetaldehyde (Sigma) and 1 ml of diphenylamine in concentrated acetic acid (4% w/v). The tubes were capped, vortexed and incubated at 30°C for 16 hrs. Absorbance of a 1 ml aliquot of each sample was then read at 595 nm with a spectrophotometer (Gilford 2600, Gilford Instrument Laboratories). Spectrophotometry was completed within 20 mins because the chromophore produced overnight fades after this time.
2.4.3 Calculation of DNA content

A DNA standard curve was constructed by plotting absorbance versus concentration and the slope used to calculate the corresponding DNA concentration in each sample. Since only 1 ml of the 1.2 ml total sample was assayed, the calculated DNA concentration was multiplied by 1.2 to yield the total DNA concentration per sample, or well.

Cell counts were also performed to verify the results obtained for the DNA assay. Cells were trypsinised, and cell count and viability assessed using a viability dye, trypan blue, and a haematocytometer.

Figure 2.4 shows a typical standard curve obtained for the DNA assay. Linear correlation was high between absorbance and DNA values between 1 µg and 20 µg (r = 0.998, p < 0.0001).
Figure 2.4 Standard curve for DNA
Figure shows a typical standard curve obtained for the DNA assay. Absorbance values were linear for DNA values between 1 μg and 20 μg. The absorbance of 1 μg of DNA was 0.009.
2.5 DETERMINATION OF TGFβ₁ PRODUCTION

The experiment described below was performed at the conclusion of these studies to provide preliminary data for future work. The methods are described here because they conclude the in vitro studies. To investigate the effect of TGFβ₁ antisense on TGFβ₁ production in vitro, murine mesothelioma cells (AC29) known to produce TGFβ₁ and TGFβ₂ (Fitzpatrick, 1994) were examined. This cell line was made available by Dr Mutsaers. RNA extracted from these cells was also used for Northern analysis (section 2.15.1).

2.5.1 Mesothelioma cell culture conditions

Culture conditions were similar to those described for HFL-1 cells (section 2.2). However, AC29 cells were cultured in 75 cm² tissue culture flasks (Costar) in DMEM containing 200 units/ml penicillin, 200 units/ml streptomycin, 4 mM glutamine, 5 ng/ml epidermal growth factor and supplemented with 5% foetal calf serum (FCS).

2.5.2 TGFβ₁ antisense oligonucleotide synthesis

The following sequence was chosen: 5' GAA GCA ATA GTT GGT GTC CA 3' (Merrilees, Scott, 1994). One phosphorothioate linkage was introduced at either end of the molecule. The oligonucleotide was prepared commercially (Pharmacia) to a concentration of 750 μg/ml and stored at -40°C prior to use.

2.5.3 Experimental conditions

One flask of AC29 cells grown to visual confluence were trypsinised as described (section 2.2.2) and resuspended in 1 ml of DMEM supplemented as above. 250 μl of cell suspension was plated onto each of five tissue culture plates and 6.19 ml of DMEM supplemented as above was added. After 24 hrs, 560 μl of TGFβ₁ antisense diluted in water to a final concentration of 1, 5 and 10 μM was added to one of three test plates. 560 μl of distilled water was added to each of the two remaining control plates. In this
way the final volume of culture medium in each plate was 7 ml.

After a further 24 hrs the medium was aspirated from the three test plates and one of the control plates and centrifuged at 2000 x g for 7 mins in an MSE centrifuge (Mistral 3000, Fisher Scientific). Supernatants were frozen at -40°C for later assay. The medium in these plates was replaced with serum-free DMEM supplemented as above. Cell counts were performed on the remaining control plate. After a further 24 hrs the medium was aspirated from the four remaining plates, spun and stored as above. Cell counts were performed on all four plates as previously described (section 2.4.3).

2.5.4 TGFB1 enzyme-linked immunoabsorbent assay

A commercial TGFB1 enzyme-linked immunoabsorbent assay (ELISA) was used (Promega) with minor modifications of the manufacturer’s protocol. Preliminary experiments by Dr Mutsaers indicated that shaking of the plate during incubations was unnecessary. One experiment using the TGFB1 standard provided failed, and the TGFB1 standard used in this experiment was the same as used in cell culture studies (section 2.3.1.1).

2.5.4.1 Plate coating

The day before the assay, 10 μl of anti-TGFB1 monoclonal antibody (coat mAb) was added to 10 ml of carbonate coating buffer (0.025 M sodium bicarbonate, 0.025 M sodium carbonate, pH 9.7) and 100 μl added to each well of a 96 well, flat bottom ELISA plate (Costar). The plate was wrapped in parafilm and incubated for 16 hrs at 4°C.

2.5.4.2 Sample preparation

5 ml push cap polypropylene tubes (Sarstedt) were labelled in duplicate to permit measurement of latent and active TGFB1 in four serial dilutions of each sample. Media samples were spun briefly at 13,000 x g and 500 μl of each pipetted into the appropriate tube. Half the samples were activated with 100 μl of 1 N HCl followed by incubation at room temperature (RT) for 15 mins. Samples were neutralised with 40
μl of 1 M NaOH. Serial dilutions of latent and active TGFB<sub>1</sub>-containing media samples were then prepared in serum-free DMEM.

2.5.4.3 Assay

28 μl of 1 x block buffer was prepared by diluting 5 x block buffer in water. The coated plate was warmed to RT, the contents of the wells flicked out and 270 μl of 1 x block buffer added to each well. The plate was then incubated at 37°C for 35 mins.

23 μl of 1 x sample buffer was prepared by diluting 10 x sample buffer in serum-free DMEM. A standard curve for TGFB<sub>1</sub> was prepared by diluting a stock solution of TGFB<sub>1</sub> in 1 x sample buffer to a starting concentration of 1 ng/ml. Following plate blocking, the contents of the wells were flicked out and 100 μl of 1 x sample buffer added to all but the first two wells in two columns down the plate. 200 μl of the prepared TGFB<sub>1</sub> standard was added to two wells in the first row of the plate. Serial two-fold dilutions were performed down the plate to produce concentrations ranging from 1000 to 0 pg/ml.

100 μl of each sample dilution was then added to the appropriate well and the plate incubated for 90 mins at 37°C wrapped in parafilm. The plate was then washed five times with wash buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween<sup>®</sup>-20) using an automated microplate washer (Wellwash 4, Denley).

10 ml of a 1:1,000 dilution of polyclonal antibody (pAb) was prepared in 1 x sample buffer and 100 μl added to each well. The plate was incubated for 2 hrs at RT. The plate was then washed as above and 10 ml of a 1:1,000 dilution of stock antibody conjugate prepared in 1 x sample buffer. 100 μl was added to each well and the plate incubated for 2 hrs at RT.

Enzyme substrate was prepared by mixing 5 ml of a proprietary chromogenic substrate (TMB) with 5 ml of peroxidase substrate. The mixture was protected from light in a foil-wrapped 15 ml polypropylene tube and kept at 4°C but warmed to RT before use. The plate was then washed as before, and 100 μl of the enzyme substrate added to each
The colour reaction was allowed to proceed at RT and was stopped within 4 mins by adding 100 μl of 1 M phosphoric acid.

The exterior base of the plate was cleaned with 70% ethanol and absorbance immediately measured at 450 nm using a Titertek microplate reader (MCC/340, Life Science International). A standard curve was obtained by plotting absorbance at 450 nm versus TGFβ1 concentration. TGFβ1 concentration in the samples was then extrapolated from the curve and expressed as pg/ml.

IN VIVO STUDIES

2.6 BLEOMYCIN MODEL OF LUNG FIBROSIS

This model has been used extensively in this laboratory (Laurent et al 1981; Hay et al 1987; Shahzeidi et al 1991). Murine lung tissue for in situ hybridisation studies was obtained and prepared by Dr McAnulty and Dr Shahzeidi. Rat lung tissue for Northern analysis was obtained with the help of Dr McAnulty and Dr Mutsaers.

2.6.1 Animals, bleomycin instillation and sacrifice

Adult mice (strain B6D2F1) aged eight weeks and weighing 24-26 g were used for in situ hybridisation studies. For Northern analysis, adult male Lewis rats weighing approximately 170 g were employed. Mice received a single dose of intratracheal saline (0.14 M) or saline containing bleomycin sulphate (6 mg/kg) in a volume of 0.05 ml and were killed 3, 10, 21 or 35 days later by pentobarbitone overdose as described previously (Shahzeidi et al 1994). Three animals were used in each group for in situ hybridisation studies.

2.6.2 Murine lung tissue preparation for in situ hybridisation

Lungs were fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde in PBS at a pressure of 25 cm H2O. The trachea was ligated just caudal to the larynx
and thoracic contents removed together. Tissue was fixed by immersion in freshly prepared 4% paraformaldehyde for 4 hrs (small samples) or 18 hrs (large samples) at RT, using 50 ml per cubic cm. It was subsequently immersed in 0.5 M sucrose in PBS overnight at RT.

The samples were then washed as follows, using fresh ethanol for dehydration.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x PBS</td>
<td>4°C</td>
<td>30 mins</td>
</tr>
<tr>
<td>0.85% NaCl</td>
<td>4°C</td>
<td>30 mins</td>
</tr>
<tr>
<td>1:1 saline/ethanol</td>
<td>RT</td>
<td>15 mins</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>RT</td>
<td>15 mins (repeated once)</td>
</tr>
<tr>
<td>85% ethanol</td>
<td>RT</td>
<td>30 mins</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>RT</td>
<td>30 mins</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>RT</td>
<td>30 mins (repeated once)</td>
</tr>
</tbody>
</table>

Paraplast paraffin for embedding was melted the day before use and then filtered through pre-heated filter paper and funnel into a pre-heated flask. It was not remelted more than twice. Preheated forceps were used to manipulate tissue in paraffin. The tissue samples were then soaked in the following:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylene</td>
<td>RT</td>
<td>30 mins (repeated once)</td>
</tr>
<tr>
<td>1:1 xylene/paraffin</td>
<td>RT</td>
<td>45 mins</td>
</tr>
<tr>
<td>paraffin</td>
<td>RT</td>
<td>20 mins (repeated twice)</td>
</tr>
</tbody>
</table>

The third paraffin treatment was done in a weigh boat and after 20 mins the paraffin was allowed to solidify at RT overnight. Blocks were stored indefinitely.

2.6.3 Rat lung tissue preparation for RNA extraction

The trachea was ligated just caudal to the larynx and thoracic contents removed together. Lung tissue was quickly minced with sterile scissors, then immediately homogenised with an RNase-free homogeniser blade and power-driven homogeniser (Ultra-Turrax T25, Fisher Scientific) in 1 ml of TRIzol Reagent (Gibco) per 100 mg of tissue. Homogenised samples were frozen immediately at -80°C prior to RNA
2.7 SELECTION OF PATIENT SAMPLES

2.7.1 Patient evaluation

2.7.1.1 Systemic sclerosis
These patients were from a cohort of over 80 patients with systemic sclerosis referred by Dr Black (Consultant Rheumatologist, Royal Free Hospital, London) to Dr du Bois (Consultant Chest Physician, Royal Brompton National Heart & Lung Hospital, London) for investigation of pulmonary involvement. All fulfilled the American Rheumatism Association preliminary criteria for the diagnosis of systemic sclerosis and none were receiving treatment for lung disease at the time of investigation.

2.7.1.2 Cryptogenic fibrosing alveolitis
These patients were from a cohort of patients referred to Dr du Bois. CFA was diagnosed following history and examination, investigations including high resolution computed tomography and pulmonary function testing, and in the absence of evidence of other risk factors for pulmonary fibrosis such as dust exposure or connective tissue disease.

2.7.2 Open lung biopsies

Open lung biopsies were performed in patients in whom the above investigations strongly supported a diagnosis of pulmonary fibrosis and in whom biopsy was considered necessary to provide further information not available from CT scanning. Biopsy was therefore employed mainly for staging the disease rather than for diagnosis. It was not performed as part of a research protocol and all patients gave informed written consent. Two biopsies were usually taken by the surgeon from sites apparently the most and least affected by fibrosis. This usually entailed biopsies being taken from the lateral segments of the right lower and middle lobes. Biopsy tissue was then processed as described above for murine lung samples (section 2.6.2).
2.8 MATERIALS

Chemicals used in the following protocols were of molecular biology grade and obtained from Gibco, Sigma or Fluka unless otherwise specified. Precautions to minimise extrinsic RNase contamination were followed, based on previously published recommendations (Maniatis et al 1989).

2.9 GENERAL MOLECULAR BIOLOGY PROCEDURES

These procedures were all based on established methods (Maniatis et al 1989), modifications being noted. They are described together because they were used routinely when preparing and analysing nucleic acid samples.

2.9.1 Spectrophotometric evaluation of nucleic acids

Quantitation of purified nucleic acid samples was performed by spectrophotometry. Nucleic acids absorb light maximally at 260 nm. At this wavelength 50 μg/ml of DNA or 40 μg/ml of RNA 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 (A_{260}/A_{280}) was performed to estimate the purity of the preparation. A ratio of 1.8 or greater was judged ideal.

2.9.2 Nucleic acid precipitation and recovery

Nucleic acid samples were precipitated with either 0.1 volume of 3 M sodium acetate (pH 5.2) or 0.1 volume of 8 M lithium chloride, followed by 2.5 volumes of chilled 100% ethanol. Samples were mixed well and frozen at -80°C for a minimum of 2 hrs. Low molecular weight nucleic acids such as riboprobes were precipitated for 72 hrs before recovery.

Nucleic acids were recovered by centrifugation at 13,500 x g at 4°C for 20 mins, the supernatant discarded and the pellet washed with 1 volume of pre-chilled 75% ethanol.
The sample was then centrifuged at 13,500 x g at 4°C for 5 mins, the supernatant discarded and the pellet dried under vacuum. RNA pellets were not dried completely, as RNA is relatively insoluble. The pellet was then resuspended in TE buffer (10mM Tris 1M pH 7.4, 1mM EDTA 0.5M pH 8) or water.

2.9.3. Agarose gel electrophoresis of nucleic acids

300 ml of 0.5 x TBE buffer was prepared from a stock solution of 5 x TBE (0.45 M Tris-borate, 0.01 M EDTA). 50 ml of a 1% solution of electrophoresis grade agarose in 0.5 x TBE was prepared by heating in a microwave oven. 2 μl of a 10 mg/ml ethidium bromide solution was added to a final concentration of 0.4 ng/ml and the agarose cooled to 60°C. The agarose solution was poured into a 10 x 6.5 cm casting tray to a thickness of 5 mm, and allowed to set for 30 mins.

Lambda DNA/Hind III fragments (0.5 μg/μl), a 100 bp DNA ladder (0.5 μg/μl) or an RNA ladder (0.24-9.5 kb, 1 μg/μl), from Gibco, and DNA or RNA samples were thawed on ice. Each sample, containing up to 10 μg, was made up to 10 μl with 0.5 x TBE buffer and 2 μl of gel loading buffer (Sigma) added. Samples were mixed, centrifuged briefly at 4°C to bring the contents of each tube to the bottom, and kept on ice. The gel was submerged in 250 ml of 0.5 x TBE, the samples loaded and the voltage applied. When the loading buffer had migrated at least 4 cm the bands were visualised under an ultraviolet (UV) transilluminator (UVP). The gel was photographed using a video camera (UVP) and image analyser (Image Store 5000, UVP) according to the manufacturer's instructions.

2.9.4 Phenol-chloroform purification of DNA samples

Removal of contaminating protein from DNA samples was performed after DNA isolation and purification when the OD 260/280 ratio was less than 1.6, and following plasmid linearisation prior to in vitro transcription. The use of Phase Lock gel II (NBL Gene Sciences) maintained at RT facilitated removal of the aqueous phase when purifying small samples. To the DNA sample in a microfuge tube on ice was added
an equal volume of water-equilibrated phenol pH 8. The mixture was vortexed thoroughly for 1 min. 2 volumes of chloroform:isoamyl alcohol 24:1 were added, 20 μl of Phase Lock gel II added with a glass Pasteur pipette tip and the sample vortexed for 1 min. The tube was centrifuged at 4°C at 13,500 x g for 5 mins, the aqueous phase removed to a fresh microfuge tube on ice and the organic phase retained for further extraction as described below.

To the aqueous phase were added a further two volumes of chloroform:isoamyl alcohol 24:1 and approximately 20 μl of Phase Lock gel. The sample was vortexed for 1 min, then centrifuged at 4°C at 13,500 x g for 5 mins. The aqueous phase was removed to a fresh microfuge tube on ice.

To the retained organic phase, 0.5 volume of water was added and the sample vortexed for 1 min. The sample was centrifuged at 4°C at 13,500 x g for 1 min, the aqueous phase removed and combined with the first one. The sample was then examined by spectrophotometry or electrophoresis and precipitated.

2.10 PLASMID PREPARATION

2.10.1 TGFβ_{1,3} probes and plasmid vectors

The TGFβ_{1,3} cDNA probes were constructed and cloned into plasmid vectors conferring ampicillin resistance by Professor Harold Moses of Nashville, Tennessee, USA. They were supplied lyophilised and were a gift. The TGFβ_{1,3} probes and the site of their insertion in the plasmid vectors are illustrated in figures 2.5-2.7. The vectors contain dual opposed SP6 and T7 promoters flanking the multiple cloning site, allowing RNA to be transcribed from either strand of the insert. Methods are based on previously published protocols (Maniatis et al 1989) unless otherwise specified.
Figure 2.5 Vector map for TGF\beta_1 probe
Plasmid designation: pmTGF\beta_1-A
Vector: pGEM 7Zf^+
Insert size: 974 bp
Figure 2.6 Vector map for TGFβ2 probe
Plasmid designation: pmTGFβ2-9A
Vector: SP72
Insert size: 442 bp
Figure 2.7 Vector map for TGFB3 probe
Plasmid designation: pmTGFB3-11b
Vector: pGEM 7Zf^+
Insert size: 609 bp
2.10.2 Preparation of bacterial plates

32 g of Lennox L agar (Gibco) was mixed with 1 litre of distilled water in a conical flask. The flask was sealed with a bung fashioned from cotton wool wrapped in gauze, and autoclaved. A sterile field was established with a bunsen burner and the flask cooled to 45°C. 100 mg of ampicillin (Gibco) was reconstituted with water and added to the cooled agar to a final concentration of 100 µg/ml. 10 ml of agarose solution was poured onto each bacterial plate (Sterilin BS 611: part 2, 1990) and allowed to set. The plates were dried upside down for 5 mins at 50°C and stored at 4°C for up to three months.

2.10.3 Bacterial transformation

This method follows the manufacturer's instructions. The bacteria are supplied competent. *E coli* cells (MAX efficiency DH5α competent *E coli*, Gibco) were kept on dry ice before thawing very briefly. Lyophilised plasmids were thawed slowly and reconstituted with water to a final concentration of 1 µg/ml. 100 µl of *E coli* suspension was pipetted into a 10 ml polypropylene Falcon tube (Falcon 2059, Marathon) kept on ice and 10 µl (10 ng) of plasmid solution added. The samples were mixed gently and the tubes incubated at 4°C for 30 mins. The cells were then heat-shocked by incubating the tubes at 42°C for 90 secs. The tubes were replaced at 4°C for 2 mins.

Maintaining a sterile field, 0.9 ml of S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂, 20 mM glucose, Gibco) was added to each tube and the samples shaken at 37°C for 1 hr. 100 µl of each sample was plated with a wire loop, the plates allowed to dry for 45 mins and then incubated upside down at 37°C overnight. The following day, two to six colonies were plated onto a fresh bacterial plate. The plates were incubated upside down at 37°C for 24 hrs and subsequently stored for one month at 4°C.
2.10.4 Freezing transformed bacteria

For long-term storage of transformed cells, one colony was removed from each plate and mixed with 2 ml of S.O.C. medium in a sterile 2059 Falcon tube. Tubes were shaken overnight at 37°C. Using a sterile field, 150 µl of filtered glycerol was pipetted into a nitrogen-fast Nunc vial (Costar), 850 µl of cell suspension added and the vials placed directly in liquid nitrogen.

2.10.5 Small scale plasmid DNA preparation

2.10.5.1 Detergent lysis

In this method, described below, bacteria are pelleted by centrifugation and lysed with EDTA, lysozyme and TRITON X. This denatures the linear bacterial chromosomal DNA but not the closely intertwined, closed circular plasmid DNA. Plasmid DNA is then recovered by centrifugation after incubation with polyethylene glycol.

40 ml of S.O.C. medium in an autoclaved 100 ml conical flask was inoculated with a single bacterial colony and incubated overnight at 37°C with vigorous shaking. The medium was then aliquoted into 1.5 ml microfuge tubes, centrifuged at 4°C at 13,500 x g for 3 mins and the supernatant discarded. The samples were recentrifuged for 10 secs and any remaining supernatant removed.

200 µl of TS buffer (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA pH 8) was added and samples vortexed for 1 min at maximum speed to solubilise the cells. 200 µl of ELT buffer (EDTA 10mM pH 8, chicken egg white lysozyme 2 mg/ml, TRITON X 0.1%), thawed on ice, was added to each tube and mixed gently. Samples were incubated for 20 mins at RT, followed by 20 mins at 70°C. Increased viscosity accompanies release of cellular proteins. The tubes were then centrifuged at 4°C at 13,500 x g for 10 mins. Precipitated proteins appear as a thick white pellet.

The supernatant was removed to a fresh tube, 400 µl of 20% polyethylene glycol added and samples incubated for 20 mins at RT followed by centrifugation at 4°C at 13,500
x g for 3 mins. The supernatant was discarded and the sample recentrifuged for 10 secs. The supernatant was aspirated and the plasmid DNA pellets were resuspended in 20 µl of TE buffer (10mM Tris 1M pH 7.4, 1mM EDTA 0.5M pH 8) and the contents of the microfuge tubes pooled and kept at 4°C. Each tube was washed with a further 10 µl of TE buffer and these washings combined with the previous ones. Plasmid DNA was examined by spectrophotometry and electrophoresis.

2.10.5.2 Alkaline lysis

The Wizard Minipreps DNA Purification System (Promega), which uses a proprietary silica-based resin, was employed to prepare the plasmid containing the TGFβ2 cDNA probe for sequencing. The method was simple and rapid. The protocol follows the manufacturer's instructions.

Two 1 ml aliquots of S.O.C. medium were inoculated with one loopful each of transformed E. coli and shaken for 8 hrs at 37°C until cloudy. Two fresh 5 ml aliquots of S.O.C. medium containing 50 µg/ml of ampicillin were inoculated with 100 µl of this culture and shaken overnight at 37°C. The culture medium was aliquoted into 1.5 ml microfuge tubes. Cells were pelleted by centrifugation at 13,500 x g for 2 mins and resuspended in Cell Resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 100 µg/ml RNase A). 200 µl of Cell Lysis solution (0.2 M NaOH, 1% SDS) was added and samples mixed gently by inversion until the solution cleared. 200 µl of Neutralization solution (1.32 M potassium acetate pH 4.8) was added and mixed by inversion. Samples were centrifuged at 13,500 x g for 5 mins and cleared supernatants containing plasmid DNA transferred to fresh microfuge tubes.

1 ml of resuspended DNA Purification Resin was added to each supernatant and mixed by inversion. The plunger was removed from a 2 ml disposable syringe and the syringe barrel attached to the luer-lock extension of a Minicolumn. The tip of this assembly was then inserted into a vacuum manifold (Vac-Man, Promega). The Resin/DNA mixture was pipetted into the syringe barrel and a vacuum applied to draw the mixture into the column. The vacuum was interrupted and 2 ml of Column Wash solution (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 55% ethanol) added to the syringe
barrel. The vacuum was reapplied to dry the Resin. The Minicolumn was transferred to a 1.5 ml microfuge tube, centrifuged at 13,500 x g for 2 mins to remove residual Column Wash solution and transferred to a new microfuge tube. 50 µl of TE buffer was applied to the Minicolumn for 1 min to elute the DNA, and the Minicolumn then removed and discarded. Plasmid DNA was examined by electrophoresis prior to storage at -80°C.

2.10.6 Large scale plasmid DNA preparation

This was performed using the Wizard Maxipreps DNA Purification System (Promega), which employs the same principles as the Miniprep System. The reagents have the same composition and the method follows the manufacturer's instructions.

Large-scale bacterial cultures were prepared in two stages. 20 ml of S.O.C. medium in a 2059 Falcon tube was inoculated with one loopful of bacteria and shaken overnight at 37°C. 1 ml of this culture was then added to 500 ml of Terrific broth (Gibco) in a sterile 1 L flask, prepared as previously. The culture was shaken for 24 hrs at 37°C. Cells were pelleted by centrifugation at 4°C at 14,000 x g for 10 mins in a Sorvall centrifuge RC5C (Du Pont) and the supernatant discarded. Cell pellets were resuspended in 15 ml of Cell Resuspension solution, 15 ml of Cell Lysis solution added and the sample stirred gently for 20 mins until the solution became clear and viscous. 15 ml of Neutralisation solution was added and the sample mixed by repeated inversion, then centrifuged at 4°C at 14,000 x g for 15 mins. The supernatant was decanted to a fresh 50 ml centrifuge tube through a funnel lined with filter paper (No. 1, Whatman). 0.6 volumes of isopropanol were added to precipitate the DNA and the sample mixed by inversion. The sample was centrifuged at 4°C at 14,000 x g for 15 mins, the supernatant discarded and the plasmid DNA pellet resuspended in 2 ml of TE buffer.

10 ml of Wizard Maxipreps DNA Purification Resin was added to the DNA solution and the tube swirled to mix. The tip of a Wizard Maxicolumn was inserted into the vacuum source, the resin/DNA mix transferred into the Maxicolumn and a vacuum applied. The centrifuge tube was washed with 13 ml of Column Wash Solution,
swirled and poured into the Maxicolumn. A vacuum was reapplied to draw the Wash Solution through the Maxicolumn. 5 ml of 80% ethanol was added to the Maxicolumn to rinse the resin and a vacuum used to draw the ethanol through the Maxicolumn. The resin was then dried in a centrifuge with a swinging bucket rotor (MSE, Mistral 3000, Fisher Scientific). This is necessary because the tube has to be in a horizontal position during centrifugation for the Resin to dry. The Maxicolumn was placed in a 50 ml Falcon tube, centrifuged at 1,300 x g for 5 mins, the Maxicolumn removed and the liquid decanted. The Maxicolumn was replaced in the tube and 1.5 ml of pre-heated (65-70°C) TE buffer applied. After 1 min, the Maxicolumn was recentrifuged at 1,300 x g for 5 mins to elute the DNA. The Maxicolumn was discarded and plasmid DNA examined by spectrophotometry and electrophoresis prior to precipitation and storage at -80°C.

2.11 DIGOXIGENIN-LABELLED RIBOPROBE PREPARATION

2.11.1 Plasmid linearisation

Purified plasmids were resuspended in water to a concentration of 1 μg/μl. Restriction endonuclease solutions of Hind III, EcoR I and Xho I (Promega) all contained 12 units/μl and were supplied with the appropriate buffer. Approximately 1 unit of enzyme activity was used per 1 μg of uncut DNA. 20 μg of plasmid was linearised in a final volume of 40 μl, and reaction mixtures prepared in sterile microfuge tubes on ice. Table 2.3 shows the restriction enzymes which were used to linearise each plasmid. In some cases alternative restriction enzymes could have been used equally well (see figures 2.5-2.7).

Reaction mixtures were incubated at 37°C for 1 hour, placed on ice, a 2 μl aliquot examined by electrophoresis to confirm linearisation and 4 μl (0.1 volume) of 200 mM EDTA added to terminate the reaction. Samples were precipitated for at least 2 hrs at -80°C. Linearised plasmids were purified with phenol-chloroform, precipitated and resuspended in water to a concentration of 1 μg/4 μl. They were stored at -40°C prior to in vitro transcription (section 2.11.2) or random prime labelling (section 2.12.2).
<table>
<thead>
<tr>
<th>Riboprobe</th>
<th>Restriction enzyme</th>
<th>RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ₁ antisense</td>
<td>Hind  III</td>
<td>T7</td>
</tr>
<tr>
<td>TGFβ₁ sense</td>
<td>EcoR I</td>
<td>SP6</td>
</tr>
<tr>
<td>TGFβ₂ antisense</td>
<td>EcoR I</td>
<td>SP6</td>
</tr>
<tr>
<td>TGFβ₂ sense</td>
<td>Xho I</td>
<td>T7</td>
</tr>
<tr>
<td>TGFβ₃ antisense</td>
<td>Hind  III</td>
<td>T7</td>
</tr>
<tr>
<td>TGFβ₃ sense</td>
<td>Xho I</td>
<td>SP6</td>
</tr>
</tbody>
</table>

Table 2.3 Restriction enzymes and RNA polymerases used to prepare TGFβ₁,₃ riboprobes.
Digoxigenin is a steroid isolated from digitalis plants (Digitalis purpurea and Digitalis lanata). The blossoms and leaves of these plants are the only natural source of digoxigenin, so that no binding of the anti-digoxigenin antibody occurs in other biological material. Digoxigenin is linked to uridine at the number 5 position of the pyrimidine ring via an 11 C atoms spacer arm. During RNA labelling, the DIG-11-UTP analogue is incorporated every 20th to 25th nucleotide.
2.11.2  

*In vitro* transcription

Nucleic acid labelling with digoxigenin (DIG) was developed by Boehringer Mannheim (Kessler, 1990), from whom the following reagents were obtained. The structure of digoxigenin-labelled uridine triphosphate used in RNA labelling is shown in fig 2.8.

To a sterile microfuge tube on ice containing 4 µl (1 µg) of linearised cDNA template were added 2 µl of 10 x transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM MgCl$_2$, 100 mM dithiothreitol, 20 mM spermidine, 100 mM NaCl, 1 U/µl RNase inhibitor), 10 µl of water, 2 µl of 10 x DIG RNA labelling mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP, pH 7.5) and 2 µl of SP6 or T7 RNA polymerase. Table 2.3 shows the RNA polymerase used for each reaction.

Samples were incubated at 37°C for 2 hrs, placed on ice and a 2 µl aliquot examined by electrophoresis to verify the size of the RNA transcript. Samples were compared with two dilutions of RNA ladder for semi-quantitative evaluation of riboprobe yield. 2 µl (0.1 volume) of 200 mM EDTA was then added to terminate transcription, followed by 0.5 µl of a 10 mg/ml solution of Brewer’s yeast tRNA to co-precipitate the riboprobe. RNA samples were then precipitated.

2.11.3  

Assessment of digoxigenin incorporation into riboprobes

The DIG Luminescent Detection kit (Boehringer) was used to confirm digoxigenin labelling of riboprobes. Digoxigenin-labelled probe is detected by an anti-digoxigenin antibody conjugated to alkaline phosphatase. Dephosphorylation of the chemiluminescent substrate Lumigen PPD, a proprietary reagent, with alkaline phosphatase generates light which is documented on X-ray film (fig 2.9). The method follows the manufacturer’s instructions.
Figure 2.9 Detection of mRNA transcripts using digoxigenin-labelled riboprobes

Digoxigenin-labelled riboprobes hybridise to complementary mRNA transcripts. After hybridisation, tissue sections or membranes are incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase, followed by an alkaline phosphatase substrate. For assessment of probe labelling and Northern analysis, a chemiluminescent substrate was used which yields light at the site of hybridised probe. This is then detected by autoradiography. For in situ hybridisation, New Fuschin Red was used, which yields a red colour at the site of hybridised probe.
2 μl of control RNA was diluted in RNA dilution buffer (5:3:2 H2O, 20 x saline sodium citrate (SSC), formaldehyde) to a concentration of 20 ng/μl. Serial dilutions (1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 0.1 pg/μl, 0.01 pg/μl) of control RNA and digoxigenin-labelled riboprobe in RNA dilution buffer were prepared in microfuge tubes on ice. 1 μl of each dilution was pipetted onto a positively charged nylon membrane (Boehringer), air dried, the membrane wrapped in Saranwrap and RNA fixed with 1200 Joules from a UV crosslinker (UVC 1000, Hoeffer Scientific Instruments).

The DIG Wash and Block Buffer Set (Boehringer) were used for subsequent steps. The blocking reagent is a proprietary compound. The membrane was sealed in a hybridisation bag (Gibco) and incubated in a shaking water bath in 1 x washing buffer (0.01 M maleic acid, 0.015 M NaCl pH 7.5, 0.3% Tween20 w/v) at 37°C for 2 mins. The solution was replaced with blocking buffer (10% blocking reagent diluted 1:10 in freshly prepared 1 x maleic acid buffer). 1 x maleic acid buffer consisted of 0.01 M maleic acid, 0.015 M NaCl pH 7.5. The membrane was incubated at 37°C with shaking for 30 mins. Anti-digoxigenin-AP, Fab fragments (Boehringer) were diluted 1:10,000 in blocking buffer and the membrane incubated in the antibody solution for at 37°C for 30 mins. The membrane was placed in a fresh hybridisation bag and washed in 1 x washing buffer at 37°C with agitation for 15 mins. This step was repeated once.

The membrane was equilibrated in 1 x detection buffer (0.01 M Tris, 0.01 M NaCl, 0.005 M MgCl2 pH 9.5) for 2 mins, drained briefly onto filter paper, and placed between two clean acetate sheets. 1 ml of Lumigen PPD diluted 1:100 in 1 x detection buffer was prepared, pipetted onto the membrane and spread evenly by wiping the top acetate sheet with a damp paper towel. The filter was incubated in this solution at RT for 5 mins and the semi-dry membrane then sealed in a fresh hybridisation bag and incubated at 37°C with shaking for 15 mins. The membrane was removed from the bag, sealed in Saranwrap and placed with a Kodak x-ray film (X-OMAT, XAR-5, Sigma) in an autoradiography cassette (Amersham) at RT for up to 24 hrs.

The film was developed by immersion in developing solution (Photosol, Ilford) for up
to 10 mins. The film was transferred to 0.17% glacial acetic acid for 2 mins to stop the reaction and then immersed in fixative (Photosol, Ilford) for up to 5 mins, rinsed and dried. Densitometry of serial dilutions of riboprobe was performed using an Image Master 1D gel and film scanner (Pharmacia) according to the manufacturer's instructions. Briefly, each area of interest was outlined, its optical density measured and the optical densities of control and test RNA dilutions compared.

2.11.4 Reduction of riboprobe length by alkaline hydrolysis

Probe hydrolysis to fragments between 100 and 250 bp has been recommended for in situ hybridisation (Angerer, Angerer, 1992; Shahzeidi et al 1993; Shahzeidi et al 1994). Limited alkaline hydrolysis of the TGFB1 probes was performed using a previously published method (Cox et al 1984). Incubation times were calculated according to the equation: $t(\text{min}) = \frac{lo - lf}{0.11 \times lo \times lf}$, where $lo =$ initial length of transcript (kb) and $lf =$ desired length (kb). Where $lo = 0.974$ and $lf = 0.1$, $t(\text{min}) = \frac{0.974 \times 0.1}{0.11 \times 0.974 \times 0.1} = 82$ mins.

Aliquots of digoxigenin-labelled TGFB1 antisense riboprobe were mixed with alkali to a final concentration of 40 mM NaHCO$_3$, 60 mM Na$_2$CO$_3$ and incubated at 60°C for 60, 82 or 100 mins. Hydrolysis was terminated by addition of 0.65 $\mu$l of concentrated acetic acid, and the RNA precipitated. Aliquots from each reaction were compared with a 1 (X ) bp size marker following electrophoresis to determine the size of the resulting probe fragments.

2.12 $^{32}$P LABELLED cDNA PROBE PREPARATION

2.12.1 Preparation of template DNA

Plasmids were resuspended in water to a concentration of 1 $\mu$g/$\mu$l. Reaction volumes were 50 $\mu$l. Appropriate restriction enzymes (Promega) were chosen to cut out the cDNA insert from the plasmid (figs 2.5-2.7). Sma I contains 8 units/$\mu$l of enzyme activity and has optimal activity at 25°C, but only 25-50% activity at 37°C.
Approximately 5 units of each enzyme were used per 1 μg of uncut plasmid. To prepare the TGFβ1 probe, Sma I was used. To prepare the TGFβ2 probe, Cla I and Xho I were used together. To prepare the TGFβ3 probe, EcoR I and Sma I were used together. All reactions were prepared in sterile microfuge tubes on ice. The sample containing the TGFβ1-A plasmid was incubated at 25°C and the other two at 37°C, each for 1 hr.

Samples were placed on ice and a 5 μl aliquot examined by electrophoresis to confirm that the insert had been generated. A 1.2% low melting point agarose gel (Gibco) was then cast into the tray of a 12 x 15 cm electrophoresis chamber (IBI). 10 μl of gel loading solution was added to each remaining sample, which was then divided into two equal aliquots. Following electrophoresis, the gel was examined under UV transillumination. Inserts were excised cleanly, transferred to pre-weighed microfuge tubes and re-weighed. 3 ml of water was added per g of gel. Samples were denatured by boiling for 5 mins, divided into 25 ng aliquots and stored at -40°C. In some experiments linearised plasmid DNA, prepared as described previously (section 2.11.1), was used directly as a template for random prime labelling. In this case the amount of DNA used in the reaction was increased in proportion to the ratio of vector to probe length.

2.12.2 Random prime labelling

The Megaprime DNA Labelling System (Amersham) was employed using the manufacturer's instructions. This employs nonamer primers which permit rapid labelling of template DNA at 37°C.

25 ng of DNA in agarose was first boiled for 30 secs. 5 μl of primers was added, followed by water to give a total volume of 50 μl in the final reaction. The sample was denatured by heating to 95°C-100°C for 5 mins and then centrifuged briefly at RT. 10 μl of labelling buffer was added followed by 5μl of Redivue α-32P dCTP (50 μCi, 1.85 MBq, Amersham) and 2 μl of Klenow enzyme. The sample was centrifuged briefly and incubated at 37°C for 10 mins, or 15-30 mins if labelling DNA in low melting point
agarose. The reaction was terminated by the addition of 5 µl of 200 mM EDTA, labelled DNA denatured by heating to 95°-100°C for 5 mins, the sample chilled on ice and used immediately in a hybridisation reaction for Northern analysis (section 2.16).

2.12.3 Calculation of labelling efficiency

This protocol was adapted from the manufacturer’s (Amersham). 1 µl aliquots of radiolabelled probe were mixed with 20 µl of water. Four 5 µl aliquots of diluted probe were pipetted onto 1 cm diameter DE81 chromatography paper circles (Whatman). Half were dried at 50°C for 10 mins. The remainder were washed twice for 5 mins each, at RT, in excess 2 x SSC with shaking. They were then rinsed briefly in distilled water and finally in 99% ethanol for 5 mins to stabilise the paper. They were then dried at 50°C for 10 mins. Dried filters were placed in scintillation vials containing 5 ml of scintillant and counted in a liquid scintillation counter (Minaxi B Tri-Carb 4000 Series, Canberra Packard) with windows adjusted for maximum ³²P counting efficiency.

2.12.4 Interpretation of results

% incorporation = \( \frac{\text{mean counts on washed filters}}{\text{mean counts on unwashed filters}} \times 100 \)

Total amount of DNA (A) ng =

\( \frac{\text{Total number of } \mu \text{Ci added} \times 13.2 \times \% \text{ incorporation}}{\text{number of radioactive dNTPs added} \times \text{average specific activity of dNTPs added}} \)

This assumes a 25% content of any dNTP in newly synthesised DNA and 25 ng of template DNA. 13.2 is four times the average molecular weight of the four dNTPs divided by 100.

Amount of radioactivity incorporated (B) in dpm =

total number of \( \mu \text{Ci added} \times 2.2 \times 10^4 \times \% \text{ incorporation} \)

Specific activity of labelled DNA = \( \frac{B}{A} \times 10^3 \) dpm per µg

113
2.13  **IN SITU HYBRIDISATION**

In this method an anti-digoxigenin antibody conjugated to alkaline phosphatase is reacted with hybridised, digoxigenin-labelled probe after the hybridisation reaction. Addition of an alkaline phosphatase substrate yields a colour at the site of hybridised probe. The principles are illustrated in fig 2.9.

2.13.1  **Prehybridisation**

The method was adapted from techniques previously published by this laboratory (Shahzeidi et al 1993; Shahzeidi et al 1994).

2.13.1.1  **Slide and coverslip preparation and tissue sectioning**

Glass slides (BDH) were washed, rinsed in distilled water and immersed in acetone for 2 mins. They were then treated for 5 mins with freshly prepared 2% 3-aminopropyltriethoxysilane in acetone, rinsed in distilled water and dried.

Boroslicate coverslips (BDH) were washed, rinsed in distilled water, methanol, water and dried. They were immersed briefly in Repelcote (BDH), air-dried, rinsed in distilled water, 100% ethanol, then dried.

5 μm tissue sections were cut in the Department of Lung Pathology at the Royal Brompton Hospital or on site with a Reichert-Jung 2035 Biocut cryotome (Leica). Using a paraffin section mounting bath (Electrothermal, BDH) they were floated onto silanised slides and dried on a slide drying bench (Electrothermal, BDH).

2.13.1.2  **Prehybridisation tissue treatments**

Sections were immersed twice in xylene for 15 mins and then in xylene: ethanol 1:1 for 10 mins to complete dewaxing. They were rinsed in 100% ethanol for 2 mins, then dehydrated by brief immersion in 100%, 95%, 70% and finally 30% ethanol. They were rinsed briefly in distilled water and immersed in 0.85% saline for 5 mins, 1 X PBS for 5 mins, freshly prepared 4% paraformaldehyde for 20 mins and twice in 1 x
PBS for 5 mins.

After ringing with a wax pen (Dako), sections were treated individually for 10 mins with 100 μl of 20 μg/ml proteinase K (Gibco) in Tris HCl pH 7.5, 5 mM EDTA. They were washed in 1 x PBS for 5 mins, refixed in 4% paraformaldehyde for 5 mins and immersed in water for 1 min.

Sections were acetylated by stirring in freshly prepared 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 mins, rinsed in 1 X PBS for 5 mins, 0.85% saline for 5 mins and distilled water. They were then dehydrated by rinsing in 30%, 70%, 95% and 100% ethanol. Finally they were rinsed in clean 100% ethanol and air-dried.

2.13.2 Hybridisation

2.13.2.1 Hybridisation buffer preparation

Hybridisation buffer was prepared in advance and stored in aliquots at -40°C. It was composed of 50% formamide (deionised immediately prior to use with mixed bead resin (Sigma) according to the manufacturer’s instructions), 300 mM NaCl, 20 mM Tris HCl pH 7.4, 5 mM EDTA, 10 mM sodium dihydrogen phosphate pH 8.0, 10% dextran sulphate, 1 X Denhardt’s and 500 μg/ml brewer’s yeast tRNA. Hybridisation buffer was not refrozen. 1 μl of RNase inhibitor (Boehringer) was added per 30 μl of thawed hybridisation buffer and the buffer heated to 50°C prior to use.

2.13.2.2 Hybridisation of tissue sections

Digoxigenin-labelled riboprobes, prepared as described (section 2.11.2) and resuspended in water to a concentration of 200 ng/ml, were thawed on ice. 30 μl of hybridisation mix was prepared per slide in sterile microfuge tubes on ice, consisting of probe and hybridisation buffer mixed in a ratio of 1:10 to give a final probe concentration of 20 ng/ml. The hybridisation mix was heated to 50°C for 10 mins, applied to tissue sections, covered with a preprepared coverslip and the slides placed in a chamber humidified with paper towels saturated with a solution containing 50% formamide and
2 x SSC. The chamber was sealed with water-resistant adhesive tape and incubated at 50°C overnight.

2.13.3 Post hybridisation treatments

Sections were immersed for 30 mins in 4 x SSC at RT followed by 30 mins in 0.2 x SSC at RT. Subsequent manipulations were performed at RT. Sections were washed for 5 mins in TBS buffer (0.1 M Tris pH 8.2, 0.15 M NaCl), then incubated with freshly prepared antibody blocking solution (5% bovine serum albumin (Sigma) in TBS containing Tween® 0.1% (Sigma) and 5% sheep serum (Sigma)) for 30 mins. They were washed twice in TBS for 5 mins each, then incubated with freshly prepared antibody solution (anti-digoxigenin antibody, Fab fragments (Boehringer) diluted 1:100 in a 1% solution of bovine serum albumin in TBS containing Tween 0.1%) for 30 mins. Sections were then washed twice with TBS for 5 mins each.

2 ml of the chromogen New Fuschin Red (Dako) was prepared according to the manufacturer’s instructions, levamisole added to a final concentration of 10 mM to inhibit endogenous alkaline phosphatase activity (Ponder, Wilkinson, 1981) and then applied to the sections for 20 mins prior to counterstaining and mounting.

2.13.4 Histological analysis

2.13.4.1 Counterstaining and mounting

Slides were rinsed in distilled water and counterstained for 2 seconds with Gills haematoxylin (Harris, BDH) or 2% methyl green in 2% acetic acid, using a modification of a published method (Bancroft, Stevens, 1996). Sections were mounted in glycerol or Aqua Polymount (Park Scientific).

2.13.4.2 Microscopy and photography

Sections were visualised with an Axioskop microscope (Zeiss) using x 100, x 200, x 400 or x 1000 magnification. Photomicrographs were taken using a Yashica 35 mm auto-exposure Single-Lens-Reflex camera (Zeiss). Kodak Ektachrome Tungsten film

116
ASA 64 was used alone. Kodak Ektachrome Daylight film, ASA 64, was also used with 80A and 82C Cokin filters (Kingsley Photographic), singly or in combination. Film was purchased from Keith Johnson & Pelling. Negatives, colour prints and photocopies were processed by the Middlesex Hospital Photography Department, London.

2.14 CHARACTERISATION OF TGFβ₂ RIBOPROBES

An unexpected finding using the TGFβ₂ probes was that positive signal was frequently obtained using the sense probe, while little signal was obtained using the antisense probe. Having checked that this did not result from an error in slide labelling or riboprobe preparation, and having confirmed the identity of the probe with Professor Moses’ laboratory, the orientation of the probe within the vector was verified. If the probe were inserted in the SP72 vector in the opposite orientation, the sequence of sense and antisense riboprobes would be reversed (fig 2.6). Probe orientation was verified in two ways. Firstly, asymmetric restriction enzyme cuts were performed using Cla I. Secondly, dideoxynucleotide sequencing was performed.

2.14.1 Asymmetric restriction enzyme digestion

The TGFβ₂ probe sequence covers 1511 through to 1953 bp (Pelton et al 1991a). Cla I recognises the sequence AT ↓ CGAT, where ↓ represents the site of cleavage. There are Cla I restriction sites at 1953 and 1770. Asymmetric restriction endonuclease digestion of the plasmid could therefore either yield a fragment of 183 bp, or a fragment of 259 bp, depending on the probe orientation within the plasmid (fig 2.10). Approximately 5 units of Cla I were used per 1 μg of the pm TGFβ₂-9A plasmid, the reaction incubated at 37°C for 1 hr and an aliquot examined by electrophoresis.

117
2.14.2 Dideoxynucleotide sequencing

The United States Biochemical Sequenase Version 2.0 T7 polymerase dideoxynucleotide sequencing kit (Amersham) was used according to the manufacturer's instructions.

2.14.2.1 Template preparation

4 µg of the pm TGFβ2-9A plasmid DNA prepared as described (section 2.10.5.2) was denatured by addition of 2 M NaOH, 2 mM EDTA to a final concentration of 0.2 M NaOH, 0.2 mM EDTA, and incubated at RT for 5 mins. The reaction was neutralised with 2 µl of 2M ammonium acetate pH 4.6, the DNA precipitated and then resuspended in 7 µl water.

2.14.2.2 Primer annealing, labelling and termination reactions

To the denatured plasmid in a sterile microfuge tube on ice were added 2 µl of annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl) and 1 µl (0.5 pmol) of T7 primer. The sample was heated to 65°C for 2 mins and then cooled to RT over 30 mins prior to chilling on ice.

2.5 µl of each termination nucleotide mixture was added to four separate wells of a Thermowell P polymerase chain reaction (PCR) plate (Costar) which was then covered with Saranwrap. Each termination mixture contains 80 µM dGTP, 80 µM dATP, 80 µM dTTP, 80 µM dCTP and 50 mM NaCl. The 'G' mixture also contains 8 µM ddGTP, the 'A'; mixture, 8 µM ddATP, the 'T' mixture, 8µM ddTTP and the 'C' mixture, 8µM ddCTP.
Figure 2.10 *Cla I* restriction mapping of pm TGFβ2-9A

Figure shows the predicted orientation of plasmid insertion in the vector, together with restriction sites for *Cla I*. If this orientation is correct, restriction digest with *Cla I* will yield a 259 bp fragment and a much larger fragment containing the vector and remainder of the plasmid sequences. If the orientation is reversed, restriction digest with *Cla I* will yield a 183 bp fragment in addition to the larger fragment.
Concentrated labelling nucleotide mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted 5-fold and the PCR plate warmed to 37°C. To the ice-cold mixture containing annealed DNA was added 1 μl of dithiothreitol (0.1M), 2 μl of diluted labelling mix, 0.5 μl of α-35S dATP (6.25 μCi, 0.23 MBq, Du Pont) and 2 μl (3 units) of Sequenase polymerase previously diluted 1:8 in enzyme dilution buffer (10 mM Tris-HCl, pH7.5, 5 mM DTT, 0.5 mg/ml bovine serum albumin). The sample was incubated at RT for 5 mins.

3.5 μl of the labelling reaction was added to each of the four wells in the PCR plate and samples incubated at 37°C for 5 mins on a thermal cycler (Techne PHC-3). Reactions were then stopped by addition of 4 μl of Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), samples mixed and chilled on ice.

2.14.2.3 DNA electrophoresis

A 4% polyacrylamide gel was prepared in advance. 15 ml of Sequagel (National Diagnostics), 52.5 ml Sequagel diluent (National Diagnostics), 7.5 ml Sequagel buffer (National Diagnostics) and 600 μl 10% ammonium persulphate were mixed and 40 μl of tetramethylethylenediamine (TEMED, Sigma) added immediately prior to pouring the gel.

Clean glass sequencing plates (Gibco) were rinsed and dried with ethanol. The smaller plate was silanised with Sigmacoat (Sigma) and the plates assembled with dividers and sharkstooth combs (5.7 mm point-to-point spacing for 0.4 mm gels, Gibco) inserted in between. The apparatus was sealed with Permacel tape, the gel cast and the plates clamped.

When the gel had set the plates were assembled in a sequencing electrophoresis tank (Gibco) to which was added 0.5 x TBE buffer (upper chamber) and 1 x TBE (lower chamber). The gel was pre-run at 1.5 Kv for 30 mins, the DNA samples heated to 95°C for 5 mins using the thermal cycler, and immediately loaded onto the sequencing gel. Samples were electrophoresed for 2 hrs until the leading edge of the dye reached
2.14.2.4 Autoradiography and sequence analysis

The buffer was drained, the plates removed and carefully separated and the lower plate and gel immersed in 10% methanol, 10% acetic acid for 15 mins. Using a filter paper cut to the size of the gel, the gel was removed from the plate, covered with Saranwrap and dried at 80°C on a slab gel dryer (model SE1160, Hoeffer Scientific Instruments). It was then placed in an autoradiography cassette with a Kodak x ray film (X-OMAT, XAR-5, Sigma) and autoradiography performed for 24 to 72 hrs. The autoradiograph was read manually and the sequence matched for homology using the FASTA database searching programme (Human Genome Mapping Project CRC, Cambridge, UK).

2.14.3 Assessment of digoxigenin labelling

To verify that the results obtained using the TGFβ2 riboprobes were not the result of increased digoxigenin labelling of the sense compared with the antisense riboprobe, digoxigenin labelling of both probes was determined. Aliquots of each probe were examined by agarose gel electrophoresis and photographed. Serial dilutions of the same probe preparation were then prepared and the chemiluminescence assay for digoxigenin performed as described previously (section 2.11.3).

Densitometry of ethidium bromide staining and digoxigenin incorporation was performed with an Image Master 1D gel and film scanner (Pharmacia). Optical density of the TGFβ2 probes was corrected for ethidium bromide staining, and the ratio of digoxigenin labelling of the antisense to sense probe calculated.

2.15 RNA EXTRACTION

2.15.1 Total RNA extraction

This method is based on a previously described one (Chomczynski, Sacchi, 1987). A monophasic solution of phenol and guanidine isothiocyanate, TRIzol Reagent (Gibco)
was used following the manufacturer's instructions.

Rat lung tissue samples were homogenised in 1ml of TRIzol Reagent per 100 mg (section 2.6.3). 1 ml aliquots of homogenate were pipetted into sterile microfuge tubes and the homogenate sheared with a 21 G needle. AC29 mesothelioma cells were cultured to visual confluence (section 2.5.1). The medium was discarded, cells immediately harvested into 2 ml of TRIzol Reagent per flask (containing 2 x 10^7 cells) and the homogenate sheared as above. Homogenised samples were incubated for 5 mins at RT to allow complete dissociation of nucleic acid and proteins.

0.2 ml of chloroform was added to each sample, samples shaken vigorously by hand for 15 seconds and incubated at RT for 3 mins. They were centrifuged at 12,000 x g for 15 mins at 4°C. The aqueous phase was transferred to a fresh tube and RNA precipitated by adding 0.5 ml of isopropyl alcohol. Samples were incubated at RT for 10 mins, centrifuged at 12,000 x g for 10 mins at 4°C, the supernatant discarded and RNA pellets washed with 1 ml of 75% ethanol. Samples were mixed by vortexing and centrifuged at 7,500 x g for 5 mins at 4°C. RNA pellets were then dried briefly and resuspended in 20 µl of sterile water prior to spectrophotometry and electrophoresis.

### 2.15.2 Polyadenylated RNA extraction

The Poly A Tract mRNA Isolation System III (Promega) was used to extract polyadenylated (poly A^+) RNA from total RNA following the manufacturer's instructions (fig 2.11).

100 to 1000 µg of total RNA was resuspended in 500 µl of water and samples heated to 65°C for 10 mins. 150 pmol (3 µl) of biotinylated oligo (dT) probe was added followed by 13 µl of 20 x SSC. Samples were cooled to RT. Streptavidin-paramagnetic particles (SA-PMPs) were resuspended, captured with a magnetic stand and the supernatant removed. SA-PMPs were then washed three times with 300 µl of 0.5 x SSC. Each time they were captured using the magnetic stand and the supernatant removed. They were then resuspended in 300 µl of 0.5 x SSC.
The cooled annealing reaction was added to the washed SA-PMPs and the mixture incubated at RT for 10 mins. The SA-PMPs were then captured using the magnetic stand and the supernatant removed. The particles were then washed four times with 300 μl of 0.1 x SSC. Poly A⁺ RNA was eluted from the SA-PMP pellet by resuspending it in 100 μl of water. The SA-PMPs were captured magnetically and the eluted mRNA transferred to a fresh tube. The elution step was repeated by resuspending the SA-PMP pellet in 150 μl of water and the eluate pooled with the first one. Eluted poly A⁺ RNA was examined by spectrophotometry using a quartz cuvette previously soaked in 50 mM NaOH and rinsed with water.

2.15.3 Concentration of polyadenylated RNA

The yield of poly A⁺ RNA from total RNA varied between 1 and 3% of the initial sample. The volume of the final eluate (250 μl) precluded direct use of samples in Northern analysis. To minimise further losses by precipitation, concentration of samples was performed.

For each sample, one microcon model 100 concentrator was used (Amicon) following the manufacturer's instructions. The sample was pipetted into the reservoir of the assembled device and centrifuged at 500 x g at 4°C for 24 mins. The sample reservoir was then separated from the vial, placed upside down in a new vial and recentrifuged at 1000 x g at 4°C for 2 mins to transfer the concentrate to the new vial. The reservoir was discarded and the sample frozen at -80°C until required. Using this method, it was routinely possible to concentrate 250 μl down to 5 μl with minimal loss of poly A⁺ RNA.
Figure 2.11 Polyadenylated RNA isolation

The biotinylated oligo(dT) probe hybridises with the 3' poly (A) region present in most mature eukaryotic mRNA species. Hybrids are captured using streptavidin coupled to paramagnetic particles (PMP) and a magnetic separation stand. They are then washed at high stringency. Finally the mRNA is eluted from the solid phase by addition of ribonuclease-free water.
2.16 NORTHERN ANALYSIS

These methods are well-established and based on previously published protocols (Maniatis et al 1989).

2.16.1 RNA electrophoresis

A 20 x solution of formaldehyde running buffer (0.4 M 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0, 0.1 M sodium acetate, 0.02 M EDTA) was prepared in a foil-wrapped bottle and autoclaved. 2g of electrophoresis grade agarose was dissolved in 162 ml water by autoclaving. When the agarose had cooled to 70°C, 10 ml of 20 x MOPS buffer, 10 ml of water and 18 ml of 37% formaldehyde were added and mixed to produce a 1% agarose solution in 1 x MOPS buffer and 1.1 M formaldehyde. It was poured into a 12 x 15 cm casting tray and gel thickness restricted to 5 mm in order to maximise subsequent efficiency of RNA transfer. A comb with wide teeth (0.6 cm) was found to produce the clearest results.

In a sterile microfuge tube on ice, a volume not exceeding 15 µl, containing up to 10 µg of poly A+ RNA, was mixed with an equal volume of RNA loading buffer (Sigma). Final volumes of all samples were kept equal. An aliquot of RNA ladder was also prepared. RNA samples were denatured by heating at 65°C for 10 mins. Meanwhile the gel was submerged in 1 litre of 1 x formaldehyde running buffer, the comb removed, and the gel pre-run at 80 V for 5 mins. Denatured RNA samples were placed briefly on ice prior to loading.

The gel was run at 80 V for approximately 5 hrs, until the leading edge of the dye had migrated to within 2-3 cm of the edge of the gel. It was photographed under UV transillumination, the top right-hand corner cut away for future orientation, and washed for 20 mins in water to remove excess formaldehyde. To determine the size of unknown RNA transcripts subsequently identified by hybridisation, a size calibration curve was constructed by plotting the log_{10} of the number of kilobases of known RNA standards against the distance these had migrated from the origin (fig 2.12).
Figure 2.12 Size calibration curve for electrophoretically separated RNA
Figure shows a typical curve used to calculate the size of unknown RNA transcripts identified by Northern analysis. The log_{10} of the number of kilobases of known RNA standards was plotted against the distance these had migrated from the origin. The size of unknown RNA transcripts was then extrapolated from the curve.
2.16.2 Northern transfer

A Hybond N nylon membrane (Amersham) was cut to the size of the gel. It was then wetted by full immersion in a plastic sandwich box containing 500 ml of 20 x SSC. The lid was placed at right angles over the box, three sheets of filter paper (Munktell grade 1F, Pharmacia) placed across it to form a wick and three filter papers the same size as the lid, previously wetted in 20 x SSC, placed on top. The gel was inverted, placed on top and covered with the membrane. Strips of Saranwrap were placed around the gel to prevent short-circuiting of buffer. Three membrane-sized filter papers, previously wetted in 20 x SSC, were placed on top. A 5 cm thick layer of disposable paper towels was placed on top, covered with another plastic sandwich box lid and a 1 litre bottle of water placed on top as a weight. Transfer was allowed to proceed for a minimum of 16 hrs.

The membrane, overlying filter papers and gel were examined under UV light to confirm that transfer had occurred. The membrane was washed briefly in 20 x SSC, drained of excess buffer and secured between two sheets of filter paper. RNA was crosslinked by brief exposure to UV light using a UV transilluminator followed by baking at 80°C for 2 hrs.

2.16.3 Prehybridisation

Conditions for prehybridisation and hybridisation using $^{32}$P labelled cDNA probes were similar to those published previously for detection of TGFβ transcripts (Pelton et al 1991a; Westergren-Thorsson et al 1993). 50 ml of a prehybridisation buffer (5 x SSPE, 5 x Denhardt's, 0.1% SDS and 50% deionised formamide) was prepared. To 25 ml was added salmon sperm DNA, previously denatured by boiling for 5 mins, to a concentration of 100 μg/ml. The remaining 25 ml of buffer was retained for hybridisation. Prehybridisation was performed in a shaking water bath at 42°C for 1 to 4 hrs.

When digoxigenin-labelled riboprobes were used for Northern analysis, DIG Easy Hyb
buffer (Boehringer) was used for both prehybridisation and hybridisation (section 2.16.4), according to the manufacturer's instructions. Briefly, the membrane was prehybridised for a minimum of 30 mins in 25 ml of buffer previously prewarmed to 42°C.

2.16.4 Hybridisation and stringency washes

Radio- or digoxigenin-labelled probe was prepared as described (sections 2.11.2 and 2.12.2). The prehybridisation buffer was replaced with the remaining 25 ml of hybridisation buffer. The probe, previously denatured by boiling for 5 mins, was added and hybridisation performed overnight at 42°C. Radiolabelled probe was used at a final concentration of 25 ng/ml and digoxigenin-labelled riboprobe at 100 ng/ml.

The membrane was washed in several changes of 2 x SSC, 0.1% SDS for 30 mins at 42°C, followed by 0.1 x SSC, 0.1% SDS at 50°C for 20 mins. It was then rinsed briefly in fresh 0.1 x SSC, 0.1 SDS.

2.16.5 Imaging

For autoradiography following hybridisation with 32P labelled probes the membrane was wrapped in Saranwrap and placed with a Kodak x-ray film (X-OMAT XAR-5, Sigma) in an autoradiography cassette at -80°C for between 8 hrs and 4 weeks. After thawing the cassette the film was developed as described previously (section 2.11.3).

Phosphoimaging was used for rapid screening of low copy number transcripts. A Fuji Bas system (1000, Raytek Scientific Ltd) was used (Amemiya, Miyahara, 1988). The imaging plate is coated with a photostimulable phosphor which stores a fraction of the absorbed incident energy from X rays. When later stimulated by visible or infrared radiation, the plate emits photostimulated luminescence whose intensity is proportional to the absorbed radiation energy. The membrane was wrapped in Saranwrap, placed against an imaging plate (Fuji Bas, Raytek) covered with Saranwrap and both placed in an exposure cassette (Fuji Bas, Raytek). This was stored at RT away from electronic
equipment for between 4 hrs and 2 weeks. The plate was then scanned using the image
analyser.

Autoradiography using digoxigenin-labelled riboprobes was performed as described
previously (section 2.11.3). Densitometry of autoradiographs and phosphoimaging
scans was performed using an Image Master 1D gel and film scanner (Pharmacia) as
described previously (section 2.11.3).
CHAPTER THREE

RESULTS


**IN VITRO STUDIES**

3.1 THE EFFECT OF TGFβ₁,₃ ON FIBROBLAST PROLIFERATION

The effect of TGFβ₁,₃ on fibroblast proliferation was examined by measurement of DNA content in confluent monolayers. Changes in cell number in response to TGFβ₁,₃ could lead to altered procollagen production without a change in individual fibroblast procollagen metabolism. It was therefore important to establish whether the TGFβ isoforms had any effect on HFL-1 proliferation before considering their effects on procollagen metabolism. HFL-1 procollagen production and degradation was then standardised and expressed in terms of pmol hydroxyproline/μg DNA/hr. The results of the DNA assay were verified by direct cell counts.

Figure 3.1 shows that none of the TGFβ isoforms altered HFL-1 DNA content over the 24 hr culture period. Cell counts performed on four replicate wells following 24 hr incubation of HFL-1 cells with TGFβ₂ at 40 pM during a separate experiment confirmed this finding. There was no difference in cell count between media control (5 x 10⁴ cells/well) and TGFβ₂ (4.8 x 10⁴ cells/well). These results show that any changes in procollagen metabolism in response to the TGFβ isoforms were not the result of an effect of TGFβ on proliferation.

131
Figure 3.1 The effect of TGFβ₁, TGFβ₂, and TGFβ₃ on DNA content of fibroblast cultures
Figure shows the effect on DNA of each of the three TGFβ isoforms at a concentration of 40 pM. Each value represents the mean ± SEM from four separate experiments, each containing four to six replicate cultures.
3.2 THE EFFECT OF TGFβ_{1,3} ON PROCOLLAGEN METABOLISM

3.2.1 Dose-response relationships for TGFβ_{1,3}

Having established that TGFβ_{1,3} did not affect fibroblast proliferation over the 24 hr culture period, dose-response studies were performed. The purpose of these was to demonstrate a dose-response relationship between each of the TGFβ isoforms and procollagen production, and to determine concentrations which would produce maximal effects. HFL-1 procollagen production was expressed as a function of TGFβ concentration in the culture medium during the 24 hr incubation period. All three isoforms significantly stimulated procollagen production in a dose-dependent manner.

Figure 3.2 shows the dose-response relationship for TGFβ_{1}. Control procollagen production was 42.75±0.99 pmol/μg DNA/h (mean ± SEM). Concentrations of 4pM and below had no effect on procollagen production, but 10 pM (0.25 ng/ml) stimulated production above media control by 53±2% (p<0.01 compared with control). No further increase was observed at concentrations above 10 pM.

Figure 3.3 shows the dose-response relationship for TGFβ_{2}. Control procollagen production was 26.97±1.22 pmol/μg DNA/h. Concentrations of 10 pM and below had no effect on procollagen production, but 20 pM (0.5 ng/ml) stimulated production above media control by 53±1% (p<0.01 compared with control). No further increase was observed at concentrations above 20 pM.

Figure 3.4 shows the dose-response relationship for TGFβ_{3}. Control procollagen production was 44.83±1.13 pmol/μg DNA/h. Concentrations of 2 pM and below had no effect on procollagen production, but 4 pM (0.1 ng/ml) stimulated production above media control by 46±2% (p<0.01 compared with control). No further increase was observed at concentrations above 4 pM.
Figure 3.2 Dose-response relationship for TGFβ₁
Figure shows HFL-1 procollagen production, assessed by measurement of hydroxyproline, as a function of TGFβ₁ concentration. Each value represents the mean ± SEM from four to six replicate cultures. * = p<0.01 compared with control.
Figure 3.3 Dose-response relationship for TGFβ₂
Figure shows HFL-1 procollagen production, assessed by measurement of hydroxyproline, as a function of TGFβ₂ concentration. Each value represents the mean ± SEM from four to six replicate cultures. * = p<0.01 compared with control.
Figure 3.4 Dose-response relationship for TGFβ3
Figure shows HFL-1 procollagen production, assessed by measurement of hydroxyproline, as a function of TGFβ3 concentration. Each value represents the mean ± SEM from four to six replicate cultures. * = p<0.01 compared with control.
Having established that all three TGFβ isoforms stimulated HFL-1 procollagen production in a dose-dependent manner, and that concentrations of 20 pM and above exerted a maximal effect, the effects of each isoform on production, intracellular degradation and total synthesis were compared at a concentration of 40 pM (1 ng/ml).

3.2.2 The effect of TGFβ₁,₃ on procollagen production

Figure 3.5 shows the effect of each of the three TGFβ isoforms on HFL-1 procollagen production. Data are the means ± SEM of four experiments, each consisting of four to six replicate cultures. Control procollagen production was 37.4±1.5 pmol/μg DNA/h. TGFβ₁,₃ stimulated procollagen production above media control by 46±3%, 46±3% and 43±2% respectively (p<0.01 in all cases).

3.2.3 The effect of TGFβ₁,₃ on procollagen degradation

Figure 3.6 shows the effect of each of the three TGFβ isoforms on intracellular HFL-1 procollagen degradation at a concentration of 40 pM (1 ng/ml). Data are the means ± SEM of four experiments, each consisting of four to six replicate cultures. Control procollagen degradation was 22±1%. There was a tendency for all three isoforms to reduce intracellular degradation, but this was only significant in the case of TGFβ₃, which reduced degradation by 17±1% (p<0.05 compared with control).

3.2.4 The effect of TGFβ₁,₃ on procollagen synthesis

Figure 3.7 shows the effect of each of the three TGFβ isoforms on HFL-1 procollagen synthesis at a concentration of 40 pM (1 ng/ml). Data are the means ± SEM of four experiments, each consisting of four to six replicate cultures. Control procollagen synthesis was 50.2±2.0. TGFβ₁,₃ stimulated procollagen synthesis above media control by 38±2%, 37±3% and 33±2% respectively (p<0.01 in all cases).
3.2.5 Summary

These results demonstrate that all three isoforms stimulated lung fibroblast procollagen production in a dose-dependent, reproducible manner. TGFB3 was the most potent isoform, exerting a maximal effect at a concentration of 4 pM, whereas the other two peptides only exerted maximal stimulation between 10 pM and 20 pM. Although TGFB3 achieved maximal stimulation at a lower concentration than TGFB1 or TGFB2, the maximal response was similar for all three isoforms, with increases of approximately 50% above media control values.

Comparison of the effects of the three isoforms shows that there were no reproducible differences between the isoforms in terms of their effects on either procollagen production or synthesis at 40 pM. TGFB3 was the only isoform to cause a significant reduction in intracellular degradation at this concentration.

Having determined that all three TGFβ isoforms were profibrotic in vitro, their role in the pathogenesis of pulmonary fibrosis was examined further by localising TGFβ1,3 gene expression in normal and fibrotic lung. The results of these studies are described in the following sections.
Figure 3.5 The effect of TGFβ₁, TGFβ₂, and TGFβ₃ on HFL-1 procollagen production

Figure shows the percentage increase in HFL-1 procollagen production above media control in response to each of the three TGFβ isoforms at a concentration of 40 pM. Procollagen production is calculated from the quantity of hydroxyproline present in the ethanol-insoluble fraction. Each value represents the mean percentage change ± SEM from four separate experiments, each containing four to six replicate cultures.

* = p < 0.01 compared with control.
Figure 3.6 The effect of TGFβ₁,₂,₃ on HFL-1 procollagen degradation

Figure shows the percentage reduction in HFL-1 procollagen degradation compared with media control in response to each of the three TGFβ isoforms at a concentration of 40 pM. Procollagen degradation (expressed as a percentage) is calculated from the amount of procollagen present in the ethanol-soluble fraction compared with that present in both the ethanol-soluble and ethanol-insoluble fractions. Each value represents the mean percentage change ± SEM from four separate experiments, each containing four to six replicate cultures. * = p<0.05 compared with control. NS = not significant compared with control.
Figure 3.7 The effect of TGFβ₁,₁,₃ on HFL-1 procollagen synthesis
Figure shows the percentage increase in HFL-1 procollagen synthesis above media control in response to each of the three TGFβ isoforms at a concentration of 40 pM. Procollagen synthesis is calculated from the sum of the quantity of hydroxyproline present in the ethanol-insoluble and ethanol-soluble fractions. Each value represents the mean percentage change ± SEM from four separate experiments, each containing four to six replicate cultures. * = p< 0.01 compared with control.
3.3 VALIDATION OF METHODS

The development of a non-isotopic method for in situ hybridisation in the lung was new to this laboratory, as were the techniques for RNA extraction and Northern analysis. The success and reproducibility of the methods used was therefore verified at various stages as outlined below.

3.3.1 Plasmid preparation

Small-scale plasmid preparation was performed initially. The resulting plasmid DNA was analysed by spectrophotometry to determine the yield and purity. When the identity of the plasmid DNA had been confirmed by agarose gel electrophoresis, large-scale plasmid preparation was performed.

3.3.1.1 Yield and purity of plasmid DNA

Table 3.1 (upper panel) shows the results of spectrophotometry of plasmid DNA prepared using the detergent lysis method for small-scale preparation. Between 120 and 170 µg of DNA were obtained from each 40 ml small-scale bacterial culture. Purity was very poor, the highest OD 260/280 ratio obtained being 0.52.

The results of spectrophotometry of plasmid DNA prepared using the Promega Wizard Maxipreps DNA Purification system are also shown in table 3.1 (lower panel). Using this system between 500 and 1000 µg of plasmid DNA was produced from each 500 ml large-scale bacterial culture. The purity of pm TGFβ-9A and pm TGFβ-11b DNA was good (OD 260/280 ratio 1.8). The purity of pm TGFβ-1A DNA was poor (OD 260/280 ratio 1.3) resulting in failure of plasmid linearisation. It was therefore purified further by phenol-chloroform extraction. The resulting DNA had an OD 260/280 ratio of 1.9, indicating that this method of purification was effective.
3.3.1.2 Electrophoresis of purified plasmids

The identity and purity of the plasmid DNA was further confirmed by agarose gel electrophoresis. Figure 3.8 shows the results of electrophoresis of each of the three purified plasmids, pm TGFβ1-A (lanes 1-3), pm TGFβ2-9A (lanes 4-6) and pm TGFβ3-11b (lanes 7-9), prepared using the Promega Wizard Maxipreps DNA Purification system. The first lane in each group shows circular plasmid DNA. The second two lanes show plasmid DNA linearised with each of the appropriate restriction enzymes prior to *in vitro* transcription and generation of sense and antisense riboprobes. Lane 10 shows lambda DNA/Hind III fragments. Size markers (bp) are given on the right.

The circular plasmid DNA in each case showed at least two major bands, superhelical and nicked DNA, migrating at different rates. Comparison of the linear plasmid DNA with molecular size standards showed that the DNA produced in each case was of the predicted size (3974 bp, 2841 bp and 3600 bp for pm TGFβ1-A, pm TGFβ2-9A and pm TGFβ3-11b DNA respectively). Successful linearisation by restriction enzymes further confirmed satisfactory purity of the plasmid DNA.
<table>
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<tr>
<th>Plasmid</th>
<th>OD 260</th>
<th>OD 280</th>
<th>OD 260/280</th>
<th>μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>pm TGFβ1-A</td>
<td>0.133</td>
<td>0.255</td>
<td>0.52</td>
<td>120</td>
</tr>
<tr>
<td>pm TGFβ2-9A</td>
<td>0.114</td>
<td>0.245</td>
<td>0.46</td>
<td>148</td>
</tr>
<tr>
<td>pm TGFβ2-11b</td>
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<td>0.42</td>
<td>170</td>
</tr>
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<td>pm TGFβ3</td>
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<td>0.047</td>
<td>1.3 (1.9)</td>
<td>897</td>
</tr>
<tr>
<td>pm TGFβ7-9A</td>
<td>0.035</td>
<td>0.019</td>
<td>1.8</td>
<td>523</td>
</tr>
<tr>
<td>pm TGFβ7-11b</td>
<td>0.07</td>
<td>0.04</td>
<td>1.8</td>
<td>1046</td>
</tr>
</tbody>
</table>

Table 3.1 Yield and purity of plasmid DNA obtained following small and large-scale preparation
Table shows results of spectrophotometry of the three plasmids prepared using the detergent lysis method (upper panel) and the Promega Wizard Maxipreps DNA Purification system (lower panel). The value in brackets was obtained after further purification by phenol-chloroform extraction.
Figure 3.8 Agarose gel electrophoresis of plasmid DNA prepared using the Promega Wizard Maxipreps DNA Purification system

Figure shows electrophoresis of the three plasmids, both circular and linearised prior to \textit{in vitro} transcription. Size markers (bp) are shown on the right. Lanes 1-3: pm TGF\(\beta_1\) A, lane 1: circular; lane 2: linearised with \textit{EcoR I}; lane 3: linearised with \textit{Hind III}. Lanes 4-6: pm TGF\(\beta_2\)-9A, lane 4: circular; lane 5: linearised with \textit{EcoR I}; lane 6: linearised with \textit{Xho I}. Lanes 7-9: pm TGF\(\beta_3\)-11b, lane 7: circular; lane 8: linearised with \textit{Xho I}; lane 9: linearised with \textit{Hind III}. Lane 10: lambda DNA/\textit{Hind III} fragments. Circular plasmid DNA shows at least two major bands, superhelical and nicked DNA, migrating at different rates.
3.3.2 Digoxigenin labelling of riboprobes

3.3.2.1 Electrophoresis of digoxigenin-labelled riboprobes

Figure 3.9 shows the result of electrophoresis of the digoxigenin labelled TGFβ₁ antisense riboprobe (lane 1). Comparison with an RNA ladder (lane 2) confirmed that the probe was of the correct size (974 bp). Size markers (kb) are shown on the right. Electrophoresis of the TGFβ₂ and TGFβ₃ antisense riboprobes showed the probes to be 442 and 609 bp respectively, as predicted. Electrophoresis of the TGFβ₁,₃ sense riboprobes showed that they were equal in size to the antisense riboprobes. Electrophoresis of the TGFβ₂ sense and antisense riboprobes is shown in figure 3.50.

3.3.2.1 Assessment of digoxigenin incorporation into riboprobes

Having determined that the riboprobes generated by in vitro transcription were of the correct length, it was important to confirm digoxigenin incorporation into the riboprobes. Figure 3.10 shows the results of chemiluminescence assay of digoxigenin labelling of the TGFβ₁,₃ sense and antisense riboprobes compared with that of a control riboprobe supplied by the manufacturer. Autoradiography of serial spot dilutions of each probe, ranging from 1000 to 0.01 pg, demonstrated digoxigenin incorporation into each of the riboprobes.

3.3.2.3 Reduction of riboprobe length by alkaline hydrolysis

Having established that digoxigenin-labelled TGFβ riboprobes could be generated successfully by in vitro transcription, the effect of alkaline hydrolysis on riboprobe length was determined. This experiment was performed using the TGFβ₁ antisense riboprobe only. Figure 3.11 shows electrophoresis of the TGFβ₁ antisense riboprobe after alkaline hydrolysis for 60 mins (lane 2), 80 mins (lane 3) and 100 mins (lane 4) compared with an RNA ladder (lanes 1 and 5). Bands corresponding to the riboprobes are indicated with arrows and size markers (bp) shown on the right. Yeast tRNA added to co-precipitate the riboprobes is seen as a prominent low molecular weight band at the leading edge of each sample. There was no difference in the size of the probe fragments hydrolysed for the different lengths of time, which were all approximately 80 bp in length. The separate aliquots were therefore combined and used together.
Figure 3.9 Agarose gel electrophoresis of digoxigenin-labelled TGFβ1 antisense riboprobe
Lane 1: digoxigenin labelled TGFβ1 antisense riboprobe; lane 2: 0.24-9.5 kb RNA ladder. Size markers (kb) are shown on the right.
Figure 3.10 Assessment of digoxigenin incorporation into riboprobes
Figure shows chemiluminescence assay of digoxigenin labelling of the TGFβ₁, sense and antisense probes and a control riboprobe ('antisense' neo RNA, Boehringer). Serial dilutions range from 1000 to 0.01 pg. a: control; b: TGFβ₁ antisense; c: TGFβ₂ antisense; d: TGFβ₃ antisense; e: TGFβ₁ sense; f: TGFβ₂ sense; f: TGFβ₃ sense.
Figure 3.11 Reduction of riboprobe length by alkaline hydrolysis
Figure shows electrophoresis of TGFβ antisense riboprobe hydrolysed with alkali for three different times. Lanes 1 and 5: 100 bp size marker; lane 2: 60 mins; lane 3: 80 mins; lane 4: 100 mins. The bands corresponding to the riboprobes are indicated by arrows and size markers (bp) shown on the right. Yeast tRNA added to co-precipitate the riboprobe appears as a prominent low molecular weight band at the leading edge of the sample.
3.3.2.4 Effect of riboprobe length on detection of mRNA transcripts by in situ hybridisation

Figure 3.12 shows *in situ* hybridisation of normal murine lung with TGFβ1 antisense riboprobe hydrolysed to a length of 80 bp (upper panel) and unhydrolysed TGFβ1 antisense riboprobe 974 bp in length (lower panel). No increase in signal was observed using hydrolysed probe. After preliminary studies, unhydrolysed digoxigenin-labelled riboprobes were therefore used for *in situ* hybridisation experiments.

3.3.3 Assessment of $^{32}$P incorporation into cDNA probes

Labelling efficiency by random prime labelling with the Megaprime DNA Labelling system was variable, and consequently so was the specific activity of the probes. $^{32}$P incorporation in eight experiments varied between 8% and 75%, and specific activity between 0.1 and $3.4 \times 10^6$ dpm/ng. Labelling efficiency was not predictably altered by the length of the labelling reaction, but tended to be lower using template DNA in agarose. Reaction conditions were therefore optimised wherever possible by using $^{32}$P on the day of delivery, when its radioactivity was greatest.

3.3.4 RNA isolation

3.3.4.1 Yield and purity of total lung and cellular RNA

Spectrophotometry of total RNA samples isolated from rat lung revealed OD ratios ranging between 1.9 and 2.5, indicating good purity. Between 130 and 250 µg were obtained from 100 mg of tissue.

OD ratios of total RNA isolated from murine mesothelioma (AC29) cells ranged from 1.5 to 2.1, indicating generally good purity. Between 80 and 230 µg of RNA was obtained from one culture flask containing $2 \times 10^7$ cells. OD ratios of poly A+ RNA isolated from total RNA ranged from 1.6 to 2.1, and yield varied from 1.2 to 3% of the initial total RNA sample.
Figure 3.12 Effect of riboprobe length on detection of mRNA transcripts by *in situ* hybridisation

Upper panel shows normal murine lung hybridised with TGFβ₁ antisense riboprobe hydrolysed to a length of 80bp.

Lower panel shows normal murine lung hybridised with unhydrolysed TGFβ₁ antisense riboprobe (length 974 bp).

Hybridised probe appears as a red colour. Original magnification: x 100. Counterstain: haematoxylin.
3.3.4.2 Electrophoresis of purified total RNA

Having determined the yield and purity of total RNA by spectrophotmetry, its integrity was verified by electrophoresis. Figure 3.13 shows 5 μg aliquots of total RNA isolated from rat lung by guanidium-acid-phenol extraction using the TRIzol reagent (lanes 2-8). Lane 1 shows a 0.24-9.49 kb RNA ladder. Size markers (kb) are shown on the right. Two prominent bands are seen in the RNA samples corresponding to 28 S (approximately 4 kb) and 18 S (approximately 2 kb) ribosomal RNA. The sharp definition of the rRNA species with minimum smearing reveals the integrity of the RNA. In lanes 6-8 transfer RNA is seen as a faint band at the leading edge. Similar results were obtained with total RNA from AC29 cells.

3.3.5 Specificity of TGFB1,3 probes

Figure 3.14 shows Northern analysis of 10 μg of poly A+ RNA from murine mesothelioma cells (AC29) using each of the 32P labelled TGFB cDNA probes (lane 1: TGFB1; lane 2: TGFB2; lane 3: TGFB3). Size markers (kb) are shown on the right. The TGFB1 probe detected a 2.4 kb transcript, the TGFB2 probe detected four transcripts between 3 to 6.5 kb and the TGFB3 probe detected a 3.5 kb transcript. These results confirm the specificity of each of the three TGFB probes.

3.3.6 Summary

These results show that good quality plasmid DNA was produced from bacterial cultures, and confirm successful digoxigenin labelling of the TGFB1 riboprobe. Alkaline hydrolysis of digoxigenin-labelled riboprobes did not enhance signal detected by in situ hybridisation and was hence not performed after preliminary studies. Random prime labelling of cDNA probes with P32 was variably efficient. High quality total and poly A+ RNA was isolated from rat lung and murine mesothelioma cells, and Northern analysis confirmed specificity of the TGFB1,3 probes.
Figure 3.13 Electrophoresis of purified total RNA
Figure shows electrophoresis of typical total RNA samples isolated from rat lung. Lane 1: 0.24-9.49 kb RNA ladder; lanes 2-8: RNA samples. Size markers (kb) are shown on the left and the position of the 28S and 18S species is indicated on the right. The sharp definition of the rRNA species with minimum smearing demonstrates the integrity of the RNA. In lanes 6-8 tRNA is seen as a faint band at the leading edge of the sample.
Figure 3.14 Northern analysis using TGFβ₃ cDNA probes
10 μg of poly A⁺ RNA from murine mesothelioma (AC29) cells was hybridised with each of the radiolabelled TGFβ cDNA probes. Size markers (kb) are shown on the left. The following transcripts were seen: TGFβ₁, 2.3 kb; TGFβ₂, multiple bands from 6 to 3 kb; TGFβ₃, 3.5 kb, confirming the specificity of the probes for each of the three isoforms.
3.4 TGFβ, AND TGFβ3 GENE EXPRESSION IN NORMAL MURINE LUNG

3.4.1 Sense and antisense controls for TGFβ, and TGFβ3

Sections of normal murine lung hybridised with sense (a) and antisense (b) TGFβ, and TGFβ3 riboprobes are shown in figures 3.15 and 3.16 respectively. Hybridisation signal appears as a red colour. There was good discrimination between sense and antisense probes, with an absence of signal in sections to which the sense probes were applied. In all cases hybridisation signal was clearly cytoplasmic and outlined the nucleus. There was faint pink staining of extracellular matrix proteins by New Fuschin Red but this did not interfere with detection of hybridisation signal. Hybridisation signal for both isoforms was prominent in bronchiolar epithelium including Clara cells.

3.4.2 Localisation of TGFβ, mRNA transcripts

Figure 3.17 shows the localisation of hybridisation signal for TGFβ,. In addition to being predominant in bronchiolar epithelial cells, it was present in alveolar macrophages (a) and in cells lining alveolar walls (b). Signal was also noted in mesenchymal cells underlying blood vessels and in pulmonary endothelial cells, as shown in figure 3.18 (a and b respectively).

3.4.3 Localisation of TGFβ3 mRNA transcripts

Figure 3.19 shows the cell types expressing TGFβ3. The distribution of TGFβ3 mRNA transcripts in normal murine lung was similar to that of TGFβ,. Hybridisation signal was predominant in bronchiolar epithelial cells and in alveolar macrophages (a) but was also detected in mesenchymal cells underlying blood vessels and in cells lining alveolar walls (b).
Figure 3.15 *In situ* hybridisation in normal murine lung: sense and antisense controls for TGFβ1
Figure shows normal murine lung hybridised with TGFβ1 riboprobes. a: sense; b: antisense. Hybridisation signal appears as a red colour. Signal is evident in bronchiolar epithelial cells (arrows in b). Original magnification: x 200. Counterstain: haematoxylin.
Figure 3.16 *In situ* hybridisation in normal murine lung: sense and antisense controls for TGFβ3.
Figure shows normal murine lung hybridised with TGFβ3 riboprobes. a: sense; b: antisense. Hybridisation signal appears as a red colour. Signal is evident in bronchiolar epithelial cells (arrows in b). Original magnification: x 200. Counterstain: methyl green.
Figure 3.17 Cellular localisation of TGFβ1 gene expression in normal murine lung (I)

Figure shows TGFβ1 mRNA transcripts in alveolar macrophages (arrows) and in cells lining alveolar walls (arrowheads). Original magnification: x 400. Counterstain: haematoxylin.
Figure 3.18 Cellular localisation of TGFβ₁ gene expression in normal murine lung (II)
Figure shows TGFβ₁ mRNA transcripts in mesenchymal cells underlying blood vessels (arrows in a) and in pulmonary endothelial cells (arrows in b). Original magnification: x 400. Counterstain: haematoxylin.
Figure 3.19 Cellular localisation of TGFβ expression in normal murine lung
Figure shows TGFβ mRNA transcripts in alveolar macrophages (arrows in a), in mesenchymal cells underlying blood vessels (arrows in b) and in cells lining alveolar walls (arrowheads in b). Original magnification: x 400 (a), x 1000 (b). Counterstain: methyl green.
3.4.4 Summary

Using digoxigenin-labelled riboprobes, TGFβ_1 and TGFβ_3 gene expression was successfully localised in normal adult murine lung. mRNA transcripts for TGFβ_1 and TGFβ_3 were identified in a wide variety of lung cell types including bronchiolar epithelium, alveolar macrophages, mesenchymal and mesothelial cells. TGFβ_1 gene expression was also detected in pulmonary endothelium.

Unexpected results were obtained using the TGFβ_2 riboprobes and these are discussed fully in section 3.8. The following sections discuss results obtained using the TGFβ_1 and TGFβ_3 riboprobes only.

The data obtained so far show that both TGFβ_1 and TGFβ_3 are profibrotic in vitro, and that a wide variety of lung cell types express both TGFβ_1 and TGFβ_3 in normal murine lung. Taken together, these results indicate that both isoforms could be implicated in the pathogenesis of pulmonary fibrosis. In order to investigate this further, TGFβ_1 and TGFβ_3 mRNA transcripts were localised during the course of bleomycin-induced murine lung fibrosis.

3.5 TGFβ_1 AND TGFβ_3 GENE EXPRESSION IN BLEOMYCIN-INDUCED LUNG FIBROSIS

3.5.1 Characteristics of murine lung following bleomycin

Data for these animals has already been published showing increased total lung collagen content following bleomycin (Shahzeidi et al 1993; Shahzeidi et al 1994). This data is summarised in table 3.2. Figure 3.20 (photographs taken by Dr Shahzeidi) shows a section of normal murine lung (a) compared with lung tissue from an animal ten days after bleomycin (b). These sections illustrate the loss of normal alveolar architecture, inflammatory cell infiltration and fibrosis which result from bleomycin injury.
3.5.1 TGFB$_1$ gene expression

TGFB$_1$ gene expression was only slightly enhanced three days after bleomycin and appeared maximally enhanced ten days after bleomycin administration. A pronounced peribronchial and perivascular inflammatory cell influx was seen at this time. Figure 3.21 shows sense (a) and antisense (b) controls for TGFB$_1$ ten days after bleomycin.

Reduced TGFB$_1$ gene expression was noted in bronchiolar epithelium following bleomycin (compare with fig 3.15) and expression was predominant in inflammatory cells including macrophages, as shown in figures 3.21 and 3.22. Figure 3.23 shows that ten days after bleomycin signal was also present in increased intensity and in greater numbers of mesenchymal cells underlying blood vessels (a) and was enhanced in mesothelial cells adjacent to areas of subpleural fibrosis (b).

Figure 3.24 shows mRNA transcripts in pulmonary endothelial (a) and alveolar type II cells (b). In addition there was intense signal throughout the interstitium consistent with expression of TGFB$_1$ by capillary endothelial cells, alveolar type I cells and fibroblasts (data not shown).

Figure 3.25 shows lung tissue examined 21 days (a: sense; b: antisense) and 35 days (c: sense; d: antisense) after bleomycin and hybridised with the TGFB$_1$ probes. Patchy fibrosis was evident at 21 days with signal predominant in alveolar macrophages. At 35 days there was a return towards the control pattern of gene expression for TGFB$_1$, with mRNA transcripts predominant in bronchiolar epithelium.
<table>
<thead>
<tr>
<th>TIME AFTER BLEOMYCIN (DAYS)</th>
<th>CONTROL ANIMALS</th>
<th>BLEOMYCIN-TREATED ANIMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.24±0.02</td>
<td>1.20±0.04</td>
</tr>
<tr>
<td>10</td>
<td>1.10±0.01</td>
<td>1.61±0.08*</td>
</tr>
<tr>
<td>21</td>
<td>1.23±0.03</td>
<td>1.76±0.14+</td>
</tr>
<tr>
<td>35</td>
<td>1.60±0.02</td>
<td>2.89±0.14+</td>
</tr>
</tbody>
</table>

Table 3.2 Time course of changes in lung collagen content during the development of bleomycin-induced pulmonary fibrosis. Table shows total lung collagen content 3, 10 and 21 days following intratracheal bleomycin. Values for collagen content are expressed in mg and represent the mean values obtained from six animals ± SEM. * = p<0.05 compared with controls; + = p<0.01 compared with controls.
Figure 3.20 Changes in murine lung following bleomycin
Normal lung (a) is compared with lung tissue taken from an animal ten days after bleomycin (b). Loss of normal alveolar architecture, inflammatory cell infiltration and fibrosis are seen following bleomycin. These morphological findings were confirmed by previously published data showing increased total lung collagen content in animals which had received bleomycin.
Figure 3.21 Sense and antisense controls for TGFβ₁ in murine lung ten days after bleomycin
Figure shows murine lung ten days after bleomycin hybridised with TGFβ₁ riboprobes. a: sense; b: antisense. Original magnification: x 100 (a); x 200 (b). Counterstain: haematoxylin.
Figure 3.22 Altered pattern of TGFβ1 gene expression in murine lung ten days after bleomycin
Figure shows a shift in predominant signal from bronchiolar epithelium (arrows in a) to inflammatory cells (arrows in b). Compare with TGFβ1 gene expression in normal bronchial epithelium, shown in figure 3.15. Original magnification: x 200. Counterstain: haematoxylin.
Figure 3.23 Enhanced TGFβ1 gene expression in mesenchymal and mesothelial cells ten days after bleomycin
Figure shows enhanced signal associated with mesenchymal cells underlying blood vessels (arrows in a) and mesothelial cells adjacent to areas of subpleural fibrosis (arrows in b). Original magnification: x 200. Counterstain: haematoxylin.
Figure 3.24 Enhanced TGFβ1 gene expression in pulmonary endothelial and alveolar type II cells ten days after bleomycin
Figure shows enhanced signal associated with pulmonary endothelial (arrows in a) and alveolar type II cells (arrows in b). Original magnification: x 1000 (a); x 400 (b). Counterstain: haematoxylin.
Figure 3.25 TGFβ, gene expression in murine lung 21 and 35 days after bleomycin

Figure shows TGFβ, gene expression 21 days (a: sense; b: antisense) and 35 days (c: sense; d: antisense) after bleomycin. At 21 days signal was primarily localised to macrophages in fibrotic foci (arrows in b). By 35 days the pattern of gene expression had returned to that seen in control animals, with predominant signal in bronchiolar epithelium (arrows in d). Original magnification: x 200. Counterstain: methyl green.
3.5.2 TGFB3 gene expression

Figure 3.26 shows sense (a) and antisense (b) controls for TGFB3 ten days after bleomycin. Lung tissue examined ten days after bleomycin administration hybridised with TGFB3 antisense probe is shown in figure 3.27. In contrast to TGFB1, TGFB3 gene expression was not enhanced at any time after bleomycin, and little or no signal was observed in either bronchiolar epithelium (a) or in inflammatory foci (b).

3.5.3 Summary

Lung tissue from mice receiving bleomycin exhibited a peribronchial and perivascular inflammatory cell influx most apparent at ten days. Following bleomycin, TGFB1 but not TGFB3 gene expression was enhanced, reaching a maximum after ten days. There was a shift from predominant signal for TGFB1 in airway epithelium to principally interstitial signal. Following bleomycin, TGFB3 gene expression was mainly localised to inflammatory cells including macrophages, but was also enhanced in mesenchymal, mesothelial, pulmonary endothelial and alveolar type II cells. Diffusely enhanced interstitial signal also suggested TGFB1 gene expression by capillary endothelial and alveolar type I cells.

Patchy fibrotic changes were apparent by 21 days and TGFB1 gene expression was most marked in macrophages within fibrotic foci. By 35 days the pattern of TGFB1 gene expression had returned to that seen in control animals. A summary of lung cell types expressing TGFB1 and TGFB3 in normal and fibrotic murine lung is shown in table 3.3.
Figure 3.26 Sense and antisense controls for TGFβ3 in murine lung ten days after bleomycin.
Figure shows murine lung ten days after bleomycin hybridised with TGFβ3 riboprobes. a: sense; b: antisense. Original magnification: x 200. Counterstain: haematoxylin.
Figure 3.27 TGFβ₃ gene expression in murine lung ten days after bleomycin
Figure shows lung tissue ten days after bleomycin hybridised with TGFβ₃ antisense probe. Little or no signal was seen in bronchiolar epithelium (a) or in association with inflammatory foci (b). Original magnification: x 200. Counterstain: haematoxylin.
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</tr>
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</tbody>
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**Table 3.3** Cells expressing TGFβ₁ and TGFβ₃ in normal and fibrotic murine lung

Table shows the cell types identified as expressing mRNA transcripts for TGFβ₁ and TGFβ₃ in normal and fibrotic murine lung. + = detected; - = not detected; +/- detected in some cells only. * inflammatory cells other than alveolar macrophages are likely to be lymphocytes and eosinophils (section 4.3.3.1).
3.6 TGFB$_1$ AND TGFB$_3$ GENE EXPRESSION IN NORMAL HUMAN LUNG

3.6.1 Characteristics of control patients

Sections were taken from two patients, one female aged 59 years with bronchial adenocarcinoma, and one male aged 44 years with mixed small cell carcinoma and adenocarcinoma. Both were smokers, as reflected by the carbon deposits seen in some sections, for example fig 3.28 (a).

3.6.2 Sense and antisense controls for TGFB$_1$ and TGFB$_3$

Sections of normal human lung hybridised with sense (a) and antisense (b) TGFB$_1$ and TGFB$_3$ riboprobes are shown in figures 3.28 and 3.29 respectively. There was good discrimination between sense and antisense probes, but some weak non-specific signal was detected in sections to which the sense probes were applied. Hybridisation signal for TGFB$_1$ was seen in bronchial epithelium and mRNA transcripts for TGFB$_3$ were evident in alveolar macrophages.

3.6.3 Localisation of TGFB$_1$ mRNA transcripts

Figure 3.30 shows further the localisation of hybridisation signal for TGFB$_1$ in normal human lung. It was predominant in alveolar macrophages (arrows in a) as well as in bronchial epithelium, but mRNA transcripts were also detected in alveolar walls (arrowheads in a) and in pulmonary endothelial cells (arrows in b). Some non-specific signal is also seen in fig 3.30 a.

3.6.4 Localisation of TGFB$_3$ mRNA transcripts

Figure 3.31 shows the cell types expressing TGFB$_3$ in normal human lung. The distribution of TGFB$_3$ RNA transcripts was more limited than that of TGFB$_1$, with signal detected only in bronchiolar epithelium (arrows in a) and alveolar macrophages (arrows
in b).

3.6.5 Summary

TGFβ3, as well as TGFβ1 mRNA was present in normal human lung. Gene expression for both isoforms was localised to bronchiolar epithelium and alveolar macrophages. TGFβ1 mRNA transcripts were also identified in alveolar walls and pulmonary endothelial cells.
Figure 3.28 *In situ* hybridisation in normal human lung: sense and antisense controls for TGFβ₁

Figure shows normal human lung hybridised with TGFβ₁ riboprobes. a: sense; b: antisense. Carbon deposits (a) reflect the history of smoking in this patient. Hybridisation signal was evident in bronchial epithelium (arrowed in b). Original magnification: x 200. Counterstain: methyl green.
Figure 3.29 In situ hybridisation in normal human lung: sense and antisense controls for TGFβ3
Figure shows normal human lung hybridised with TGFβ3 riboprobes. a: sense; b: antisense. Hybridisation signal is seen in an alveolar macrophage (arrow in b). Original magnification: x 400 (a); x 1000 (b). Counterstain: methyl green.
Figure 3.30 Cellular localisation of TGFβ; gene expression in normal human lung

Figure shows TGFβ; mRNA transcripts localised to alveolar macrophages (arrows in a) and alveolar walls (arrowheads in a), and pulmonary endothelial cells (b). Original magnification: x 400 (a); x 1000 (b). Counterstain: methyl green (a); haematoxylin (b).
Figure 3.31 Cellular localisation of TGFβ3 gene expression in normal human lung
Figure shows TGFβ3 mRNA transcripts localised to bronchial epithelium (arrows in a) and alveolar macrophages (arrows in b). Original magnification: x 400 (a); x 1000 (b). Counterstain: methyl green.
3.7 TGFβ₁ AND TGFβ₂ GENE EXPRESSION IN FIBROTIC HUMAN LUNG

3.7.1 Patient characteristics

Biopsies were taken from five patients with SSc and three patients with CFA. The patients with SSc were all female and ranged in age from 36 to 55 years. Two of the patients with CFA were male, aged 64 and 66 years, and the third was a female aged 42 years. Three SSc patients had a predominantly cellular pattern and two had a predominantly fibrotic pattern of disease. Two of the three patients with CFA had advanced fibrosis and one had patchy fibrosis.

3.7.2 TGFβ₁ gene expression

Figure 3.32 shows lung tissue from a patient with SSc hybridised with sense (a) or antisense (b) TGFβ₁ riboprobes. Although there was good discrimination between the sense and antisense probes, some non-specific signal was obtained using the sense probe. Loss of the normal alveolar architecture together with a striking inflammatory cell infiltrate was apparent in these sections. Hybridisation signal for TGFβ₁ was seen in association with the inflammatory focus (arrows in b). The accumulation of connective tissue, stained a faint pink by New Fushcin Red, was also evident (arrowheads in b).

There was no difference in the localisation of TGFβ₁ gene expression between patients with SSc and patients with CFA. In comparison with control lung tissue, TGFβ₁ gene expression was slightly increased in two out of five patients with SSc (as an example, compare figs 3.28b and 3.32b). Of these, one showed a predominantly cellular pattern and one had predominant fibrosis. TGFβ₁ gene expression appeared slightly increased in the CFA patient with patchy fibrosis, while in one of the two patients with advanced fibrosis it was unchanged and in the other one hybridisation signal was undetectable.

Figure 3.33 shows TGFβ₁ mRNA transcripts localised to bronchial epithelium (a) and
alveolar macrophages (b). Figure 3.34 also shows TGFβ₁ gene expression in type I alveolar epithelial (a) and mesothelial (b) cells. In contrast, figure 3.38 shows a section taken from a patient with CFA and advanced fibrosis hybridised with the TGFβ₁ antisense probe. No hybridisation signal was detected in sections from this biopsy.
Figure 3.32 *In situ* hybridisation in fibrotic human lung: sense and antisense controls for TGFβ.

Figure shows lung tissue from a patient with SSc hybridised with sense (a) and antisense (b) TGFβ riboprobes. Hybridisation signal is evident in association with an inflammatory cell infiltrate (arrows in b). The faint pink staining of connective tissue is also clearly seen (arrowheads in b). Original magnification: x 400. Counterstain: methyl green.
Figure 3.33 TGFβ₁ gene expression in bronchial epithelium and alveolar macrophages in fibrotic human lung

Figure shows TGFβ₁ mRNA transcripts localised to bronchial epithelium in lung tissue from a patient with SSc (a) and to alveolar macrophages in lung tissue from a patient with CFA (b). No differences were detected in the localisation of TGFβ₁ gene expression between patients with SSc and patients with CFA. Original magnification: x 400. Counterstain: methyl green.
Figure 3.34 TGFβ1, gene expression in alveolar epithelial and mesothelial cells in fibrotic human lung

Figure shows TGFβ1 mRNA transcripts localised to type I alveolar epithelial (arrows in a) and mesothelial (arrows in b) cells in the same patient with SSc. Original magnification: x 400. Counterstain: methyl green.
Figure 3.35 Absence of hybridisation signal for TGFβ₁ in a patient with CFA and advanced fibrosis.
Figure shows lung tissue from a patient with CFA and advanced fibrosis hybridised with the TGFβ₁ antisense probe. No hybridisation signal was detected in sections examined from this biopsy. Original magnification: x 400. Counterstain: methyl green.
3.7.3  TGFβ3 gene expression

Figure 3.36 shows lung tissue from a patient with SSc hybridised with sense (a) or antisense (b) TGFβ3 riboprobes. As for TGFβ1, some weak non-specific signal was obtained with the sense probe. TGFβ1 and TGFβ3 gene expression co-localised in fibrotic human lung. This is illustrated by comparison of figs 3.32 (b) and 3.36 (b) which represent consecutive sections.

There was no difference in the localisation of TGFβ3 gene expression between patients with SSc and patients with CFA. In comparison with control tissue, TGFβ3 gene expression was not increased in any of the patients with SSc. In one patient with a predominantly cellular pattern it appeared reduced, in one patient with predominant fibrosis it was undetectable and in three it appeared unchanged. TGFβ3 gene expression appeared slightly increased in the CFA patient with patchy fibrosis, while in one patient with advanced fibrosis it was unchanged and in the other with advanced fibrosis hybridisation signal was undetectable. These changes paralleled those for TGFβ1 described above.

Figure 3.37 shows TGFβ3 gene expression localised to bronchial epithelium (a) and mesothelial cells (b). TGFβ3 gene expression was also noted in alveolar macrophages and alveolar type II cells, as illustrated in figure 3.38. Figure 3.39 shows lung tissue from a patient with CFA and advanced fibrosis hybridised with the TGFβ3 antisense probe. No hybridisation signal was detected in sections from this biopsy.

3.7.4  Summary

TGFβ3 as well as TGFβ1 mRNA transcripts were detected in fibrotic human lung and the distribution of the transcripts appeared identical. There was no difference in the localisation of TGFβ1 and TGFβ3 gene expression between patients with SSc and CFA. Table 3.4 summarises the lung cell types expressing TGFβ1 and TGFβ3 in normal and fibrotic human lung. TGFβ1 and TGFβ3 mRNA transcripts were localised to bronchial epithelium, alveolar macrophages, mesothelial and alveolar epithelial cells. Gene
expression for both isoforms was predominantly localised to areas of mild fibrosis, regions of dense acellular fibrosis being characterised by a paucity or complete absence of hybridisation signal.

Table 3.5 summarises the quantitative changes seen in gene expression for both isoforms in patients with SSc and CFA. TGFβ₁ gene expression was slightly increased in two out of five patients with SSc, and in one of three patients with CFA. In one patient with CFA mRNA transcripts for TGFβ₁ were undetectable. In the remaining four patients TGFβ₁ gene expression appeared to be unchanged.

TGFβ₃ gene expression was increased in one of the three patients with CFA but was not increased in any of the patients with SSc. In one patient with SSc it was reduced and in another it was undetectable. In one patient with CFA mRNA transcripts for TGFβ₃ were undetectable. In the remaining four patients TGFβ₃ gene expression appeared to be unchanged.

Changes in isoform gene expression relative to the histological pattern can be summarised as follows. In the two patients with a predominant cellular pattern, TGFβ₁ gene expression was increased in one and was unchanged in the other. TGFβ₃ gene expression was decreased in one and unchanged in the other. In the patient with a mixed fibrotic and cellular pattern, gene expression for both isoforms was increased. In the remaining five patients with a predominant fibrotic pattern, gene expression for both isoforms was either unchanged or undetectable, with the exception of one patient where TGFβ₁ gene expression was enhanced.
Figure 3.36 *In situ* hybridisation in fibrotic human lung: sense and antisense controls for TGFβ3

Figure shows lung tissue from a patient with SSc hybridised with sense (a) and antisense (b) TGFβ3 riboprobes. Hybridisation signal is seen in association with an inflammatory cell infiltrate (arrows in b). The faint pink staining of connective tissue is also clearly seen. Original magnification: x 400. Counterstain: methyl green.
Figure 3.37 TGFβ3 gene expression in bronchial epithelium and mesothelial cells in fibrotic human lung.
Figure shows TGFβ3 mRNA transcripts localised to bronchial epithelium (a) and mesothelial cells (b) in lung tissue from a patient with SSc. Original magnification: x 400. Counterstain: methyl green.
Figure 3.38 TGFβ3 gene expression in alveolar macrophages and alveolar epithelial cells in fibrotic human lung

Figure shows hybridisation signal for TGFβ3 in intra-alveolar macrophages (arrowheads) and in cuboidal type II alveolar epithelial cells lining the alveolar surface (arrows). This lung tissue was taken from a patient with SSc.
Figure 3.39 Absence of hybridisation signal for TGFβ3 in a patient with CFA and advanced fibrosis.

Figure shows lung tissue from a patient with CFA and advanced fibrosis hybridised with the TGFβ3 antisense probe. No hybridisation signal was detected in sections examined from this biopsy. Original magnification: x 400. Counterstain: methyl green.
### Table 3.4 Cells expressing TGFβ₁ and TGFβ₃ in normal and fibrotic human lung

Table shows the cell types identified as expressing mRNA transcripts for TGFβ₁ and TGFβ₃ in human lung. + = detected; - = not detected. There was no difference in localisation between SSc and CFA.

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**NUMBER OF SAMPLES WITH INCREASED SIGNAL**

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**Table 3.5 Quantitative changes in TGFβ₁ and TGFβ₃ gene expression in fibrotic human lung**

Table summarises the quantitative changes in TGFβ₁ and TGFβ₃ gene expression in patients with SSc and CFA. Histology is summarised as being predominantly fibrotic, predominantly cellular or mixed (both fibrotic and cellular areas seen). ↑ = increased gene expression; ↓ = decreased; → = unchanged compared with control human lung tissue. - = not detected.
3.8 IN SITU HYBRIDISATION WITH TGFβ2 RIBOPROBES

3.8.1 Normal murine lung

Figure 3.40 shows the results of in situ hybridisation in normal murine lung with sense (a) and antisense (b) TGFβ2 riboprobes. Unexpected results were obtained using the TGFβ2 probes. Hybridisation with the sense probe consistently yielded positive signal, while hybridisation with the antisense probe yielded little or no signal. These results were obtained on two separate occasions and with two different batches of freshly prepared probe. The distribution of hybridisation signal obtained using the TGFβ2 sense probe was similar to that observed with the TGFβ1 and TGFβ3 antisense probes, with signal predominant in bronchiolar epithelium and alveolar macrophages.

3.8.2 Bleomycin-induced murine lung fibrosis

Similar results were obtained in mice following bleomycin, although the intensity of hybridisation signal was variable. Figure 3.41 shows results of in situ hybridisation in murine lung ten days after bleomycin using sense (a) and antisense (b) TGFβ2 riboprobes. At this time the difference in hybridisation signal obtained using the two probes was less striking, but signal was still evident in inflammatory cells (arrows in a).

Figure 3.42 shows results of in situ hybridisation in murine lung 21 days after bleomycin using sense (a) and antisense (b) TGFβ2 riboprobes. At this time the difference in signal obtained using the two probes was marked. Hybridisation signal was localised to interstitial cells within developing fibrotic foci (arrows in a).
Figure 3.40 *In situ* hybridisation in normal murine lung: sense and antisense controls for TGFβ

Figure shows normal murine lung hybridised with TGFβ riboprobes. a: sense; b: antisense. Original magnification: x 100. Counterstain: methyl green. The results obtained using these probes were unexpected. Hybridisation with sense probe produced positive signal but hybridisation with antisense probe yielded little or no signal. Hybridisation signal obtained with the sense probe was predominant in bronchiolar epithelium and alveolar macrophages.
Figure 3.41 *In situ* hybridisation with TGFβ₂ probes in murine lung ten days after bleomycin

Figure shows murine lung ten days after bleomycin hybridised with sense (a) and antisense (b) TGFβ₂ riboprobes. Positive signal, localised to inflammatory cells, was obtained with sense probe (arrows in a). No signal was obtained with antisense probe. Original magnification: x 200. Counterstain: haematoxylin.
Figure 3.42 *In situ* hybridisation with TGFβ2 probes in murine lung 21 days after bleomycin

Figure shows murine lung 21 days after bleomycin hybridised with sense (a) and antisense (b) TGFβ2 riboprobes. Positive signal obtained with sense probe was localised to interstitial cells within developing fibrotic foci (arrows in a). No signal was obtained with antisense probe. Original magnification: x 200. Counterstain: methyl green.
3.8.3 Normal rat lung

In order to determine whether this phenomenon was species-dependent, *in situ* hybridisation with the TGFβ2 riboprobes was repeated in normal rat lung. Similar results were obtained although the difference in hybridisation signal between the two probes was less marked than in normal murine lung. Figure 3.43 shows the results obtained with sense (a) and antisense (b) probes. Hybridisation signal was localised to bronchial epithelium and alveolar macrophages.

3.8.4 Normal human lung

*In situ* hybridisation was also performed in normal human lung. As in murine and rat lung, hybridisation signal was obtained with sense probe but little was obtained with antisense probe. The difference in hybridisation signal was once again less marked than in murine lung. Figure 3.44 shows the results obtained using sense (a) and antisense (b) probes. Hybridisation signal was localised to alveolar macrophages.

3.8.5 Summary

*In situ* hybridisation studies using the TGFβ2 riboprobes yielded unexpected results. Use of the sense probe yielded positive signal, while little or no signal was obtained using the antisense probe. These results were confirmed on two separate occasions and with two different, freshly prepared batches of probe. The difference in hybridisation signal between the two probes was most marked in normal murine lung and 21 days after bleomycin, but it was also detectable ten days after bleomycin, and in normal rat and human lung.
Figure 3.43 In situ hybridisation with TGFβ₂ probes in normal rat lung
Figure shows normal rat lung hybridised with sense (a) and antisense (b) TGFβ₂ riboprobes. Hybridisation with sense probe yielded signal localised to bronchiolar epithelium and alveolar macrophages. Some signal was also obtained using antisense probe. Original magnification: x 200. Counterstain: methyl green.
Figure 3.44 *In situ* hybridisation with TGFβ2 probes in normal human lung

Figure shows normal human lung hybridised with sense (a) and antisense (b) TGFβ2 riboprobes. Hybridisation with sense probe yielded positive signal localised to alveolar macrophages. Little signal was obtained using antisense probe. Original magnification: x 200. Counterstain: haematoxylin.
Having obtained unexpected results using the TGFβ₂ riboprobes for *in situ* hybridisation studies, possible interpretations were investigated. The first explanation explored was that the probe might be inserted in the vector in the opposite orientation to that predicted. In this case the sequences of the sense and antisense probes would be reversed, and this would be expected to generate the results described above. This possibility was investigated by asymmetric restriction enzyme mapping (section 3.9.1) and sequencing (section 3.9.2).

### 3.9.1 Determination of TGFβ₂ probe orientation by asymmetric restriction enzyme mapping

Figure 3.45 shows electrophoresis of pm TGFβ₂-9A (lane 2) following asymmetric restriction enzyme digest with *Cla I*, with a 100 bp DNA ladder for comparison (lane 1). Size markers (bp) are given on the left. A fragment of approximately 259 bp length was produced following restriction digest, indicating that the orientation of the probe in the vector was as originally predicted (see figure 2.7). Incorrect orientation would have generated a fragment of approximately 183 bp.

### 3.9.2 Determination of TGFβ₂ sense riboprobe sequence

The results obtained above were verified by sequencing. Sequencing was also performed in order to exclude the possibility that the TGFβ₂ sense riboprobe was hybridising with an unrelated mRNA species. Figure 3.46 shows the sequence homology obtained between the TGFβ₂ sense probe and mRNA for murine TGFβ₂, using data derived from the FASTA database searching programme (Human Genome Mapping Project CRC, Cambridge, UK). The percent identity was 86.301.

### 3.9.3 Assessment of digoxigenin incorporation into TGFβ₂ riboprobes

Assessment of digoxigenin incorporation into the TGFβ₂ sense and antisense riboprobes
was performed in order to exclude the possibility that signal obtained with the sense but
not the antisense probe was the result of disproportionately greater digoxigenin
incorporation into the sense probe.

Figure 3.47 shows electrophoresis of the digoxigenin-labelled TGFβ₂ sense and
antisense riboprobes. Size markers (kb) are given on the right. A single discrete band
was obtained for each probe. The probes were of equal size and were 442 bp in length
as predicted.

Figure 3.48 shows the results of chemiluminescence assay of digoxigenin incorporation
into the TGFβ₂ sense and antisense probes (upper panel). The aliquots analysed were
from the same sample as those electrophoresed in figure 3.47. Densitometry of the
blots obtained, corrected for ethidium bromide staining, is shown in the lower panel.
The mean ratio of digoxigenin incorporation into the antisense compared with the sense
probe, corrected for ethidium bromide staining on electrophoresis, was 1.3:1.
Figure 3.45 Cla I restriction digest of pm TGFβ2-9A
Figure shows electrophoresis of pm TGFβ2-9A following asymmetric restriction digest with Cla I. Size markers (bp) are shown on the left. Lane 1: 100 bp DNA ladder; lane 2: pm TGFβ2-9A following Cla I digest. A fragment nearly 300 bp in size (arrow) is seen at the leading edge of the gel.
Figure 3.46 Dideoxynucleotide sequencing of pm TGFβ2-9A
Figure shows the sequence homology between the TGFβ2 sense probe (lower strand) and mRNA for murine TGFβ2 (upper strand). Data was derived from the FASTA database searching programme (Human Genome Mapping Project CRC, Cambridge, UK). Percent identity: 86.301.
Figure 3.47 Electrophoresis of TGFβ₂ riboprobes
Figure shows electrophoresis of digoxigenin-labelled TGFβ₂ sense and antisense riboprobes. Size markers (kb) are shown on the right. Lane 1: TGFβ₂ antisense probe; lane 2: TGFβ₂ sense probe. A single discrete band was obtained for each probe, which were of equal length (442 bp).
Figure 3.48 Digoxigenin incorporation into TGFβ, riboprobes.
Upper panel shows chemiluminescence assay of digoxigenin incorporation into the TGFβ sense and antisense riboprobes. Serial dilutions range from 1000 to 1 pg.

Lower panel shows mean densitometry of each dilution (expressed as optical density x area). The mean ratio of digoxigenin labelling of the antisense to the sense probe, corrected for ethidium bromide staining on electrophoresis, was 1.3:1.
3.9.4 Summary

The possibility that probe reversal within the vector could have produced the unexpected results obtained with the TGFβ2 riboprobes was excluded following verification of probe orientation within the vector by two independent methods. The possibility that the sense probe was hybridising with an unrelated species was excluded as far as possible by sequencing. That disproportionately greater digoxigenin labelling of the sense probe with respect to the antisense probe could have been responsible for these results was excluded by chemiluminescence assay and densitometry. The nature of the mRNA transcript with which the TGFβ2 sense riboprobe was hybridising was therefore investigated further by Northern analysis. The results are described in the next section.

3.10 IDENTIFICATION OF TRANSCRIPTS DETECTED BY THE TGFβ2 SENSE RIBOPROBE

3.10.1 Identification of a transcript in rat lung

Figure 3.49 shows results of Northern analysis of 10 μg of poly A+ RNA from rat lung using TGFβ2 antisense (lane 1) or TGFβ2 sense (lane 2) riboprobes. The antisense probe detected four transcripts of 4, 5, 5.9 and 6.9 kb each. The sense probe detected one 3.5 kb transcript, the optical density of which exceeded that of the other four transcripts.

3.10.2 Identification of a transcript in murine mesothelioma cells

Results obtained were similar to those obtained for rat lung and are shown in fig 3.50. The antisense probe detected four transcripts of 3.7, 4.8, 5.8 and 6.7 kb each. The sense probe detected one 3.6 kb transcript. Its optical density exceeded that of the other four transcripts.
3.10.3 Summary

The TGFβ2 antisense riboprobe hybridised with multiple transcripts ranging from 3.7 to 6.9 kb in rat lung and murine mesothelioma cells. In both species the sense probe hybridised with a single but prominent transcript.
Figure 3.49 Northern analysis in rat lung with digoxigenin-labelled TGFβ2 riboprobes

Upper panel shows the results of hybridisation of 10 μg of poly A+ RNA from rat lung with antisense (lane 1) or sense (lane 2) TGFβ2 riboprobe. Hybridised probe was detected by chemiluminescence assay. The antisense probe detected four mRNA transcripts. The sense probe detected one 3.5 kb transcript whose peak optical density was more than double that of the other four transcripts.

Lower panel shows the densitometry of each transcript (expressed as optical density x area).
Transcript size (kb) | 3.6 | 3.7 | 4.8 | 5.8 | 6.7  
--- | --- | --- | --- | --- | ---
Optical density   | 1.616 | 0.343 | 0.990 | 0.640 | 0.134

Figure 3.50 Northern analysis in murine mesothelioma cells with digoxigenin-labelled TGFβ₂ riboprobes

Upper panel shows the results of hybridisation of 10 µg of poly A⁺ RNA from murine mesothelioma cells with antisense (lane 1) or sense (lane 2) TGFβ₂ riboprobe. Hybridised probe was detected by chemiluminescence assay. The antisense probe detected four mRNA transcripts. The sense probe detected one 3.6 kb transcript whose peak optical density was twice that of the other four transcripts.

Lower panel shows the densitometry of each transcript (expressed as optical density x area).
3.11 PRELIMINARY STUDIES: EFFECT OF TGFβ₁ ANTISENSE ON MESOTHELIOMA CELL GROWTH AND TGFβ₁ SECRETION

3.11.1 Murine mesothelioma cell morphology and cell number

Figure 3.51 shows normal murine mesothelioma (AC29) cells in culture (a) compared with AC29 cells after 24 hrs' incubation with 10 μM of TGFβ₁ antisense (b). Cells (unstained) were photographed using an inverted phase-contrast light microscope. Untreated cells remained confluent while many of those incubated with antisense rounded up and detached from the culture plate, resulting in a non-confluent culture pattern. Similar but less striking changes were seen after incubation with 1 and 5 μM of TGFβ₁ antisense.

Figure 3.52 shows the effect of 1, 5 and 10 μM of TGFβ₁ antisense on AC29 cell number over 24 hrs. Total cell count at t₀ was 8.26 x 10⁶, and all three concentrations inhibited cell growth. No statistical analysis was performed on these preliminary data obtained from one experiment.

3.11.2 Murine mesothelioma cell TGFβ₁ production

Figure 3.53 shows the effect of 1, 5 and 10 μM of TGFβ₁ antisense on AC29 cell TGFβ₁ production as measured by ELISA. All concentrations reduced latent TGFβ₁ production when corrected for cell number. 1 and 10 μM antisense also reduced active TGFβ₁ production.

3.11.3 Summary

A 20 mer TGFβ₁ antisense oligonucleotide reduced cell number and TGFβ₁ production at concentrations of between 1 and 10 μM when incubated for 24 hrs with AC29 cells. Cell morphology was also strikingly altered.
Figure 3.51 Morphology of murine mesothelioma (AC29) cells
Figure shows normal morphology of confluent murine mesothelioma (AC29) cells (a) compared with that following 24 hrs' incubation with 10 μM of TGFβ1 antisense (b). Cells were unstained and photographed using an inverted phase-contrast light microscope. Following incubation with antisense many cells became rounded and detached from the plate.
Figure 3.52 Effect of TGFβ₁ antisense on AC29 cell number
Figure shows the effect of TGFβ₁ antisense (5'-GAA GCA ATA GTT GGT GTC CAG-3') on cell number. AC29 cells were incubated with antisense for 24 hr. Each value represents a single culture in one experiment.
Figure 3.53 Effect of TGFβ1 antisense on TGFβ1 production
Figure shows the reduction in latent (solid bars) and active (hatched bars) TGFβ1 production by AC29 cells following incubation with TGFβ1 antisense (5'-GAA GCA ATA GTT GGT GTC CA-3') for 24 hr. TGFβ1 in conditioned medium was measured by ELISA. Each value represents a single culture in one experiment.
CHAPTER FOUR

DISCUSSION
IN VITRO STUDIES

4.1 THE EFFECT OF TGFβ ISOFORMS ON PROCOLLAGEN METABOLISM

The initial phase of this study examined the effects of TGFβ₁,₂ on fibroblast procollagen synthesis and degradation. The results demonstrate that TGFβ₂ and TGFβ₃ share the ability of TGFβ₁ to promote collagen deposition by stimulating fibroblast procollagen synthesis. This is consistent with their high degree of homology and previous assays demonstrating similar biological effects (Seyedin et al 1985; Ohta et al 1987; Graycar et al 1989; ten Dijke et al 1990a).

In the experiment shown in fig 3.3 the basal level of fibroblast procollagen production was lower than that for experiments with TGFβ₁ and TGFβ₃, but there was a clear increase in response to TGFβ₂. There is some variation in the basal rate of procollagen production between experiments which is in part accounted for by cells being of different passage number. The experiments shown in figs 3.2-3.4 were designed to demonstrate a dose response relationship between each of the TGFβ isoforms and procollagen production, and to determine concentrations which would produce maximal effects. It was not designed to demonstrate absolute rates of production. It was felt, therefore, that it was inappropriate to repeat dose-response experiments continually merely to obtain a set of data with similar basal rates, especially as these studies are extremely time-consuming.

The dose-response relationship studies show that TGFβ₃ was ten times more potent than the other two isoforms in stimulating procollagen production. Furthermore, TGFβ₃ was the only isoform to reduce intracellular procollagen degradation. These findings are consistent with the observation that TGFβ₃ is more potent than TGFβ₁ in stimulating collagen production by foetal rat bone cells (ten Dijke et al 1990a), and may reflect differing receptor affinities for TGFβ₁,₂. Previous studies in this department have shown that TGFβ₁ reduces intracellular procollagen degradation by foetal rat fibroblasts (McAnulty et al 1991a). That TGFβ₁ did not reduce HFL-1 procollagen degradation
significantly may reflect the fact that the control level of procollagen degradation in these cells was relatively low (22%) compared with that in foetal rat fibroblasts (38%). The low baseline degradation rate could make any reduction difficult to assess.

**IN VIVO STUDIES**

4.2 USE OF DIGOXGENIN-LABELLED RIBOPROBES

4.2.1 Previous *in situ* hybridisation studies

To date few studies have employed digoxigenin-labelled probes for *in situ* hybridisation in the respiratory tract. At the time of developing this method, there were only two reports of the use of digoxigenin-labelled riboprobes, both to detect cytokine gene expression in nasal biopsy tissue (Ying et al 1993; Ying et al 1994). Four studies in lung tissue had used digoxigenin-labelled probes (Durham et al 1993; Fukuda et al 1994; Black et al 1993; Morey et al 1992) but all employed oligomers or DNA probes. One study, using digoxigenin-labelled riboprobes to detect gene expression in pulmonary interstitium, has been published since then (Santana et al 1995), but the results shown demonstrate faint hybridisation signal, possible reasons being discussed later (section 4.2.3). The protocols developed during this thesis therefore represent the optimum technique currently available for this particular application. Its advantages are discussed below.

4.2.2 Advantages over radiolabelled probes

The speed of execution is a major advantage of this method, since the procedure presented here can be completed within 48 hours. In contrast, isotopic methods require generation of fresh radiolabelled probe for each experiment and time-consuming autoradiographic detection techniques, lasting between weeks and months for isotopes such as $^3$H which provide the greatest degree of resolution. The data presented here show that digoxigenin incorporation is predictable and synthesis of digoxigenin-labelled riboprobes efficient, with up to 10 μg of digoxigenin-labelled riboprobe being
synthesised from 1 μg of starting template. Handling of non-toxic reagents is simpler and safer, and waste disposal is greatly facilitated. Long-term storage of probe, for at least two years, is also possible using this method.

A further advantage is enhanced tissue resolution. Comparison of my results with previous in situ hybridisation studies of pulmonary gene expression (Pelton et al 1991a; Shahzeidi et al 1993; Shahzeidi et al 1994; Zhang et al 1995) suggests that mRNA transcripts can be localised more precisely and sensitively in lung tissue using a non-radioactive technique. In the past, digoxigenin-labelled probes have been shown to be less sensitive or only equally sensitive as radiolabelled probes. This probably reflects the fact that until recently it was only possible to generate end-labelled probes. Only one anti-digoxigenin antibody will bind to each end-labelled probe. The number of antibody molecules which bind to probes labelled along their entire length is greater, and hybridisation signal correspondingly enhanced. The sensitivity of this method is discussed in more detail below (sections 4.2.3 and 4.3.1).

Little non-specific signal was observed, as shown by the cytoplasmic nature of the hybridisation signal and the high degree of cellular resolution obtained. This is an important feature because high levels of non-specific binding seen with radiolabelled probes may confound identification of labelled cells. The Dako New Fushcin reagent provides a readily identifiable colour signal and has the advantage, in the setting of fibrosis, of staining extracellular matrix proteins in a way that is distinguishable from hybridisation signal. Its other advantage is that it is relatively permanent. Preliminary experiments with alternative alkaline phosphatase substrates yielded colours which faded and became granular in appearance with time (unpublished observations). Finally, the use of non-isotopically labelled probes will in future enable immunohistochemistry to be performed on the same sections, or simultaneous detection of more than one nucleic acid target using different probe labels.

4.2.3 Advantages over biotinylated probes

The digoxigenin system offers important advantages over the other non-isotopic
technique available, which involves labelling with biotin. Earlier comparisons of
digoxigenin with biotin labelling have revealed that the latter more frequently generates
false positive signal (Morris et al 1990; Morey et al 1992) and is up to 10 times less
sensitive (Morris et al 1990; Furuta et al 1990; Egger et al 1994). Biotin-labelled
probes may also yield false positive results because of the presence of endogenous
biotin in certain tissues, such as mitochondria. Since digoxigenin is unique to Digitalis,
such false positive results do not arise.

4.2.4 Probe hydrolysis

Hydrolysis of probes to 50 to 500 bp is sometimes recommended for in situ hyridisation
(Tautz et al 1992), shorter probes being thought to penetrate more easily into cross-
linked tissues than longer ones. In these studies signal was not enhanced by probe
hydrolysis, suggesting that if lung tissue is sufficiently permeabilised, hydrolysis of
probes not exceeding 1000bp is unnecessary. The longer the probe, the greater the
number of digoxigenin molecules incorporated. Anti-digoxigenin antibody binding is
correspondingly amplified and signal intensity enhanced. Given these findings, I would
recommend that the longest probes which can reproducibly penetrate the tissue are used
to achieve maximum sensitivity. Digoxigenin-labelled oligonucleotides may
consequently not be appropriate for detecting mRNA transcripts present in low copy
number in lung tissue.

4.2.5 Use in Northern analysis

Digoxigenin-labelled riboprobes were found to be equally valuable for Northern
analysis. Northern analysis with 32P labelled cDNA probes was found to be relatively
insensitive for detection of TGFβ mRNA in lung tissue. The time required for
autoradiography therefore extended to several weeks and frequently necessitated the use
of phosphoimaging. In contrast, the chemiluminescent detection method adopted
following Northern analysis with digoxigenin-labelled riboprobes generated signal after
only 10 mins exposure at room temperature. These findings confirm those of a
previous study in which the chemiluminescent detection system was found to afford
equivalent sensitivity to the $^{32}$P system (Puchhammer-Stoeckl et al 1993) and illustrate vividly the speed of this technique.

4.2.6 Summary

At the time this method was developed, digoxigenin-labelled riboprobes had not been used previously to study cytokine gene expression in pulmonary interstitium, and the results presented demonstrate that this technique offers numerous advantages over other existing methods, including speed, reproducibility, safety, sensitivity and specificity. Using this method, riboprobe hydrolysis was not found necessary. The method was equally useful for Northern analysis. The advantages of enhanced sensitivity and specificity conferred by the use of digoxigenin-labelled riboprobes for in situ hybridisation are illustrated further by the findings discussed in detail below.

4.3 IN SITU HYBRIDISATION IN MURINE LUNG

4.3.1 TGF$\beta_1$ and TGF$\beta_3$ expression in normal lung

I have shown that mRNA transcripts for TGF$\beta_1$ and TGF$\beta_3$ are present in adult murine lung and predominantly localised to bronchiolar epithelial cells, including Clara cells, and alveolar macrophages. They are also present in mesenchymal, mesothelial and alveolar lining cells. TGF$\beta_1$ mRNA transcripts were also observed in pulmonary endothelial cells. Relatively few large blood vessels were present in the sections of mouse lung examined, which may account for TGF$\beta_3$ mRNA transcripts not being detected in pulmonary endothelium. With this exception, TGF$\beta_1$ and TGF$\beta_3$ mRNA transcripts co-localised in normal murine lung.

In an earlier study, Pelton and colleagues used $^{35}$S labelled probes to localise TGF$\beta$ isoform gene expression in murine lung (Pelton et al 1991a). They demonstrated TGF$\beta_1$ and TGF$\beta_3$ gene expression in mesenchymal cells underlying bronchiolar epithelium, but did not detect signal in bronchiolar epithelial, alveolar or endothelial cells. A study in sheep lung also failed to demonstrate TGF$\beta$ mRNA transcripts in
bronchiolar epithelium (Perkett et al 1994). Both these studies used riboprobes of identical sequence to mine. A study of TGFβ₁ gene expression during bleomycin-induced lung injury in rats using a radiolabelled 30-mer oligonucleotide probe failed to demonstrate any significant gene expression in control animals (Zhang et al 1995).

Endothelial cells express TGFβ₁ mRNA and secrete TGFβ₁ in vitro (Phan et al 1992), but TGFβ₁ gene expression by endothelial cells in vivo has not to my knowledge been reported before. Taken together, these data suggest that digoxigenin-labelled riboprobes may be more sensitive than ³⁵S-labeled riboprobes. Certainly a recent report showed that digoxigenin-labelled riboprobes are at least as sensitive as ³⁵S-labelled riboprobes when used to detect interleukins in T lymphocytes (Karr et al 1995).

Cultured bronchial epithelial cells secrete TGFβ (Sacco et al 1992) and immunostaining shows the TGFβ isoforms to be preferentially localised in bronchiolar epithelial cells in murine lung (Pelton et al 1991b). These authors suggested that the presence of TGFβ protein but not mRNA transcripts in bronchiolar epithelium indicated a paracrine mode of action for this peptide. I propose instead that gene expression and synthesis of TGFβ₁ and TGFβ₃ are co-localised in bronchiolar epithelial cells, permitting autocrine as well as paracrine modes of action.

4.3.2 Functions of TGFβ₁ and TGFβ₃ in normal lung

The wide variety of cells in the lung expressing the TGFβ₁ and TGFβ₃ genes supports the hypothesis that these peptides have important roles in normal lung homeostasis. As discussed in the introduction, multiple in vitro biological effects are ascribed to TGFβ₁, including regulation of cell proliferation, immune response and extracellular matrix metabolism. TGFβ₁ inhibits epithelial and endothelial cell proliferation in vitro, and transformed bronchial epithelial cells are characterised by a loss of sensitivity to growth inhibition by TGFβ₁ (Fynan, Reiss, 1993). In normal mature lung TGFβ₁ and TGFβ₃ may therefore act in autocrine fashion in bronchiolar epithelial and pulmonary endothelial cells to maintain physiological homeostasis by regulating cell proliferation and subsequent differentiation. Clara cells can participate in epithelial repair by
division and differentiation (Evans et al 1978), and type II cells perform a similar function in the alveoli (Adamson, Bowden, 1974a), thus possibly explaining why these cell types express TGFβ.

As previously outlined (section 1.5.4.1), TGFβ probably plays a role in normal immune defence. Activated human macrophages express TGFβ1 mRNA (Assoian et al 1987) and constitutive TGFβ1 gene expression by alveolar macrophages in normal human lung has been documented (Shaw et al 1991). This may reflect continuous macrophage activation, which is also exhibited by non-specific pathogen-free mice. In view of the various effects of TGFβ1 on macrophage function, TGFβ gene expression by macrophages in normal lung may indicate a vital role in preventing tissue injury following repeated exposure to inhaled irritants and pathogens. This is also supported by the findings in animals in which TGFβ1 expression is compromised (Shull et al 1992; Kulkarni, Karlsson, 1993). In bronchiolar epithelial cells TGFβ may have a further immunomodulatory role consistent with the ability of TGFβ1 to stimulate IgA secretion by B lymphocytes (van Vlasselaer et al 1992). Finally, since TGFβ is also a potent stimulator of extracellular matrix protein synthesis, TGFβ in bronchiolar epithelial, pulmonary endothelial, mesothelial and mesenchymal cells may play a further role in regulating extracellular matrix metabolism.

4.3.3 TGFβ1 and TGFβ3 during development of bleomycin-induced pulmonary fibrosis

4.3.3.1 Altered localisation of TGFβ1 expression

A striking finding ten days following bleomycin was the shift from predominant signal in airway epithelium of normal animals to principally interstitial signal. This may reflect a combination of epithelial cell damage, inflammatory cell influx and increased TGFβ1 gene expression by interstitial cells including fibroblasts. It was not seen in control animals, indicating that it was specific to bleomycin injury. Increased TGFβ1 gene expression by pulmonary interstitial cells has been reported in rats three to 14 days after bleomycin-induced injury (Zhang et al 1995). Following bleomycin, TGFβ1 gene expression was mainly localised to inflammatory cells. Seven to 14 days after
bleomycin in rats, the majority of inflammatory cells expressing TGFβ₁ have been identified as eosinophils, macrophages and lymphocytes (Zhang et al 1995). Macrophages are recognised as an important source of TGFβ₁ in pulmonary fibrosis (Khalil et al 1989; Broekelmann et al 1991). However, my results demonstrate that signal in mesenchymal, pulmonary endothelial and mesothelial cells was also enhanced after bleomycin. Furthermore, the generalised increase in interstitial signal suggests that alveolar epithelial and microvascular endothelial cells were also expressing TGFβ₁.

### 4.3.3.2 Mesenchymal cell TGFβ₁ expression

Mesenchymal cells expressing TGFβ₁ may be fibroblasts, myofibroblasts or smooth muscle cells. Zhang’s study identified significant numbers of myofibroblasts, staining strongly for α-smooth muscle actin, expressing TGFβ₁ after bleomycin (Zhang et al 1995). Lung fibroblasts produce TGFβ₁ in vitro (Kelley et al 1991) and auto-induction can occur (Kelley et al 1993), probably mediated by AP-1 binding to the promoter (Kim et al 1990a). Fibroblast and endothelial cell TGFβ₁ gene expression increases after in vitro bleomycin exposure (Phan et al 1991b; Cutroneo et al 1991), and TGFβ₁ augments endothelial matrix protein synthesis (Müller et al 1987). In the light of this evidence, my data suggest that increased TGFβ₁ gene expression by mesenchymal cells, pulmonary and capillary endothelial cells after bleomycin administration contributes to interstitial matrix accumulation.

### 4.3.3.3 Mesothelial cell TGFβ₁ expression

Cultured mesothelial cells express TGFβ₁ mRNA (Gerwin et al 1987; Bermudez et al 1990) and protein (Offner et al 1996) but to my knowledge this is the first study to show this in vivo. Mesothelial cells have not been shown to express TGFβ₁ mRNA or protein previously. Earlier studies from this department have demonstrated procollagen gene expression in the subpleural region of fibrotic mouse lung (Shahzeidi et al 1993; Shahzeidi et al 1994). Mesothelial cells may thus participate in the pathogenesis of pulmonary fibrosis by producing TGFβ₁ which stimulates subpleural fibroblast collagen synthesis. TGFβ₁ also stimulates mesothelial cell proliferation in vitro (Franzen, Dahlquist, 1994; Gabrielson et al 1988; Mutsaers et al 1996). TGFβ₁ produced by these cells may therefore also act in autocrine fashion to stimulate their replication after
injury. Previous evidence suggests that mesothelial cells actively participate in repair following pleural injury (Davila, M., Crouch, 1993; Owens, Grimes, 1993; Adamson et al 1994). These findings suggest that mesothelial cells also respond to paracrine mediators, including TGFβ1, released as a consequence of proximal lung injury.

4.3.3.4 Type II cell TGFβ1 expression

Cells lining alveolar walls expressing TGFβ1 may be alveolar type II cells or adherent macrophages. Bleomycin induces proliferation and metaplasia of type II cells (Adamson, Bowden, 1979). However, TGFβ1,3 inhibit alveolar type II cell replication and TGFβ secretion by these cells after bleomycin, as measured by bioassay, is inversely related to their proliferation (Khalil et al 1994). TGFβ1 production by metaplastic type II cells may therefore act in autocrine manner to regulate their proliferation and differentiation following injury.

4.3.3.5 Time course of TGFβ1 expression

Following bleomycin, TGFβ1 gene expression increased to a maximum at ten days. The intensity of signal then declined to that seen in control animals by 35 days. At 21 days hybridisation signal was predominant in fibrotic foci within the interstitium, but by 35 days the pattern of TGFβ1 gene expression had returned to that seen in control animals, with mRNA transcripts predominant in bronchial epithelium and in alveolar macrophages. The time course for TGFβ1 gene expression observed in this study is similar to that observed by Zhang and colleagues (Zhang et al 1995) and earlier studies which quantified TGFβ1 mRNA by Northern analysis (Hoyt, Lazo, 1988; Raghow et al 1989; Phan, Kunkel, 1992).

4.3.3.6 TGFβ3 expression

In contrast to TGFβ1, TGFβ3 gene expression was not enhanced ten days after bleomycin nor associated with macrophage influx. Little signal was detected in bronchiolar epithelium or inflammatory cells following bleomycin, either at three, ten or 21 days. 35 days after bleomycin the pattern and intensity of TGFβ3 gene expression was indistinguishable from that seen in control animals.
Gene expression of TGFβ₁ and TGFβ₃ is therefore differentially regulated. The basis for differential gene expression is probably structural differences between the TGFβ₁ and TGFβ₃ promoters. The TGFβ₁ gene lacks a TATA box but includes a GC-rich region containing several Sp1 binding sites just upstream of the first transcriptional start site (Kim et al 1989b). AP-1 sites, located upstream of each of the two distinct transcriptional start sites, bind the jun/fos protein complex and are the main positive regulatory sequences involved in the upregulation of TGFβ₁ gene expression (Kim et al 1989c; Kim et al 1990a; Kim et al 1990b; Kim et al 1989a).

In contrast, the TGFβ₃ contains a TATA box 21 bp upstream of the transcription start site but no AP-1 sites (Potts et al 1991). Transcriptional control of both the TGFβ₂ and TGFβ₃ promoters appears to be mediated by cAMP responsive elements (CRE) and AP-2 binding sites. The main differences between the promoter regions of the three mammalian TGFβ isoforms are illustrated in fig 4.1.
Figure 4.1 The mammalian TGFβ promoters
Figure shows the main differences in the TGFβ₁-₃ promoters. Adapted from Roberts et al 1991.

226
4.3.4 TGFB₁ and TGFB₃ expression in wound healing

These findings suggest that TGFB₁ but not TGFB₃ is implicated in the pathogenesis of bleomycin-induced pulmonary fibrosis. Evidence for differential TGFB isoform gene expression and protein synthesis exists in a variety of other models of injury and wound healing, including embryonic and adult tissues such as skin, liver and heart (Jakowlew et al. 1991; Flanders et al. 1993; Levine et al. 1993; Martin et al. 1993; Schmid et al. 1993b; Schmid et al. 1993a; Nath et al. 1994; Frank et al. 1996). The isoforms have also been proposed to play different roles in wound healing, with exogenous TGFB₃ apparently having an anti-scarring effect (Shah et al. 1995).

In the majority of these studies, TGFB₁ was implicated in acute repair following injury to adult tissues, while upregulation of TGFB₂ and TGFB₃ expression was variable. The available evidence to date therefore suggests that TGFB₁ is somewhat more consistently implicated in wound healing and repair following injury than is TGFB₃, although further studies, using standardised models of adult and embryonic wound healing, will be required before definitive conclusions can be reached.

Consistent with our observations are the results of recent studies of bleomycin-induced lung fibrosis showing that TGFB₁ but not TGFB₂ or TGFB₃ secretion by alveolar macrophages and alveolar epithelial type II cells increases during the evolution of this disease (Khalil et al. 1993; Khalil et al. 1994). Furthermore, increased mRNA for TGFB₁ but not TGFB₂ has been demonstrated in bleomycin-treated mice (Baecher-Allan, Barth, 1993).

One study of TGFB gene and protein expression during bleomycin-induced pulmonary fibrosis has reported that gene expression of TGFB₁,₂,₃ increases after bleomycin. The authors therefore propose that all three isoforms are implicated in the pathogenesis of lung fibrosis (Santana et al. 1995). Several factors may account for the different results obtained in this study. Firstly, these authors used male Sprague-Dawley rats, so species variation in the TGFB response may be important. Secondly, the hybridisation signal illustrated in all cases was weak, probably because a lower concentration of probe was applied to tissue sections (0.8 ng/µg compared with 20 ng/µl) and a post hybridisation
RNase A wash was performed. Thirdly, no sense controls were shown for comparison. These two latter factors are likely to obscure any quantitative differences between the isoforms. They probably also explain why no hybridisation signal for any of the isoforms was observed in normal bronchial epithelium, despite the fact that digoxigenin-labelled riboprobes were employed of identical sequence to the ones I used.

One study has reported an increase in mRNA for TGFB1,3 in the murine radiation model of lung fibrosis (Finkelstein et al 1994). These authors used Northern analysis to quantitate changes in mRNA at days 1 and 14 after radiation. Differences in the type of injury induced in this model may account for the discrepancy between my results and these.

4.3.5 Summary

I have successfully developed a technique for in situ hybridisation in lung tissue using digoxigenin-labelled riboprobes. The technique is rapid, reproducible, safe, sensitive and specific. Using this method I have localised TGFB isoform gene expression in normal murine lung and during bleomycin-induced lung fibrosis. In normal lung TGFB1 and TGFB3 mRNA transcripts were identified in a wide variety of cells including bronchiolar epithelium, alveolar macrophages, pulmonary endothelial, mesenchymal and mesothelial cells. The widespread distribution of TGFB1 and TGFB3 mRNA transcripts in adult murine lung adds significant new information to previous data using radiolabeled probes which showed gene expression for TGFB1,3 to be limited to smooth muscle cells and fibroblasts. These findings suggest that digoxigenin-labelled riboprobes are a more sensitive tool for cytokine detection than radiolabelled ones. They also suggest that TGFB1 and TGFB3 play important roles in normal lung homoeostasis consistent with the recognised regulatory effects of TGFB1 on epithelial cell proliferation and differentiation, immunomodulation and matrix protein turnover.

Following bleomycin, TGFB1 gene expression was maximally enhanced at ten days and predominantly localised to macrophages and monocytes. TGFB3 gene expression was not enhanced after bleomycin. Differential gene regulation of the isoforms during the course of bleomycin-induced pulmonary fibrosis is consistent with data emerging from
other models of wound healing. Recognised structural differences in the promoter regions presumably underlie differential regulation of the two isoforms. These results presented here are consistent with the hypothesis that, while TGFβ₁ is involved in the pathogenesis of bleomycin-induced lung fibrosis, TGFβ₃ is not. The role of TGFβ₂ is discussed later (section 4.4).

4.4 IN SITU HYBRIDISATION IN HUMAN LUNG

4.4.1 TGFβ₁ and TGFβ₃ expression in normal lung

Few studies to date have examined TGFβ₁ gene expression in normal adult human lung. TGFβ₁ mRNA transcripts have been localised to alveolar macrophages (Broekelmann et al 1991; Shaw et al 1991) using isotopic in situ hybridisation, and Northern analysis has demonstrated TGFβ₁ gene expression in the airways of healthy smokers (Aubert et al 1994). As far as I am aware this is the first report of TGFβ₃ gene expression in adult human lung.

The data presented here show that mRNA transcripts for both TGFβ₁ and TGFβ₃ are present in normal human lung and predominantly co-localised to bronchiolar epithelium and alveolar macrophages. TGFβ₁ gene expression was also observed in pulmonary endothelial and mesenchymal cells. Immunohistochemical studies in normal human lung and healthy smokers have demonstrated TGFβ in bronchial epithelium, alveolar macrophages, smooth muscle cells and blood vessels (Magnan et al 1994; Aubert et al 1994), but some workers report little or no TGFβ₁ in normal lung (Corrin et al 1994; Khalil et al 1996b). The latter study demonstrated TGFβ₃ protein in alveolar macrophages, epithelial and smooth muscle cells in normal human lung. Taken together, these data suggest that as in murine lung, gene and protein expression for TGFβ₁ and TGFβ₃ are co-localised, indicating an autocrine as well as paracrine mode of action. As in murine lung, the diversity of cells expressing these genes is consistent with the wide variety of biological functions ascribed to these peptides.
4.4.2 TGFβ₁ and TGFβ₃ expression in fibrotic lung

4.4.2.1 Differential TGFβ₁ and TGFβ₃ expression

TGFβ₁ gene expression appeared to be increased in a total of three out of eight patients, while TGFβ₃ gene expression was increased in only one of these patients. In patients with dense fibrosis there was a paucity of hybridisation signal for either isoform. These results confirm the findings in murine bleomycin-induced lung fibrosis, namely that the isoforms are generally co-localised but that differential gene regulation occurs. Although the numbers of patients are too small for definitive conclusions to be drawn, there was a tendency for TGFβ₁ to be the predominant isoform expressed in fibrotic lung. Since TGFβ₁ production is increased in many tumours, and since the control lung tissue was obtained from tumour resections, the increases observed in TGFβ₁ and TGFβ₃ gene expression may be an underestimate.

Human lung tissue was taken from current or previous smokers, raising the possibility that smoking might alter TGFβ gene expression. To date no studies have addressed this question directly. However, in a study of TGFβ₁ gene expression in human airways, no differences were found between smokers and non-smokers (Aubert et al 1994).

These data suggest that TGFβ₁ may be more consistently implicated in the pathogenesis of pulmonary fibrosis associated with SSc or CFA than is TGFβ₃. This is consistent with the findings of Khalil and colleagues (Khalil et al 1996b). They reported ubiquitous TGFβ₂ and TGFβ₃ protein in normal human lung but little TGFβ₁. In contrast, TGFβ₁ was widespread in macrophages, epithelial cells and extracellular matrix in fibrotic lung. TGFβ₂ and TGFβ₃ deposition was unchanged in fibrotic lung, suggesting that the presence of TGFβ₁ is a marker of chronic fibrosis. However, given the greater profibrotic potential of TGFβ₃ in vitro, the possibility that this isoform does play an important role in some patients, or at a particular stage of the disease, cannot yet be excluded.

4.4.2.2 Localisation of gene expression

Both TGFβ₁ and TGFβ₃ mRNA transcripts were observed in fibrotic lung and their distribution appeared identical. Gene expression for both isoforms was observed in
alveolar macrophages, bronchial epithelium, alveolar walls and mesothelial cells. No
differences in localisation of TGFβ1 and TGFβ3 gene expression were detected between
patients with CFA or SSc. This reflects the affinity between these conditions in terms
of their histology and radiology, and suggests that aspects of their pathogenesis,
including their profile of TGFβ induction, are very similar. In this context it is
interesting that a recent study examining TGFβ1,3 protein deposition in fibrotic lung
found no difference between patients with IPF, asbestosis, hypersensitivity or non­
specific pneumonitis (Khalil et al 1996b).

The prognosis of pulmonary fibrosis associated with SSc is better than for patients with
CFA (Alton, Turner-Warwick, 1988; Wells et al 1994a). This may be because
pulmonary fibrosis in patients with SSc is detected at an earlier stage and patients with
SSc are generally younger. However, it may also be that upregulation of TGFβ gene
and protein expression, while important in the pathogenesis of the disease, is not the
sole determinant of prognosis and that other factors are important.

In contrast with the murine model, a switch from predominant TGFβ1 gene expression
in bronchial epithelium to predominantly interstitial expression following lung injury
was not observed in human fibrotic lung. There are several possible explanations for
this. Firstly, the bleomycin model is a model of acute lung injury rather than slowly
progressive disease. Changes in localisation of gene expression may therefore be more
subtle and less easily detected in the human disease. Secondly, human airway epithelial
cells may be subject to a greater degree of activation than those of laboratory mice,
leading to higher basal TGFβ gene expression. Finally, patients with pulmonary
fibrosis often present relatively late. It is therefore possible that an obvious switch does
occur early on in the disease, but was no longer apparent by the time biopsies were
taken.

One previous study has examined TGFβ1 gene expression in human fibrotic lung
(Broekelmann et al 1991). This study, employing a radiolabelled probe, localised
TGFβ1 gene expression to macrophages adjacent to fibrotic foci but did not detect other
cells expressing the gene. This again suggests that digoxigenin-labelled riboprobes may
be a more sensitive tool than radiolabelled ones for detecting cytokine gene expression
in lung tissue. Immunohistochemical studies of TGFβ in pulmonary fibrosis have localised the protein to fibroblastic foci (Broekelmann et al 1991), alveolar macrophages, bronchiolar epithelial and hyperplastic alveolar type II cells (Khalil et al 1991; Corrin et al 1994). As in murine lung, these data, taken together, suggest a predominantly autocrine mode of action for TGFβ.

4.4.2.3 Gene expression in dense fibrosis

Hybridisation signal for both TGFβ₁ and TGFβ₃ was scarce in biopsies characterised by areas of dense, acellular fibrosis. This may reflect the fact that the predominant source of TGFβ is inflammatory cells, so that when few of these cells are present, little gene expression is observed. This would be consistent with Broekelmann's data showing that mRNA transcripts for TGFβ₁ were associated with macrophages while TGFβ₁ was associated with areas of extracellular matrix deposition.

An alternative explanation is that TGFβ gene expression is downregulated by matrix protein accumulation. To my knowledge the only evidence for this comes from experiments showing that TGFβ₁ promoter transcription and gene expression by mammary epithelial cells is downregulated when these cells are cultured in contact with extracellular matrix rather than on plastic (Streuli et al 1993). This phenomenon was not observed with TGFβ₂ and no data are available for TGFβ₃. However, if this kind of regulation of TGFβ₁ gene expression occurs in vivo it would be consistent with the recognised ability of various extracellular matrix components to bind and sometimes inactivate active TGFβ₁, including betaglycan (Andres et al 1989), decorin (Yamaguchi et al 1990) and type IV collagen (Paralkar et al 1991). It might also provide a mechanism whereby tissue injury, involving disruption of the interaction between cells and their basement membranes, causes upregulation of TGFβ₁ gene expression. If this is the case, the absence of TGFβ₁ mRNA transcripts in murine lung bronchial epithelial cells following bleomycin must presumably reflect injury severe enough to inhibit gene transcription.

Finally, biopsies characterised by dense fibrosis may simply represent an advanced stage of the disease where the stimulus for increased TGFβ gene expression is no longer present. If this is the case, a very low level of TGFβ gene expression is presumably
sufficient to maintain increased extracellular matrix deposition. It is likely that a combination of the above factors accounts for the reduction in TGFβ gene expression observed in association with densely fibrotic lung tissue.

4.4.3 Summary

In summary, I have localised TGFβ 1 gene expression in normal and fibrotic human lung and demonstrated TGFβ 3 gene expression in normal adult and fibrotic human lung for the first time. In normal lung the isoforms are predominantly co-localised to bronchiolar epithelium and alveolar macrophages. TGFβ 1 gene expression was also observed in pulmonary endothelial and mesenchymal cells. In fibrotic lung, gene expression for both isoforms was observed in alveolar macrophages, bronchial epithelium, alveolar walls and mesothelial cells. No differences in localisation of TGFβ 1 and TGFβ 3 gene expression were detected between patients with CFA or SSc. TGFβ 1 gene expression was more consistently enhanced in fibrotic lung than was TGFβ 3 gene expression. In patients with dense fibrosis there was a paucity of hybridisation signal for either isoform.

These data confirm the sensitivity of digoxigenin-labelled riboprobes for in situ hybridisation detection of cytokine gene expression in human lung tissue. In conjunction with previously published immunohistochemical studies they suggest an autocrine as well as paracrine mode of action for TGFβ in human lung and are consistent with the view that TGFβ plays multiple roles in normal human pulmonary homoeostasis.

As in the murine model, the results show that the isoforms can be regulated differentially, but suggest that the two different forms of pulmonary fibrosis behave very similarly in terms of their profiles of TGFβ induction. TGFβ 1 and TGFβ 3 gene expression was apparent in association with cellular, inflammatory patterns of disease but was scarce in densely fibrotic patterns.
4.5 TGFβ₂ EXPRESSION

Using the antisense riboprobe, TGFβ₂ mRNA was not detectable in normal murine, human or rat lung, or in fibrotic murine lung. Signal obtained using the sense probe raises several possibilities, amongst them that a naturally-occurring antisense molecule is present in lung tissue. This section discusses the progress that has already been made towards resolving this question, the evidence currently available to support this possibility, and addresses potential future studies.

4.5.1 In situ hybridisation

Positive hybridisation signal obtained with the TGFβ₂ sense but not the antisense probe could have a variety of explanations. Firstly, the probe may have been inserted incorrectly in the vector. Secondly, digoxigenin labelling of the sense probe may be much greater than that of the antisense probe, so that non-specific signal obtained with the sense probe is more readily visualised than specific hybridisation signal with the antisense probe. Finally, an endogenous TGFβ₂ antisense molecule may be present in excess in the lung. These are discussed in turn below.

4.5.2 Characterisation of TGFβ₂ riboprobes

In order to address the first possibility, asymmetric restriction enzyme digests and sequencing of the plasmid DNA containing the probe were performed. The results show that the probe was correctly inserted in the plasmid vector, so that the sense and antisense probes did indeed correspond to their predicted sequences. In order to address the second possibility, chemiluminescence assay of digoxigenin labelling of the sense and antisense probes was performed. This showed that the antisense probe was slightly more heavily labelled with digoxigenin than the sense probe.

4.5.3 Northern analysis

Northern analysis confirmed previous data (Pelton et al 1991a; Perkett et al 1994) demonstrating that all three probes are specific for each of the TGFβ isoforms.
Northern analysis of RNA from rat lung or murine mesothelioma cells using TGFβ2 riboprobes showed that the sense probe detected a 3.5 or 3.6 kb transcript respectively. This provides further evidence that an endogenous TGFβ2 antisense transcript may be present in normal and fibrotic lung. Since Pelton and colleagues were able to demonstrate murine TGFβ2 mRNA transcripts with identical TGFβ2 probes to those I used (Pelton et al 1991a), the appearance of a natural antisense may be strain or age-specific.

4.5.4 Endogenous antisense transcripts

4.5.4.1 Antisense transcripts in prokaryotes

The possibility of an endogenous TGFβ2 antisense RNA is potentially very exciting because in prokaryotes endogenous antisense RNAs have been shown to bind to a complementary region of a target RNA and affect its function (Eguchi et al 1991). Regulation by antisense RNA was first discovered while studying replication of the Escherichia coli plasmid ColE1. Plasmid replication is dependent on the formation of an RNA primer whose precursor RNA is functional only when it adopts a certain structure during its synthesis. Interaction of a small antisense RNA to the primer precursor inhibits formation of this structure, and consequently prevents plasmid replication. Since then, a number of examples of regulation by antisense transcripts have been reported. Levels at which control is exerted include primer formation, as in the example given above, transcription termination, mRNA stability or translation. Functions controlled include replication, lysis and protein synthesis. Antisense RNAs and their target RNAs are usually transcribed from the complementary strands of the same region of DNA, but the antisense may be transcribed from a region away from the target gene. Alternatively, both molecules may be transcribed from the same promoter, when the shorter antisense RNA probably interacts with a distal region of the target RNA.

4.5.4.2 Antisense transcripts in eukaryotes

To date no naturally occurring antisense RNA regulation has been proven in eukaryotes. However, there are several reports in eukaryotes of both strands of a DNA segment being transcribed, or of complementary RNAs being detected. Examples in vertebrates
include complementary sequences to chicken myosin heavy chain (Heywood, 1986) and murine dihydrofolate reductase (Farnham et al 1985). The former can inhibit translation of myosin heavy chain mRNA in vitro.

4.5.4.3 Cytokine antisense transcripts

More recently, antisense transcripts for cytokines have also been described. An antisense RNA transcript for the human basic fibroblast growth factor (bFGF) gene was identified and characterised in 1994 (Murphy, Knee, 1994). bFGF is overexpressed in human glioma cells and increased mRNA stability has been shown to contribute to elevated FGF mRNA levels in these cells. The authors found that normal rat tissues and human breast cancer cells contained low levels of bFGF mRNA, but high levels of the antisense RNA molecule. In contrast, the antisense transcript was undetectable in tissues overexpressing bFGF mRNA. The reciprocal relationship between bFGF mRNA and antisense expression supports a role for the antisense transcript in regulating bFGF gene expression.

4.5.4.4 TGFβ antisense transcripts

An endogenous TGFβ antisense also has a precedent since a naturally occurring antisense RNA to TGFβ3 mRNA has been reported in chick heart (Potts et al 1992). In this study, spatial and temporal analysis revealed that TGFβ3 mRNA is concentrated in the atrioventricular (AV) canal tissue where valve formation occurs. The antisense transcript is also expressed in the AV canal. Expression of the antisense transcript increased during the period of development examined. The increase coincided with the loss of capacity of the AV canal myocardium to induce the epithelial-mesenchymal transformation of endothelial cells which initiates valve formation. The temporal expression of the antisense transcript during this stage of development suggests that it may play a role in the regulation of TGFβ3 production during cardiac valve formation. At present there are no published data on antisense transcripts in the lung or data showing that TGFβ antisense transcripts are playing roles in regulating matrix production in vivo.

In the in situ hybridisation studies, signal obtained with the TGFβ3 sense probe was most intense in control animals and during the phase of resolution, 21 days after
bleomycin. Signal was weakest at the height of the inflammatory response, that is, ten
days after bleomycin, coincident with the peak in TGF\(\beta_1\) gene expression. It is
tempting to speculate that a natural antisense transcript negatively regulates TGF\(\beta_2\)
production under normal conditions, but is downregulated following injury, thereby
facilitating enhanced TGF\(\beta_2\) gene expression, protein synthesis and accumulation of
extracellular matrix. Such a view must remain tentative until further studies resolve
this issue. However, it seems likely that in future, other endogenous cytokine antisense
transcripts will be identified. It may therefore become increasingly important to
identify them and develop means of examining their roles \textit{in vivo}.

\subsection*{4.5.4.5 Future studies and potential applications}

Future studies should examine whether a natural TGF\(\beta_2\) antisense is present in normal
lung and fibrotic lung, and whether it may play a role in regulating TGF\(\beta_2\) production.
These could include using RNase protection assays and vector deletions to define the
sequence which hybridises to the TGF\(\beta_2\) sense probe, amplifying this sequence by PCR
and finally cloning the antisense transcript. \textit{In vitro} studies could then be performed
to determine whether the antisense transcript is able to inhibit TGF\(\beta_2\) transcription
and/or translation. Using the murine bleomycin model of pulmonary fibrosis, Northern
analysis and immunohistochemistry of normal and fibrotic lung tissue could be
performed in order to determine whether there is a relationship between levels of
TGF\(\beta_2\) mRNA, TGF\(\beta_2\) antisense RNA and TGF\(\beta\) protein synthesis which might indicate
an \textit{in vivo} role for the antisense. Furthermore, my data demonstrate the presence of the
antisense transcript in lung tissue from three species. It would therefore be of interest
to determine whether it is present in other tissues.

A significant challenge facing researchers designing antisense treatments is that of
developing safe and effective delivery systems. Viral vectors carry potential hazards
and liposome-mediated delivery, although safe, remains relatively inefficient.
Modulation of an endogenous inhibitory antisense could provide a safe and effective
means of limiting TGF\(\beta\) synthesis and further collagen deposition. This is a distant goal
at present but information on natural regulatory mechanisms is essential to further our
understanding of pathogenesis and enable effective therapies to be designed.
4.5.5 Summary

Hybridisation signal obtained with the TGFβ₂ sense but not the antisense probe raises the possibility that a natural TGFβ₂ antisense RNA transcript may be present in normal lung tissue. Natural antisense transcripts have been documented in both prokaryotes and eukaryotes and may play a role in regulating mRNA translation and thereby protein synthesis. Endogenous antisense transcripts have been reported for bFGF and TGFβ₃, but their function in vivo remains to be confirmed. The possibility that endogenous antisense transcripts might regulate TGFβ production during the pathogenesis of pulmonary fibrosis is an exciting concept and could provide a novel therapeutic target.

4.6 Future Perspectives

This section summarises the results of the preliminary in vitro studies using TGFβ₁, discusses possible explanations and considers potential future applications.

4.6.1 The effect of TGFβ₁ antisense in vitro

Incubation of murine mesothelioma cells with a 20 mer TGFβ₁ antisense phosphorothioate oligodeoxynucleotide for 24 hrs altered cell morphology, reduced cell number and decreased TGFβ₁ production.

Following antisense treatment, a substantial proportion of cells became rounded and detached from the culture plate. It is possible that the antisense was toxic to the cells. Toxicity might result from inhibition of TGFβ₁ production per se, or from the negatively charged sulphur atom introduced in the phosphorothioate oligomer to increase resistance to nuclease degradation. In cultured cells, this substitution can lead to the oligomer binding to proteins or other unintended targets, sometimes with unwanted consequences. A particular example is that which results in an anti-adhesive effect. Some phosphorothioate antisense molecules interact with laminin to prevent its binding to its ligand, sulphatide, thereby disrupting the extracellular matrix on which cells spread and grow. This effect is particularly noted with antisense molecules containing four contiguous guanosine residues (the 'G-quartet'), but research to date has
not yet excluded the possibility that it could arise with other sequences (Stein, 1995). Further studies would be required to ascertain whether this antisense is toxic.

An alternative explanation for these findings could be that, by inhibiting TGFβ₁ production, the antisense reduced synthesis and deposition of extracellular matrix required for cell attachment and growth. Antisense inhibition of TGFβ expression by these cells can certainly reduce anchorage-independent growth (Fitzpatrick, 1994).

Both latent and active TGFβ₁ secretion were reduced by treatment with antisense, although the reduction in active TGFβ₁ production was less predictable. All three concentrations of antisense were effective. Although cell number was also reduced, as discussed above, TGFβ₁ production was reduced even when corrected for final cell number, indicating that it was a real effect.

The 20 mer I used has previously been shown to inhibit induction of proteoglycan synthesis by TGFβ₁ (Merrilees, Scott, 1994) and does not contain a 'G quartet' sequence. It is therefore likely that it reduced TGFβ₁ production via a sequence-specific effect. It is thought that antisense oligonucleotides pair with complementary mRNA to inhibit translation or reduce stability. However, it is becoming clear that many effects of phosphorothioate antisense oligomers are unrelated to Watson-Crick base pair hybridisation (Stein, 1995). As polyanions they are capable of binding to various proteins, binding being dependent not only on charge but also to some extent on base sequence. In order to determine whether the effect seen in this experiment is sequence-specific it would be important to repeat it using several control oligomers including 'sense' and 'nonsense' sequences.

It is also becoming apparent that if the concentration of antisense is kept below 5 μM, and preferably below 1 μM, the majority of non sequence-specific effects may be abolished. It would therefore be valuable to repeat this experiment at concentrations of 1 μM and below. Finally, the use of short oligomers (no longer than 15-17 bases) may also reduce non sequence-specific effects (Stein, 1995). It would therefore be of interest to repeat this experiment with several different antisense sequences in order to determine whether the effects are sequence-specific.
4.6.2 TGFβ₁ antisense as a therapeutic strategy in pulmonary fibrosis

Antisense oligonucleotides are among the first of the new 'genetic' therapies to have reached clinical trials. It was the discovery that natural antisense RNA transcripts existed which regulated gene expression in prokaryotes which first led to the idea that antisense oligonucleotides could have therapeutic value. Taken together, the data suggesting that TGFβ₁ is the predominant isoform involved in the pathogenesis of pulmonary fibrosis, that a natural TGFβ₂ antisense transcript exists in lung tissue, and that TGFβ₁ antisense oligonucleotides can regulate TGFβ₁ production in vitro, raise the possibility that TGFβ₁ antisense might be an appropriate therapy for pulmonary fibrosis.

In view of the debate, outlined above, over whether antisense oligomers exert sequence-specific effects, the use of antisense molecules as a therapeutic approach has been criticised. However, additional motifs are being discovered which exert unpredictable biological effects, such as the CpG motif (Krieg et al 1995) which can murine trigger B cell activation. It is therefore becoming apparent that antisense molecules may have other, valuable biological effects, which even though they are not sequence-specific, are nevertheless worth investigating and possibly exploiting.

A number of studies of antisense therapy are emerging. Antisense therapy has been shown to be effective in suppressing B cell lymphomas in mice (Cotter et al 1994) and clinical studies are now in progress (Cotter, personal communication). Angiotensinogen antisense oligodeoxynucleotides reduce hypertension transiently in rats (Tomita et al 1995), and TGFβ₁ antisense gene therapy can eradicate established intracranial rat gliomas (Fakhrai et al 1996). The use of TGFβ₁ antisense as therapy for a number of conditions characterised by fibrosis, including pulmonary disease, is thus becoming a credible prospect.

4.6.3 Summary

In summary I have shown that a 20 mer TGFβ₁ antisense can reduce TGFβ₁ production by murine mesothelioma cells in vitro, even when corrected for an accompanying reduction in cell number. Whether this is a sequence-specific effect remains to be
determined. These results, together with data emerging from other studies, suggest that TGFβ antisense might be a valuable therapeutic strategy for the future treatment of pulmonary fibrosis.

4.7 SUMMARY AND CONCLUSIONS

The findings presented in this thesis have fulfilled the specific aims set out previously (section 1.8.2) as follows.

I have shown that TGFβ1, TGFβ2 and TGFβ3 all stimulate fibroblast procollagen production. TGFβ3 is the most potent. In addition to increasing procollagen synthesis, TGFβ3 also reduces intracellular procollagen degradation.

I have developed a method using digoxigenin-labelled riboprobes to localise cytokine gene expression in lung tissue. I have shown that this technique is quick, reproducible, sensitive and specific. It offers several technical advantages over isotopic methods and provides improved tissue resolution with a greater potential for specific signal localisation.

Using this technique it has been possible to identify a wide variety of lung cells not previously recognised to express TGFβ1 and TGFβ3 genes in vivo. I have documented widespread cellular gene expression of both isoforms, confirmed in human as well as murine lung. This suggests significant roles for both peptides in normal pulmonary homeostasis. These are likely to include regulation of cellular proliferation, growth and differentiation, immune function and extracellular matrix metabolism. Future studies utilising this technique should provide further information concerning the role of individual genes during lung development and in the pathogenesis of a wide variety of lung diseases.

I have demonstrated the cellular localisation of TGFβ1 and TGFβ3 gene expression during the course of bleomycin-induced lung fibrosis. Following bleomycin, TGFβ1 gene expression is maximally enhanced after ten days when it is predominantly localised to macrophages and inflammatory cells. In contrast, TGFβ3 gene expression is not
enhanced after bleomycin. These results suggest that TGFβ₁ but not TGFβ₃ is involved in the pathogenesis of murine bleomycin-induced lung fibrosis. The role of TGFβ₂ remains unclear.

I have localised TGFβ₁ and TGFβ₃ gene expression in human pulmonary fibrosis, both CFA and fibrosis arising in association with SSc. In fibrotic human lung, TGFβ₁ and TGFβ₃ mRNA transcripts were again found in a wide variety of cells, and no differences were detected between patients with CFA or SSc. TGFβ₁ gene expression was more consistently enhanced in fibrotic lung than TGFβ₃ gene expression, suggesting that TGFβ₁ may be the predominant isoform involved in the pathogenesis of this disease. In densely fibrotic tissue there was little hybridisation signal for either isoform. If these results are confirmed in larger numbers of patients, TGFβ₁ will become the key target for anticytokine therapies.

An unexpected finding was that mRNA transcripts for TGFβ₂ were not detected at any stage. Hybridisation signal obtained with the sense probe raises the possibility that a natural antisense transcript is present and may regulate TGFβ₂ gene expression. If expression of the TGFβ₂ antisense transcript were confirmed in normal and fibrotic lung, it would have important implications for our understanding of the regulation of TGFβ gene expression in vivo. Such an antisense transcript may be regulating TGFβ₂ gene expression in various ways, and it is likely that other antisense RNA transcripts will be discovered. If confirmed, the implications of an antisense RNA molecule regulating TGFβ₂ gene expression will be important not only for our understanding of regulation of its gene expression and its role in the pathogenesis of fibrosis, but also for the design of future therapies directed at modifying TGFβ function in a variety of fibrotic disorders.

Finally, a 20 mer TGFβ₁ antisense oligonucleotide reduced TGFβ₁ production by murine mesothelioma cells in vitro. The results of this preliminary study, together with data emerging from other groups, suggest that TGFβ₁ antisense may have potential as a therapeutic agent in the treatment of pulmonary fibrosis. They also highlight the importance of elucidating the existence and role of endogenous TGFβ antisense transcripts in the lung.
In conclusion, all three TGFβ isoforms are profibrotic in vitro, but differential gene expression during the development of pulmonary fibrosis suggests that TGFβ₁ may be the predominant isoform implicated. Further studies are required to clarify this issue in human disease. TGFβ₁ and TGFβ₃ are expressed by a wide variety of pulmonary cells in normal and fibrotic lung and probably play important roles in lung homoeostasis. A natural TGFβ₂ antisense transcript may be present in normal and fibrotic lung, and could regulate TGFβ₂ gene expression in vivo. Preliminary data suggests that exogenous TGFβ₁ antisense can reduce TGFβ₁ production in vitro, raising the possibility that this could represent a potential therapeutic approach in lung fibrosis. The nature and functions of natural TGFβ antisense transcripts in the lung remain to be elucidated.
APPENDIX
MANUFACTURERS AND SUPPLIERS

American Type Culture Collection Rockville MD 20852 USA
Amersham International Plc Little Chalfont Buckinghamshire UK
Amicon Stonehouse Gloucestershire UK
Beckman High Wycombe Buckinghamshire UK
Bio-Rad Laboratories Ltd Hemel Hempstead Hertfordshire UK
BDH/Merck Lutterworth Leicestershire UK
Boehringer Mannheim Lewes East Sussex UK
Canberra Packard Pangbourne Berkshire UK
Costar High Wycombe Buckinghamshire UK
Dako Ltd High Wycombe Berkshire UK
Du Pont Ltd Stevenage Hertfordshire UK
Fisher Scientific Loughborough Leicester UK
Fluka Chemicals Ltd Gillingham Dorset UK
Gibco BRL Life Technologies Paisley Scotland UK
Gilford Instrument Laboratories Inc Oberlin Ohio USA
Hoeffer Scientific Instruments San Francisco California USA
IBI Ltd (Eastman Kodak Co) Cambridge UK
Ilford Ltd Knutsford Cheshire UK
Imperial Laboratories Andover Hampshire UK
Institute of Cancer Research Fulham Road London UK
John Lewis Oxford Street London UK
Jones Chromatography Hengoed Mid Glamorgan Wales UK
Keith Johnson & Pelling Ltd Drummond Street London UK
Kingsley Photographic Tottenham Court Road London UK
Laboratory Impex Teddington Middlesex UK
Leica Milton Keynes Buckinghamshire UK
Life Science International Basingstoke Hampshire UK
LKB/Pharmacia Milton Keynes Buckinghamshire UK
Marathon Unit Park Royal Road London UK
Millipore Watford Hertfordshire UK
National Diagnostics Hull North Yorkshire UK
NBL Gene Sciences Ltd Cramlington Northumberland UK
Olympus Optical Company Honduras Street London UK
Park Scientific Ltd Northampton UK
Pharmacia Biotech St Albans Hertfordshire UK
Promega Southampton Hampshire UK
Raytek Scientific Ltd Sheffield South Yorkshire UK
R&D Systems Abingdon Oxfordshire UK
Sartorius Ltd Epson Surrey UK
Sarstedt Leicester UK
Sigma Chemical Co Ltd Poole Dorset UK
Sterilin Ashford Middlesex UK
Techne Ltd Cambridge UK
Trivector Sandy Bedfordshire UK
UVP International Cambridge UK
Whatman International Ltd Maidstone Kent UK
Zeiss Welwyn Garden City Hertfordshire UK
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257


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296


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