TRANSFORMING GROWTH FACTOR-
BETA IN PLEURAL FIBROSIS

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Abstract

The pleura is involved in a wide range of pulmonary and systemic disorders. Patients with pleural diseases commonly present with pleural effusions. As many as 1 in 1500 adults each year undergo pleurodesis, the iatrogenic induction of pleural fibrosis to obliterate the pleural space, for the management of recurrent pleural effusions and pneumothoraces. Chemical pleurodesis is performed by intrapleural injection of a sclerosing agent (e.g., talc or tetracycline derivatives) that induces acute inflammatory response and subsequent fibrosis. Currently available agents are either sub-optimal in efficacy or have significant side effects, especially pain and fever from acute pleural inflammation.

Transforming Growth Factor (TGF)-β is a unique cytokine with potent profibrotic and immunomodulatory functions. Its effect in the pleura is unknown. The hypothesis that TGFβ can induce pleurodesis effectively, promptly and safely without causing significant pleural inflammation was investigated.

Using a validated rabbit model, intrapleural injection of a single dose of TGFβ2 was sufficient to stimulate rapid deposition of collagen with resultant effective pleural symphysis, and did so at a rate significantly faster than talc. Macroscopic pleural thickening and adhesions were evident within 24 hours after TGFβ2 administration.

Intrapleural use of TGFβ2 was safe. When administered to sheep, which have a thick visceral pleura resembling that of humans, TGFβ2 produced excellent pleurodesis without causing any acute haemodynamic disturbance or significant systemic absorption. Histologically, no extrapleural abnormalities were seen at 14 days.

In contrast to conventional agents, all of which elicit acute pleural inflammation, no significant inflammation was seen microscopically after TGFβ2 pleurodesis. The
pleural fluid induced after its injection had significantly lower inflammatory indices (leukocyte count and LDH) than those induced by talc and doxycycline. Furthermore, TGFβ2 induced a dose-dependent suppression of pleural fluid interleukin-8 levels. While steroids inhibit talc and doxycycline pleurodesis, high dose corticosteroids did not affect the efficacy of TGFβ2 pleurodesis - lending further support that TGFβ induced pleural fibrosis without necessitating significant inflammation.

Interestingly, intrapleural TGFβ2 stimulated the transient accumulation of large amounts of pleural fluid in a dose-dependent manner. The amount of fluid decreased as fibrosis ensued and did not affect the effectiveness of the pleurodesis.

To explain the in vivo findings, the effect of TGFβ2 on primary culture of pleural mesothelial cells was examined. TGFβ2 upregulated collagen mRNA synthesis in mesothelial cells, significantly more so than talc and doxycycline. Talc and doxycycline induced a dose-dependent increase in mesothelial cell IL-8 production while TGFβ2 did not increase the IL-8 levels over control. In the rabbit model, TGFβ2 induced a dose-dependent increase in the pleural fluid levels of vascular endothelial growth factor (VEGF), a potent vascular permeability factor, which in turn correlated significantly with the volume of the effusion. In vitro, TGFβ2 also stimulated a dose-dependent release of VEGF from mesothelial cells.

In summary, TGFβ2 has potent fibrogenic and anti-inflammation effects in the pleura in vivo and on mesothelial cells in vitro. TGFβ2 can be a novel pleurodesing agent and may have important advantages over currently used compounds.
Acknowledgements

I would like to thank my supervisors, Professors Philip Thompson (University of Western Australia) and Richard Light (Vanderbilt University, Tennessee, USA), and our scientist, Dr Kirk Lane, for their supervision, advice and patience over the three years during which this project was completed. Not only was the time spent most productive, but it has also been very enjoyable.

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Candidate’s Contribution in the Research Work

I hereby declare that this submission is my own work (including both animal and cell culture studies) and that, to the best of my knowledge, it contains no material that to any substantial extent has been accepted for the award of a degree or diploma from any university. I also declare that the intellectual content of this thesis was the product of my own work. These studies were performed at Vanderbilt University, Nashville, Tennessee, U.S.A.

I would like to acknowledge the collaboration and help received during the course of this project. In Chapter 3, the early animal experiments were performed by a co-worker (Dr D S Cheng, Vanderbilt University). Dr R E Parker performed the anesthesia for the surgery in sheep, and Mr D J Clinton assisted in the rabbit experiments. The collagen analysis and histological examinations in Chapters 4 to 6 were performed by my collaborators (Dr T R Leixeira, University of Sao Paulo and Dr J E Johnson, Vanderbilt University respectively).
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Abbreviations

Acute respiratory distress syndrome ARDS
Connective Tissue Growth Factor CTGF
Dulbecco's Modified Eagle's Medium DMEM
Enzyme-linked immunosorbent assay ELISA
Food and Drug Administration FDA
Fibroblast Growth Factor FGF
Functional residual capacity FRC
Hank’s Balanced Salt Solution HBSS
Hematoxylin-eosin H&E
Intercellular Adhesion Molecules ICAM
Interferon Gamma IFNγ
Interleukin IL
Interleukin-1 receptor antagonist IL-1 RA
Lactate dehydrogenase LDH
Latency-associated Protein LAP
Monocyte Chemoattractant Protein MCP
Metalloproteinase MMP
Nuclear Factor kappa B NFKB
Plasminogen Activator Inhibitor PAI
Standard deviation SD
Standard error of the mean SEM
Tissue Inhibitor of Matrix Metalloproteinases TIMP
Total Lung Capacity TLC
Transforming Growth Factor-beta TGFβ
Tumour Necrosis Factor-alpha TNFα
Vascular Endothelial Growth Factor VEGF
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1.1 The Pleura

1.1.1 Function

The pleural space is the coupling system between the lung and the chest wall and is accordingly a crucial feature of the breathing apparatus. Under normal circumstances, the pleural space contains a small amount of clear fluid that facilitates the expansion of the lungs and chest wall during inspiration (Boggs and Kinasewitz, 1995). The pleura also represents a crossroad of exchanges of cells and fluids and is often involved in lung disease as well as systemic illness. In spite of this, our understanding of the pathogenesis of pleural diseases and treatment of pleural disorders are limited, especially when compared with other areas of respiratory medicine.

1.1.2 Anatomy

1.1.2.1 Gross Anatomy

1.1.2.1.1 Structure

The pleura is the serous membrane that covers the lung parenchyma, the diaphragm, the mediastinum, and the rib cage. It is divided into the visceral and the parietal pleurae. The visceral pleura covers the lung parenchyma, while the parietal pleura lines the inside of the thoracic cavities. The latter is subdivided into the costal, diaphragmatic, and mediastinal parietal pleura. The visceral and the parietal pleurae meet at the lung root (Light, 2001; Wang, 1998). The total areas of the two pleural surfaces are approximately equal (2000cm$^2$ in a 70kg man) (Sahn, 1988).
The pleural space is the space that exists between the visceral and parietal pleurae. It is approximately 10-20\(\mu\)m in width, and is lined by a thin film of pleural fluid of 0.1-0.2mL/kg body weight in volume (Sahn, 1988). In humans, the left and right pleural cavities are separated by the mediastinum and the pericardial cavity (Wang, 1998). No communication normally exists between the two pleural cavities (Light, 2001).

The parietal pleura tends to be uniform in thickness between species, in contrast to the visceral pleura, the thickness of which is extremely variable. Human, sheep and cattle have thick visceral pleura, while dog, cat and rabbit have thin visceral pleurae (McLaughlin, 1983). The difference in thickness of the visceral pleura is inherent to its blood supply and plays a role in pleural fluid formation and absorption (see below).

1.1.2.1.2 Blood Supply

In humans, the parietal pleura receives its blood supply from the systemic capillaries. Small branches of the intercostal arteries supply the costal pleura, whereas the diaphragmatic pleura is supplied by the superior phrenic and musculophrenic arteries. The mediastinal pleura is supplied principally by the pericardiacophrenic artery (Light, 2001). The diaphragmatic pleura drains into the inferior vena cava and branchiocephalic trunk (Leak, 1979).

The blood supply to the visceral pleura is different among species. In animals with a thin pleura, e.g. dog, cat, and rabbit, blood supply to the visceral pleura originates from the pulmonary circulation. In animals with a thick pleura, including human, sheep,
cattle, pig, and horse, the visceral pleura is supplied by the systemic circulation via the bronchial arteries (McLaughlin, 1983). The terminal arteries and arterioles end in a capillary network larger than those of the alveolar capillaries (Krahl, 1964). The venous return from the subpleural capillaries drain largely into the pulmonary veins (Albertine et al., 1982).

1.1.2.1.3 Lymphatic Drainage

The parietal lymphatic system carries a principal part in the drainage of pleural fluids during normal and disease states. On the contrary, lymphatics in the visceral pleura in man play no significant role.

Fluid and particulate matter in the pleural space are drained via round or slit-like stomata of 2 to 6 μm in diameters in the parietal pleura into the lymphatic vessels (Wang, 1975). The stomata are grouped in clusters and empty into an extensive network of dilated submesothelial lymphatic spaces in the lymphatic vessels called lacunas (Wang, 1975). The stomata are found mostly on the mediastinal pleura, the intercostal surface of the anterior lower thorax (Albertine et al., 1984; Wang, 1975), and the diaphragm where the density of stomata can reach 8000/cm³ (Miserocchi, 1997). Experiments with labelled particles have confirmed that the stomata with their associated lacunas and lymphatic vessels form the main pathway for the egress of particulate matter from the pleural space (Albertine et al., 1984; Wang, 1975).
In the parietal pleura, lymphatic vessels of the costal pleura drain ventrally toward nodes along the internal thoracic artery and dorsally toward the internal intercostal lymph nodes. The lymphatic vessels of the diaphragmatic pleura pass to the parasternal, middle phrenic, and posterior mediastinal nodes. The lymphatic vessels of the mediastinal pleura drain to the tracheobronchial and mediastinal lymph nodes (Light, 2001).

A plexus of intercommunicating lymphatic vessels drains lymph from the visceral pleura to the lung root (Light, 2001). No stomata have been found in the visceral pleura, and the lymphatic vessels of the visceral pleura are separated from the mesothelial cells by connective tissues (Wang, 1975). These observations support the current concept that fluid in the pleural space is predominantly drained via the parietal pleura.

1.1.2.1.4 Nerve Supply

Sensory nerve endings are present in the costal and diaphragmatic parietal pleura, but not in the visceral pleura. Hence, the presence of pleuritic chest pain indicates inflammation or irritation of the parietal pleura. The intercostal nerves supply the costal pleura and the peripheral part of the diaphragmatic pleura. When either of these areas is stimulated, pain is felt at the adjacent chest wall. In contrast, the central portion of the diaphragm is innervated by the phrenic nerve, and stimulation of this pleura causes referred pain to the ipsilateral shoulder (Light, 2001).

1.1.2.2 Histology
1.1.2.2.1 Structure

Normal pleura is smooth and semi-transparent and can be divided into five layers. From the pleural surface, they are: (1) a monolayer of mesothelial cells, (2) a thin submesothelial connective tissue layer with a basal lamina, (3) an elastic layer, (4) a loose, irregular connective tissue layer, and (5) a deep fibroelastic layer. The loose connective tissue (layer 4) contains adipose tissue, vessels, nerves, and lymphatics. This layer often serves as the cleavage plane for pleurectomy. The fibroelastic layer (layer 5) is tightly adhered to the underlying lung parenchyma, diaphragm, mediastinum or chest wall. There are inter-species differences in the thickness of each layer, and sometimes regional differences within the same species (Wang, 1974; Wang, 1998).

1.1.2.2 Mesothelial Cells

The visceral and the parietal pleurae are lined with a monolayer of flattened mesothelial cells of 16 to 42 μm in diameter and of <1 to 4 μm in thickness (Wang, 1998). They are connected to each other by tight junctions on the luminal side and by desmosomes on the basal portion of the intercellular junction (Wang, 1985). Microvilli are present diffusely over the entire mesothelial monolayer, especially in the inferior portions of the visceral and mediastinal pleurae (Wang, 1974). They are approximately 0.1 μm in diameter, and can be up to 3 μm in length (Gaudio et al., 1988). Microvilli enmesh glycoproteins rich in hyaluronic acid to lessen the friction between the lung and the chest wall (Andrews and Porter, 1973; Wang, 1974). Impingement of the microvilli from one pleural surface into the opposing pleural surface can help maintain the thin rim of fluid in the pleural space (Miserocchi and Agostoni, 1980).
The mesothelial layer is fragile and focal denudation of mesothelial cells is common in humans (Peng et al., 1994). Mesothelial cells adjacent to the wound and on the opposing serous surface exfoliate, proliferate and repopulate the area (Efrati and Nir, 1976; Herbert, 1986; Peng et al., 1994). When irritated, the mesothelial cells retract, but maintain continuity with adjacent cells by projections termed cellular bridges. Dislodged mesothelial cells are commonly seen in pleural fluids. They become round or oval, and are rich in organelles. They may transform into macrophages capable of phagocytosis (Efrati and Nir, 1976) and may have an immunological role (Bakalos et al., 1974).

1.1.2.2.3 Connective Tissue Matrix of the Pleura

Histologically, a thick visceral pleura is composed of two layers: the mesothelium and connective tissue. Blood and lymph vessels and nerves are located in the connective tissue. Animals with a thick visceral pleura have a layer of dense connective tissue of varying thickness interposed between the mesothelium and the blood vessels (Albertine et al., 1982). This layer limits the exchange of fluid and particulate matter between the pleural space and the blood vessels and lymphatics in the visceral pleura. In sheep, the visceral pleura ranges in thickness from 25 to 83 μm (10-25 μm for the parietal pleura) and the distance from the microvessels to the pleural space ranges from 18 to 56 μm (10-12 μm for the parietal pleura) (Albertine et al., 1984).

Collagen accounts for 70-80% of the dry weight of the extracellular pleura. Most are type I though type III and IV collagen fibres are probably also present. Two
important glycoproteins, fibronectin and laminin, help to anchor cells to the pleural matrix. Fibronectin also regulates the collagen fibril formation (Rennard et al., 1985).

1.1.2.3. Metabolic Activities of Mesothelial Cells

Pleural mesothelial cells have important biologically activities. Resting mesothelial cells are flattened but become cuboidal when stimulated (Wang, 1998). They contain enzymes for the pentose pathway (Whitaker et al., 1980).

Mesothelial cells probably have a role in fibrosis of the pleura and loculation of pleural fluids. They have been shown to produce collagen, and express intermediate filaments typical of both epithelial cells and fibroblasts (Antony et al., 1992). In addition, mesothelial cells from rats can produce pro-fibrotic cytokines, including transforming growth factor beta (TGFβ) – generally regarded as the most potent pro-fibrotic cytokine (Border and Noble, 1994) – as well as basic fibroblast growth factor (FGF), fibronectin and laminin (Bermudez et al., 1990). Mesothelial cells also contain pro-coagulant as well as fibrinolytic activities and can produce collagenase (Idell et al., 1992; Wang, 1998). As mesothelial cells outnumber the fibroblasts in the pleural tissues, it is believed that mesothelial cells play a major role in pleural fibrosis (Rennard et al., 1985).

Mesothelial cells also orchestrate the local inflammatory response through the production of key mediators such as interleukin (IL)-8 and nitric oxide (Owens et al., 1995; Owens et al., 1996), as well as the expression of intercellular adhesion molecule (ICAM)-1 on the mesothelial surface (Goodman et al., 1992; Jonjic et al., 1992).
The normal mesothelium is a non-thrombotic surface (Nicholson et al., 1984), and production of plasminogen activators, such as urokinase and tissue plasminogen activator, by the mesothelial cells help prevent intrapleural thrombosis (Whitaker et al., 1982). Thromboxanes and prostacyclins produced by the mesothelial cells contribute to the local regulation of pleural blood flow (Boggs and Kinasewitz, 1995). Mesothelial cells are also responsible for the production of the hyaluronic acid and other glycosaminoglycans found in the pleural fluid (Schuler et al., 1988).

1.1.2 Pleural Fluid Formation

A film of pleural fluid is normally present between the parietal and visceral pleurae. It acts as a lubricant and allows the visceral pleura to slide along the parietal pleura during respiratory movements.

1.1.2.1 Characteristics of Normal Pleural Fluid

1.1.3.1.1 Volume and Thickness

In humans, the total amount of pleural fluid within both pleural cavities is about 0.26 ± 0.1 mL/kg in the normal state (Noppen et al., 2000). In rabbits, the pleural space contains about 0.4 - 1.0 ml of pleural fluid (Miserocchi and Agostoni, 1971; Sahn et al., 1979), and that in dogs about 2.4 ml (Miserocchi and Agostoni, 1971). This physiological pleural fluid is distributed relatively evenly throughout the pleural space. There is usually no contact between the visceral and parietal pleurae. The friction between the lungs and chest wall is low as the microvilli and the visceral and parietal mesothelial cells
do not interdigitate (Albertine et al., 1991). Increased accumulation of pleural fluid is associated with many disease conditions, and will be discussed further.

1.1.3.1.2 Cells

Normal pleural fluid, in rabbits, dogs, and humans alike, contains significant numbers of leukocytes and few erythrocytes, Table 1.1. In humans, macrophages account for 75% of the total white blood cells, followed by lymphocytes (23%) (Noppen et al., 2000). In rabbits, mononuclear cells (>60%) predominate, followed by mesothelial cells and lymphocytes (Miserocchi and Agostoni, 1971; Sahn et al., 1979).

1.1.3.1.3 Physicochemical Factors

The mesothelium plays an important role in determining the composition of fluid bathing its luminal surface. The important normal pleural fluid values are summarized in Table 1.1. A small amount of protein (1.0-1.5g/dL) is normally present in the pleural fluid (Miserocchi and Agostoni, 1971). Protein electrophoresis demonstrates that the electrophoretic pattern for pleural fluid is similar to that of the corresponding serum, except that low-molecular-weight proteins such as albumin are present in relatively greater quantities in the pleural fluid.

Interestingly, the pH of the pleural fluid is slightly alkaline with respect to plasma due to a bicarbonate gradient across the pleural membranes (Boggs and Kinasewitz, 1995). The major cation (Na⁺) is lower by 3-5%, and the major anion (Cl⁻) is lower by 6-
9% compared with that of plasma. The $K^+$ and glucose concentrations in the pleural fluid and plasma appears to be nearly identical (Rolf and Travis, 1973).

**TABLE I.1** Normal pleural fluid values (Boggs and Kinasevitz, 1995)

<table>
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<tr>
<th>PARAMETERS</th>
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<tr>
<td>Volume</td>
<td>0.1-0.2 mL/kg</td>
</tr>
<tr>
<td>Cells</td>
<td>1000-5000 / mm³</td>
</tr>
<tr>
<td>% mesothelial cells</td>
<td>3-70%</td>
</tr>
<tr>
<td>% monocytes</td>
<td>30-75%</td>
</tr>
<tr>
<td>% lymphocytes</td>
<td>2-30%</td>
</tr>
<tr>
<td>% granulocytes</td>
<td>10%</td>
</tr>
<tr>
<td>Protein</td>
<td>1.0-2.0 g/dL</td>
</tr>
<tr>
<td>% albumin</td>
<td>50-70%</td>
</tr>
<tr>
<td>Glucose</td>
<td>approximate plasma level</td>
</tr>
<tr>
<td>LDH</td>
<td>&lt; 50% of plasma level</td>
</tr>
<tr>
<td>pH</td>
<td>&gt; or = plasma level</td>
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</table>
1.1.2.2 Formation of Pleural Fluid in Normal Physiological State

In normal individuals, the parietal pleura is the "business end" of liquid and protein exchange in the pleural space (Sahn, 1988). The parietal pleura is supplied by the systemic circulation and a pressure gradient exists from the pleural interstitium to the pleural space where the pressure is subatmospheric (Sahn, 1988). Most of the physiological pleural fluid originates from the capillaries in the parietal pleura (Broaddus et al., 1991). This low protein filtrate then enters the interstitial space of the parietal pleura and subsequently leaks through the mesothelial layer. The microvascular endothelium is the main barrier to water and solute exchange in the pleural space. Both the fluid and protein then exit the parietal pleura by the stomata (Sahn, 1988), as discussed above.

In humans, the visceral pleura plays little part in pleural fluid formation in the normal state for several reasons. It is thick, and the distance between the pleural microvessels and the pleural space is relatively far. Also, the filtration pressure is lower in the visceral pleural microcirculation as bronchial venules empty into the lower pressure pulmonary veins (Sahn, 1988).
Figure 1.1. A. Schematic representation of pleural fluid flux within the pleural cavity under physiologic conditions. Fluid is filtered from the parietal pleura into the pleural cavity. A minute amount may be reabsorbed via the visceral pleura (small arrows) while the majority flows towards the gradient dependent regions of the pleural cavity and is removed by the parietal lymphatics. B. Enlarged view of the movement of pleural fluid from the parietal pleural capillaries to the visceral pleural microvessels and parietal lymphatic stoma.
1.1.2.3 Formation of Pleural Effusions in Pathological States

Pleural effusions, the abnormal accumulation of pleural fluid, are associated with many disease conditions. Pleural fluid accumulates when the rate of pleural fluid formation exceeds the maximal rate of its absorption. The main factors that lead to increased formation or decreased absorption of pleural fluid are summarised in Table 1.2.

**TABLE 1.2** General causes of pleural effusions

**Increased pleural fluid formation**

- Increased interstitial fluid in the lung
  - *Left ventricular failure*

- Increased vascular permeability from inflammation
  - *Pneumonia, malignancy, collagen vascular diseases, acute respiratory distress syndrome*

- Increased intravascular pressure in pleura
  - *Left ventricular failure, superior vena caval obstruction*

- Decreased pleural pressure
  - *Lung atelectasis*

- Increase fluid in peritoneal cavity
  - *Ascites, peritoneal dialysis*

- Disruption of the thoracic duct
  - *Chylothorax*

**Decreased pleural fluid absorption**

- Obstruction of the lymphatics draining the parietal pleura
  - *Malignancy including lymphoma, lung transplantation*

- Elevation of systemic vascular pressures
  - *Superior vena caval obstruction*
Fluid movement between the pleural capillaries and the pleural space is governed by Starling's law of transcapillary exchange: 

\[ Q = L \times A \times \left[ (P_{cap} - P_{pl}) - \sigma_d (F_{cap} - F_{pl}) \right] \]

where \( Q \) is the liquid movement; \( L \) is the filtration coefficient per unit area or the hydraulic water conductivity of the membrane; \( A \) is the surface area of the membrane; \( P \) and \( F \) are the hydrostatic and oncotic pressures, respectively, of the capillary (cap) and pleural (pl) space; and \( \sigma_d \) is the solute reflection coefficient for protein, a measure of the membrane's ability to restrict the passage of large molecules (Kinasewitz et al., 1984). The magnitude of these forces in the pleural cavity is shown in Figure 1.2a.

**Increased Pleural Fluid Formation:** Many factors can lead to increased pleural fluid formation (Table 1.2). In most conditions, more than one factor is involved. In the majority of cases of exudative effusions, increased vascular permeability is believed to play the principal role underlying the increase in pleural fluid formation. Although the exact mechanism is not understood, it is generally believed that the increased pleural fluid filtration occurs at the parietal pleura. There the filtration rate can increase by two orders of magnitude in pathological conditions, therefore saturating the maximum capacity of fluid removal by the parietal lymphatics, resulting in accumulation of pleural effusions (Miserocchi, 1997).

The mechanism of transudate pleural fluid formation in left ventricular failure (LVF) is different from that of exudative pleural effusions described above. In LVF, increased interstitial fluid in the lung and/or intravascular pressure in the pleura can be
the predominant mechanism for the formation of pleural effusions (Kinasewitz and Eid, in press). This is presented in a simplified diagram in Figure 1.2b. Allen et al and Broaddus et al both demonstrated that pleural fluid is formed during experimental left heart failure, as a result of pulmonary oedema (Allen et al., 1989; Broaddus et al., 1990). Pleural effusions did not develop unless alveolar oedema was present. The resolution of the oedema requires the reabsorption of the alveolar fluid into the interstitium of the lung for subsequent removal by the pulmonary lymphatics (Kinasewitz and Eid, in press). This process raises the lung interstitial pressure and promotes leakage of fluid from the subpleural lung into the pleural space via the visceral pleura. Hence, in the particular case of LVF, pleural fluid orginates from the visceral side rather than the parietal pleura.

Decreased pleural pressure can also lead to increased pleural fluid formation through its influence on Starling's equation such as with lung atelectasis or trapped lung syndrome (Light, 2001).

Free fluid in the peritoneal cavity can lead to pleural fluid accumulation through fenestrations in the diaphragm (Mouroux et al., 1996). Likewise, chyle can accumulate in the pleural space following disruption of the thoracic duct.
FIGURE 1.2. (a) Various pressures that normally influence the movement of fluid in and out of the pleural space in humans. All values are in cmH₂O.

<table>
<thead>
<tr>
<th>PARIETAL PLEURA</th>
<th>PARIETAL SPACE</th>
<th>VISCERAL PLEURA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrostatic Pressure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+30</td>
<td>-5</td>
<td>+24</td>
</tr>
<tr>
<td>→→→→</td>
<td>→→→→</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

**Oncotic Pressure**

<table>
<thead>
<tr>
<th>+34</th>
<th>+5</th>
<th>+34</th>
</tr>
</thead>
<tbody>
<tr>
<td>←←←←</td>
<td>←←←←</td>
<td>←←←←</td>
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<tr>
<td>29</td>
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</tbody>
</table>

**Net Gradient:**

<p>| | | |</p>
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<tbody>
<tr>
<td>→→→→</td>
<td>- - - -</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

(b) Various pressures that normally influence the movement of fluid in and out of the pleural space in humans. All values are in cmH₂O.

<table>
<thead>
<tr>
<th>PARIETAL PLEURA</th>
<th>PARIETAL SPACE</th>
<th>VISCERAL PLEURA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrostatic Pressure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+30</td>
<td>-5</td>
<td></td>
</tr>
<tr>
<td>→→→→</td>
<td>←←←←</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Net Gradient:**

| ←←←←|
| Fluid exudation from lung via visceral pleura into the pleural space |
Decreased Pleural Fluid Absorption: Fluid and particulate matter can be removed by the parietal lymphatics (Broaddus et al., 1988; Wang, 1975) at a rate as high as 0.2-0.4 ml/kg/hour in one pleural space (Leckie and Tothill, 1965; Stewart, 1963). In sheep the capacity for lymphatic drainage can be increased to 20-fold over baseline. At its maximal rate, the lymphatic clearance is 28 times as high as the normal rate of pleural fluid formation (Broaddus et al., 1988).

Obstruction of the lymphatics draining the parietal pleura is the most common cause of decreased pleural fluid absorption and is a major factor in the development of malignant effusions (Leckie and Tothill, 1965). Since the lymphatics drain into the systemic venous circulation, elevation of the pressures in the central veins, such as in superior vena cava obstruction, will decrease the lymphatic flow. The amount of pleural fluid accumulated increased exponentially as the pressure rose (Allen et al., 1988).

1.1.3 Pleural Effusions

1.1.4.1 Clinical Conditions

The presence of a pleural effusion affects approximately 0.32% of the general population each year (Marel et al., 1993). This translates to 60,000 cases in Australia and 800,000 in the United States each year. It may represent a local response to disease within or adjacent to the pleural membranes or be a manifestation of a systemic illness. Pleural effusions are commonly categorised into transudates and exudates by Light's Criteria, based on the serum and pleural fluid levels of protein and lactate dehydrogenase (LDH), Table 1.3 (Light et al., 1972). While other laboratory criteria to separate
transudates and exudates have been proposed (Heffner et al., 1997; Heffner et al., 2002),
none appear convincingly superior to Light’s criteria (Metintas et al., 1997;
Paramothayan and Barron, 2002), which remains as the most widely used criteria
worldwide for pleural fluid categorization.

TABLE 1.3 Distinguishing transudative and exudative effusions (Light et al., 1972)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Transudates</th>
<th>Exudates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleural Fluid to Serum Protein Ratio</td>
<td>&lt; 0.5</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>Pleural Fluid to Serum LDH Ratio</td>
<td>&lt; 0.6</td>
<td>&gt; 0.6</td>
</tr>
<tr>
<td>Pleural Fluid LDH to Upper limit of Normal Serum LDH</td>
<td>&lt; 2/3</td>
<td>&gt; 2/3</td>
</tr>
</tbody>
</table>
Transudates develop when the balance of the Starling forces is disturbed such that the rate of fluid filtration exceeds its absorption. In these conditions, the pleura remains normal. Increased capillary hydrostatic pressure (e.g. in left ventricular failure) and decreased capillary oncotic pressure (e.g. in hypoalbuminaemia from nephrotic syndrome or hepatic cirrhosis) account for most of the transudative effusions (Boggs and Kinasewitz, 1995).

Exudates develop because the rate of protein entry into the pleural cavity is increased and/or the lymphatic drainage is impaired. It is usually associated with acute inflammation of the pleura that increases the permeability of pleural capillaries to protein and cells (Herbert, 1986). Parapneumonic effusions and malignant pleural effusions are common examples (Light, 2001).

The common causes of pleural effusion are summarised in Table 1.4.
**TABLE 1.4** Common clinical causes of pleural effusions. (The incidence was based on the epidemiological data from Marel et al collected over a 1-year period in a well-defined region in Eastern Europe (Marel et al., 1993). The incidence of different types of pleural effusions varies according to geographical locations.

<table>
<thead>
<tr>
<th>Transudative Effusions</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiac failure</td>
<td>45.8%</td>
</tr>
<tr>
<td>Renal Failure</td>
<td>1.4%</td>
</tr>
<tr>
<td>Hepatic cirrhosis</td>
<td>&lt;0.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exudative Effusions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant Effusions</td>
<td>21.8%</td>
</tr>
<tr>
<td>Parapneumonic Effusions (including empyema)</td>
<td>17.0%</td>
</tr>
<tr>
<td>Pulmonary Embolism</td>
<td>5.6%</td>
</tr>
<tr>
<td>Haemothorax</td>
<td>4.2%</td>
</tr>
<tr>
<td>Subdiaphragmatic Abscesses/Inflammation</td>
<td>2.8%</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>0.7%</td>
</tr>
<tr>
<td>Connective Tissues Diseases</td>
<td>0.7%</td>
</tr>
<tr>
<td>Tuberculous Pleuritis</td>
<td>*</td>
</tr>
<tr>
<td>Drug-induced Pleuritis</td>
<td>&lt;0.5%</td>
</tr>
<tr>
<td>Post-Coronary Artery Bypass Grafting</td>
<td>&lt;0.5%</td>
</tr>
<tr>
<td>Chylothorax and Pseudochoylothorax</td>
<td>&lt;0.5%</td>
</tr>
<tr>
<td>Yellow Nail Syndrome</td>
<td>&lt;0.5%</td>
</tr>
</tbody>
</table>

* Varied among different geographic regions
1.1.4.2 Presentations and Physiologic Effects of a Pleural Effusion

Patients with pleural effusions typically present with dypsnoea, especially on exertion. The presence of fluid in the pleural space produces restrictive ventilatory dysfunction. When saline is instilled into the pleural spaces of dogs, both the functional residual capacity (FRC) and total lung capacity (TLC) decrease with increasing amounts of saline injected. However, the decrease in FRC and TLC are about 33% and 20% that of the added saline volume respectively. This is because the majority of the volume is accommodated by an increase in the size of the thoracic cage, particularly from downward displacement of the diaphragm. Accordingly, there was no decrease in the inspiratory capacity (Krell and Rodarte, 1985). In humans, relief of dyspnea following thoracentesis is accompanied by a reduction in the size of the thoracic cage, which allows the inspiratory muscles to operate on a more advantageous portion of their length-tension curve (Estenne et al., 1983).

The arterial oxygen saturation is usually decreased and the alveolar-arterial oxygen gradient increased in patients with pleural effusions, though these changes are often in part a result of the underlying lung disease(s) (Light, 2001). Large pleural effusions may also lead to right ventricular diastolic collapse and an associated decrease in cardiac output (Light et al., 1980; Vaska et al., 1992).

1.1.5 Pleurodesis

1.1.5.1 Clinical Use
Pleurodesis is an important and frequently employed option in the management of recurrent pleural effusions. Pleurodesis refers to the iatrogenic induction of pleural adhesions and fibrosis to obliterate the pleural cavity such that there remains no space for the accumulation of fluid or air.

It is estimated that over 200,000 patients in the United States received pleurodesis each year (Light, 2001). Pleurodesis is usually performed in patients with malignant pleural effusions or in those with recurrent pneumothoraces. Recent evidence suggests that it can also be successfully performed and be beneficial in selected patients with benign recurrent effusions in whom conventional management strategies failed (Aelony et al., 1991; Glazer et al., 2000; Jiva et al., 1994; Nordkild et al., 1986; Vargas et al., 1994).

Chemical pleurodesis is performed by injecting a sclerosing agent into the pleural space, usually via a chest tube. Surgical pleurodesis by pleural abrasion and/or pleurectomy are effective but carries significant mortality (>12%) (Fry and Khandekar, 1995) and morbidity (Martini et al., 1975), and is seldom recommended.

1.1.5.2 Mechanisms of Chemical Pleurodesis

Conventional pleurodesing agents act by inducing direct injuries to the pleura. This is evident by the acute pleural inflammation and denudement of mesothelial cells observed soon after pleurodesis (Kennedy et al., 1995). Following the injection of pleurodesing agents, e.g. talc, an IL-8 mediated neutrophil influx into the pleural space occurs, followed by macrophage accumulation (van den Heuvel et al., 1998). The activated macrophages also release IL-8 and monocyte chemoattractant protein (MCP)-1
and, together with the adhesion molecules on the mesothelial cells, serve to amplify the inflammatory response (Nasreen et al., 1998). In humans, the levels of pro-inflammatory cytokines, such as IL-8, tumour necrosis factor (TNF)-α and nitric oxide, in the pleural fluids are significantly elevated following chemical pleurodesis (Argenius et al., 1994; van den Heuvel et al., 1998). Conversely, co-administration of parental corticosteroids reduces the inflammation and significantly inhibits pleurodesis (Xie et al., 1998c).

The inflammation elicited may resolve (failed pleurodesis) or progress to chronic inflammation and fibrosis. If sufficient fibrosis is produced, symphysis develops between the visceral and parietal surfaces and the pleural space is obliterated such that no fluid or air can accumulate (successful pleurodesis). The summary of this process is presented in Figure 1.3.
FIGURE 1.3. The intrapleural administration of conventional pleurodesing agents induces an acute pleural injury and inflammation. This process is inhibited by corticosteroids. Pain and fever associated with pleurodesis are presumed to be a result of the acute pleural inflammation. The inflammation may heal with restoration of normal pleura (failed pleurodesis). However if the inflammation is sufficiently intense, it will progress to chronic inflammation and pleural fibrosis (successful pleurodesis).

Mechanism of Pleurodesis

Injection of Pleurodesing Agent

| Inhibited by Steroid |

Pleural Injury: Inflammation

Pain + Fever

Pleural Fibrosis / Pleurodesis
Most existing pleurodesing agents produce chest pain and fever (Walker-Renard et al., 1994), which are likely the results of the acute pleural injury and intense inflammation of the pleura. For instance, fever occurred in up to 62% of patients following talc pleurodesis (Walker-Renard et al., 1994) and may last for 72 hours (Sahn, 1997). Severe pain was reported in over 50% of patients who received tetracycline pleurodesis for pneumothorax (Light et al., 1990).

In general, pleurodesis is only recommended in patients with a reasonable short-term prognosis who do not have trapped lungs. Trapped lung syndrome refers to the condition when the underlying lung fails to fully re-expand after complete drainage of the effusion. It can be a result of endobronchial obstruction with distal atelectasis or due to extensive tumour involvement of the visceral pleura and resultant encasement of the lung. With a trapped lung, the pleural surfaces are separated and pleurodesis is unlikely to be successful (Lee et al.).

1.1.5.3 Commonly Used Agents: Effectiveness and Complications

The ideal pleurodesing agent has yet to be found. Currently available compounds either have sub-optimal efficacy or carry significant, or potentially lethal, side effects. This is highlighted by the recent controversy regarding the safety of talc (Light, 2000; Sahn, 2000). Talc, tetracycline derivatives, and bleomycin are the most commonly used agents worldwide (Sahn, 1997).
**Talc:** Talc is a heterogeneous compound with the chemical formula of \( \text{Mg}_3(\text{Si}_2\text{O}_5)(\text{OH})_2 \). Sterilized talc can be instilled into the pleural space either as an aerosol (insufflation) via thoracoscopy or thoracotomy or a suspension (slurry) through chest tubes. Numerous studies have attested to the effectiveness of talc, consistently reporting a success rate of over 90% (Alder and Sayek, 1976; Hartman *et al.*, 1993; Kennedy *et al.*, 1994; Noppen *et al.*, 1997; Webb *et al.*, 1992). In direct comparison trials, talc is more effective than bleomycin (Hamed *et al.*, 1989; Hartman *et al.*, 1993), tetracycline (Fentiman *et al.*, 1986) and mustine (Fentiman *et al.*, 1983). In animal studies, talc is as effective as surgical pleural abrasion (Bresticker *et al.*, 1993).

However, a growing number of reports cast increasing doubts over the safety of talc pleurodesis. Talc-induced acute respiratory distress syndrome (ARDS) and systemic embolization are the main concerns. There are over 32 reported cases of talc-induced ARDS (Bouchama *et al.*, 1984; Kennedy *et al.*, 1994; Marel *et al.*, 1998; Migueres and Jover, 1981; Milanez *et al.*, 1997; Nandy, 1980; Rehse *et al.*, 1999; Rinaldo *et al.*, 1983; Todd *et al.*, 1980), which often resulted in respiratory failure, and in eight instances, death (Marel *et al.*, 1998; Milanez *et al.*, 1997; Nandy, 1980; Rehse *et al.*, 1999; Rinaldo *et al.*, 1983). These complications are not limited to either form of talc (slurry or insufflated), nor to high doses (Milanez *et al.*, 1997).

Systemic embolization of talc following its intrapleural administration has been observed in both humans and in animal studies. Talc particles were found in lungs, liver, spleen, kidney, and brains of all the rats given intrapleural talc injections (Kennedy *et al.*, 1994; Noppen *et al.*, 1997; Webb *et al.*, 1992).
In humans, talc particles have been found in bronchoalveolar fluids, heart, liver, kidney, and even the brain following talc pleurodesis (Milanez et al., 1997; Rinaldo et al., 1983). The long-term consequences of systemic deposition of talc particles have not been studied. However, it has been hypothesized that the systemic absorption of intrapleural talc may elicit inflammatory reactions that underlie the development of ARDS (Light, 2000).

The aetiology for the ARDS after intrapleural talc remains unknown. Talc is often inhomogeneous, and differences in physical characteristics of the talc particles in different commercial preparations have been well documented (Ferrer et al., 2001). The size of talc particles vary significantly among commercial preparations, Table 1.5 (Ferrer et al., 2001). This may account for the variations in the incidence of acute lung injury. It has been hypothesized that smaller talc particles are more likely to be absorbed into the systemic circulation via the parietal lymphatics and induce more systemic inflammatory responses as well as ARDS (Light, 2000). In addition, commercial talc preparations frequently contain impurities such as quartz, kaolinite, calcite or chlorite. It is possible that these contaminants, rather than talc itself, can be the cause of the ARDS.

While it is generally accepted that talc can induce ARDS, the extent of this problem is a matter of intense debate (Light, 2000). The highest incidence was that reported by Rehse et al (Rehse et al., 1999). In 89 talc pleurodesis procedures in 78 patients, 9% of patients developed ARDS requiring mechanical ventilation and one died.
(Rehse et al., 1999). On the contrary, no cases of ARDS were observed in 360 patients following talc pleurodesis in another series (Weissberg and Ben-Zeev, 1993).

**TABLE 1.5** Particle sizes of talc in different preparations as tested by Ferrer et al. (Ferrer et al., 1996)

<table>
<thead>
<tr>
<th>Talc Source</th>
<th>DIAMETER (µm)</th>
<th></th>
<th>10-90 percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td></td>
</tr>
<tr>
<td>USA Preparation A</td>
<td>10.8</td>
<td>7.8</td>
<td>2.4-22.7</td>
</tr>
<tr>
<td>USA Preparation B</td>
<td>19.4</td>
<td>13.2</td>
<td>3.2-46.8</td>
</tr>
<tr>
<td>USA Preparation C</td>
<td>20.1</td>
<td>13.5</td>
<td>3.1-49.5</td>
</tr>
<tr>
<td>Spain</td>
<td>20.1</td>
<td>14.8</td>
<td>3.7-45.7</td>
</tr>
<tr>
<td>USA Preparation D</td>
<td>20.4</td>
<td>13.9</td>
<td>3.1-49.4</td>
</tr>
<tr>
<td>Brazil</td>
<td>25.4</td>
<td>21.5</td>
<td>6.4-50.5</td>
</tr>
<tr>
<td>Taiwan</td>
<td>32.3</td>
<td>28.7</td>
<td>7.2-64.4</td>
</tr>
<tr>
<td>France</td>
<td>33.6</td>
<td>31.3</td>
<td>10.5-60.6</td>
</tr>
</tbody>
</table>

*Tetracycline Derivatives:* Tetracycline is effective for pleurodesis, but parenteral tetracycline is no longer available in most countries. Doxycycline has now replaced tetracycline in many countries. In five studies of a total of 110 patients, doxycycline produced an overall success rate of 83% (Heffner et al., 1994; Mansson, 1988; Pulsiripunya et al., 1996; Robinson et al., 1993; Seaton et al., 1995). Chest pain and
fever are both common side effects (Walker-Renard et al., 1994). Another tetracycline
derivative, minocycline, has also been shown to be effective (Walker-Renard et al., 1994).
However, it has been associated with severe complications including vestibular
disturbances, serum sickness and drug-induced lupus (Rodriguez-Panadero, 1997).

**Bleomycin:** Bleomycin is less effective than talc or doxycycline in both human
and animal studies (Vargas et al., 1993). In a review of eight studies with a total of 199
patients using bleomycin for treatment of malignant pleural effusions, the overall success
rate was only 54% (Walker-Renard et al., 1994). By six months, bleomycin pleurodesis
failed to control the effusions in 65% of patients (Diacon et al., 2000). Also, bleomycin is
about 20 and 300 times more expensive than doxycycline and talc respectively (Walker-
Renard et al., 1994).

**Other agents:** Numerous other agents have been evaluated as potential
pleurodesing agents over the years. Anti-cancer drugs other than bleomycin, including
nitrogen mustard, mitoxantrone, 5-fluorouracil, cytarabine and decarbazine have been
studied for pleurodesis (Walker-Renard et al., 1994). None of them are suitable because
of ineffectiveness or toxicity.

Dried killed *Corynebacterium parvum*, an anaerobic gram-positive bacterium, has
been evaluated in nine reports (total 169 patients) with an overall success rate of 76%
(Walker-Renard et al., 1994). OK-432 is obtained from *Streptococcus pyogenes* and has
immunostimulating and cytotoxic properties similar to *C. parvum*. The availability of *C.*
parvum and OK-432 worldwide are very limited. Large-scale clinical trials are lacking to ascertain their effectiveness. Interferon gamma (IFNγ) (Sartori et al., 1998), IL-2 and TNFα (Lissoni et al., 1995) have been used in small clinical trials with modest results.

In view of the above-mentioned limitations of currently available pleurodesing agents, there is a strong need for a better understanding of the pleurodesis process with the goal of developing better agents that can provide safe and effective pleurodesis.

1.1.5.4 Experimental Models of Pleurodesis

No in vitro models are available for evaluation of clinical efficacy of pleurodesing agents. Experimental studies of pleurodesis are therefore mostly conducted using animal models. Large animals have greater anatomical similarity with humans, but are expensive. Conventionally, rabbits are the most commonly used model for pleurodesis (Light et al., 1996; Mitchem et al., 1999; Vargas et al., 2000; Xie et al., 1998c), although dogs (Colt et al., 1997) and rats (Werebe et al., 1999) have occasionally been used. Smaller animals, e.g. mice, have not been used due to difficulties in delivering agents into their pleural cavities. Also, in mice, unlike in humans, the pleural space of each hemithorax is not separated but exist as one single cavity.

Sheep are often used for the study of pleural fluid exchange because sheep have a thick visceral pleura resembling that of humans (Albertine et al., 1984; Broaddus et al., 1988; Broaddus et al., 1990). However, sheep have not previously been used for the study of pleurodesis.
1.2 Transforming Growth Factor beta

1.2.1 General Description

TGFβ is a family of multi-functional growth-modulating protein cytokines notable for their prodigious capacity to modulate a wide range of cellular behaviours. Virtually all cells can produce and have receptors for TGFβ. Their broad spectrum of actions makes TGFβ one of the most complex groups of cytokines yet recognised (Blobe et al., 2000).

1.2.1.1 TGFβ Isoforms

Humans possess three TGFβ isoforms: β₁, β₂ and β₃. Two other isoforms TGFβ₄ and TGFβ₅ have been identified and cloned in chick and frog embryo sources respectively. Most of the TGFβ activities can be accounted for by TGFβ₁ and β₂ (Kelley, 1993), which share 70% homology in their amino acid sequences and an overlapping range of biological actions (Cheifetz et al., 1987). The role of TGFβ₃ is less well defined.

While in general the TGFβ isoforms bear similar biological properties (Border and Noble, 1994), differences in their temporal and spatial distributions have been reported (Pelton et al., 1991). Each isoform is regulated by a different promoter region (Khalil et al., 1993) and TGFβ₁ and -β₂ knockout mice showed no phenotype overlap indicating numerous non-compensated functions between the two isoforms (Sanford et
All three isoforms are highly conserved in mammals, suggesting a critical biologic function for each isoform (Blobe et al., 2000).

1.2.1.2 Structure

TGFβ₁ molecules from human, primate, porcine, and bovine sources are completely identical in amino acid sequence, suggesting an essential selective pressure against mutational variation. Inter-species conservation of TGFβ₂ sequences has also been confirmed (Kelley, 1993).

1.2.1.3 Distribution

TGFβ is ubiquitous and is particularly abundant in bone, kidneys and lungs in humans. In blood, platelets are the richest source of circulating TGFβ (Border and Noble, 1994; Kelley, 1993).

1.2.2 Biosynthesis and Activation

1.2.2.1 Gene Structure

TGFβ₁ gene has been localised to human chromosome 19q13, and mouse chromosome 7 (Fujii et al., 1986). The TGFβ₁ gene consists of seven exons containing about 1200 bases (Derynck et al., 1987). At least five polymorphisms have been identified in the TGFβ₁ gene, and are associated with variation in circulating TGFβ levels (Awad et al., 1998).

1.2.2.2 Control of Gene Expression

35
The amount of active TGFβ released by cells can be regulated at any of the regulatory points from transcriptional control to activation following secretion (see below). Molecular dissection of the TGFβ1 upstream promoter reveals a number of distinct regulatory elements, including an enhancer region, a positive regulatory region, and at least two separate negative regulatory elements. Delayed translation has been observed in TGFβ gene expression in lymphocytes (Kehrl et al., 1986). This interesting property adds the dimension of time to TGFβ gene expression and subsequent cellular actions, and may have an important role in lymphocytic modulation of inflammation (Kelley, 1993).

1.2.2.3 Latent vs Active Form

TGFβ1 is synthesized as a large prepropeptide. It is secreted as an inactive protein complex consisting of a 391 amino acid precursor and requires activation by proteases such as plasmin to yield peptide fragments and a 112 amino acid subunit. Active TGFβ1 is a 25 kD dimeric protein composed of two subunits linked by a disulphide bond. TGFβ1 is secreted in a latent form in which TGFβ1 is non-covalently bound to the latency-associated peptide (LAP), which is formed from the cleavage fragments of the TGFβ1 precursor. Latent TGFβ1 is stored at the cell surface and in the extracellular matrix (Border and Noble, 1994). The post-translational activation of the latent high molecular mass TGFβ1 represents the most important controlling step in the regulation of its action (Tucker et al., 1984). Other TGFβ types, including TGFβ2, are also secreted in latent form, and require activation (Kelley, 1993).
1.2.2.4 Activation of TGFβ

Secreted latent TGFβ may be activated in vitro by various non-physiological treatments such as extreme pH, detergents, or proteolytic enzymes (Lyons et al., 1988). Activated TGFβ is over 200 times more potent than latent TGFβ proteins in binding TGFβ receptors and inducing phenotypic changes in target cells (Kelley, 1993).

TGFβ can be released as "preactivated" by certain cell types including human polymorphonuclear leukocytes and monocytes (Grotendorst et al., 1989), presumably by prompt proteolytic processing at the cell surface. In platelets, TGFβ is stored in intracellular granules and released on platelet degranulation (Baas et al., 1997). In contrast, in rats, TGFβ produced by alveolar and interstitial polymorphonuclear leukocytes and macrophages are largely in latent form (Kelley, 1993).

1.2.2.5 Autoinduction

Exogenous TGFβ induces the production and secretion of TGFβ by cultured cells. Autoinduction describes this ability of cytokines to induce their own expression by certain cells. It is a specialised variant of the autocrine hypothesis and a form of positive feedback that serves to amplify a weak paracrine signal through an autocrine mechanism. Also, since different TGFβ types have contrasting effects on diverse cell populations, autoinduction may be seen as a way to modify the incoming autocrine signal at the local level (Kelley, 1993).
The gene expressions for the several TGFβ types are differentially regulated by TGFβ1 and TGFβ2 in a complex pattern. The particular gene enhancement pattern induced by each TGFβ type may result from transcription, message stabilization, or post-translational mechanisms. Moreover, the pattern of regulation and the magnitude of response vary among cell types (Bascom et al., 1989).

1.2.3 Receptors

There are three commonly known TGFβ receptors: I, II and III, which exist on virtually all cells (Border and Noble, 1994). Importantly, the receptor proteins are not cognate receptors for TGFβ isoforms of the same number. Receptors I and II are glycoproteins of 53kD and 73-95kD respectively. Both have greater affinity for TGFβ1 than for TGFβ2. They interact with one another and facilitate each other's signalling (Ebner et al., 1993). Deletion of receptor I has been shown to ablate the responsiveness of cells to TGFβ (Boyd and Massague, 1989). Receptor III is a large 300kD glycoprotein and can bind both TGFβ1 and TGFβ2 isoforms equally well (Andres et al., 1989). It acts mainly to present TGFβ to the other receptors (Lopez-Casillas et al., 1994).

1.2.4 Signalling Pathway

It is believed that the Smad proteins are the most important intracellular signalling mediators for TGFβ (Xiao et al., 2000). Other systems, including the G-protein second-messenger and MAP kinase pathways contribute to the mediation of certain TGFβ functions (Kelley, 1993; Miyazono, 2000).
On binding with its receptors on the cell surface, TGFβ initiates signalling through a ligand-dependent activation of a complex of heteromeric transmembrane serine/threonine kinases (Derynck and Feng, 1997). Activated receptors phosphorylate and activate Smad 2 and/or Smad 3 (the "signalling mediators"), which then induce the formation of complexes with Smad 4 (a "common-mediator"). The Smad protein complexes then translocate to the nucleus where they act as transcription factors to regulate a host of target genes (Derynck et al., 1998).

The TGFβ-Smad system can be inhibited by the nuclear factor kappa B (NFκB)-dependent activation of Smad 7 (an "inhibitory SMAD"). Smad 7 gene expression can be induced by TGFβ itself, thus forming an auto-inhibitory loop (Nakao et al., 1997). A variety of pathogenic and pro-inflammatory stimuli as well as mechanical stress and IFNγ are capable of increasing the gene transcription and the intracellular level of Smad 7. Smad 7 then binds to the ligand-activated receptors and interferes with their phosphorylation, as well as the nuclear translocation and DNA binding of the Smad transcriptional activator complexes (Bitzer et al., 2000; Miyazono, 2000).

Smads are degraded by the ubiquitin-proteasome pathway in both ligand-dependent and -independent fashions. Once reaching the nucleus, TGFβ signalling can be modulated by various transcriptional corepressors (Miyazono, 2000). These areas are still subject to ongoing investigation.

1.2.5 Pharmacokinetics
The half-life of activated TGFβ in the systemic circulation is about four minutes (LaMarre et al., 1991). Free TGFβ in serum binds to α2-macroglobulin and is inactivated (Danielpour and Sporn, 1992). The α2-macroglobulin, its receptors and the hepatic proteases thus act as a concerted system to clear TGFβ rapidly from serum. It may serve the function of inactivating any TGFβ that moves away from its intended site of action (Kelley, 1993). Binding to α2-macroglobulin also differentially modulates the activity of TGFβ1 and -β2.

1.2.6 Functions in Normal and Disease States

1.2.6.1 Tissue Fibrosis

Fibrosis represents a pathologic excess of normal tissue repair. It is now well accepted that excessive or sustained production of TGFβ is a key factor in tissue fibrosis in a wide variety of fibrotic conditions in humans, Table 1.5. In particular, the role of TGFβ in pulmonary, renal and hepatic fibrosis have been well established (Border and Noble, 1994).
**TABLE 1.5.** Disease characterised by excessive matrix accumulation and prominent tissue fibrosis, in which TGFβ plays an important pro-fibrotic role.

<table>
<thead>
<tr>
<th>Diseases</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>LUNG</strong></td>
<td></td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>(Anscher <em>et al.</em>, 1993; Broekelmann <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td>Pulmonary sarcoidosis</td>
<td>(Salez <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>Autoimmune fibrosis</td>
<td>(Deguchi, 1992)</td>
</tr>
<tr>
<td>Radiation-induced fibrosis</td>
<td>(Li <em>et al.</em>, 1999b)</td>
</tr>
<tr>
<td><strong>HEART</strong></td>
<td></td>
</tr>
<tr>
<td>Cardiac fibrosis</td>
<td>(Li <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td><strong>LIVER</strong></td>
<td></td>
</tr>
<tr>
<td>Hepatic cirrhosis</td>
<td>(Murawaki <em>et al.</em>, 1998; Tsushima <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>(Martinez <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td><strong>KIDNEY</strong></td>
<td></td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>(Yoshioka <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>(Yamamoto <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td><strong>BONE MARROW</strong></td>
<td></td>
</tr>
<tr>
<td>Myelofibrosis</td>
<td>(Kimura <em>et al.</em>, 1989; Rameshwar <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td><strong>SKIN</strong></td>
<td></td>
</tr>
<tr>
<td>Scleroderma</td>
<td>(Kulozik <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td>Keloid/hypertrophic scars</td>
<td>(Peltonen <em>et al.</em>, 1991)</td>
</tr>
</tbody>
</table>
After tissue injury, a repair process is initiated with increased expression of TGFβ mRNA and protein by parenchymal cells, infiltrating macrophages and lymphocytes (Branton and Kopp, 1999). Latent TGFβ is also activated within one hour, followed by a second wave of activation several days later (Yang et al., 1999). TGFβ induces fibrosis by increasing the expression ± synthesis of most extracellular matrix proteins, as summarized in Table 1.6.

**TABLE 1.6.** TGFβ increases the mRNA ± protein synthesis of the following extracellular matrix components (Branton and Kopp, 1999).

*Collagen Fibres*

- Type I, II, III, IV, V, VII and XVI

*Basement Membrane*

- Laminin
- Entactin
- Perlecanc

*Interstitial Matrix*

- Fibronectin
- Thrombospondin
- Tenascin
- Osteonectin
- Elastin
- Biglycan
TGFB not only increases matrix production but also inhibits its degradation. Under normal circumstances, matrix synthesis is balanced by protein breakdown. Plasminogen is converted to plasmin - a process regulated by tissue and urokinase plasminogen activators and plasminogen activator inhibitors (PAI). Plasmin then activates latent collagenases and metalloproteinases (MMP) as well as degrades fibrin, fibronectin and laminin (Branton and Kopp, 1999). TGFB enhances tissue fibrosis by inducing inhibitors of matrix-degrading MMPs and PAIs (Border and Noble, 1994; Branton and Kopp, 1999; Miyazono, 2000). The fibrotic actions of TGFB is summarized in Figure 1.4.

The potent pro-fibrotic functions of TGFB may have clinical implications. Firstly, direct application of TGFB may enhance fibrosis and be beneficial in the healing of wounds (Beck et al., 1993; Sporn and Roberts, 1993) and fractures (Rosier et al., 1998). Secondly, strategies to block TGFB activity may prevent the development and/or severity of fibrotic diseases. Significant reduction in bleomycin-induced lung fibrosis (Wang et al., 1999), renal fibrosis (Border et al., 1990), and scar formation (Shah et al., 1992) have been achieved in animal models by neutralising TGFB functions.

The potential roles of TGFB in pleural diseases, especially in the formation of loculation and fibrosis in the pleural space have not been studied.
FIGURE 1.4. TGFβ in tissue fibrosis. ROS = reactive oxygen species.

**Activating Factors**

Tissue Injury; Irradiation; Mechanical Stress
TGFβ; Angiotensin II; Thromboxane
Glucose and Glycosylation products

---

**Parenchymal cells**
Platelets; Macrophages; Lymphocytes

---

**Activators**
Plasmin
Thrombospondin
ROS

---

**TGFβ**

---

↑ Matrix Synthesis
↓ Matrix Degradation

---

Fibrosis
1.2.6.2 *Immune Modulation*

TGFβ is a pivotal immunomodulatory cytokine with multiple faces in the regulation of the inflammatory process. Under some circumstances TGFβ can act to promote inflammation, especially through monocyte chemotaxis and their production of pro-inflammatory cytokines (Roberts and Sporn, 1990). However, in the majority of situations, TGFβ serves as a potent anti-inflammatory agent (Chen and Wahl, 1999).

TGFβ<sub>1</sub> and -β<sub>2</sub> significantly inhibit both T- and B-lymphocytes. TGFβ is 10,000 times more potent than cyclosporine in lymphocyte inhibition (Kehrl *et al.*, 1986; Roberts and Sporn, 1990). TGFβ can inhibit natural killer cell functions and the generation of lymphokine-activated killer cells (Chen and Wahl, 1999; Kelley, 1993). While there is conflicting data regarding the effect of TGFβ on Th cell differentiation, TGFβ can inhibit the production of, and the response to, cytokines associated with both Th1 and Th2 cells (Letterio and Roberts, 1998). TGFβ<sub>1</sub> knockout mice died shortly after birth from overwhelming systemic inflammation mediated by lymphocytes (Diebold *et al.*, 1995), reiterating the importance of the anti-inflammatory functions of TGFβ.

TGFβ also inactivates macrophage and neutrophil functions by suppressing the production of most pro-inflammatory cytokines (discussed below) (Tsunawaki *et al.*, 1988). Furthermore, systemic TGFβ can inhibit E-selectin expression and block adhesion and targeting of leukocytes to inflammatory areas (Gamble *et al.*, 1993).
Since most autoimmune and chronic inflammatory diseases are mediated by lymphocytes, particularly Th1 cells, and effected by macrophages and neutrophils, TGFβ may have a potential therapeutic role in these diseases (Chen and Wahl, 1999). In animals, TGFβ1 reduced the incidence and severity of experimental allergic encephalomyelitis in multiple sclerosis (Johns et al., 1991; Racke et al., 1993). This led to a phase I clinical trial of the use of TGFβ2 in multiple sclerosis, which demonstrated the safety of regular intravenous infusion of TGFβ2 (Calabresi et al., 1998). Systemic administration of exogenous TGFβ has also shown therapeutic benefits in rodent arthritis models (Thorbecke et al., 1992). Intramuscular injection of naked DNA plasmid encoding the TGFβ1 gene could successfully inhibit the chronic arthritic inflammation in animal studies (Song et al., 1998). This resulted in the ongoing development of TGFβ-based gene therapy (Chen and Wahl, 1999).

1.2.6.3 Cell Proliferation and Carcinogenesis

TGFβ is a potent inhibitor of cell proliferation. It stimulates cyclin-dependent protein kinase inhibitor p15 and inhibits cell-cycle regulators, arresting the cell cycle in the G1 phase. In normal human cells, TGFβ acts as a tumour suppressor by inhibiting cellular proliferation and/or by promoting cellular differentiation or apoptosis (Blobe et al., 2000).

TGFβ bears a "love-hate" relationship with cancer. During the early stage of carcinogenesis, TGFβ can act as a potent tumour suppressor and may mediate the actions of chemotherapy agents, such as tamoxifen. However, at the advanced stage of tumour...
development, malignant cells often become resistant to the TGFβ-mediated growth arrest, and may increase their own production of TGFβ. This resistance is believed to be the consequence of inactivating mutations in genes that encode signalling intermediates, including TGFβ receptors type I and II, and several Smad proteins (Blobe et al., 2000; Reiss, 1997).

Once resistance develops, the more aggressive forms of cancer cells (whose growth is now not inhibited by TGFβ) may become even more aggressive in response to the increased TGFβ levels (Blobe et al., 2000; Gold, 1999). These cancer cells can make use of several properties of TGFβ to enhance their growth and metastasis.

Firstly, TGFβ can stimulate angiogenesis (Dickson et al., 1995; Pepper, 1997), which may explain in part how TGFβ enhances the growth of late-stage tumours. Secondly, TGFβ possesses potent immunosuppressive functions (as discussed above), which may allow cancer cells to suppress the activities of infiltrating immune cells, thereby escaping immune surveillance (Blobe et al., 2000). Thirdly, the pro-fibrotic nature of TGFβ may enable the cancer cells to increase extracellular matrix synthesis. This may strengthen the invasiveness of these malignant cells by increasing their proteolytic activity, and promoting their binding to cell-adhesion molecules, thereby facilitating distant metastasis (Maehara et al., 1999).

1.2.6.4 Other Functions
Studies continue to reveal increasingly diverse roles of TGFβ in human diseases. Evidence from knockout mice highlights the importance of TGFβ in embryonic development. TGFβ2 knockout mice have cardiac, lung, craniofacial and urogenital defects (Sanford et al., 1997), while mice lacking TGFβ3 have cleft palates (Proetzel et al., 1995).

TGFβ can function as an inhibitor of atherosclerosis by inhibiting proliferation and migration of smooth muscle and endothelial cells (Grainger et al., 1994; Grainger et al., 1995). TGFβ has a probable role in transplant rejection. High levels of TGFβ1 mRNA in tissue are associated with chronic rejection in lung transplant recipients (Charpin et al., 1998), and with cardiac allograft vasculopathy after heart transplantation (Aziz et al., 2000). TGFβ receptors play an essential role in angiogenesis, and is important in the pathogenesis of hereditary hemorrhagic telangetasia (Blobé et al., 2000).

TGFβ may also serve as a disease predictor. Early increase in serum TGFβ2 predicts a treatment response to tamoxifen in breast cancer patients (Kopp et al., 1995). High serum TGFβ levels are correlated with the development of post-radiotherapy fibrosis (Li et al., 1999b), chronic graft-versus-host disease, idiopathic interstitial pneumonitis, and veno-occlusive disease in transplant recipients (Anscher et al., 1993; Liem et al., 1999).
Polymorphisms in TGFβ1 gene have been linked to fibrosis (Awad et al., 1998), osteoporosis (Yamada et al., 1998; Yamada et al., 2000), myocardial infarction (Yokota et al., 2000) and hypertension (Li et al., 1999a).

1.2.7 Relationships with Other Cytokines

1.2.7.1 Effect of TGFβ on Production of Other Cytokines

The interactions of TGFβ with other cytokines are often complicated and dependent on the particular cell type and the experimental environment.

In keeping with its anti-inflammatory roles described above, TGFβ suppresses the pro-inflammation cytokines release in most cell lines. It consistently reduces the expression of TNFα, IL-1 (Karres et al., 1996; Link et al., 1995; Zissel et al., 1996) and IL-1 receptor antagonist (IL-1 RA) (Bry and Lappalainen, 1994; Muzio et al., 1994) and IL-8 (Smith et al., 1996). TGFβ can increase IL-6 production under some circumstances (Gautam et al., 1993; McGee et al., 1992; Moller et al., 1994), but reduces it in others (Chen and Manning, 1996; Karres et al., 1996; Link et al., 1995). TGFβ can also suppress the expression of IFNγ, IL-4 (Link et al., 1995), IL-5 (Enokihara et al., 1994), and IL-10 (Reinhold et al., 1995). On the other hand, TGFβ can promote IL-2 (Han et al., 1998) and IL-11 expression (Tang et al., 1998).

Furthermore, TGFβ is one of the most potent stimulators of vascular endothelial growth factor (VEGF) production in various cell lines (Berse et al., 1999; Koochekpour et
al., 1996; Pertovaara et al., 1994), confirming its role in increasing vascular permeability and angiogenesis.

1.2.7.2 Effect of Other Cytokines on TGFβ Production

TGFβ can induce its own production via autoinduction (Kelley, 1993). IL-1 can stimulate TGFβ expression in fibroblasts and in peritoneal mesothelial cells (Denk et al., 2000; Offner et al., 1996). Moreover, IL-12 and IFNγ have also been shown to increase TGFβ expression (Marth et al., 1997; Wilkinson et al., 2000).

1.3 TGFβ and Pleural Disease

1.3.1 Pleural Fluid Levels

Both TGFβ1 and -β2 isoforms have been demonstrated in human pleural effusions of various aetiologies. Their levels were both significantly higher in exudative than in transudative effusions. The pleural fluid levels of these two isoforms correlate with each other, as well as with that of VEGF (Cheng et al., 2000a). The TGFβ1 levels were higher in malignant effusions from mesothelioma than those from lung cancers (Maeda et al., 1994). Pleural fluid TGFβ levels were higher in tuberculous pleuritis than in non-tuberculous benign effusions (Maeda et al., 1993).

1.3.2 Effects on Mesothelial Cells

TGFβ have been shown to stimulate a variety of biological reactions from pleural and peritoneal mesothelial cells. TGFβ induces the synthesis of fibronectin (Kinnula et al., 1998), MMP-1, MMP-9 and tissue inhibitor of matrix metalloproteinases (TIMP)-2
from mesothelial cells (Ma et al., 1999; Rougier et al., 1997). The mesothelial production of PAI-1 and PAI-2 are also increased in the presence of TGFβ (Idell et al., 1992). In keeping with its anti-inflammatory role, TGFβ suppresses nitric oxide production from mesothelial cells in vivo (Owens et al., 1996).

There was conflicting data on the effect of TGFβ on the growth of mesothelial cells. TGFβ has been shown to stimulate DNA synthesis in mesothelial cells in vitro (Gabrielson et al., 1988), but no effect was observed in vivo (Mutsaers et al., 1997). On the other hand, inhibition of mesothelial cell growth by TGFβ has been reported, and the effect can be blocked with anti-TGFβ antibodies (Ikubo et al., 1995).

Interestingly, treatment of cultured mesothelial cells with TGFβ can induce distinct morphological changes. With increasing doses of TGFβ, the mesothelial cells and their nuclei become swollen. The separation between the mesothelial cells, and thereby their permeability, increases with TGFβ in a dose-dependent fashion (Ikubo et al., 1995). This observation suggests that TGFβ may have a role in pleural fluid formation.
SUMMARY

- The pleura is often affected in lung and systemic diseases. Pleural effusion is the most common clinical presentation of disorders involving the pleura.

- Pleurodesis remains an important management option for recurrent pleural effusions. However, none of the currently available pleurodesing agents is ideal.

- Chemical pleurodesis conventionally involves the intrapleural administration of an agent that induces acute pleural inflammation, followed by fibrosis and symphysis. The intense pleural inflammation frequently causes fever and pain.

- TGFβ is a ubiquitous multi-functional cytokine, particularly known for its pro-fibrotic and immunomodulatory properties.

- Mesothelial cells are biologically active and play a critical role in inflammation and fibrosis of the pleura. TGFβ can act on mesothelial cells, which in turn can also produce TGFβ.
2.1 Preparations

2.1.1 TGFβ₂
2.1.2 Talc Slurry
2.1.3 Bleomycin

2.2 Animal Models

2.2.1 Rabbit Pleurodesis Model
2.2.2 Sheep Pleurodesis Model
2.2.3 Pleurodesis Grading
2.2.4 Histologic Grading
2.2.5 Collagen Measurement

2.3 Pleural Fluid Analysis

2.3.1 Biochemical Parameters
2.3.2 Cytokine Measurements
2.3.3 Leukocyte Counts

2.4 Primary Culture of Pleural Mesothelial Cells

2.4.1 Pleural Mesothelial Cell Harvesting from Mice
2.4.2 Pleural Mesothelial Cell Harvesting from Rabbits
2.4.3 Culture of Mesothelial Cells
2.4.4 Verification of Mesothelial Origin of the Cultured Cells
2.4.5 Evaluation of Mesothelial Cell Integrity

2.5 Cytokine Measurements
2.1 Preparations

2.1.1 Transforming Growth Factor beta-2 (TGFβ2)

A recombinant human TGFβ2 (Genzyme Corp., Framingham, MA, USA), produced in Chinese hamster ovary cells was used. TGFβ2 was formulated in a vehicle consisting of 20mM sodium phosphate, 130mM sodium chloride, 15% (w/w) propylene glycol and 20% (w/w) polyethylene glycol 400. The pH of the solution was 7.2. The vehicle was prepared using USP/NF grade reagents in water for injection and sterile-filtered through a 0.2-micron filter. TGFβ2 concentration was determined by a sandwich enzyme-linked immunosorbent assay utilizing two monoclonal antibodies that cross-react with both TGFβ2 and -β3. The activity of TGFβ2 was determined using a mink lung cell (Mv1Lu) anti-proliferation assay, modified from the method described by Ogawa et al (Ogawa and Seyedin, 1991).

2.1.2 Talc Slurry

Commercially available asbestos-free talc powder (Sigma, St. Louis, MO, USA) was sterilized by either of the following methods. In the rabbit studies and in the cell culture experiments, talc powder was sterilized by standard autoclaving. Talc used in the sheep study was gas sterilized using ethylene oxide and then aerated for 96 hours before use. In animal studies, the sterilized talc was then diluted in 0.9% NaCl (Baxter, Deerfield, IL, USA) into a talc slurry.

2.1.3 Bleomycin

Bleomycin sulphate (Bleoxane, Nippon Kayaku, Tokyo, Japan) was used. The powder was diluted in 0.9% NaCl (Baxter) under sterile conditions before use.
2.2 Animal Models

All animal experiments had been approved by the Vanderbilt University Institutional Animal Care and Use Committee.

2.2.1 Rabbit Pleurodesis Model

New Zealand white rabbits (Myrtle Rabbitry, Nashville, TN, USA) weighing 1.5 – 2.0 kg were anesthetized with an intramuscular injection of 35mg/kg of ketamine hydrochloride (Fort Dodge Animal Health, IA, USA) and 5mg/kg of xylazine hydrochloride (Fermenta, Kansas City, MO, USA). The chest was shaven and the skin sterilized with 10% povidone iodine (Baxter). The rabbit was placed in the lateral decubitus position and a small (<3cm) skin incision was made midway between the tip of the scapula and the sternum approximately 2 cm above the costal margin. Chest tubes were made from intravenous solution set tubes (Baxter) with three extra openings near the distal end of the tube to enhance drainage. The chest tube was inserted by blunt dissection into the right pleural cavity. The left pleural cavity was used for control. The chest tube was secured at the muscle layers with purse-string sutures (3.0 ethilon). The proximal end of the chest tube was then tunneled underneath the skin and drawn out through the skin posteriorly and superiorly between the two scapulae. The exterior end of the chest tube was sealed with a one-way valve with cap (Medexine, Hilliard, OH, USA) via an adapter and sutured to the skin using a 2.0 silk suture. A three-way stopcock was attached to the end of the chest tube through which any aspirated air was immediately evacuated from the pleural space.
The pleurodesing agent, diluted to the standard volume of 2.5 mL, was injected via the chest tube followed by the instillation of 1.0 mL of 0.9% NaCl solution (Baxter) to clear the dead space. After the intrapleural injection, the chest tube was aspirated at 24-hour intervals for any pleural fluid. The chest tube was removed under light sedation when the pleural fluid drainage was <5 mL over the preceding 24 hours.

At the time of sacrifice, the rabbits were sedated and euthanised with carbon dioxide. The thorax was removed en bloc. The lungs were expanded by the injection of 50 mL of 10% neutral-buffered formalin (Sigma Diagnostics, St. Louis, MO, USA) into the exposed trachea via a plastic catheter (6mm diameter). The trachea was then ligated and the entire thorax submerged into the formalin solution for at least 48 hours.

2.2.2 Sheep Pleurodesis Model

Yearling sheep of mixed breeds (Ligon Sheep, Nashville, TN, USA) were used. The sheep was anesthetized with an intravenous injection of 2.5% sodium thiopental (Abbott, North Chicago, IL, USA) at 20 mg/kg. The chest and the right neck was then shaved and the skin was sterilized with 2% chlorhexadine (DVM, Miami, FL, USA) and then with 10% povidone iodine. Using a laryngoscope, an endotracheal tube (8.5 mm internal diameter) was inserted with an attached plastic “bite block” and secured with tape. Anesthesia was maintained with a gaseous mixture of room air, oxygen, and 1.5-2.5% halothane at a ventilation rate of 10 breath cycles/minute with a volume of 15 ml/kg per breath cycle. The sheep was placed on its side on a surgical table and the feet secured to the table with rope.
Blood Gas Measurements: The right carotid artery was cannulated through a right neck incision. A transverse incision of approximately 3cm was made at mid-length on the right side of the neck and a 5cm section of the carotid artery isolated. A 2-0 silk suture was used to ligate the vessel as distal from the heart as possible and a second 2-0 silk suture was loosely placed around the vessel 1 cm proximal to the first suture. A silastic catheter was inserted within the blood vessel and the second suture was tightened. The first suture was also placed around the catheter and tied to provide additional support. The wound was closed with a 3-0 ethilon monofilament nylon suture and the catheter secured to the neck of the sheep with a 3-0 nylon suture. Through this arterial line, arterial blood samples were collected for blood gas analysis. The line was flushed with 2000 U of heparin (Elkins-Sinn, Cherry Hill, NJ, USA) and 8 mg of gentamicin (American Pharmaceutical Partners, Los Angeles, CA, USA) in 2.5mL every day and also after each blood sample collection.

Chest Tube Insertion and Pleurodesis: To insert the chest tube, a 5cm incision was made in the lateral chest wall at the 7th intercostal space. By blunt dissection, a 18G French Foley balloon-catheter with 30 mL balloon volume (Bard, Covington, GA, USA) was inserted into the pleural space under aseptic conditions and tunneled underneath the skin and brought to the surface just lateral to the vertebrae. The tube was secured at the skin with purse string sutures. The same procedure was then performed on the other side to insert chest tube into the contralateral pleural space. The sheep was then ventilated with a positive end expiratory pressure of 15 cmH₂O. A three-
way stopcock was attached to the end of the Foley catheter through which all air was evacuated from the pleural space immediately after the chest tube insertion.

All sheep received a single intrapleural injection of the pleurodesing agent via the chest tube 24 hours after the surgery. The buffer or vehicle was injected to the contralateral side and served as the control. After the injection of pleurodesing agents, the chest tube was aspirated (with the Foley catheter balloon inflated) every 24 hours for any pleural fluid produced. The volume of the fluid was recorded. The chest tubes were removed when the drainage was <10mL for 2 consecutive days, but not earlier than 72 hours. The sheep were sacrificed 14 days after the chest tube insertion with an intravenous injection of sodium phenobarbital (Euthanasia-5 solution, Henry Schein, Port Washington, NY, USA).

Arterial blood were collected at baseline and then at ½, 1, 2, 4, 8, 24, 48 and 72 hours after the injection of pleurodesing agents. The arterial blood gas measurements were performed using a blood gas analyser (Ciba Corning, Essex, England) which was calibrated daily.

2.2.3 Pleurodesis Grading

The pleural cavity was carefully exposed following methodology previously described (Light et al., 1994). A consensus grading was reached by two blinded investigators on the degree of macroscopic pleurodesis using a previously published semi-
quantitative scheme (Light et al., 1994; Sassoon et al., 1995; Xie et al., 1998c), as detailed below. Any evidence of hemothorax, infection or empyema was recorded if present.

The degree of pleurodesis was graded as 0 to 4 in the initial study:

Grade 0 = normal pleural space
Grade 1 = 1-3 small adhesions in the pleural space;
Grade 2 = >3 scattered adhesions in the pleural space but lung easily separated from the chest wall;
Grade 3 = generalised scattered adhesions with areas where the lung can be separated from the chest wall only with difficulty;
Grade 4 = complete obliteration of the pleural space by adhesions.

The grading scheme was later modified such that the degree of pleurodesis was graded in more details, on a scale of 1 to 8:

1=No adhesions between the visceral and parietal pleurae;
2=Rare adhesions between the visceral and parietal pleurae with no symphysis;
3=Scattered adhesions between the visceral and parietal pleurae with no symphysis;
4=Many adhesions between the visceral and parietal pleurae with no symphysis;
5=Many adhesions between the visceral and parietal pleurae with symphysis involving < 5% of the hemithorax;
6=Many adhesions between the visceral and parietal pleurae with symphysis involving 5 - 25% of the hemithorax;
7=Many adhesions between the visceral and parietal pleurae with symphysis involving 25 - 50% of the hemithorax;
8=Many adhesions between the visceral and parietal pleurae with symphysis involving > 50% of the hemithorax.
**Adhesions** were defined as fibrous connections between the visceral and parietal pleura. **Symphysis** was present if the visceral and parietal pleura were difficult to separate as a result of adhesions.

### 2.2.4 Histologic Grading

At the time the pleura was evaluated grossly, samples of the visceral pleura and lung from each hemithorax were obtained and placed in 10% neutral buffered formalin (Sigma). The tissue samples were stained with hematoxylin-eosin (H&E) for histologic examination. The degree of microscopic inflammation and fibrosis were graded separately from the H&E slide by an experienced examiner blinded to the treatment agent as 0-4: 0 = none; 1 = equivocal; 2 = mild; 3 = moderate; or 4 = severe.

The thickness of the pleura was measured using the Leica Q500IW Imaging Workstation, Image Processing and Analysis System (Leica Imaging Systems Ltd., Cambridge, UK). With this system, the image obtained was transformed from pixels to μm. Measurements were obtained at 10 different points on each sample and the mean result was reported.

### 2.2.5 Collagen Measurement

Collagen fibres were subdivided into mature (thick) and immature (thin) fibers using picrosirius staining as reported by Andrade et al (Andrade *et al.*, 1997). The tissue blocks of the pleural tissues obtained at the time of necropsy were sectioned at 5 μm and stained for one hour in a 0.2% solution of Sirius Red, Direct Red 80 (Aldrich, Milwaukee,
WI, USA) dissolved in aqueous saturated picric acid (Montes, 1996). The enhancement of collagen birefringence elicited by picrosirius staining is specific for collagen and discloses its distinct patterns of physical aggregation. Immature (thin) fibres, as those present in early granulation tissue, are shown as weakly birefringent green structures, while mature (thick) fibres, characteristic of mature fibrotic lesions, are identified by their strong birefringence and their yellow or red colour. The areas covered by mature and immature collagen fibres were measured by the Leica Q500IW Imaging Workstation, Image Processing and Analysis System (Leica). This system detects all pixels in the image that are equivalent to, or nearly equivalent to, the colour levels of the select area. The total area of collagen deposition per high power (100x) field was the sum of the areas covered by mature and immature fibres in the same field and is expressed as the percentage of the total area of the pleural surface. For each sample, readings were taken from six representative fields and the mean result used for analysis.

2.3 Pleural Fluid Analysis

2.3.1 Biochemical Parameters

The volume of the pleural fluid collected was recorded. For biochemical measurements, pleural fluid was transported in a plain container and centrifuged at 3,000 r.p.m. for 15 minutes at room temperature. The supernatant was collected and analysed for protein, glucose and lactate dehydrogenase (LDH) levels using an automated analyser (Johnson & Johnson, Rochester, NY, USA).

2.3.2 Cytokine measurements
The pleural fluid was collected in citrated tubes, kept on ice, and then centrifuged at 3,000 r.p.m. for 15 minutes at -4°C. The supernatant was stored immediately at -70°C until assay.

2.3.3 Leukocyte Counts

Approximately 1mL of the pleural fluid was collected in an EDTA tube. Total leukocyte count was measured using an automated counter (Coulter Electronics, Luton, England) which was calibrated daily. The first reading was ignored and the mean of the next three readings was recorded.

2.4 Primary Culture of Pleural Mesothelial Cells

2.4.1 Pleural Mesothelial Cell Harvesting from Mice

Pleural mesothelial cells were obtained from wild type mice. After the mice were euthanized with carbon dioxide, the abdomen was opened to expose the diaphragm. 1.0 mL of Hank’s Balanced Salt Solution (HBSS) (Life Technologies, Green Island, NY, USA) was injected into the pleural cavity from beneath the diaphragm under direct vision and then aspirated after 2 minutes. Approximately 1.0 mL of 0.25% trypsin-EDTA solution (Life Technologies) was then injected into the pleural cavity from beneath the diaphragm. The solution was left in the pleural cavities for 10 minutes during which the mice were rotated. The solution, with the mesothelial cells, was then aspirated and transported in 10.0 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) on ice. The solution was then centrifuged at 1,000 r.p.m. for five minutes at -4°C. The supernatant was discarded and the cell pellet was re-suspended in DMEM for plating.
2.4.2 Pleural Mesothelial Cell Harvesting from Rabbits

Pleural mesothelial cells were also obtained from New Zealand white rabbits (1.5-2.0 kg) in a similar method as used in mice (see above). The exceptions were that in rabbits, 10.0 (instead of 1.0) mL of HBSS and trypsin-EDTA solution were injected into the pleural cavity. The method of cell collection, centrifugation and plating were identical to the harvest of murine mesothelial cells.

2.4.3 Culture of Mesothelial Cells

The cells were plated in 75cm² cell culture flasks (Costar, Cambridge, MA, USA) and cultured with DMEM, supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal calf serum (Life Technologies). The cells were incubated at 37°C with 95% of air and 5% of CO₂. The medium was changed the following day to remove non-adherent cells. The mesothelial cells were grown to confluence before the experiment. For murine mesothelial cells, passages 2-3 were used, whereas for rabbit mesothelial cells, passages 2-7 were used. This is because murine mesothelial cells showed senescence after passage 3, but rabbit mesothelial cells are known to maintain fast growth rate and activities up to at least 10 passages. The cells were then trypsinised and transferred to tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) 24 hours prior to the cell experiments.

2.4.4 Verification of Mesothelial Origin of the Cultured Cells
The purity of the cultured cells was verified prior to the experiments by the following methods: (1) The cells demonstrated the typical cobblestone morphology of mesothelial cells as described by Antony et al (Antony et al., 1989). (2) The cells were prepared on glass slides and stained with low molecular weight cytokeratin. One hundred cells were counted and the number of cytokeratin-positive and cytokeratin-negative cells were recorded. The average result from three different high power fields was taken. It was considered satisfactory if 95% or more of the cultured cells were cytokeratin-positive, confirming their epithelial origin.

2.4.5 Evaluation of Mesothelial Cell Integrity

At the end of the experiments, the supernatant were aspirated and immediately stored at -70°C until assay. The wells were washed with 2.0 mL of HBSS, which was collected. The cells were then trypsinised. Any remaining cells still adherent to the wells were scrapped off with a cell lifter (Fisher Scientific, Pittsburgh, PA, USA) or a cell scrapper (Costar). Each well was examined under phase contrast microscopy to ensure that all the cells were completely removed. All the cells collected in the HBSS and trypsin were transported on ice and then centrifuged at 3000 r.p.m. for 10 minutes at -4°C. The supernatant was discarded and the cell pellet re-suspended in 1.0mL of DMEM.

Mesothelial cell integrity at the end of the experiments was assessed by (1) direct visual inspection using phase contrast microscopy and (2) with trypan blue exclusion (Nasreen et al., 2000). In the latter, 100μL of the solution of re-suspended cells (as described in 2.4.4) was mixed with equal volume of 0.4% trypan blue (Sigma) and the
resultant solution was loaded onto a hemacytometer (Propper Manufacturing, Long Island City, NY, USA). In this method, non-viable cells were stained blue while viable cells were not. One hundred cells were counted under high power view with light microscopy. The average result from two different high power fields was taken and the percentage of mesothelial cell viability was calculated. The concentration of cells on each well was measured using a hemacytometer. The average counts from two different loadings were taken.

2.5 Cytokine Measurements

Cytokine levels, including TGF\( \beta_1 \), \( \beta_2 \), IL-8 and vascular endothelial growth factor (VEGF), were determined using an enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN, USA). Measurements were made using the Microplate Manager 4.0 (Bio-Rad Laboratories Inc., USA) at the wavelength of 450nm (reference wavelength 540nm). In the case of TGF\( \beta \), all samples were acidified to convert all TGF\( \beta \) present to the immunoreactive form for measurement. There was no detectable cross-reactivity between the TGF\( \beta_1 \) and \( \beta_2 \) isoforms using this commercial method (Mozes et al., 1999).
CHAPTER 3

INTRAPLEURAL ADMINISTRATION OF 
TRANSFORMING GROWTH FACTOR $\beta_2$
IN A RABBIT MODEL
3.1 Introduction

The treatment of a recurrent pleural effusion or a recurrent pneumothorax frequently involves the creation of a pleurodesis. With a pleurodesis, fusion of the visceral and parietal pleura occurs such that there is no space for the collection of pleural fluid or air. In clinical practice, pleurodesis is most commonly created by the intrapleural administration of a pleurodesing agent through a chest tube. The agents most commonly used are talc slurry, the tetracycline derivatives (doxycycline or minocycline) and bleomycin. None is ideal. The injection of talc can lead to the development of the acute respiratory distress syndrome and death (Milanez et al., 1997; Rehse et al., 1999) (Rehse et al., 1999). The injection of the tetracycline derivatives is at times extremely painful (Light et al., 1990). Bleomycin is relatively expensive and is less efficacious than talc or the tetracycline derivatives (Walker-Renard et al., 1994). Accordingly, the search continues for an ideal pleurodesing agent.

The exact mechanisms responsible for pleurodesis are unknown. It is believed that the intrapleural injection of an irritant produces inflammation (Kennedy et al., 1995; van den Heuvel et al., 1998), which in turn initiates a cascade of events that culminates at times in the creation of a pleurodesis. Cytokines are unquestionably involved in the processes of pleurodesis. Theoretically, it might be possible to create a pleurodesis by the intrapleural injection of a cytokine, which would avoid the necessity of producing an injury to the pleura.
If a solitary cytokine could produce a pleurodesis, the following characteristics of TGFβ make it a good candidate. (A) TGFβ is a potent fibrogenic cytokine which regulates extracellular matrix production - in situations where there is too much TGFβ, fibrosis results (Grande, 1997). The transient over-expression of TGFβ in the rat lung leads to marked pleural and interstitial fibrosis (Sime et al., 1997). (B) Once present, TGFβ may induce its own transcription through auto-induction (Border and Noble, 1994; Kelley, 1993) - in which case, a single injection may be sufficient. (C) Mesothelial cells express and secrete TGFβ (Offner et al., 1996); therefore one intrapleural injection of TGFβ might result in prolonged secretion of TGFβ which could result in pleurodesis. (D) The incubation of human pleural mesothelial cells with TGFβ results in secretion of increased levels of PAI-1 (Idell et al., 1992) - this could facilitate pleurodesis since inhibition of the fibrinolytic system is thought to be necessary for the production of a pleurodesis (Rodriguez-Panadero et al., 1995).

In this and the following chapters, the effect of intrapleural administration of TGFβ2, its speed of action and its safety would be explored. As discussed in Chapter 1, TGFβ1 and β2 have significant overlaps in their biological properties, and both are believed to possess potent pro-fibrotic and immunomodulatory properties. Human recombinant TGFβ2 can now be manufactured in sufficient purity and quantities for animal experiments, and a phase I clinical trial has confirmed the safety of the systemic administration of TGFβ2 in humans (Rodriguez-Panadero et al., 1995).
The purpose of this first study was to determine if the intrapleural injection of TGFβ2 could result in a pleurodesis. This was based upon the hypothesis that the intrapleural injection of TGFβ2 would result in a pleurodesis and produce less inflammation than is produced by other usual pleurodesing agents.
3.2 Methods

The method used was that described in Chapter 2.2.1, in accordance with a previously described rabbit model of pleurodesis (Cheng et al., 2000b). In brief, chest tubes were inserted into the right pleural cavities of New Zealand white rabbits under sedation. The left hemithorax received no chest tube and no injection and served as a control. Human recombinant human TGFβ2 was used and the method of production was that detailed in Chapter 2.1.1.

The primary goal of this research was to evaluate whether pleurodesis could be produced by the intrapleural injection of TGFβ. The study was performed in two phases. The initial phase was a pilot study to determine if the intrapleural injection of TGFβ2 could produce a pleurodesis. The second phase was a dose response study of the production of pleurodesis by single doses of TGFβ2 in the dose range determined from the results of the pilot study.

Ten rabbits were included in the pilot study (Table 3.1). The first two rabbits served as controls and received 1 or 4 injections of the buffer. Two rabbits received 1 or 4 injections of TGFβ2 5.0 μg, two rabbits received 1 or 4 injections of TGFβ2 10.0 μg, and two rabbits received 1 or 4 injections of TGFβ2 10.0 μg. For the last two rabbits, one rabbit each received 2 and 3 injections of 20.0 μg TGFβ2. When rabbits received multiple injections, the injections were separated by 24 hours. All injections had a total volume of 2 ml and were followed by 1.0 ml of 0.9% NaCl to clear the dead space of the chest tube.
The pleural space was aspirated via the chest tube every 24 hours after the initial pleural injection, and the pleural fluid analysed as described (Chapter 2). In the pilot study, if an animal was scheduled to have an additional intrapleural instillation, the aspiration was performed immediately before the instillation. Chest tubes were left in place for 96 hours following the initial pleural injection or until the volume of pleural fluid aspirated over the preceding 24 hours was <3 ml, whichever was later.

The second phase was a dose-response study. Based on the results of the initial pilot study, rabbits in the second phase were given single intrapleural injections of TGFβ2 at 5.0 μg (n = 12), 1.67 μg (n = 10), 0.50 μg (n = 10), 0.167 μg (n = 4) or the buffer alone (n = 5). The animal experiments and pleural fluid collection were performed in the same fashion as in the pilot study.

Rabbits were sacrificed 14 days after the intrapleural injection of TGFβ2. (Rabbits that received multiple doses were sacrificed 14 days after the first injection.) The thorax were preserved en block as described in Chapter 2. At the time of grading the pleurodesis, each pleural cavity was exposed by making bilateral incisions through the diaphragms, and through all the ribs at the midclavicular line. In this manner, the sternum and the medial portions of the anterior ribs were removed so that the lung and pleural cavities could be evaluated. In this study, the degree of pleurodesis observed grossly was graded from 0 to 4 according to the scheme outlined in Chapter 2.2.3 by two blinded investigators.
In addition, the degree of pleurodesis and the characteristics of the pleural fluid after the administration of 5.0 μg of TGFβ2 were compared to results obtained 28 days after the intrapleural administration of 400 mg/kg talc or 10 mg/kg doxycycline which have been reported previously (Rogers et al., 1998). The protocol that had been followed in the rabbits that had been given doxycycline and talc was essentially identical to that which was used for the single-dose TGFβ2 injections.

Statistical Analysis: All data are expressed as the mean ± standard error of the mean unless otherwise stated. The pleurodesis scores and pleural fluid volume, LDH, white blood cell counts and differential cell counts in the different groups were compared using two way repeated measures analysis of variance (ANOVA). The means in the various groups were compared using the Student-Newman-Keuls Method. If the data failed tests of normality or equal variance, the medians were compared using the Kruskal-Wallis one way ANOVA on ranks. The medians were compared using Dunn’s method (Sigma Stat, SPSS, San Raphael, CA, USA). Differences in the results were considered significant when p<0.05.
3.3 Results

The pilot study demonstrated that the intrapleural injection of TGFβ2 can produce a pleurodesis (Table 3.1). All rabbits that received TGFβ2 intrapleurally achieved the maximum pleurodesis score of 4. In contrast, the two rabbits that received one or four injections of the buffer alone had pleurodesis scores of only 0 and 1. In addition, the pilot study showed that a single injection of TGFβ2 at a dose of 5, 10 or 20 μg could produce a pleurodesis. The fibrous reaction with the higher doses was actually more than would be ideal in producing a pleurodesis. All the rabbits, except the one that received a single dose of 5 μg, had some adhesions in the contralateral pleural space. The rabbit that received four separate doses of 20 μg TGFβ2 had a fibrin ball in the peritoneal cavity, 112 mL of straw colored peritoneal fluid, 28 mL bloody pleural fluid in the right pleural space and a pleurodesis score of 3 on the left (control) side. The rabbit that received three separate doses of 20 μg TGFβ2 had similar findings but had no fibrin balls in the peritoneal cavity. The rabbit that received four separate doses of 10 μg TGFβ2 had no fibrin balls in the peritoneal cavity but did have 100 mL of straw colored peritoneal fluid, 8 mL of pleural fluid and a pleurodesis score of 2 on the control side. The rabbit that received a single dose of 5 μg TGFβ2 had no pleural or peritoneal fluid and had no adhesions on the control side.

In the pilot study, the intrapleural injection of TGFβ2 resulted in the formation of large amounts of pleural fluid characterised by relatively low leukocyte counts and LDH levels (Table 3.1). Several of the rabbits had more than 20 mL pleural fluid present at the 24 hour time period.
**TABLE 3.1.** Results from the pilot study for the intrapleural injection of TGFβ₂.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Dose</th>
<th># Inj</th>
<th>R</th>
<th>L</th>
<th>24h</th>
<th>Total</th>
<th>24h</th>
<th>24h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3.5</td>
<td>19</td>
<td>34362</td>
<td>6023</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>Buffer</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>19</td>
<td>10156</td>
<td>13601</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>5µg</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>55</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>5µg</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>24</td>
<td>82</td>
<td>915</td>
<td>1799</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>10µg</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>35</td>
<td>78</td>
<td>1467</td>
<td>4665</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>10µg</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>11</td>
<td>120</td>
<td>975</td>
<td>7232</td>
<td>3.1</td>
</tr>
<tr>
<td>7</td>
<td>20µg</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>24</td>
<td>70</td>
<td>1376</td>
<td>4620</td>
<td>3.3</td>
</tr>
<tr>
<td>8</td>
<td>20µg</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>22</td>
<td>107</td>
<td>1508</td>
<td>4500</td>
<td>2.1</td>
</tr>
<tr>
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<td>4</td>
<td>3</td>
<td>15</td>
<td>131</td>
<td>1377</td>
<td>7343</td>
<td>3</td>
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<td>20µg</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>17</td>
<td>122</td>
<td>1147</td>
<td>1342</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Abbreviations: # Inj = number of injections; R = right; L = left; LDH = Lactate dehydrogenase; WBC = white blood cell; Prot = protein.
In the second phase, single intrapleural injections of TGFβ2 induced pleurodesis in a dose-dependent manner (Figure 3.1). The mean pleurodesis score was significantly higher in the group that received 5.0μg TGFβ2 (3.6±0.9) than in the groups given 1.67μg (2.6±1.4, p<0.05), 0.50μg (1.5±1.0, p<0.001), 0.167μg (0.6±0.9, p<0.001) or the buffer (0.3±0.1, p<0.001). The mean score for the group that received 1.67μg was significantly higher than that for the group that received 0.50μg (p<0.05), 0.167μg (p<0.01) or buffer (p<0.001). In none of the rabbits was the pleurodesis score on the left (control) side higher than 1. Since the pleurodesis scores were very low and did not differ significantly between the control and the TGFβ2 0.167μg groups, these groups were combined for statistical analysis of the pleural fluid results.
**FIGURE 3.1.** Intrapleural injection of TGFβ₂ induced pleurodesis in a dose-dependent manner.

The horizontal bar indicated the mean pleurodesis score of the group.
In the dose-response study, the intrapleural injection of the higher doses of TGFβ2 resulted in a high volume pleural effusion (Figure 3.2). The fluid collected at 24 hours was an exudate in that the mean pleural fluid LDH exceeded 2000 IU, although the mean protein levels at 24 hours were in the 2.7 to 3.0 g/dL range (data not shown). The amount of pleural fluid was significantly (p<0.02) greater in the groups that received the three higher doses of TGFβ2 than it was in the control group at 24 hours. The group that received 5.0μg of TGFβ2 had significantly higher fluid volumes at 48 and 72 hours. When the total amount of fluid (including that obtained at autopsy) was compared, the mean amount of fluid was significantly more in the rabbits that received 5.0μg TGFβ2 than in all the other groups (p<0.05). The increased fluid had decreased to <2mL/day in almost all rabbits by day 5 and none of the rabbits had pleural fluid present at day 14.

Although the intrapleural injection of TGFβ2 induced the accumulation of a large amount of pleural fluid, the fluid did not appear to be particularly inflammatory (Figures 3.3 and 3.4). The pleural fluid leukocyte and LDH levels were both significantly lower in the group that received 5.0μg TGFβ2 than they were in the control group on all three days.
FIGURE 3.2. Mean amounts of pleural fluid produced during each of the first three days and the total amount of fluid produced (including at autopsy) after the intrapleural injection of different dosages of TGFβ2 or the TGFβ2 buffer only.

<table>
<thead>
<tr>
<th></th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ug TGFβ2</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>1.67 ug TGFβ2</td>
<td></td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 ug TGFβ2</td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

* p<0.05 when compared with control
** p<0.05 when compared with 5.0ug TGFβ2

n = 10-14 for each TGFβ2 treatment group; n = 5 for buffer controls. Error bar = S.E.M.
FIGURE 3.3. Mean pleural fluid white blood cell counts on the first three days after the intrapleural injection of different dosages of TGFβ2 or the buffer alone.

* \( p < 0.05 \) when compared with control

\( n = 10-14 \) for each TGFβ2 treatment group; \( n = 5 \) for buffer controls. Error bar = S.E.M.
FIGURE 3.4. Mean pleural fluid LDH levels on the first three days after the intrapleural injection of different dosages of TGFβ₂ or the buffer alone.

$p<0.05$ when compared with control.

$n = 10-14$ for each TGFβ₂ treatment group; $n = 5$ for buffer controls. Error bar = S.E.M.
**TABLE 3.2.** Differential cell counts at 24, 48 and 72 hours after the injection of buffer and increasing dosages of TGF\(\beta_2\). The differential cell counts are expressed as percentage of total leukocyte counts.

<table>
<thead>
<tr>
<th></th>
<th>Buffer (n = 5)</th>
<th>0.167 µg (n = 4)</th>
<th>0.50µg (n = 9)</th>
<th>1.67µg (n = 10)</th>
<th>5.0µg (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>58.4 ± 4.4</td>
<td>66.7 ± 5.9</td>
<td>60.1 ± 2.8</td>
<td>57.5 ± 4.1</td>
<td>61.1 ± 6.4</td>
</tr>
<tr>
<td>48 h</td>
<td>31.3 ± 3.3</td>
<td>50.0 ± 9.1</td>
<td>33.4 ± 3.3</td>
<td>25.0 ± 3.0</td>
<td>32.2 ± 4.9</td>
</tr>
<tr>
<td>72 h</td>
<td>28.0 ± 2.1</td>
<td>27.8 ± 3.3</td>
<td>27.4 ± 5.4</td>
<td>34.5 ± 6.4</td>
<td>25.7 ± 5.5</td>
</tr>
<tr>
<td>Monocyte %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>41.2 ± 4.6</td>
<td>32.3 ± 6.0</td>
<td>39.3 ± 2.9</td>
<td>41.5 ± 4.2</td>
<td>37.8 ± 6.3</td>
</tr>
<tr>
<td>48 h</td>
<td>65.3 ± 2.6</td>
<td>46.8 ± 9.1</td>
<td>63.7 ± 3.2</td>
<td>71.7 ± 3.6</td>
<td>66.7 ± 4.8</td>
</tr>
<tr>
<td>72 h</td>
<td>68.3 ± 2.3</td>
<td>68.0 ± 3.3</td>
<td>68.6 ± 5.3</td>
<td>62.5 ± 5.9</td>
<td>71.4 ± 5.6</td>
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<tr>
<td>Lymphocyte %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.4 ± 0.4</td>
<td>1.0 ± 0.6</td>
<td>0.6 ± 0.2</td>
<td>1.0 ± 0.6</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>48 h</td>
<td>3.3 ± 0.9</td>
<td>3.3 ± 1.1</td>
<td>2.8 ± 1.2</td>
<td>3.3 ± 1.1</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>72 h</td>
<td>3.7 ± 2.7</td>
<td>4.3 ± 2.7</td>
<td>4.0 ± 1.3</td>
<td>3.0 ± 1.6</td>
<td>2.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM.
We next compared the results of the intrapleural injection of 5.0µg TGFβ2 with the previous documented results of the injection of doxycycline 10 mg/kg or talc slurry 400 mg/kg. Although there were no significant differences in the pleurodesis scores (Figure 3.5), the pleural fluids that resulted from intrapleural injections of doxycycline and talc were much lower in volume (Figure 3.6) but more inflammatory (with higher total leukocyte counts and LDH levels) than those from intrapleural injection of TGFβ2 (Figures 3.7 and 3.8). At 24 hours post-injection, the mean pleural fluid leukocyte counts after the intrapleural injections of TGFβ2, talc and doxycycline were 830; 6430 and 29,149 cells/mm³ respectively. At this same time-point, the mean pleural fluid LDH levels after the intrapleural injections of TGFβ2, talc and doxycycline were 2,965; 26,435 and 29,593 lU/dL respectively.
**FIGURE 3.5.** Mean degree of pleurodesis after intrapleural injection of TGFβ2 (5.0ug), talc slurry (400mg/kg) and doxycycline (10mg/kg).

The horizontal bar indicated the mean pleurodesis score of the group.
FIGURE 3.6. Mean amounts of pleural fluid produced during each of the first three days and the total amount of fluid produced (including at autopsy) after the intrapleural injection of TGFβ2 (5μg), doxycycline (10mg/kg) and talc slurry (400mg/kg). Note that there is significantly more fluid produced after the intrapleural injection of TGFβ2.

\[ \begin{array}{c|c|c|c|c} 
 & 24 hours & 48 hours & 72 hours & Total \\
TGFβ2 5μg & * & * & * & * \\
Doxycycline 10mg/kg & 0 & 0 & 0 & 0 \\
Talc 400mg/kg & 0 & 0 & 0 & 0 \\
\end{array} \]

\[ p<0.05 \text{ when compared with TGFβ2} \]

n=12 for TGFβ2 group; n=9 for talc group; n=7 for doxycycline group. Error bar = S.E.M.
FIGURE 3.7. Mean WBC counts in the pleural fluid during the first three days after the intrapleural injection of TGFβ₂ (5μg), doxycycline (10mg/kg) and talc slurry (400mg/kg).

* p<0.05 when compared with TGFβ₂

n=12 for TGFβ₂ group; n=9 for talc group; n=7 for doxycycline group. Error bar = S.E.M.
**FIGURE 3.8.** Mean pleural fluid LDH levels during each of the first three days after the intrapleural injection of TGFβ2 (5ug), doxycycline (10mg/kg) and talc slurry (400mg/kg).

*n=12 for TGFβ2 group; n=9 for talc group; n=7 for doxycycline group. Error bar = S.E.M.*
3.4 Discussion

The present study demonstrates that a single intrapleural injection of TGFβ2 produces an excellent pleurodesis in rabbits. This pleurodesis is at least as good as that which results from intrapleural injections of doxycycline (10 mg/kg) or talc slurry (400 mg/kg). Interestingly, intrapleural injections of TGFβ2 resulted in the production of significantly more pleural fluid with a lower leukocyte count and LDH level than did the injections of doxycycline or talc. These observations suggest that induction of a pleurodesis by the intrapleural injection TGFβ2 might involve less injury to the pleura than does the injection of doxycycline or talc slurry, and therefore might produce less chest pain and systemic symptoms.

The mechanism by which various agents produce a pleurodesis is largely unknown. The initial event in the production of a pleurodesis is usually an injury to the pleura. An acute exudative pleural effusion develops within 12 hours of the instillation of essentially all of the agents that are presently used for pleurodesis including talc (Xie et al., 1998d), tetracycline derivatives (Wu et al., 1998), bleomycin (Sahn and Good, 1981) and mitoxantrone (Vargas et al., 1998). Subsequently, the resolution of the pleural injury may or may not result in a pleurodesis (Sahn and Good, 1981).

It is likely that cytokines in the pleural space are responsible for determining whether or not a pleurodesis will result from a pleural injury. It was reasoned that if this were true, then the intrapleural injection of a cytokine might produce a pleurodesis
without requiring the non-specific injury to the pleura. Amongst the cytokines, TGFβ appears to be a promising candidate for the production of a pleurodesis. TGFβ is a member of a superfamily of polypeptide factors that control development and tissue homeostasis in organisms from Drosophila to humans (Grande, 1997). TGFβ is found in inflammatory cells (especially macrophages) and platelets, and is very abundant in the cells of the lung. TGFβ stimulates extracellular matrix accumulation, is chemotactic for fibroblasts and monocytes and is involved in many lung diseases in which fibrosis plays a part including idiopathic pulmonary fibrosis (Coker et al., 1997; Sime et al., 1997), asbestos lung disease (Jagirdar et al., 1997) and radiation-induced lung fibrosis (Martin et al., 2000).

TGFβ2 has several characteristics that suggest that it might be a suitable agent for inducing pleurodesis. Pleurodesis results from fibrosis, and the excessive production of TGFβ2 can lead to fibrosis. When rats are transfected with an adenovirus vector expressing an active form of TGFβ1, the transient over-expression of TGFβ1 results in prolonged and severe interstitial and pleural fibrosis (Sime et al., 1997). In humans there are at least five polymorphisms of the TGFβ1 gene. The lymphocytes of individuals with different genotypes produce varying amounts of TGFβ1. In the population of lung transplant patients, individuals with the genotypes associated with a higher production of TGFβ1 had more fibrosis in their native lung (Awad et al., 1998).

Mesothelial cells express and secrete TGFβ2 (Gerwin et al., 1987). Since TGFβ can potentially induce its own transcription (Border and Noble, 1994; Kelley, 1993), one
intrapleural injection of TGFβ2 can induce the mesothelial cells to express and secrete TGFβ2. Inhibition of the fibrinolytic pathway is thought to be necessary for the production of a pleurodesis (Rodriguez-Panadero et al., 1995); the incubation of human pleural mesothelial cells with TGFβ results in increased levels of PAI-1 (Idell et al., 1992), which should inhibit the fibrinolytic system.

What is the mechanism by which the intrapleural injection of TGFβ2 produces a pleurodesis? Although most agents that produce a pleurodesis induce a pleural injury, we speculate that such a pleural injury is not necessary when TGFβ2 is used to produce a pleurodesis. The low pleural fluid WBC counts and LDH levels after TGFβ2 suggest that the pleura was not severely injured. Rather, the intrapleural injection of TGFβ2 induces the mesothelial cells and fibroblasts to produce collagen and more TGFβ2, which in turn leads to the production of additional collagen.

Theoretically, it is possible that pleurodesis resulting from the intrapleural injection of TGFβ2 could represent an immune response to a non-species specific product since we used human TGFβ2 in rabbits. We believe that this is unlikely for the following reasons. Firstly, the primary structure of the TGFβ isoforms is remarkably conserved among mammalian species (Kelley, 1993; Letterio and Roberts, 1998) such that there is only one amino acid difference between activated human and murine TGFβ1. Secondly, there is complete interspecies cross-reactivity of TGFβ isoforms with their cognate receptors in all mammals (Letterio and Roberts, 1998). Human TGFβ1, β2, and β3 have all shown similar biological activity in tissue studies in the mouse, rat, dog, rabbit, and
pig. Thirdly, the immunosuppressive properties of TGFβ make it unlikely that the molecule would evoke a classic immune response in the host. Indeed, it is very difficult to raise antibody against this molecule (Letterio and Roberts, 1998).

Intrapleural injections of TGFβ₂ led to the production of much larger volumes of pleural fluid than did intrapleural injections of doxycycline or talc. What is the mechanism by which the intrapleural injection of TGFβ₂ leads to the production of the large amounts of pleural fluid? In vitro, TGFβ₂ can alter the morphology of mesothelial cells, increase the size of the intercellular spaces and hence the permeability of mesothelial cell monolayers (Ikubo et al., 1995). TGFβ is also one of the most potent stimulators of the production of vascular endothelial growth factor (VEGF) (Berse et al., 1999) – a cytokine well known for its ability to increase vascular permeability (Ferrara and Keyt, 1997). In some studies, VEGF is more potent than histamine in increasing vascular permeability (Collins et al., 1993). VEGF has been postulated to be an important mediator in the formation of pleural and peritoneal fluid (Cheng et al., 1999; Kraft et al., 1999; Thickett et al., 1999). When quiescent cultures of mouse embryo-derived cells or human lung adenocarcinoma cells are treated with TGFβ, VEGF mRNA and protein are induced (Pertovaara et al., 1994). In addition, when human synovial fibroblasts are incubated with various cytokines, TGFβ is the strongest inducer of VEGF secretion (Berse et al., 1999). Therefore, if TGFβ induced VEGF in the pleural space, the increased levels of VEGF could certainly lead to additional pleural fluid formation. This hypothesis will be further explored later (see Chapter 8).
The present study demonstrates that the intrapleural injection of TGFβ produces a pleurodesis, but what are the possible side effects? Our pilot study demonstrates that repeated intrapleural injections of large doses of TGFβ2 results in development of pleurodesis on the contralateral side and the accumulation of fibrous balls in the peritoneal cavity. In humans with multiple sclerosis who were given up to 2.0 μg/kg TGFβ2 intravenously three times a week for 4 weeks, there was a mild deterioration of renal function and mild anaemia which were reversible after the drug was discontinued. Patients in that study who received lower doses did not develop any significant side effect (Calabresi et al., 1998).

What are the clinical implications of the present study? Certainly, none of the available agents for pleurodesis are ideal. The three primary agents used for pleurodesis at the present time are talc, the tetracycline derivatives and bleomycin. In the United States, the only two agents approved by the Food and Drug Administration are talc and bleomycin. The major concern regarding talc is its safety. It is now accepted that talc can induce ARDS, though there is still considerable debate as to how often it occurs (Antunes and Neville, 2000; Light, 2000). In a retrospective study of 89 talc pleurodesis procedures in 78 patients, the incidence of respiratory complications or death was 33%: 8 patients developed ARDS, one patient died, 6 patients developed dyspnea and 3 patients developed re-expansion pulmonary edema (Rehse et al., 1999). Systemic embolization following intrapleural talc injections is also well documented though the long-term consequences of this remain unknown (Light, 2000). The intrapleural injection of a tetracycline derivative is at times very painful, and recent animal studies demonstrate
that in rabbits it produces elevations of liver enzymes and tissue toxicity (Mitchem et al., 1999). Bleomycin is expensive and is relatively ineffective compared with other sclerosing agents (Walker-Renard et al., 1994). Also since it does not produce pleurodesis in animals with normal pleura (Vargas et al., 1993), it is unlikely to produce a pleurodesis in a normal human.

Pain and fever from the intense pleural inflammation are common side effects following pleurodesis. The low inflammatory indices in the pleural fluids in this study suggested that the degree of pleural inflammation following intrapleural TGFβ2 injection was significantly less that following talc or doxycycline pleurodesis. This point and its underlying pathophysiology will be further explored in Chapter 7.

In conclusion, the intrapleural instillation of TGFβ2 in rabbits produced effective pleurodesis. In the following chapters, additional studies are performed to delineate the speed at which TGFβ2 can induce effective pleurodesis compared with talc, and the safety of intrapleural administration of TGFβ2.
SUMMARY

• The intrapleural injection of TGFβ2 induced effective pleurodesis in rabbits in a dose-dependent manner.

• Doses of 5.0 and 1.7μg of TGFβ2 were effective. Higher doses, especially when given repeatedly, could result in systemic side effects with contralateral pleural fibrosis and peritoneal fibrosis.

• The injection of TGFβ2 induced the production of a large volume of pleural fluid. This effect was transient and did not hinder the production of effective pleurodesis.

• The pleural fluid produced was low in inflammatory markers, suggesting that TGFβ2 might stimulate pleurodesis without inducing significant inflammation of the pleura.

• When compared with historic data, TGFβ2 was at least as effective as conventional pleurodesing agents such as talc and bleomycin at 14 days.
CHAPTER 4

TEMPORAL EVOLUTION OF
TRANSFORMING GROWTH FACTOR $\beta_2$-INDUCED
AND TALC-INDUCED PLEURODESIS
4.1 Introduction

The ideal pleurodesing agent should produce pleurodesis effectively, safely and in the shortest possible time.

Talc is the most effective of the currently available pleurodesing agents. However, there is increasing concern of its safety, as mentioned in Chapter 3. The speed at which talc produces pleurodesis in humans is unknown. However, various animal studies have shown that talc, even in high doses, is slow in inducing pleural fibrosis - requiring up to four weeks to produce a satisfactory pleurodesis (Xie et al., 1998d). Conventional pleurodesing agents, including talc, act indirectly by inducing pleural injury which results in acute inflammation and subsequent fibrosis (Kennedy et al., 1995).

While we have demonstrated that TGFβ2 is effective in producing pleurodesis in rabbits (Chapter 3), the rate at which it creates pleurodesis is not known. TGFβ2 is a potent pro-fibrotic cytokine. It increases extracellular matrix by stimulating collagen and fibronectin synthesis as well as by inhibiting matrix degradation (Border and Noble, 1994; Kelley, 1993). The direct fibrogenic action of TGFβ2 may allow it to produce pleurodesis faster than talc by bypassing the pleural injury and subsequent inflammatory processes.

The purpose of the present study was to compare the temporal evolution of TGFβ2- and talc-induced pleurodesis. It was hypothesized that the intrapleural
administration of TGFβ2 would (i) produce an effective pleurodesis faster than the intrapleural administration of talc; (ii) stimulate more collagen deposition than talc and (iii) induce less inflammation than talc.
4.2 Methods

The production methods of the recombinant human TGFβ2 (Genzyme) used and the animal experiment procedures were the same as those described in Chapter 2.1 and 2.2 respectively.

Thirty New Zealand white rabbits were divided into two groups of 15. Rabbits in the TGFβ2 group received an intrapleural injection of 1.7 μg of TGFβ2 in 2.5 mL via the chest tube while rabbits in the talc group received 400mg/kg of sterilized talc slurry (Sigma, St. Louis, MO, USA) in 2.5mL via the chest tube. Talc at 400mg/kg was shown to be the most effective dosage in a previous dose-response study of talc pleurodesis in rabbits (Light et al., 1995). It is the most common dosage of talc used in rabbit pleurodesis studies (Xie et al., 1998b; Xie et al., 1998c). The dose of TGFβ2 used was the lowest effective dose as determined in the dose-response study in Chapter 3.

After the intrapleural injection, the chest tube was aspirated at 24-hour intervals for any pleural fluid. The chest tube was removed under light sedation when the pleural fluid drainage was <5 mL over the preceding 24 hours. The fluid was collected and processed as described in Chapter 2.

Five rabbits in each group were sacrificed at each of the following time-points: days 1, 4 and 7. At the time of sacrifice, the rabbits were sedated and euthanised with carbon dioxide. The thorax was removed en bloc and preserved as described in Chapter 2.2.1. The pleural cavity was carefully exposed as outlined in Chapter 3, and following
methodology previously described (Light et al., 1994). A more detailed semi-quantitative scheme of scores 1 (no adhesions) to 8 (symphysis of 50% of the hemithorax) was used, as detailed in Chapter 2.2.5.

Samples of the visceral pleura and lung from each hemithorax were obtained. The tissue samples were assessed for the degree of microscopic inflammation and fibrosis (0 to 4) by a blinded examiner. The thickness of the pleura was measured and the amount of mature (thick) and immature (thin) collagen fibers were measured as described in Chapter 2.

**Statistical analysis**

Student's $t$ test (for parametric data) and Mann-Whitney Rank Sum test (for non-parametric data) were used to compare the values between subgroups. Two-way ANOVA and the Tukey test were used to compare the values between talc and TGFβ2 groups, using the pleurodesing agent and the days after injection as the two factors for analysis. If the values were not normally distributed, the data were log transformed before analysis with the two-way ANOVA. A $p$ value of < 0.05 was considered significant. Data were presented as mean ± S.D. (standard deviation) unless otherwise stated. All data were analyzed with Sigma Stat V2.03 statistic software program (SPSS).
4.3 Results

**Pleurodesis Scores**

The intrapleural injection of TGFβ2 produced a more rapid pleurodesis than did the intrapleural injection of talc slurry. The mean pleurodesis scores were significantly higher in rabbits that received TGFβ2 than those injected with talc slurry at all three time-points studied, Figure 4.1. By day 7, rabbits in the TGFβ2 group had achieved excellent pleurodesis while those rabbits in the talc group had developed significantly less pleurodesis (pleurodesis scores 6.2±2.2 vs 2.4±1.8, p<0.02).

One rabbit in the TGFβ2 group developed an empyema 48 hours after the chest tube injection and was sacrificed and replaced. No other rabbits died before the time of sacrifice. One rabbit in the TGFβ2 group (sacrificed at day 7) had a mild hemothorax occupying <15% of the hemithorax.
FIGURE 4.1. Pleurodesis Scores of the TGFβ2 (▲) and Talc (△) Groups at Different Time-points. The differences between the pleurodesis scores of the TGFβ2 and the talc groups were significant at all time-points: Day 1 $p=0.01$; Day 4 $p<0.001$; Day 7 $p<0.02$. 
Microscopic Pleural Histology

The pleural fibrosis score, pleural thickness, and degree of inflammation are presented in Table 4.1. The pleural fibrosis and pleural thickening scores increased with time in both groups. In keeping with the macroscopic pleurodesis score, the TGFβ2 group had higher levels of pleural fibrosis and pleural thickening at all three time-points than the talc group. When analysed as a group, rabbits that received TGFβ2 had significantly higher pleural fibrosis score and pleural thickness than rabbits in the talc group (p=0.01, p<0.05 respectively). Although TGFβ2 produced substantially more pleural fibrosis and thickening, the degree of pleural inflammation was similar in the two groups at each of the time-points.

The amount of collagen deposition (expressed as a percentage of the total area of the pleura) and the proportion of mature and immature fibres between the two groups at different time-points are shown in Figure 4.2. Significantly more total collagen, as well as mature (thick) and immature (thin) fibre depositions were seen in the pleura of rabbits that received TGFβ2 when compared with those that received talc (p<0.01 for all three collagen groups). The amount of collagen deposition was approximately five folds higher in the TGFβ2 group than in the talc group consistently at all time-points studied. As expected, there were more total collagen, mature and immature collagen fibre depositions (p<0.001; p<0.05; p<0.01 respectively) at day 7 than in the earlier time-points. The
The proportion of mature and immature collagen fibres deposited were comparable in each group.

**TABLE 4.1.** Histologic comparison between the TGFβ2 and the talc groups at various time-points. (n=5 for each group at each time point)

*p<0.05 and ** p<0.01 between the TGFβ2 and the talc groups.

Mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Talc</td>
<td>TGFβ2</td>
<td>Talc</td>
</tr>
<tr>
<td>Pleural Inflammation (0-4)</td>
<td>1.4 ± 0.5</td>
<td>1.8 ± 0.8</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>Pleural Fibrosis (0-4) *</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.4</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Pleural thickness (μm) *</td>
<td>44 ± 29</td>
<td>42 ± 30</td>
<td>100 ± 61</td>
</tr>
<tr>
<td>% Pleura Covered by Collagen**</td>
<td>0.5 ± 0.3</td>
<td>2.6 ± 2.7</td>
<td>1.0 ± 0.7</td>
</tr>
</tbody>
</table>
**FIGURE 4.2.** The amount of mature and immature collagen deposition in the TGFβ₂ and talc groups at different time-points. (n=5 for each group at each time point)

(The differences in total collagen, mature collagen and immature collagen deposition between the TGFβ₂ and talc groups were all statistically significant, p<0.01.)
**Pleural Fluid Analysis**

The pleural fluids collected at 24 hours after the injection of TGFβ₂ or talc slurry were compared (Table 4.2). The TGFβ₂ group of rabbits produced significantly more fluid than the talc group at 24 hours post-injection. The fluid produced after TGFβ₂ injection was characterised by lower LDH levels, though the protein levels were similar in both groups. The amount of fluid produced after TGFβ₂ decreased rapidly over the following days. None of the rabbits sacrificed at day 4 had pleural fluid present at the time of necropsy.

**TABLE 4.2.** Pleural fluid characteristics following intrapleural administrations of TGFβ and talc at 24 hours after injection. (Mean ± S.D.) (n=15 for each group)

<table>
<thead>
<tr>
<th>Effusion Indices</th>
<th>TGFβ₂</th>
<th>Talc</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>16.5 ± 9.0</td>
<td>1.8 ± 1.0</td>
<td>≤0.001</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>3156 ± 1426</td>
<td>12733 ± 5249</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>2.96 ± 0.51</td>
<td>3.00 ± 0.46</td>
<td>=0.89</td>
</tr>
</tbody>
</table>
4.4 Discussion

This present study demonstrated that TGFβ2 produces pleurodesis significantly faster than talc. Rabbits in the TGFβ2 group achieved excellent pleurodesis within seven days. Intrapleural administration of TGFβ2 induced more collagen deposition and fibrosis with comparable degree of pleural inflammation when compared with talc slurry.

An ideal pleurodesing agent is one that produces pleurodesis effectively, safely and quickly. While talc is the most effective agent currently available (Antunes and Neville, 2000), it is slow in inducing pleurodesis as shown in various animal studies. Even in doses >5 times higher than that used in humans, talc does not usually produce significant pleurodesis in rabbits until four weeks after administration (Xie et al., 1998d). Talc produces pleurodesis by provoking acute pleural injury, inflammation and hence fibrosis (Kennedy et al., 1995). The more intense the insult, the more successful is the pleurodesis. Pain and fever – secondary to the pleural inflammation - are common side effects with talc pleurodesis (Walker-Renard et al., 1994). Talc-induced ARDS (Campos et al., 1997; Rehse et al., 1999) (Rehse et al., 1999) and systemic talc embolization (Kennedy et al., 1995; Werebe et al., 1999) are two major clinical concerns.

The faster action of TGFβ over talc may imply a clinical advantage. In patients with pneumothorax or rapidly re-accumulating pleural effusions, the ongoing accumulation of air or fluid in the pleural space separates the visceral pleura from the parietal surface, and may hinder the development of pleurodesis. This is especially the
case if the pleurodesing agent acts slowly. Hence, the findings of this study would favour the use of TGFβ over talc in clinical settings.

It was shown in Chapter 3 that TGFβ2 is effective in producing pleurodesis in rabbits, and at least as efficacious as historic data of talc. In this study, the rabbits that received intrapleural TGFβ2 had significantly more collagen (both mature and immature fibers) deposition, thicker pleural membranes, more fibrosis in keeping with the known fibrogenic effects of TGFβ, and developed effective pleurodesis promptly. The rate of pleurodesis induced by TGFβ2 is significantly faster than all other agents previously studied, including talc (Xie et al., 1998a; Xie et al., 1998c; Xie et al., 1998d), doxycycline (Wu et al., 1998) and mitoxantrone (Vargas et al., 1998) – all of which required 28-60 days to achieve satisfactory pleurodesis in animal models.

The potent and direct fibrogenic actions of TGFβ can explain the effective and rapid pleurodesis seen after intrapleural injection of TGFβ2 in our study. TGFβ is more potent than any other growth factor in stimulating extracellular matrix production (Kelley, 1993). It directly induces matrix deposition by stimulating the transcription of the genes of matrix proteins, thereby increasing the synthesis of collagen, fibronectin, proteoglycan and other matrix proteins by several folds (Border and Noble, 1994; Fine and Goldstein, 1991). It also simultaneously inhibits the synthesis of matrix-degrading proteases, increases the production of their inhibitors (Roberts and Sporn, 1992) and limits fibrinolysis by increasing the levels PAI-1 (Idell et al., 1992).
Bypassing the pleural inflammation process may also explain why TGFβ2 can elicit pleurodesis significantly faster than talc. Intrapleural administration of conventional pleurodesing agents leads to pleural injury, inflammation and subsequent fibrosis. In the case of TGFβ, however, significantly more fibrosis and pleurodesis were produced and yet the degree of pleural inflammation was similar to that induced by talc and that the pleural fluid had significantly lower inflammatory indices when compared with talc. This is most likely explained by the fact that TGFβ, unlike talc, produces pleurodesis without inducing acute pleural injury as well as the known potent anti-inflammatory properties of TGFβ described in Chapter 1.2.6.

TGFβ2 stimulated the production of significant amount of pleural fluid with low inflammatory indices in the first 24 hours after injection, similar to our previous observation in Chapter 3. In this study, the amount of pleural fluid produced decreased rapidly after the first two days and no fluid was present by day 4. In this study, chest tube was left in situ for pleural fluid drainage, as per common clinical practice. Given the large volume of effusion generated after TGFβ2 injection, it is likely that the pleurodesis would be impaired if these fluids were not removed.

One possible explanation for the differences in the results between the two treatment groups is that the chest tubes remained in place longer in the TGFβ2 group, as there was more pleural fluid induced after TGFβ2 injection. In the protocol used, the chest tubes were left in place until the pleural fluid drainage was <5mL in the preceding 24 hours. However, it is unlikely that the better pleurodesis seen in the TGFβ2 group was
due to the longer duration of chest tubes placement. Rabbits sacrificed at day 1 in both
groups had chest tubes for the same duration, and the differences between the two
groups were already significant at that time-point. Also, in previous studies, the presence
of chest tubes for up to 96 hours in rabbits did not lead to the development of significant
pleural adhesions (Devin et al., in press).

In the histological analysis of this study, only the visceral pleura was examined (as
in the case of most previous studies using the same animal model). This is because of the
technical difficulties frequently encountered in separating the parietal pleura from the
chest wall, especially in the presence of significant pleural fibrosis/symphysis. It is
however believed that the visceral and parietal pleural thickening should run parallel to
each other. The histological examination results of the visceral pleura in this study
mirrored closely the macroscopic pleurodesis scores, confirming that the examination of
the visceral pleura is appropriate.

In conclusion, intrapleural administration of TGFβ2 produced excellent
pleurodesis in rabbits at a rate faster than talc slurry and all other pleurodesing agents
that had been investigated before. TGFβ2 stimulated more collagen deposition without
inducing excess inflammation when compared with talc slurry. If this data can be
extrapolated to humans, TGFβ2 will have an advantage over talc slurry in preventing
rapid re-accumulation of pleural effusion and in stopping air leak in patients with
pneumothorax.
While TGFβ2 can produce effective pleurodesis and do so significantly faster than the intrapleural injections of talc, the safety of intrapleural injections of TGFβ2 need to be evaluated.
SUMMARY

• TGFβ₂ induced effective pleurodesis in rabbits at a rate faster than talc slurry and all other pleurodesing agents that had previously been investigated.

• TGFβ₂ stimulated more collagen deposition, more pleural thickening and fibrosis than talc slurry at days 1, 4 and 7. It did so without inducing excess inflammation when compared with talc slurry.

• Intrapleural administration of TGFβ₂ resulted in the transient production of a large amount of pleural fluid, significantly more than that induced by talc. The fluid induced by TGFβ₂ had lower inflammatory indices than that induced by talc.

• As a pleurodesing agent, TGFβ₂ may have an advantage over talc slurry in preventing rapid re-accumulation of pleural effusion and in stopping air leak in patients with pneumothorax.
CHAPTER 5

THE EFFECT OF CORTICOSTEROIDS ON TRANSFORMING GROWTH FACTOR $\beta_2$ INDUCED PLEURODESIS
5.1 Introduction

In Chapters 3 and 4, the data clearly demonstrates that TGFβ₂ can produce pleurodesis effectively and rapidly in rabbits. As previously discussed, commonly used pleurodesing agents produce pleurodesis by inducing acute pleural injury and hence inflammation and fibrosis (Kennedy et al., 1995). Co-administration of high doses of parental corticosteroids has been shown to reduce the pleural inflammation and inhibited talc and doxycycline pleurodesis in rabbits, confirming that the intense inflammatory process is essential to pleurodesis (Xie et al., 1998c). The pleural inflammation is of clinical significance as it is likely to be the cause of chest pain and fever, which frequently complicates chemical pleurodesis (Walker-Renard et al., 1994). Furthermore, many patients with malignant pleural effusions are taking corticosteroids, and it is likely that the efficacy of chemical pleurodesis with common pleurodesing agents is reduced in these patients (Xie et al., 1998c).

The ideal pleurodesing agent should be capable of producing fibrosis without inducing pleural inflammation, and remain effective in the presence of corticosteroids. We postulate that TGFβ, with its unique capabilities of being a strong fibrogenic activator as well as a potent immunomodulatory cytokine, was likely to fulfill these criteria. In the previous chapters, the leukocyte counts and LDH levels in the pleural fluid (surrogate measures of the degree of pleural inflammation) were significantly lower after intrapleural injection of TGFβ₂ when compared with talc and doxycycline.
In the present study, the effect of high dose systemic corticosteroids on TGFβ₂-induced pleurodesis was investigated. It was hypothesised that TGFβ₂ produces pleurodesis without inducing significant pleural inflammation and hence systemic corticosteroids would have little or no inhibitory effect on TGFβ₂-induced pleurodesis.
5.2 Methods

Thirty New Zealand white rabbits were divided into two groups of 15. Rabbits in the steroid group received an intramuscular injection of triamcinolone diacetate (Fujisawa Inc., Deerfield, IL, USA) at 0.8 mg/kg at the time of chest tube insertion and then weekly thereafter. This dose of corticosteroids was chosen as it had been shown to significantly inhibit talc pleurodesis (Xie et al., 1998c). Rabbits in the control group received no injections of steroid or placebo. All rabbits received an intrapleural injection of human recombinant TGFβ2 on the day following the chest tube insertion. The preparation of TGFβ2 and the methods of animal surgery were those used in Chapters 3 and 4.

Ten rabbits in each group received a TGFβ2 dose of 5.0 μg in 2.5 mL while the remaining five in each group received a lower dose of 1.7 μg in 2.5 mL via the chest tube. These doses of TGFβ2 were chosen as they were shown to be the lowest effective doses in the previous dose-response study (Chapter 3).

Rabbits that developed signs of inflammation or infection at the skin wound were given daily intramuscular injections of 12.5 mg of gentamicin (Fermenta, Kansas City, Mo., USA) for two consecutive days. Rabbits that showed evidence of reduced oral intake or dehydration after anesthesia were given subcutaneous injections of 50 mL of 5% dextrose and 50 mL of 0.9% NaCl solution every 24 hours.
Pleural fluids were collected and analysed as described in previous chapters. The animals were weighed at baseline and before sacrifice. The rabbits were sacrificed at 14 days after the injection of TGFβ2. The procedure of sacrifice and the scale for macroscopic grading of pleurodesis (0-8) were those used in the Chapter 4. Samples of the pleural tissues were collected in each rabbit and graded by a blinded examiner for the following parameters: the degree of pleural fibrosis, pleural inflammation, alveolar fibrosis and alveolar inflammation on the same semi-quantitative scale (0-4) used in Chapter 4. The thickness of the pleura and the amount of collagen deposition were measured as previously described.

Statistical analysis: Student’s t test (for parametric data) and Mann-Whitney Rank Sum test (for non-parametric data) were used to compare the values between subgroups. Data were presented as mean ± S.D. (standard deviation) unless otherwise stated. A p value of <0.05 was considered significant. All data were analysed with Sigma Stat V2.03 statistic software program (SPSS).
5.3 Results

Intrapleural injection of TGFβ2 at both 5.0μg and 1.7μg doses produced effective pleurodesis in rabbits with mean pleurodesis scores of 7.3±1.3 and 6.9±1.2 respectively. There was no difference in the pleurodesis scores between rabbits that received TGFβ2 and systemic corticosteroids and rabbits that received TGFβ2 alone (7.2±1.3 vs 7.1±1.2 respectively, p=NS). The results of the rabbits in each subgroup are presented in Table 5.1.

<table>
<thead>
<tr>
<th></th>
<th>TGFβ2 (5.0μg) + Steroid</th>
<th>TGFβ2 (5.0μg) only</th>
<th>p value</th>
<th>TGFβ2 (1.7μg) + Steroid</th>
<th>TGFβ2 (1.7μg) only</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>10</td>
<td>10</td>
<td></td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Pleurodesis Score</td>
<td>7.3±1.3</td>
<td>7.3±1.3</td>
<td>NS</td>
<td>6.8±1.3</td>
<td>7.0±1.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
The injection of TGFβ2 stimulated the production of large quantities of pleural fluid in all rabbits. The effusion produced was relatively non-inflammatory as evidenced by the low protein, LDH and total leukocyte counts, Table 5.2. The production of the fluid was transient and did not hinder the development of effective pleurodesis.

Rabbits that received TGFβ2 and corticosteroids showed no significant difference from those given only TGFβ2 in the amount of effusion produced and the biochemical analysis of the pleural fluid, Table 5.2. The only exception was that the effusion protein level was higher in the steroid group (mean 3.1mg/dL vs 2.9mg/dL, p=0.02), though the difference is unlikely to be of clinical significance. There was no difference in the other markers of inflammation (LDH and total WBC counts) between the two groups.

There was no difference in the effusion protein, LDH and total leukocyte counts between rabbits given 5.0μg of TGFβ2 and those given 1.7μg. Interestingly, although the mean volumes of effusion at 24 hours were not different (28.0mL for the 5.0μg group and 26.7mL for the 1.7μg group), rabbits that received the higher dose of TGFβ2 had a significantly lower amount of total effusion drained (44.0mL vs 65.6mL respectively, p=0.02).
**TABLE 5.2.** Pleural fluid characteristics. (The WBC and biochemistry values were those measured in the pleural fluids collected at 24 hours after intrapleural TGFβ₂ injections.)
(Mean ± S.D.)

<table>
<thead>
<tr>
<th></th>
<th>TGFβ₂ + Steroid</th>
<th>TGFβ₂ only</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n =</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Effusion Volume in 24 hr (mL)</td>
<td>28.0±10.5</td>
<td>26.7±8.8</td>
<td>NS</td>
</tr>
<tr>
<td>Total Effusion Volume (mL)</td>
<td>55.0±26.0</td>
<td>47.3±23.0</td>
<td>NS</td>
</tr>
<tr>
<td>WBC (/mm³)</td>
<td>1107±387</td>
<td>1376±581</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>3.1±0.3</td>
<td>2.9±0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>176±38</td>
<td>182±50</td>
<td>NS</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>478±232</td>
<td>502±123</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
Comparing pleural tissues of the rabbits that received TGFβ2 and steroid with pleural tissues of the rabbits given only TGFβ2, there was no significant differences in the microscopic grading scores for pleural fibrosis, pleural inflammation (Figure 5.1), or in pleural thickness (Figure 5.2) and collagen deposition (Figure 5.3). The degree of alveolar inflammation or alveolar fibrosis was minimal in both groups (Figure 5.1).

**FIGURE 5.1.** Comparison of histologic changes in rabbits given TGFβ2 and corticosteroids vs rabbits given TGFβ2 only.

- Pleural Fibrosis
- Pleural Inflammation
- Alveolar Fibrosis
- Alveolar Inflammation

n = 15 for each group. Error bar = S.D.
FIGURE 5.2. Pleural thickness of rabbits given TGFβ2 and corticosteroids vs rabbits given TGFβ2 only.

n = 15 for each group. Error bar = S.D.
FIGURE 5.3. Collagen deposition in rabbits given TGFβ2 and corticosteroids vs rabbits given TGFβ2 only.

<table>
<thead>
<tr>
<th>Steroid Group</th>
<th>No Steroid Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant by Mann-Whitney Rank Sum test

n = 15 for each group.  Error bar = S.D.
Four rabbits died and were replaced. Three of them died from causes related to surgery (e.g. pneumothorax or pulmonary contusion) during or within 48 hours of chest tube insertion. The fourth rabbit had a blocked chest tube and died from a tension hydrothorax three days after the injection of TGFβ2 (5.0μg). The overall incidence of hemothorax was low in all groups and the median (25%-75% range) score of hemothorax was 0 (0.0-2.7) in rabbits given TGFβ2 and corticosteroids and 0 (0.0-2.0) in rabbits given TGFβ2 only. None of the rabbits had evidence of empyema at necropsy examination.

The weight gain was comparable in rabbits that received corticosteroids and those that did not (0.18±0.16kg vs 0.22±0.19 respectively, p=NS). Rabbits that received the lower dose gained more weight than rabbits that received the higher dose of TGFβ2 injection (0.14kg vs 0.34 kg, p=0.001). Three rabbits (all in 5.0μg group; two had corticosteroids) required gentamicin for skin wound infections. One rabbit (in the no steroid group) required supplementary subcutaneous fluid treatment.
5.4 Discussion

This study demonstrated that systemic corticosteroids, at a dose that inhibits talc pleurodesis, does not affect the efficacy of TGFβ₂-induced pleurodesis nor the inflammatory indices in the effusion fluid. The data provides further evidence that TGFβ₂ induces pleurodesis via a pathway that involves minimal inflammation of the pleura and thus remains effective in the presence of high dose parenteral corticosteroids.

The results demonstrated two further advantages of TGFβ₂ over conventional pleurodesing agents such as talc and tetracycline derivatives. Firstly, unlike conventional pleurodesing agents, TGFβ may present a novel means to produce pleurodesis without injuring the pleura or inducing significant pain or fever - a distinct advantage as pleurodesis is mainly performed for symptomatic comfort in patients with incurable malignancies. Secondly, many of the patients with malignant effusions are on corticosteroids, for example for long-term management of co-existing chronic obstructive airways disease or for palliation (e.g. to stimulate appetite). Concurrent use of corticosteroids can markedly decrease the inflammatory reaction and inhibit talc and doxycycline pleurodesis (Teixeira et al., 2002)(Xie et al., 1998c). However, the results of this study showed that TGFβ₂ remains highly effective despite corticosteroids - another probable clinical advantage over talc and doxycycline.

Besides its pro-fibrotic properties, TGFβ is also a potent immunomodulatory cytokine. It can reduce or prevent excessive inflammation by regulating T and B
lymphocyte activities and their production of TNFα and IL-1 (Kelley, 1993). TGFβ, under suitable circumstances, is also capable of down-regulating IL-6 and IL-8 (Chen and Manning, 1996; Smith et al., 1996), and can also modulate the cytotoxicity of macrophages by suppressing their superoxide and nitric oxide production (Border and Noble, 1994; Vodovotz et al., 1993). TGFβ1 knockout mice have markedly elevated TNFα and IL-1 levels and die rapidly after birth from autoimmune-like diseases (Kulkarni et al., 1993; Shull et al., 1992). There is increasing evidence that the immunosuppressive actions of glucocorticoids (Almawi et al., 1996) and cyclosporine (Khanna et al., 1999) are mediated at least in part via TGFβ.

In the present study, direct administration of TGFβ2 to the pleura created excellent pleurodesis. Pleurodesis induced by 5.0μg of TGFβ2 was not inhibited by systemic corticosteroids at a dose that blocks talc pleurodesis. Corticosteroids did not inhibit the TGFβ2-induced pleurodesis even when applied to a lower dose of TGFβ2 (1.7μg), which was known to produce lower pleurodesis scores. The results of this study provide further evidence that TGFβ2 induces pleurodesis without necessitating pleural injury and inflammation. This is also confirmed by the low inflammatory indices (protein, LDH levels and leukocyte counts) in the pleural fluids induced after TGFβ2 injection.

As in previous experiments, intrapleural administration of TGFβ2 induced the production of large volume of pleural fluid. Interestingly, the concurrent administration of corticosteroids made no difference to the amount of fluid produced, indicating that the
production of fluid after TGFβ injection is unlikely to be due to inflammation. The total amount of effusion drained was significantly higher in rabbits receiving lower dose of TGFβ2 than in those receiving the higher dose, although there was no difference in the amount drained at 24 hours. It seems feasible that higher dose of TGFβ2 may have induced more fibrosis and loculations in the first few days, which then impair the pleural fluid drainage.

TGFβ is multifunctional and its action on target cells are critically dependent on the cell type, its state of differentiation and the particular set of growth factors and hormones acting on the cell (Sporn and Roberts, 1988). Its interactions with corticosteroids are complex. While TGFβ can reduce cortisol (Feige et al., 1991) and mineralocorticoid (Gupta et al., 1993) synthesis, glucocorticoids in turn are potent regulators of the expression of TGFβ isoforms (Koli and Keski-Oja, 1996). These regulations are again cell- and stimulus-specific (Almawi et al., 1996; Koli and Keski-Oja, 1996). For example, glucocorticoids enhance accumulation of TGFβ mRNA in some cell lines, including fibroblasts (Wang et al., 1995), osteoblasts (Oursler et al., 1993) and T lymphocytes (Ayanlar-Batuman et al., 1991; Correale et al., 1998), but inhibit the production of TGFβ in others (Baumgartner et al., 1996; Daniepourn et al., 1991).

This study is the first to examine the effects of corticosteroids on TGFβ in pleural tissues in vivo. Previous studies of the in vivo effects of corticosteroids and TGFβ in animal models of fibrosis have yielded conflicting results. In one study dexamethasone blocked the pro-fibrotic effect of TGFβ in the abdominal wounds of rats (Meisler et al.,
In another study using a rat model of bleomycin-induced pulmonary fibrosis, the alveolar macrophage production of TGFβ was not inhibited by high concentrations of corticosteroids (Khalil et al., 1993). Conversely, other authors have also demonstrated that both local (Pierce et al., 1989; Slavin et al., 1992) and systemic (Beck et al., 1993) administration of TGFβ can reverse the steroid-induced impairment in wound healing.

In summary, TGFβ2 induces pleurodesis through a novel pathway whereby it induces pleural fibrosis without provoking excessive inflammation. This was confirmed by demonstrating that corticosteroids, at the same dose which blocks talc pleurodesis, did not impair the effectiveness of TGFβ2 in inducing pleurodesis. If these data can be extrapolated to humans, then TGFβ2 - as a non-inflammatory pleurodesing agent – may be less likely to induce pain and fever than talc and will remain effective in patients receiving corticosteroids.
SUMMARY

- TGFβ₂ induced effective pleurodesis in the presence of high dose parental corticosteroids, which has previously been shown to inhibit talc- and doxycycline-induced pleurodesis. This lends indirect evidence to support the postulate that TGFβ₂ stimulates pleurodesis in a way that does not necessitate pleural injury and inflammation.

- The results of this study suggest that TGFβ₂ may have an advantage over conventional pleurodesing agents in that it may induce less fever and chest pain. Also, it is likely to remain effective in patients who are receiving concurrent corticosteroids treatment, while talc or doxycycline will not.

- The induction of pleural fluid formation following the intrapleural administration of TGFβ₂ was not inhibited by co-administration of corticosteroids.
CHAPTER 6

THE SAFETY AND EFFICACY OF INTRAPLEURAL INJECTION OF TRANSFORMING GROWTH FACTOR $\beta_2$ IN A NEW SHEEP MODEL
6.1 Introduction

An ideal pleurodesing agent should be effective, fast-acting and safe. In previous chapters, it has been demonstrated that a single intrapleural injection of TGFβ2 can produce excellent pleurodesis in rabbits at a rate faster than any other agent previously studied, including talc slurry. However, two important questions remain to be investigated. Firstly, the safety of TGFβ2 when administered intrapleurally needs to be studied. TGFβ is a potent pro-fibrotic cytokine and its over-expression plays a pathogenic role in fibrotic diseases, especially glomerulosclerosis and pulmonary fibrosis. Also, in the studies outlined in Chapter 3, extremely high doses of intrapleural TGFβ2 could induce fibrosis in the contralateral pleural space as well as in the peritoneum. Systemic fibrosis was not seen at autopsy in rabbits that received low doses of TGFβ2 (Chapters 4 and 5).

Secondly, an animal model that closer resembles humans need to be evaluated. While the rabbit model is the one most commonly used for the study of pleurodesis, rabbits have a thin visceral pleural membrane that is different from humans, which have a thick visceral pleura (see Chapter 1). The pleura of rabbits receives its blood supply from the pulmonary circulation whereas the blood supply of sheep and human pleurae is derived from the systemic circulation (Albertine et al., 1982). Sheep have a thick pleura that resembles the human pleura more closely than does the rabbit pleura. Hence, sheep have commonly been used for the study of pleural fluid dynamics (Broaddus et al., 1990; Wiener-Kronish et al., 1984). The efficacy of TGFβ2, as compared with talc and
bleomycin, in inducing pleurodesis in animals with thick visceral pleura requires investigation.

To determine the efficacy and safety of TGFβ₂ as a novel pleurodesing agent in animals with a thick visceral pleura resembling humans, a new sheep model for pleurodesis was developed. It was hypothesized that TGFβ₂ administered intrapleurally would produce an effective pleurodesis without inducing significant side effects.

This study was performed in two parts. In the first part, the efficacy of intrapleural TGFβ₂ as a pleurodesing agent in sheep was assessed and the histopathological changes in extra-pulmonary organs were evaluated at 14 days. In the second part of the study, we compared the effectiveness of TGFβ₂ (at the optimal dose established in the Part I of the study) was compared with talc and bleomycin in producing pleurodesis in sheep; and the acute physiological effects and systemic TGFβ levels following intrapleural administration of these agents was investigated.
6.2 Methods

In both parts of the study, yearling sheep of mixed breeds (22 to 35 kg) were used.

PART I:

To establish the efficacy and to determine the dose response relationship of TGFβ2-induced pleurodesis in sheep, 12 sheep were divided into four groups. A single intrapleural injection of TGFβ2 at 1.0 (Group A), 0.50 (Group B), 0.25 (Group C) and 0.125 (Group D) µg/kg was administered to each sheep of the four groups. These doses were chosen based on results of a pilot study in which sheep receiving 2 µg/kg of TGFβ2 developed complete pleural symphysis.

The design of this new animal model was that described in Chapter 2.2.2. In brief, all sheep received a chest tube in the right pleural space under anaesthesia, through which TGFβ2 was administered as a single intrapleural injection 24 hours after its insertion. The volume of injection was standardized at 1.0mL/kg. The sheep in Groups C and D also received a chest tube in the left pleural space through which equal volume of the buffer was injected to serve as the control.

On subsequent days the chest tube was aspirated for any pleural fluid produced. The collection and analysis of the pleural fluids were performed in the same way as per the rabbit experiments described in Chapter 2.3. The chest tube was removed when the
pleural fluid drainage was <10 mL per day on two consecutive days. The sheep were sacrificed 14 days after the intrapleural injection of TGFβ₂.

At the time of sacrifice, a consensus grading was reached by two blinded investigators on the degree of pleurodesis using the same semi-quantitative (0-8) scheme described in Chapters 4 and 5. At necropsy, macroscopic examination and biopsies were performed on the ipsilateral and contralateral pleura and lungs, the pericardium, liver, spleen, diaphragm, kidneys, adrenals, ureter, urinary bladder, omentum, the small intestine and (in female sheep) the ovaries and fallopian tubes. The tissue samples were fixed in 10% neutral buffered formalin and processed by routine methods, embedded in paraffin, sectioned and stained with H&E stain. Histomorphologic evaluation was performed by an experienced animal pathologist. All histologic changes were graded as 0 (no abnormality), 1 (minimal), 2 (mild), 3 (moderate) or 4 (marked).

PART II:

Twelve sheep were divided into three groups, and each receive one of the following: intrapleural TGFβ₂, talc or bleomycin. All sheep had bilateral chest tubes inserted under anaesthesia following the same protocol as in Part I of the study. In addition, an arterial line was inserted (as described in Chapter 2) in each sheep through which arterial blood samples were collected for blood gas and cytokine analysis.

All sheep received a single intrapleural injection of the pleurodesing agent via the chest tube 24 hours after the surgery. The side of injection of the active agent was
randomised such that half of the animals in each group receive the active agent in the
left and the other half in the right pleural cavity. The buffer (for sheep in the TGFβ2
group) or 0.9% NaCl solution (for sheep in the talc and bleomycin groups) was injected
to the contralateral side to serve as the control.

Arterial blood was collected at baseline and then at ½, 1, 2, 4, 8 and 24 hours after
the injection of pleurodesing agents. Respiratory and pulse rates were also measured at
these time points. The arterial blood gas measurements were performed using a blood
gas analyser (Ciba Corning, Essex, UK) that was calibrated daily.

Blood samples were collected in citrated tubes at baseline (24 hours after surgery
and immediately before injection of pleurodesing agents), 24, 48 and 72 hours for
cytokine measurement. Blood was separately collected from three other sheep that did
not receive any surgical intervention for comparison. The blood samples were
centrifuged at 3,000 r.p.m. for 15 minutes at -4°C, and the supernatant stored
immediately at -70°C until the time of assay.

After the injection of pleurodesing agents, the chest tube was aspirated every 24
hours for any pleural fluid produced. The chest tubes were removed after 72 hours. The
sheep were sacrificed 14 days after the chest tube insertion with an intravenous injection
of sodium phenobarbital (Henry Schein, Port Washington, NY, USA).

Pleurodesing Agents: The preparation protocols for TGFβ2, talc slurry and bleomycin
were those described in Chapter 2. TGFβ2 was administered in 0.25μg/kg, basing on the
results from Part I of the study. Five grams of sterilized talc (Sigma) and 60 IU of bleomycin sulphate (Nippon Kayaku) were used. These were the commonly employed dosages of these agents in humans (Walker-Renard et al., 1994).

Pleurodesis Grading Scheme: The grading of macroscopic pleurodesis, the sampling of the visceral pleura and lung and the processing of the tissue samples were performed as described in Part I of the study. Histomorphological evaluation was performed on hematoxylin and eosin (H&E) slides by an experienced pathologist blinded to the treatment groups.

In sheep that died before 14 days, a post-mortem examination was performed in the presence of an experienced veterinarian to determine the cause of death. Tissues of the lung and extra-pulmonary organs were collected for histological examination as described above.

Statistical analysis

One way ANOVA and one way ANOVA on ranks were used to compare the values among subgroups in parametric and non-parametric data respectively. Tukey Test was used to perform multiple comparison procedures. Correlation between variables was measured using Pearson correlation test. A p value <0.05 was considered significant. All data were analyzed with Sigma Stat V2.03 statistic software program (SPSS).
6.3 Results

PART I:

Pleurodesis: The intrapleural injection of TGFβ2 was very effective in inducing pleurodesis. The intrapleural TGFβ2 at 1.0µg/kg, 0.5µg/kg and 0.25µg/kg (Groups A, B and C) produced a maximum pleurodesis score of 8 in all nine sheep receiving these doses. The three sheep receiving 0.125µg/kg (Group D) all had a pleurodesis score of 6. At the time of sacrifice, the pleura of the treatment side were grossly thickened in all sheep. The lungs were tightly adhered to the chest wall and could only be separated from the chest wall after intense blunt dissection. Nevertheless, after the lung was freed from the chest wall, it could be easily inflated with positive pressure.

In the six sheep (Groups C and D) that had buffer injection into the left (control) pleural space, there was essentially no pleurodesis with scores of 1 to 2 in all the animals. One sheep (Group D) had evidence of a small loculated empyema in the left pleural space (control side) at the post-mortem examination. There was no hemothorax in any of the sheep.

The volume of effusion produced at 24 hours and in total, the biochemical analysis and the total leukocyte count of the pleural fluid from the treatment side of the sheep in each dosage group were summarised in Table 6.1. All six sheep receiving 0.125 and 0.25µg/kg (Groups C and D) of TGFβ2 had no significant (<5mL) pleural fluid production while sheep receiving higher doses produced large volume of effusion (mean >700mL for both the 0.5 and 1.0µg/kg groups). Of the remaining six sheep receiving
either 0.5 or 1.0 μg/kg (Groups A and B) of TGFβ2, there was no significant drainage after 72 hours. None of the sheep developed more than 2 mL of effusion in the first 72 hours on the control side. The volume was considered insignificant and the fluid was not analysed. The effusions produced were exudative with mean protein concentrations of 3.9 mg/dL in Group A and 3.6 mg/dL in Group B and mean LDH levels of 958 IU/L and 896 IU/L respectively. The white cell counts were relatively low but increased with decreasing doses of TGFβ2 (1352/mm³ in Group A, 1938/mm³ Group B and 3475/mm³ in Group C).
**TABLE 6.1.** Results of pleurodesis score, volume of effusion produced and analysis of pleural effusion in sheep after intrapleural TGFβ2 administration.

(Mean ± S.D.)

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=3</td>
<td>n=3</td>
<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
<td>TGFβ2 Dose (µg/kg)</td>
<td>1.0</td>
<td>0.5</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>Pleurodesis Score (1-8) Δ</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Effusion Volume at 24 Hour (mL)</td>
<td>600±521</td>
<td>643±127</td>
<td>0.3±0.6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Total Effusion Volume Drained (mL)</td>
<td>715±341</td>
<td>965±383</td>
<td>0.7±0.6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>3.9±0.2</td>
<td>3.6±0.3</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>958±80</td>
<td>896±156</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>72.7±5.9</td>
<td>62.0±1.0</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Total Leukocyte Count (/mm³)</td>
<td>1352±1052</td>
<td>1938±936</td>
<td>3475±108</td>
<td>#</td>
</tr>
</tbody>
</table>

# = Insufficient fluid for analysis
Δ = Median score reported
Histology: The pleura of the treated side of all sheep demonstrated significant pleural thickening with fibrovascular proliferation with a minimal inflammatory component, Figures 6.1 and 6.2 (see colour illustrations at the back of the thesis). There were no differences noted in the nature or intensity of the pleural reaction among animals that received different doses of TGFβ2. Minor inflammatory changes were noted in the underlying lung including interstitial non-suppurative inflammation, peribronchial lymphoid aggregates and localised small patchy consolidation. These findings were considered spontaneous and incidental, commonly observed in sheep and unlikely to be related to the intrapleural administration of TGFβ2.

Specific changes that can occur with TGFβ were specifically examined for. In the kidneys, tuberlointerstitial chronic inflammation and periglomerular sclerosis were either not present or minimal (grade 1) in all the samples. Likewise, in the adrenal gland, medullary lymphoid infiltration was occasionally seen (median grade 0 or 1 in all dosage groups). Urinary bladder samples showed no abnormality. Two of the sheep were female and both had normal ovaries and uteri. Liver samples showed minimal chronic inflammation (median grade =1) while splenic congestion were present in mild degree (median =2) in all samples. One sheep had evidence of focal necrotising enteritis and four other had very mild coccidiosis: these findings were common in post-mortem examination of sheep and were considered incidental.
PART II:

Of the 12 sheep, nine (three from each group) survived to the designated end-point of day 14. In those sheep, no macroscopic abnormality in the lungs or extra-pulmonary organs was observed at autopsy except for one sheep (in the talc group) that developed pneumonia in the control side. Culture of the lung aspirates revealed heavy (>1000 organisms/mL) growth of Pasteurella haemolytica. None of the sheep had > 2mL of pleural fluid at 24 or 48 hours after the administration of the pleurodesing agents. Since the dead space of the chest tube is 10mL, the small amount of pleural fluid aspirated was considered insignificant and was not analysed. The weight of the sheep in different treatment groups remained relatively stable over the 14-day period. The amount of weight gain (expressed as percentage increase over baseline weight) was 0.8±3.6% in the TGFβ group, 4.3±7.7% in the talc group and 0.0±2.7% in the bleomycin group (p=NS).

Three sheep (one from each group) died before day 14. One sheep died seven days after receiving intrapleural injection of bleomycin. At post-mortem, there was evidence of bilateral hemorrhagic pulmonary edema. A sheep in the TGFβ2 group died at day 9. Post-mortem and histological examination of the extra-pulmonary organs failed to demonstrate any obvious cause of death. Histologically, there was evidence of liver necrosis and acute tubular necrosis, though no obvious precipitating event was identified. One sheep died two days after intrapleural talc injection. At autopsy, the sheep had extensive intestinal parasitic infestation with trichostrongyloidea species and was anemic. Microscopically, there was extensive hepatic necrosis and granular fibrin debris.
associated with talc granulomas in the liver. The lungs of that sheep also showed mild intra-alveolar edema.

**Macroscopic Pleurodesis**

At day 14, the pleurodesis scores of the TGFβ2 (7.7±0.6) and talc (7.0±1.7) groups were both higher than that of the bleomycin group (3.3±2.3) in the treatment side, Figure 6.3. In the control side, the pleurodesis score was no greater than 2 in all the sheep except the one that had pneumonia. In that specific sheep (talc group), there was evidence of a complicated para-pneumonic effusion and significant adhesions in the control hemithorax with a resultant score of 7.

![Fig. 6.3 Pleurodesis Scores of Sheep That Received Different Pleurodesing Agents](image)

* p < 0.05 when compared with TGFβ2
Cardiorespiratory Responses

There were no significant differences in the respiratory rates, heart rates and arterial blood gases results between the sheep of the three groups, Tables 6.1 and 6.2.

There were no differences in the vital signs or the arterial blood gas results of the three sheep that died before 14 days when compared with those that survived.

*Table 6.2. Heart and respiratory rates in sheep after injection of different pleurodesing agents. (Mean ± S.D.)*

<table>
<thead>
<tr>
<th>Heart Rate (/min)</th>
<th>TGFβ2</th>
<th>Talc</th>
<th>Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>101±11</td>
<td>118±7a</td>
<td>104±13</td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>101±27</td>
<td>111±22</td>
<td>100±12</td>
</tr>
<tr>
<td>1 Hour</td>
<td>96±6</td>
<td>97±24</td>
<td>99±13</td>
</tr>
<tr>
<td>2 Hours</td>
<td>97±6</td>
<td>116±29</td>
<td>96±27</td>
</tr>
<tr>
<td>4 Hours</td>
<td>82±15</td>
<td>110±33</td>
<td>105±21</td>
</tr>
<tr>
<td>8 Hours</td>
<td>104±8</td>
<td>106±23</td>
<td>93±16</td>
</tr>
<tr>
<td>24 Hours</td>
<td>85±13</td>
<td>114±52</td>
<td>96±12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Respiratory Rate (/min)</th>
<th>TGFβ2</th>
<th>Talc</th>
<th>Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>44±7</td>
<td>40±13</td>
<td>40±17</td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>47±12</td>
<td>37±8</td>
<td>46±14</td>
</tr>
<tr>
<td>1 Hour</td>
<td>43±9</td>
<td>36±11</td>
<td>38±8</td>
</tr>
<tr>
<td>2 Hours</td>
<td>37±13</td>
<td>40±7</td>
<td>36±4</td>
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<tr>
<td>4 Hours</td>
<td>42±7</td>
<td>33±3</td>
<td>41±14</td>
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<tr>
<td>8 Hours</td>
<td>37±10</td>
<td>43±14</td>
<td>47±18</td>
</tr>
<tr>
<td>24 Hours</td>
<td>41±10</td>
<td>39±12</td>
<td>36±12</td>
</tr>
</tbody>
</table>
Table 6.3. Arterial blood gas analysis in sheep after injection of pleurodesing agent. (Mean ± S.D.)

<table>
<thead>
<tr>
<th>pH</th>
<th>TGFβ2</th>
<th>Talc</th>
<th>Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>7.47±0.00</td>
<td>7.52±0.05</td>
<td>7.50±0.04</td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>7.49±0.02</td>
<td>7.53±0.05</td>
<td>7.51±0.05</td>
</tr>
<tr>
<td>1 Hour</td>
<td>7.47±0.00</td>
<td>7.54±0.04</td>
<td>7.51±0.03</td>
</tr>
<tr>
<td>2 Hours</td>
<td>7.46±0.01</td>
<td>7.54±0.06</td>
<td>7.51±0.04</td>
</tr>
<tr>
<td>4 Hours</td>
<td>7.51±0.02</td>
<td>7.54±0.05</td>
<td>7.52±0.02</td>
</tr>
<tr>
<td>8 Hours</td>
<td>7.50±0.01</td>
<td>7.56±0.05</td>
<td>7.51±0.01</td>
</tr>
<tr>
<td>24 Hours</td>
<td>7.50±0.02</td>
<td>7.50±0.03</td>
<td>7.51±0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pO₂ at Baseline</th>
<th>80.5±16.3mmHg</th>
<th>86.8±13.2mmHg</th>
<th>81.7±12.3mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Change from Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>3.1±24.3</td>
<td>-16.7±8.7</td>
<td>-3.7±15.7</td>
</tr>
<tr>
<td>1 Hour</td>
<td>-12.5±17.7</td>
<td>-8.6±13.5</td>
<td>-3.2±16.9</td>
</tr>
<tr>
<td>2 Hours</td>
<td>-8.0±13.3</td>
<td>-1.0±14.4</td>
<td>-6.5±12.4</td>
</tr>
<tr>
<td>4 Hours</td>
<td>4.7±9.7</td>
<td>-2.0±15.2</td>
<td>6.0±15.2</td>
</tr>
<tr>
<td>8 Hours</td>
<td>-7.1±22.3</td>
<td>-0.6±16.2</td>
<td>-6.6±16.3</td>
</tr>
<tr>
<td>24 Hours</td>
<td>-24.8±0.3</td>
<td>-10.1±13.9</td>
<td>2.0±7.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pCO₂ at Baseline</th>
<th>28.0±5.7mmHg</th>
<th>30.3±2.9mmHg</th>
<th>29.3±5.5mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Change from Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>-9.4±13.3</td>
<td>2.7±4.4</td>
<td>-4.6±9.8</td>
</tr>
<tr>
<td>1 Hour</td>
<td>0.5±22.8</td>
<td>-2.0±6.4</td>
<td>-7.5±6.6</td>
</tr>
<tr>
<td>2 Hours</td>
<td>-1.6±19.9</td>
<td>-7.4±10.3</td>
<td>-3.2±10.7</td>
</tr>
<tr>
<td>4 Hours</td>
<td>1.0±10.3</td>
<td>2.0±10.1</td>
<td>-9.3±18.0</td>
</tr>
<tr>
<td>8 Hours</td>
<td>22.9±50.0</td>
<td>-2.9±12.6</td>
<td>-2.2±10.4</td>
</tr>
<tr>
<td>24 Hours</td>
<td>39.6±2.9</td>
<td>4.0±11.5</td>
<td>11.9±31.7</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Bicarbonate</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>19.5±4.3</td>
<td>25.0±4.1</td>
<td>22.5±2.6</td>
</tr>
<tr>
<td>0.5 Hour</td>
<td>18.7±1.7</td>
<td>26.0±4.6</td>
<td>21.8±2.9</td>
</tr>
<tr>
<td>1 Hour</td>
<td>20.2±0.5</td>
<td>25.7±5.8</td>
<td>21.5±3.6</td>
</tr>
<tr>
<td>2 Hours</td>
<td>19.2±0.4</td>
<td>24.1±6.6</td>
<td>22.5±2.4</td>
</tr>
<tr>
<td>4 Hours</td>
<td>22.6±3.2</td>
<td>26.7±4.8</td>
<td>21.1±3.3</td>
</tr>
<tr>
<td>8 Hours</td>
<td>25.2±5.2</td>
<td>26.6±6.0</td>
<td>23.0±2.6</td>
</tr>
<tr>
<td>24 Hours</td>
<td>30.0±6.7</td>
<td>24.0±2.7</td>
<td>25.1±2.3</td>
</tr>
</tbody>
</table>
Systemic TGFβ Levels

No significant differences were seen in the plasma levels of TGFβ1 and TGFβ2 in the sheep that received TGFβ2 and those that received talc or bleomycin at 24, 48 and 72 hours after the injection of the pleurodesing agents, Figures 6.4 and 6.5. The plasma levels of TGFβ1 and TGFβ2 were closely correlated (r=0.80, p<0.0001), Figure 6.6.

*FIGURE 6.4. Plasma TGFβ1 levels in sheep*

(Error bar = S.E.M.)
FIGURE 6.5. Plasma TGFβ2 levels in sheep

* 24 hrs post-op

* 24 hrs post-op After Pleurodesis

(Error bar = S.E.M.)
FIGURE 6.6. Plasma TGFβ1 and TGFβ2 Levels in All Sheep

$r = 0.80$
$p < 0.0001$
Histology

Histological examination of the lungs and extra-pulmonary organs revealed dramatically more pleural thickening in the sheep that received TGFβ than those given talc or bleomycin. Splenic congestion, a common post-mortem finding in sheep, was seen in most sheep of all three groups. No other abnormalities were seen in the extra-pulmonary organs of the sheep in the TGFβ group that survived to the designated end-point of 14 days. In the one sheep in the TGFβ group that died after nine days, significant pleural fibrosis had already developed, which was not histologically different from the pleural changes observed in those sheep sacrificed at day 14.

In the talc group, one sheep had significant pneumonia on the contralateral lung as mentioned above. In the other sheep in the talc group, BALT and prominent mononuclear infiltrates were seen in the lung parenchyma of one, and hepatic mononuclear infiltration in another. In the bleomycin group, one sheep had mild hepatic portal infiltration by mononuclear cells, while another had prominent eosinophilic infiltration in the portal area of its liver, without associated evidence of parasites. The sheep in the bleomycin group that died prematurely (day 7) had bilateral massive acute pulmonary hemorrhage and pulmonary edema. Only mild proliferation was observed in the pleura of this sheep compared with the others in the bleomycin group that survived to day 14.
6.4 Discussion

The present study demonstrates that TGFβ2 is effective in producing pleurodesis in sheep – an animal species with a thick pleural membrane similar to that of humans – with no evidence of significant systemic absorption or extra-pulmonary histological abnormalities. In this new sheep model, TGFβ2 was capable of inducing pleurodesis in a dose-dependent fashion. At its lowest effective dose, TGFβ2 was as effective as talc slurry and more effective than bleomycin in inducing pleurodesis. There were no significant differences in cardio-respiratory changes after the intrapleural injection of TGFβ2, as compared with talc and bleomycin. Also, the intrapleural administration of TGFβ2 did not result in any demonstrable increase in systemic levels of TGFβ1 or TGFβ2. At 14 days after intrapleural injection of TGFβ2, there were no significant histological abnormalities in any of the extra-pulmonary organs examined.

This study is the first to establish the use of a sheep model to study the effect of pleurodesing agents. The rabbit is the most common animal model used to study pleurodesis (Kennedy et al., 1995; Light et al., 1995; Vargas et al., 1995; Vargas et al., 1993), although dogs (Colt et al., 1997; Gallagher et al., 1990), rats (Werebe et al., 1999) and pigs (Cohen et al., 1996) have occasionally been studied. The visceral pleurae of small animals (e.g. rabbits, dogs and cats) are thin and different in structure when compared with pleurae of larger animals (e.g. sheep, horses and cows) (Albertine et al., 1982). Pleurae of the latter group are more similar to human pleura which is relatively
thick. Results of pleurodesis studies using animals with a thin pleura have at times
demonstrated findings different from that in human. For example, bleomycin was
completely ineffective in creating pleurodesis in rabbits (Vargas et al., 1993) and a much
higher dose (>5 fold) of talc is required to produce satisfactory pleurodesis in rabbits
(Light et al., 1995) than in humans. The sheep model described can provide a useful
method for future studies of pleurodesis.

TGFβ₂ was effective in generating pleurodesis in sheep as it was in rabbits. All
animals receiving 0.25µg/kg or above reached the maximum pleurodesis score indicating
symphysis of over 50% of the chest wall. This minimal effective dose in sheep was only
10% of that necessary in rabbits.

Interestingly, intrapleural injection of higher dose of TGFβ₂ induced the
production of a large volume of effusion that was dose-related, as observed in the rabbit
studies. At lower doses, minimal amounts of pleural fluid were produced. The
production of effusion neither hindered nor was necessary for the production of
pleurodesis as excellent pleurodesis was generated in sheep independent of whether they
developed any sizable effusion. Also, the effusion induced had low inflammatory indices,
in keeping with the observations in rabbits. Furthermore, the pleural biopsy samples at
day 14 showed no evidence of pleural inflammation. These findings are in keeping with
the hypothesis that TGFβ₂ produces pleural fibrosis by direct stimulation of extracellular
matrix deposition and inhibition of its degradation without inducing significant pleural
injury and inflammation.
While the effectiveness of TGFβ2 as a novel pleurodesing agent had been demonstrated in previous chapters, this is the first time the safety of its intrapleural administration has been assessed. In sheep and human, the visceral pleurae are thick and the pleural space is drained by the lymphatic system via stroma in the parietal pleura which opens eventually to the systemic circulation (Albertine et al., 1984). The systemic absorption of TGFβ2 following its intrapleural injection has not been previously studied.

In the current study, there were no significant differences in the physiologic changes in sheep that received the three different agents. Human and animal studies have suggested that high circulating levels of TGFβ predispose to the development of organ fibrosis (Border and Noble, 1994; Kopp et al., 1996; Liem et al., 1999; Tsushima et al., 1999). It is therefore important to note that the systemic levels of TGFβ1 and -β2 in sheep that received TGFβ2 were no different from the plasma levels in sheep given talc or bleomycin. The pharmacokinetics of TGFβ2 after its intrapleural administration is unknown. Free TGFβ in serum binds to α2-macroglobulin and is inactivated (Danielpour and Sporn, 1992). The half-life of TGFβ in systemic circulation is extremely short (<5 minutes) (LaMarre et al., 1991). It is therefore possible that there had been transient increase in the systemic levels of TGFβ in between the time points sampled. The data did confirm that there was no sustained systemic elevation of TGFβ levels. It was also reassuring that there were no histological abnormalities in extra-pulmonary organs of sheep up to 14 days after TGFβ pleurodesis. Hence, it is unlikely that a transient spike of TGFβ2 (if any) after its intrapleural administration would be of significance.
Further reassurance can be derived from a clinical study in patients with multiple sclerosis that involved repeated intravenous infusions of TGFβ2 and showed no major side effects, except for reversible reduction in renal blood flow at the highest dosage group (Calabresi et al., 1998).

Previous studies evaluating the systemic effects of TGFβ administration have yielded different results. When TGFβ2 was applied topically to rats in doses up to 800μg/kg, there was no evidence of systemic absorption (Zioncheck et al., 1994). No acute systemic or local reactions were seen after intravenous TGFβ2 administration in animal studies (Kelly et al., 1999). Other animal studies have shown that administration of extremely high doses of TGFβ could lead to fibrotic changes in the liver and kidneys. Rabbits given intravenous TGFβ1 at 1000μg/kg/day developed periportal fibrosis and marked centrilobular degeneration in the liver (Terrell et al., 1993). Rats treated with 800μg/kg of TGFβ2 together with volume-depletion developed subcutaneous fibrous nodules and thickened vessel walls in the kidneys (Kelly et al., 1999). These changes were not seen in rabbits or rats receiving chronic systemic administration of lower doses of TGFβ (Racke et al., 1993; Terrell et al., 1993). In the current study, none of the sheep demonstrated any of these changes. Importantly, the potential side effects of hepatic, renal and peritoneal fibrosis were not seen in any of the sheep. There were also no significant differences in the heart rate, respiratory rates and in gaseous exchange among the sheep in the three groups. Of note, however, was a decrease in pO2 with a
corresponding rise in pCO$_2$ in the TGFβ$_2$ group at the 24-hour time point. The significance of this isolated result needs further investigations.

In conclusion, the present study has established that the intrapleural injection of TGFβ$_2$ is effective in producing a pleurodesis in sheep - a species with thick visceral pleura similar to that of humans. The intrapleural injection of TGFβ$_2$ stimulated significant pleural fibrosis but with minimal pleural inflammation. High doses of TGFβ$_2$ induced the production of a large amount of pleural fluid. Low doses of TGFβ$_2$ produced excellent pleurodesis without inducing pleural fluid formation. We also demonstrated that the intrapleural administration of TGFβ$_2$ was safe and did not result in any significant acute cardio-respiratory disturbances or elevation in plasma TGF levels, and no systemic histological changes were detected up to 14 days.
SUMMARY

- A new animal model of pleurodesis using sheep, which has a thick visceral pleural membrane similar to that of humans, has been developed. This model may better evaluate the effects of pleurodesing agents than models of animals with thin pleura.

- Intrapleural administration of TGFβ2 was effective in producing pleurodesis in a dose-dependent fashion. At its lowest effective dose, it was at least as effective as talc and significantly more so than bleomycin in inducing pleurodesis.

- Intrapleural administration of high doses of TGFβ2 induced the production of a large quantity of pleural fluids with low inflammatory indices in the sheep, as seen in the previous rabbit experiments.

- Lower doses of TGFβ2 did not produce any significant pleural fluid formation. This demonstrates that:
  (a) The success of pleurodesis does not depend on the production of pleural fluid; and
  (b) At a suitable dose, TGFβ2 can induce pleurodesis without stimulating the production of large pleural effusions.

- There was no evidence of significant acute physiological disturbance or systemic effects of TGFβ after its intrapleural injection. The plasma levels of TGFβ1 and -β2 were comparable in sheep that received TGFβ2 and those given talc or bleomycin. There were no significant histological abnormalities in extra-pulmonary organs in sheep up to 14 days after intrapleural TGFβ2.
CHAPTER 7

TRANSFORMING GROWTH FACTOR-β INCREASES COLLAGEN SYNTHESIS WITHOUT INDUCING IL-8 RELEASE FROM PLEURAL MESOTHELIAL CELLS
7.1 Introduction

In the previous chapters, direct intrapleural injection of TGFβ2 has been shown to produce pleurodesis effectively, promptly and with no short-term complications, in both the rabbit and sheep models (Chapters 3-6). In this and the following chapter, the underlying mechanisms of the prominent features observed after the intrapleural administration of TGFβ2, namely: the induction of fibrosis and the suppression of inflammation (this chapter), and the stimulation of pleural fluid formation (Chapter 8) will be further investigated.

While the pathophysiology of tissue fibrosis has not been entirely elucidated, it is generally assumed that organ fibrosis is initiated by an insult to the tissue which leads to acute inflammation and the release of a cascade of inflammatory mediators, including potent chemokines such as IL-8. This is further supported by recent evidence that administration of inflammatory cytokines (eg IL-1β) can induce pulmonary inflammation followed by fibrosis.

Previous studies on pleural fibrosis have also shown that conventional pleurodesing agents, such as talc and tetracycline derivatives, induce acute pleural inflammation before fibrosis ensues. Intrapleural injection of talc in humans induces a rapid increase in IL-8 (a critical chemotactic factor for neutrophils) in the pleural cavity (van den Heuvel et al., 1998) and an associated influx of polymorphonuclear cells. Similar findings have been reported in animal studies after talc and tetracycline pleurodesis (Miller et al., 1999; Utsunomiya et al., 1996). Conversely, neutralisation of
IL-8 has been shown to abolish the pleural inflammatory process. Administration of high dose corticosteroids can also inhibit the acute inflammation and significantly reduce the subsequent pleural fibrosis (Teixeira et al., 2002)(Xie et al., 1998c).

TGFβ is a unique cytokine with both potent pro-fibrotic and immunomodulatory properties. While intrapleural injection of TGFβ in vivo stimulates collagen deposition in the pleura (Chapter 4) and macroscopic pleural symphysis (Chapters 3-6), the cellular origin of the collagen production is not known. Previous studies on pleural fibrosis have often focused on the effect of fibroblasts. Mesothelial cell is the predominant cell type in the pleural space, but their role in pleural fibrosis has seldom been studied. In addition, the pleural fluid induced after TGFβ injection has low inflammatory indices when compared with those induced by talc or doxycycline (Chapter 3). However, the IL-8 levels induced after TGFβ administration either intrapleurally in vivo or to mesothelial cells in vitro have not been studied.

In this chapter, the effect of TGFβ, talc and doxycycline on mesothelial cell collagen synthesis and IL-8 production is investigated. We hypothesized that TGFβ, unlike talc and doxycycline, can stimulate collagen synthesis without inducing IL-8 production from pleural mesothelial cells.
7.2 Material and Methods

Reagents

For the animal study, TGF\( \beta_2 \) (Genzyme) was diluted in its buffer, while talc (Sigma) and doxycycline (Fujisawa, Deerfield, IL) were diluted with 0.9% NaCl (Baxter) for intrapleural instillation. Asbestos-free talc powder (Sigma) was gas sterilized using ethylene oxide and then aerated for 96 hours before use. All reagents were diluted in serum-free DMEM for the cell culture experiments.

In Vitro Experiments

Rabbit Pleural Mesothelial Cells Preparation

Primary culture of rabbit pleural mesothelial cells was used in the in vitro experiments. The method of harvesting, verification of the purity of the mesothelial cells and the cell culture protocol were described in Chapter 3. Cells were grown to confluence, and then transferred to 12-well tissue culture plates (well area = 4 cm\(^2\)) 24 hours prior to the experiments.

Mesothelial Cell Survival Study

At the onset of the experiment, the media was changed to serum-free DMEM. TGF\( \beta_2 \) (0.0001-10ng/cm\(^2\)), talc (0.1-10000\( \mu \)g/cm\(^2\)) and doxycycline (0.01-1000\( \mu \)g/cm\(^2\)) were administered in incremental log doses to the mesothelial cells (2-5 wells for each dose). Cells exposed to serum-free DMEM only were used as controls. Cell survival was
measured using trypan blue exclusion method (see Chapter 2) and was expressed as percentage over results of the controls.

Mesothelial Cell IL-8 Production

The experiment was then repeated using log doses below the toxic dosages identified from the cell survival study. In a preliminary experiment, TGFβ₂, talc and doxycycline were administered to pleural mesothelial cells for 4 to 24 hours (3 wells each) and significant differences in IL-8 production was observed at 4 but not at 24 hours. Following that, a full study was performed in which TGFβ₂ (0.01-1ng/cm²), talc (0.1-10μg/cm²), doxycycline (0.01-1.0μg/cm²) and serum-free culture media only were administered to the mesothelial cells (6 wells each) for 4 hours. The supernatant was collected at the end of the experiment and stored at -70°C until assay. The cells in each well were then lysed with 500μL of a lysis agent containing 0.5% SDS. The protein of the lysate was measured with a BCA protein assay (Pierce Chemical Co., Rockford, IL), and represented the amount of cells in each well. Cytokine levels were normalised to the protein concentrations to adjust for any variation in the number of cells in individual wells.

Mesothelial Cell Collagen Production

Rabbit pleural mesothelial cells (passage 2) were plated in 6-well plates (area = 9.6cm²). TGFβ₂ (0.4ng/cm²), talc (10μg/cm²), and doxycycline (1μg/cm²) were each administered to 3 wells of cells at confluence. Ascorbic acid (400μM) was added to each well at the start of the experiment and thereafter every 24 hours. At 48 hours, the cells
were harvested for RNA extraction. Briefly, the cells were washed twice with RNAase-free phosphate buffered saline and RNA was extracted using a Qiagen Rneasy Mini-kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA). The total RNA (7µL) was run in a denaturing agarose gel overnight at 20V. In the following day, the samples were transferred with 6xSSC to a positive-charged membrane (Schleicher & Schuell, Keene, NH, USA). After transferring, the membrane was washed briskly in 2x SSC and then exposed to ultraviolet light for cross-linking. The membrane was pre-hybridised for 3 hours in 10mL of ULTRAhyb solution (Ambion, Austin, TX, USA) at 42°C. After replacing the old pre-hybe solution, 10mL of fresh solution plus the probe were added and hybridisation was carried on overnight. The membrane was washed the following morning following the protocol for a commercial non-radioisotopic detection kit (Ambion) and exposed to a radiography film (Kodak, Rochester, NY, USA). The probe used was a 250 base-pair PCR product of cDNA obtained from the RT-PCR reaction from mesothelial cell RNA. The primers were 20mers (IDT, Coralville, IA, USA) of the sequences: 5' GGC AAC TTG AAC AAG GCT GT and 3' CGA TGT CCA AAG GTG CAA TA.

**In Vitro Experiments**

**Intrapleural Administration of Agents in Rabbit Model**

Pleural fluids (n=27) collected in previous experiments were used, and included samples from rabbits given intrapleural TGFβ2 at 5.0µg (n=5), 1.7µg (n=5) or 0.5µg (n=5), talc slurry at 400mg/kg (n=5), doxycycline at 10mg/kg (n=3) or the buffer for TGFβ2 (n=4). All samples were collected by aspiration via the chest tubes 24 hours after
the intrapleural injections. The total leukocyte counts, protein and LDH levels in these samples were determined as described in Chapters 3 to 5. IL-8 levels were measured using ELISA kits (R&D).

Statistical Analysis

Differences among treatment groups were compared using one way analysis of variance (ANOVA). Multiple comparisons among the various groups were compared using the Dunnett's Method. In non-parametric data, the medians were compared using one-way ANOVA on-ranks and multiple comparisons among groups were performed using Dunn's method. The values of the IL-8 and other biochemical parameters were log transformed for the linear regression analysis, and the correlation was expressed using Pearson's correlation coefficient. $p<0.05$ was considered significant. Data were analysed with a Sigma Stat V2.03 program (SPSS) and expressed as the mean ± standard error of the mean (S.E.M.).
7.3 Results

In Vitro Collagen Synthesis

The Northern blot showed that the pleural mesothelial cells were capable of synthesising mRNA for collagen I. This synthesis was upregulated in the presence of all the sclerosants applied. However, at the optimal dose of each reagent, TGFβ₂ was more potent in upregulating collagen mRNA synthesis than talc and doxycycline, Figure 7.1.

FIGURE 7.1. Northern blot analysis of collagen I mRNA synthesis by rabbit pleural mesothelial cells following stimulation with TGFβ₂, talc and doxycycline.
In Vivo IL-8 Induction

Intrapeural injection of TGFβ2 induced a dose-dependent suppression of IL-8 levels in the pleural fluids, Figure 7.2. The pleural fluid IL-8 level was lowest after the injection of TGFβ2 5.0μg, followed by 1.7μg and 0.5μg (88±26, 169±46, 358±143 pg/mL respectively), all of which were lower than the pleural fluid IL-8 levels in the controls (414±68 pg/mL).

The IL-8 levels were significantly lower in the pleural fluids induced after intrapeural injections of TGFβ2 5.0μg (88±26 pg/mL) than in those induced by talc 400mg/kg (4334±580 pg/mL, p<0.05) and doxycycline 10mg/kg (706±83 pg/mL), Figure 7.3.

The IL-8 levels correlated significantly with the total leukocyte counts (r=0.79, p<0.00001) and LDH (r=0.73, p<0.0001) in the pleural fluids, Figures 7.4 and 7.5 respectively. The pleural fluid IL-8 levels were inversely correlated with the volume of the pleural fluids induced at 24 hours (r=−0.70, p<0.0001). No significant correlation was observed between pleural fluid IL-8 and protein levels (r=0.36, p=0.08).
**FIGURE 7.2.** Intrapleural injection of TGFβ2 suppressed the IL-8 levels in the pleural fluids induced in a dose-dependent manner. (Error bar = S.E.M.)

* $p < 0.05$ when compared with Buffer controls.
**FIGURE 7.3.** The IL-8 levels in the pleural fluids was significantly lower after intrapleural injection of TGFβ₂ when compared with talc and doxycycline. (Error bar = S.E.M.)

* p < 0.05 when compared with TGFβ₂
FIGURE 7.4. The IL-8 levels and the total leukocyte counts in the pleural fluids correlated significantly.

$\text{WBC } mm^{-3}$

$\text{IL-8 pg/mL}$

$r = 0.79$

$p < 0.00001$
FIGURE 7.5. The IL-8 levels and the LDH concentrations in the pleural fluids correlated significantly.
In Vitro IL-8 Induction

In vitro studies were performed to measure the IL-8 production by pleural mesothelial cells in response to TGFβ2, talc and doxycycline. Cell survival studies were performed in primary culture of rabbit pleural mesothelial cells to establish the lethal doses of each reagent, Table 7.1.

**TABLE 7.1** Survival of rabbit pleural mesothelial cells after treatment with incremental log doses of different agents for 24 hours. (n=2-5 for each dose)

<table>
<thead>
<tr>
<th>AGENT</th>
<th>DOSE</th>
<th>Survival (% over Controls) Mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ2</td>
<td>10ng/cm²</td>
<td>83.9±8.7</td>
</tr>
<tr>
<td></td>
<td>1ng/cm²</td>
<td>94.5±1.9</td>
</tr>
<tr>
<td></td>
<td>0.1ng/cm²</td>
<td>96.5±7.3</td>
</tr>
<tr>
<td></td>
<td>0.01ng/cm²</td>
<td>98.2±4.6</td>
</tr>
<tr>
<td></td>
<td>1pg/cm²</td>
<td>98.6±0.2</td>
</tr>
<tr>
<td></td>
<td>0.1pg/cm²</td>
<td>105.1±2.0</td>
</tr>
<tr>
<td>Talc</td>
<td>100μg/cm²</td>
<td>77.5±7.6</td>
</tr>
<tr>
<td></td>
<td>10μg/cm²</td>
<td>91.5±4.3</td>
</tr>
<tr>
<td></td>
<td>1μg/cm²</td>
<td>95.2±6.1</td>
</tr>
<tr>
<td></td>
<td>0.1μg/cm²</td>
<td>100.4±0.9</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>100μg/cm²</td>
<td>Cell fragmentation</td>
</tr>
<tr>
<td></td>
<td>10μg/cm²</td>
<td>Cell fragmentation</td>
</tr>
<tr>
<td></td>
<td>1μg/cm²</td>
<td>94.5±0.5</td>
</tr>
<tr>
<td></td>
<td>0.1μg/cm²</td>
<td>115.5±0.5</td>
</tr>
<tr>
<td></td>
<td>0.01μg/cm²</td>
<td>117.0±2.0</td>
</tr>
</tbody>
</table>
In the *in vitro* studies, talc and doxycycline induced a significant increase in IL-8 production in a dose-dependent fashion when compared with the controls, whereas TGFβ2 did not stimulate any significant increase in IL-8 levels over controls (culture media only).

**FIGURE 7.6.** Talc (a) and doxycycline (b) induced an increase in IL-8 in a dose-dependent manner from rabbit pleural mesothelial cells. On the contrary, TGFβ2 (c) did not increased IL-8 production. The experiments were repeated with similar results. The data presented were the combined results of two experiments to a total of 6 to 9 wells for each agent and dose. (Error bar = S.E.M.)

(a) IL-8 production after talc
(b) IL-8 production after doxycycline

(c) IL-8 production after TGFβ2
7.4 Discussion

In this study, TGFβ2 was more potent than talc or doxycycline in upregulating collagen synthesis from pleural mesothelial cells. Importantly, talc and doxycycline, while stimulating collagen production, increased the IL-8 accumulation in the rabbit pleural space in vivo and the IL-8 production from pleural mesothelial cells in vitro. In contrast, TGFβ2 stimulated collagen synthesis without inducing mesothelial cell IL-8 production in vitro. In vivo, TGFβ2 suppressed the IL-8 accumulation in the pleural fluid in a dose-dependent manner.

The pathogenic process of tissue fibrosis is not completely understood, and controversy exists on the role of inflammation in the fibrotic process, with idiopathic pulmonary fibrosis (IPF) being a good example. It is commonly believed that inflammation plays a role in the initiation of the fibrotic process. Recent evidence suggests that administration of IL-1β, a central regulator of acute inflammation, can lead to progressive pulmonary fibrosis in animals, even after the inflammation has subsided (Kobe et al., 2001). This has led to the speculation that the acute inflammatory process disrupts the ability of tissues to repair damage, allowing an unregulated increase in TGFβ activity, which results in ongoing fibrosis (Sheppard, 2001).

The current belief in the pathophysiology of pleural fibrosis is very similar to that of pulmonary fibrosis described above. Instillation of sclerosants into the pleural space has been shown to induce acute pleural injury and inflammation. Talc, when given intrapleurally in humans for pleurodesis, induces a significant increase in IL-8 and
polymorphonuclear cells in the pleural space which peak after 3-24 hours (van den Heuvel et al., 1998). In experimental animals, injection of talc or tetracycline derivatives induced acute inflammation and a neutrophillic pleural effusion (Kennedy et al., 1995). Steroid inhibits the acute inflammation and reduces the subsequent pleural fibrosis (Teixeira et al., 2002)(Xie et al., 1998c). Such evidence implies that the inflammatory process is crucial in the successful induction of pleural symphysis by these conventional pleurodesing agents.

In this chapter, mesothelial cells have been shown to produce collagen and that TGFβ2, along with known pleural sclerosants (talc and doxycycline), upregulated collagen mRNA expression in vitro. TGFβ2 was more potent than talc and doxycycline in stimulating collagen mRNA synthesis. This is in keeping with our previous data that TGFβ2 induced more mature and immature collagen deposition in the pleura (Chapter 4) as well as more effective pleurodesis than talc (Chapters 3, 4 and 6) in both rabbits and sheep. While fibroblasts are no doubt important in pleural fibrosis, this is the first study to show that common pleurodesing agents also upregulate the collagen expression in the resident mesothelial cells. This is important as the mesothelial cell is the predominant cell type in the pleura, and their contribution to pleural fibrosis should not be overlooked.

Importantly, while talc and doxycycline induced collagen mRNA synthesis, they also stimulated significant increase in IL-8 accumulation in the pleural space in rabbits and in pleural mesothelial cells in vitro. This is in accordance with the belief that conventional pleurodesing agents induce acute pleural inflammation followed
subsequently by pleural fibrosis and symphysis. This inflammatory process is believed to be the cause of the pain and fever commonly experienced after talc and doxycycline pleurodesis. TGFβ, however, is unique in that it induces collagen synthesis and yet suppresses IL-8 release. This challenges the traditional concept that pleural inflammation is essential in the initial induction of pleurodesis.

IL-8 is a potent chemotactic factor and is central to inflammation. It is present in high concentrations in exudative pleural effusions, especially parapneumonic effusions and empyema (Segura et al., 1998). The IL-8 concentration also correlates with the neutrophil counts in human pleural effusions (Ceyhan et al., 1996). Direct intrapleural injection of IL-8 causes neutrophil infiltration in the pleural cavity in rats in a dose-dependent manner (Utsunomiya et al., 1996). Conversely, addition of IL-8 antibodies neutralises neutrophil chemotaxis (Antony et al., 1993; Boylan et al., 1994). Most studies have confirmed that IL-8 levels are significantly higher in the pleural fluids than in matching serum samples (Ceyhan et al., 1996; Stam et al., 2000), suggesting that most of the cytokine is locally produced. This is consistent with the observations with this study that mesothelial cells are capable of producing IL-8 (Antony et al., 1995; Nasreen et al., 1998), which is significantly increased in the presence of talc or doxycycline.

This study is the first to show the effect of TGFβ on mesothelial IL-8 production in vivo or in vitro. Other studies have shown that the effect of TGFβ on IL-8 production is cell-specific. IL-8 levels are increased by TGFβ stimulation in alveolar epithelial cells (Kumar et al., 1996) and uterine fibroblasts (Winkler et al., 2000). In contrast, TGFβ
downregulates IL-8 production in endothelial cells (Mao et al., 1997), endometrial stromal cells (Arici et al., 1996) and melanoma cells (Gutman et al., 1995). TGFβ₂ appears to have anti-inflammatory effect in the pleural space, and may have a role in regulation of excessive inflammation. In previous chapters (Chapter 3 and 4), the pleural fluids induced following intrapleural injection of TGFβ₂ had very low inflammatory indices (total leukocyte count and LDH). The IL-8 levels correlated significantly with both of these indices. The in vitro results also confirmed that TGFβ₂ did not stimulate IL-8 release from mesothelial cells compared with controls. While TGFβ₂ did not suppress the baseline IL-8 release from mesothelial cells in vitro, TGFβ induced a dose-dependent suppression of IL-8 levels in pleural fluids after its intrapleural injections in vivo. One possible explanation is that TGFβ may have inhibited the release of inflammatory cytokines from infiltrating cells in the pleural space in vivo, such as neutrophils and macrophages, and hence reduced the pleural fluid IL-8 concentrations. This possibility requires further investigation.

In conclusion, these experiments provided an explanation at the cellular level for the findings seen in previous chapters – that TGFβ can induce more effective pleurodesis without inducing excessive pleural inflammation. It shows that pleurodesis can be achieved without necessitating acute pleural inflammation.
SUMMARY

• TGFβ2, talc and doxycycline were all capable of stimulating collagen mRNA synthesis in pleural mesothelial cells. At the optimal dose of each agent, TGFβ2 was more potent in upregulating collagen mRNA synthesis than the other agents.

• The IL-8 levels in the pleural fluids induced after intrapleural injection of TGFβ2 was significantly lower than those induced by talc and doxycycline. TGFβ2 induced a dose dependent reduction in IL-8 levels in the pleural fluids when compared with the buffer controls.

• In vitro, both talc and doxycycline stimulated a dose dependent increase in IL-8 release from pleural mesothelial cells, whereas TGFβ2 did not induce IL-8 production.
CHAPTER 8

TRANSFORMING GROWTH FACTOR-β INDUCES VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION FROM PLEURAL MESOTHELIAL CELLS IN VIVO AND IN VITRO
8.1 Introduction

In previous chapters, intrapleural administration of TGFβ2 produced effective pleurodesis, but also stimulated the production of a large volume of pleural fluid in the first few days, in both the rabbit and sheep models (Chapters 3-6). The mechanism underlying this interesting observation has not been explored.

Pleural effusion is very common in clinical practice and affects over 3000 people per million population each year (Marel et al., 1993). Pleural fluid accumulates as a result of increased pleural fluid formation, and/or reduced drainage (Light, 2001). The pathophysiology of increased pleural fluid formation remains poorly understood. Understanding how TGFβ stimulates pleural effusion formation may provide insight into the mechanism of pleural fluid accumulation.

Current evidence suggests that vascular endothelial growth factor (VEGF) plays an important role in effusion formation (Ferrara, 1999). VEGF is a cytokine known for its potent ability to induce vascular leakage, and hence formation of effusion and ascites. It has been shown that tumour cells implanted in the pleural (Yano et al., 2000b) or peritoneal cavity (Yeo et al., 1993) in mice secrete VEGF, which increases the permeability of microvessels and results in development of pleural effusion and ascites respectively. Conversely, blockade of phosphorylation of the VEGF receptors inhibits the formation of malignant pleural effusions from lung adenocarcinoma in a murine model (Yano et al., 2000a). In humans, pleural fluid VEGF levels are significantly higher in exudates than in transudates, and VEGF receptors are present in high density in the
pleura (Cheng et al., 1999; Thickett et al., 1999), suggestive that VEGF plays a functional role in the development of effusions. However, the factors responsible for the accumulation of VEGF in the pleural space remain largely unknown.

There is evidence that TGFβ can stimulate VEGF release in some epithelial and cancer cell lines (Boussat et al., 2000; Donovan et al., 1997; Koochekpour et al., 1996), though its effect on mesothelial cells has not been studied. Recently it has been shown that the levels of both TGFβ1 and -β2 concentrations correlate with the VEGF levels in all common types of pleural effusions in humans (Cheng et al., 2000a), but a causal relationship has not been established.

Hence, it was hypothesized that TGFβ induces VEGF in pleural fluid in vivo and in vitro. The aim of this study was (i) to compare the VEGF production in the pleural space in vivo after intrapleural injection of TGFβ as compared with other pro-fibrotic agents, and (ii) to assess the effect of TGFβ on VEGF production of pleural mesothelial cells in vitro.
8.2 Methods

Reagents

The production of TGFβ2 and its buffer (Genzyme) were as previously described. Asbestos-free talc powder (Sigma) was gas sterilized using ethylene oxide and then aerated for 96 hours before use. Talc and doxycycline (Fujisawa, Deerfield, IL) were diluted with 0.9% NaCl (Baxter) in the animal studies and with Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) in cell culture experiments. To neutralise the effect of TGFβ, a murine monoclonal anti-TGFβ antibody, 1D11, (Genzyme and R&D Systems) was used, which binds with the active forms of TGFβ1, -β2, and -β3 (Dasch et al., 1989).

In Vivo Experiments

Intrapleural Administration of Agents in Rabbit Model

Pleural fluids (n=33) collected in previous experiments were used. This included samples collected in rabbits given intrapleural injections of TGFβ2 at 5.0μg (n=9) or 1.7μg (n=9), talc slurry at 400mg/kg (n=9), doxycycline at 10mg/kg (n=3) or the TGFβ2 buffer (n=3). All samples were collected by aspiration via the chest tubes 24 hours after the intrapleural injections. The total leukocyte counts, protein and LDH levels in these samples were determined as described in Chapters 3 to 5. To assay for VEGF, the pleural fluid samples were collected in citrated tubes on ice, centrifuged at 3,000 r.p.m. for 15 minutes at -4°C, and the supernatant stored immediately at -70°C.
In order to compare the systemic and pleural fluid VEGF levels, plasma (at baseline and 24 hours) and pleural fluid (at 24 hours) were collected after intrapleural TGFβ2 (5.0μg) administration in another five rabbits. Blood was drawn from the marginal ear veins, centrifuged and stored in the same fashion as for the pleural fluid samples described above.

*In Vitro* Experiments

**Pleural Mesothelial Cell Harvesting and Culture from Mice**

Pleural mesothelial cells were obtained from C57BL/6 wild type mice and cultured using the methods described in Chapter 3. The mesothelial cells were grown to confluence, and then trypsinised and transferred to 6-well (in the preliminary study) or 24-well (in the full study) tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) 24 hours prior to the experiments. The area of each well is 9.6 and 1.9cm² for the 6- and 24-well plates respectively.

The purity of the cultured mesothelial cells was verified by (a) their typical cobblestone morphology (Antony *et al.*, 1989), and by (b) cytokeratin staining (Chapter 3). On average, 96% of the cultured cells were cytokeratin-positive, confirming their epithelial origin.

*Pilot Study to Establish the Toxic Doses*

A pilot study was performed to establish the toxic and the optimal doses for VEGF production by each agent. TGFβ2 (0.01-100ng/cm²), talc (1-100μg/cm²), doxycycline (0.1-
1000\( \mu \text{g/cm}^2 \) in half-log increments as well as the TGF\( \beta \) buffer were applied to the murine mesothelial cells on 6-well plates (two wells for each dose) for 4, 8 and 24 hours. At the end of the experiments, the culture media were aspirated and stored at -70°C until assay. Cells in the wells were removed with trypsin and a cell scraper. Each well was examined under phase contrast microscopy to ensure complete removal of cells. Cell viability was tested by trypan blue dye exclusion (Chapter 3), similar to the method used for rabbit mesothelial cells described in Chapter 7.

**Full Study**

In the full study, murine pleural mesothelial cells (primary culture) were plated in 24-well plates. Immediately prior to the study, the media was changed to serum-free DMEM, as fetal bovine serum (FBS) is known to contain measurable amounts of TGF\( \beta \) isoforms. TGF\( \beta_2 \) (0.1\( \text{ng/cm}^2 \)), talc (10\( \mu \text{g/cm}^2 \)), doxycycline (3\( \mu \text{g/cm}^2 \)), buffer or media alone were applied to the wells. The doses used were that determined from the pilot study. The supernatant was collected at 4 hours, and stored at -70°C until assay. The cells in each well were then lysed with 500\( \mu \text{L} \) of a lysis agent containing 0.5% SDS. The protein of the lysate was measured with a BCA protein assay (Pierce Chemical Co., Rockford, IL, USA) and represented the amount of cells in each well. All cytokine measurements were normalized to the protein concentration to ensure that the differences were not due to variation of cell numbers in individual wells. In the in vitro study, VEGF levels were expressed as fold-increase over the media control group.

The experiment was performed similarly for 8-hour and 24-hour time-points. The full study was repeated to a total of 8 (for the 4- and 8-hour studies) and 10 (for the 24
hours studies) wells for each agent at each time-point. The results of the pilot and the repeated full studies were highly consistent.

**Dose Response of TGFβ2-induced VEGF Production**

To establish the dose response of VEGF production from TGFβ2, mesothelial cells (five wells in each group) were stimulated with TGFβ2 (0.04, 0.1 and 0.4ng/cm²) for 24 hours and the supernatant collected for VEGF measurement. The cell experiment was performed in the same manner as in the Full Study.

**Anti-TGFβ Antibody Study**

To further establish the role of TGFβ in the production of VEGF, TGFβ2, talc, doxycycline, buffer and media alone were each applied to 10 wells (24 well-plates) of murine pleural mesothelial cells. The protocol and doses used were that described above. Anti-TGFβ antibodies (1D11) were added at 47μg/mL to half of the wells of each group five minutes before the different reagents were applied. The supernatants were collected at 24 hours, and the cells of each well were lysed as described.

To ensure that the inhibitory effect seen was not a result of non-specific inhibition, murine gamma globulin (Sigma) was used as a control antibody for comparison. VEGF was measured in supernatants of the wells of mesothelial cells treated with TGFβ2 alone, TGFβ2 with anti-TGFβ antibodies (47μg/mL), TGFβ2 with murine gamma globulin (47μg/mL), media with mice gamma globulin, and media alone (five wells in each group).
Cytokines Measurements

TGFβ₁, -β₂ and VEGF concentrations were determined using enzyme-linked immunosorbent assay kits (R&D). In the case of TGFβ, all samples were acidified to convert all TGFβ present to the immunoreactive form for measurement, following the instructions of the manufacturer. There was no detectable cross-reactivity between the TGFβ₁ and -β₂ isoforms using this commercial method (Mozes et al., 1999).

Statistical Analysis

All data are expressed as the mean±standard error (S.E.M.). The differences among groups were compared using one-way ANOVA or one-way ANOVA on-ranks for parametric and non-parametric data respectively. Multiple comparisons were performed using the Tukey (pairwise comparison) or Dunnett's (vs control) Methods. Student's t test was used to compare the VEGF levels with or without anti-TGFβ antibodies. Pearson's correlation was used to measure linear regression. p<0.05 was considered significant. Data were analyzed with a Sigma Stat V2.03 program (SPSS).
8.3 Results

*In vivo VEGF production*

Intrapleural injection of TGFβ2 induced a dose-dependent increase in the pleural fluid VEGF concentrations, Figure 8.1a. The pleural fluid VEGF level was significantly higher in the rabbits that received 5.0μg of TGFβ2 than those given 1.7μg, which in turn had higher pleural fluid VEGF levels than rabbits receiving buffer alone (3195±275 vs 1512±129 vs 1135±215 pg/mL respectively), p<0.05. The VEGF level in the pleural fluid induced after intrapleural injection of 5.0μg of TGFβ2 (3195±275 pg/mL) was also significantly higher than the VEGF levels after the injection of 400mg/kg of talc (748±98 pg/mL), and 10mg/kg of doxycycline (1127±77 pg/mL), Figure 8.1a.
FIGURE 8.1a. The VEGF levels in pleural fluids induced after the intrapleural injection of different pleurodesing agents and the TGFβ buffer in rabbits. The VEGF levels were higher in both the TGFβ2 5.0μg and 1.7μg groups when compared with its buffer controls ($p<0.05$ for both, one-way ANOVA). (n=9 for each of the TGFβ groups and for the talc group. n=3 for the doxycycline and the buffer groups.) Error bar = S.E.M.
To establish whether the VEGF was produced locally within the pleural space or as a result of filtration from systemic circulation, plasma (at baseline and at 24 hours) and pleural fluid (at 24 hours) was collected after intrapleural injection of 5.0μg of TGFβ2 in another five rabbits. There was no difference in the plasma VEGF levels at baseline and at 24 hours (37.2±1.6 and 38.0±2.1 pg/mL respectively) after intrapleural TGFβ2 injections. Pleural fluid VEGF (2795±272 pg/mL) was about 70 folds higher than the plasma VEGF level at either baseline or at 24 hours (p<0.01 for both comparisons), Figure 8.1b.

**FIGURE 8.1b.** Plasma and pleural fluid VEGF levels in rabbits given intrapleural TGFβ2 (5.0μg) injections. (n=5) (Error bar = S.E.M.)
The higher VEGF production induced by TGFβ2 over other agents paralleled the development of significantly larger amount of pleural fluid induced at 24 hours after the injection of TGFβ2, Figure 8.2. The pleural fluid VEGF concentrations were strongly correlated with the volume of pleural fluid induced after the injection of different agents ($r=0.79, p<0.00001$), Figure 8.3. The pleural fluid VEGF levels correlated inversely with LDH or leukocyte concentrations in the effusion ($p<0.00001$ for both). There was no significant correlation between the pleural fluid VEGF concentration and the effusion protein levels (Table 8.1).

**TABLE 8.1. Correlation values of VEGF with pleural fluid parameters.**

* = Pearson's Correlation Coefficient. $n=33.$

<table>
<thead>
<tr>
<th></th>
<th>Correlation with Pleural Fluid VEGF Levels*</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>-0.03</td>
<td>NS</td>
</tr>
<tr>
<td>LDH</td>
<td>-0.84</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Total Leukocyte Count</td>
<td>-0.71</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>
FIGURE 8.2. The volume of pleural fluids induced in rabbits 24 hours after the intrapleural injection of different pleurodesing agents and the control buffer. The volume of the effusions were higher in both the TGFβ2 5.0μg and 1.7μg groups than the buffer controls (p<0.05, one-way ANOVA), when the TGFβ2 treatment groups were compared with the buffer group. (n=9 for each of the TGFβ groups and for the talc group. n=3 for the doxycycline and the buffer groups.) Error bar = S.E.M.
FIGURE 8.3. Correlation of the pleural fluid VEGF concentrations and the volume of pleural fluid induced in rabbits at 24 hours.
In vitro VEGF production

To assess the ability of mesothelial cells to produce VEGF, primary culture of murine pleural mesothelial cells were harvested. The cells were stimulated with TGFβ2, and compared with the other pro-fibrotic agents. The pilot study demonstrated that pleural mesothelial cells were capable of producing VEGF. The lethal doses of these agents on the pleural mesothelial cells were shown in Figures 8.4a, b and c. Interestingly, even at extremely high doses of TGFβ2, none of the cells took up the trypan blue dye. However, at concentrations >1ng/cm², the cells demonstrated obvious morphological changes with severe fragmentation. This morphological change corresponded with a reduction of VEGF protein production, confirming cell injury. Cells cultured with 0.9% saline, the TGFβ buffer or the culture media alone all had ≥95% survival at 24 hours. The optimal doses for VEGF production were determined (TGFβ2 0.1ng/cm²; talc 10ug/cm²; doxycycline 3µg/cm²) for each reagent. In general, the optimal dose was approximately one log dose lower than the lethal concentration.
FIGURE 8.4. Percentage survival of murine pleural mesothelial cells at 24 hours after exposure to (a) TGF\(\beta_2\), (b) talc, and (c) doxycycline as measured by trypan blue dye exclusion method. The arrow in Figure 8.4a indicated the dosage at which significant morphological changes were observed. (n=2 wells for each dose)

(a) Murine pleural mesothelial cells with TGF\(\beta_2\)
(b) *Murine pleural mesothelial cells with talc slurry*
(c) Murine pleural mesothelial cells with doxycycline
TGFβ2 stimulated significantly higher VEGF production from the pleural mesothelial cells *in vitro* than talc, doxycycline, or the controls (buffer or culture media only) as early as 4 hours (Figure 8.5a). The levels of VEGF for each group increased over time, but the same pattern was observed at 8 hours and 24 hours (Figure 8.5b), with the TGFβ2 group inducing the highest amount of VEGF. Results of the preliminary study and the two full studies were highly consistent.

To further confirm the effect of TGFβ2 on VEGF production, a dose response study was performed by applying TGFβ2 at half log increments (0.04, 0.1, 0.4, 1, and 4 ng/cm²) to the mesothelial cells. The concentration of VEGF in the supernatant increased in a dose dependent manner, Figure 8.6. At concentrations higher than 0.4ng/cm², the VEGF production reached a plateau, and then decreased with further increase in TGFβ2 concentrations (not shown), mirroring the results observed in the survival study.
FIGURE 8.5. VEGF production by murine pleural mesothelial cells at (a) 4 hours and (b) 24 hours. The results represented data from two experiments of a combined total of 8-10 wells in each group. At both 4 and 24 hours, TGFβ2 induced significantly higher VEGF production than its buffer controls ($p<0.05$, Student’s $t$ test). VEGF levels in the talc and doxycycline groups were lower than their media control ($p<0.05$, one-way ANOVA) at 4 hours, but no differences was observed at 24 hours ($p=NS$, one-way ANOVA). Error bar = S.E.M.

(a) VEGF production at 4 hours.
(b) VEGF production at 24 hours.
To further confirm the effect of TGFβ$_2$ on VEGF production, a dose response study was performed by applying TGFβ$_2$ at half log increments (0.04, 0.1, 0.4, 1, and 4 ng/cm$^2$) to the mesothelial cells. The concentration of VEGF in the supernatant increased in a dose dependent manner, Figure 8.6. At concentrations higher than 0.4ng/cm$^2$, the VEGF production reached a plateau, and then decreased with further increase in TGFβ$_2$ concentrations (not shown), mirroring the results observed in the survival study.

FIGURE 8.6. Dose-dependent increase in VEGF following TGFβ$_2$ stimulation in murine pleural mesothelial cells. (n=5 wells in each group) Error bar = S.E.M.
The concentration of TGFβ₂ was below detectable range (50pg/mL) in all groups at all time-points, including the group that received TGFβ₂ (0.1ng/cm² or 2ng/mL) at 4 and 24 hours. TGFβ₁ levels were measurable in all groups, but did not differ significantly at 4 hours. At 24 hours, a significantly higher TGFβ₁ level was present in the TGFβ₂ group when compared with the talc or doxycycline groups, p<0.05, Table 2.

**TABLE 8.2.** TGFβ₁ levels in murine mesothelial cells following treatment. All values were normalized to the amount of cells (protein) in each well.

<table>
<thead>
<tr>
<th></th>
<th>TGFβ₁ pg/mL per μg protein (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells treated for 4 hrs with</strong></td>
<td></td>
</tr>
<tr>
<td>TGFβ₂</td>
<td>1.69±0.15</td>
</tr>
<tr>
<td>Talc</td>
<td>1.26±0.16</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>1.53±0.16</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.87±0.13</td>
</tr>
<tr>
<td>Culture Media</td>
<td>1.20±0.08</td>
</tr>
<tr>
<td><strong>Cells treated for 24 hrs with</strong></td>
<td></td>
</tr>
<tr>
<td>TGFβ₂</td>
<td>2.93±0.18</td>
</tr>
<tr>
<td>Talc</td>
<td>* 2.07±0.17</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>* 2.10±0.14</td>
</tr>
<tr>
<td>Buffer</td>
<td>2.65±0.17</td>
</tr>
<tr>
<td>Culture Media</td>
<td>2.58±0.20</td>
</tr>
</tbody>
</table>

* p <0.05 compared with TGFβ₂ group at 24 hrs.
The VEGF production from the mesothelial cells were significantly reduced when TGFβ2 was administered with anti-TGFβ antibodies, but not with murine gamma globulin (IgG), confirming that the reduction in VEGF is a result of specific TGFβ2 inhibition, Figure 8.7.

**FIGURE 8.7.** VEGF production in mesothelial cells at 24 hours in the presence of anti-TGFβ antibodies or murine gamma globulin. (n=5 wells for each group) Error bar = S.E.M.
When TGFβ2, talc, doxycycline and TGFβ buffer were administered to the pleural mesothelial cells, the VEGF concentrations were reduced in all groups with the co-application of the anti-TGFβ antibody, Figure 8.8.

**FIGURE 8.8.** VEGF production at 24 hours by murine pleural mesothelial cells in the presence of anti-TGFβ antibodies. (n=10 wells for each group) Error bar = S.E.M.

*** p < 0.001; ** p < 0.005; * p < 0.05.
8.4 Discussion

The present study demonstrated that the direct intrapleural administration of TGFβ2 induced the accumulation of VEGF in pleural fluid in a dose-dependent fashion in vivo. This increase in VEGF production was associated with the development of large pleural effusions. This study also demonstrated that pleural mesothelial cells are capable of producing VEGF, and mesothelial cell production of VEGF increases in a dose-dependent fashion following stimulated by TGFβ2. This is the first study to demonstrate the in vivo induction of VEGF by TGFβ, and the first to show that mesothelial cells play a role in VEGF production in the pleural space.

Pleural effusion is a common clinical problem and affects 0.32% of the general population each year (Marel et al., 1993). Pleural effusion develops when the rate of pleural fluid formation exceeds its rate of absorption (Light, 2001). Recent evidence suggests that VEGF plays a critical role in the formation of pleural effusion and ascites (Ferrara, 1999; Neufeld et al., 1999). VEGF is a potent inducer of vascular hyperpermeability, and can also stimulate vasodilatation and induce fenestrations in endothelial cells in vitro and in vivo (Ferrara, 1999). These properties contribute to a leakage of plasma and proteins from the vascular space. In animal studies, tumour cells implanted by intrathoracic (Yano et al., 2000b) or intraperitoneal (Yeo et al., 1993) injections produced VEGF and resulted in fluid accumulation. Deletion of the VEGF gene in tumour cells before implantation significantly reduced its expression and effusion formation (Yano et al., 2000b). Likewise, inhibition of VEGF receptor phosphorylation
decreased the formation of malignant pleural effusion in mice (Yano et al., 2000a). In humans, VEGF was present in significantly higher concentrations in exudative effusions. Also, VEGF (fms-like tyrosine kinase-1) receptors are present in human pleural mesothelial cells in high densities, implying an active biological role for VEGF in the pleural space (Thickett et al., 1999).

Little however is known about the factors that induce VEGF production, or the principal source of VEGF in the pleural space. TGFβ itself does not affect vascular permeability (Murohara et al., 1998), but results of this present study have shown that TGFβ can stimulate VEGF accumulation in the pleura, which is associated with the development of large pleural effusions in rabbits. Also, TGFβ2 stimulates significantly more VEGF production than do talc or doxycycline both in vivo and in vitro. This explains the observation from previous chapters that while TGFβ induced more pleural fibrosis, it was associated with the production of larger amount of pleural fluids than talc or doxycycline.

It is interesting that although TGFβ stimulated the accumulation of VEGF and large pleural effusions, it was effective in inducing pleurodesis. TGFβ is a potent pro-fibrotic agent and its intrapleural administration can stimulate significant pleural adhesions even within 24 hours (Chapter 4). By day 4, most rabbits have developed partial symphysis. This rapid induction of pleural fibrosis allows early obliteration of the pleural space that inhibits accumulation of pleural fluid. Also, the results of Chapter 6 have shown that at low doses, TGFβ2 can induce pleurodesis without stimulating
significant pleural fluid accumulation. Given the similarity of the pleural structure of sheep to man, it may therefore be possible that TGFβ2 can be employed in humans to create pleurodesis without inducing excessive fluid formation.

What is the source of the VEGF in the pleural fluid induced after intrapleural injections of TGFβ? The accumulation of VEGF in the pleural or peritoneal space is believed to be a result of local production rather than passive diffusion from systemic circulation (Kraft et al., 1999; Zweers et al., 1999). This is supported by the observation that in humans the VEGF levels in malignant effusions were up to 10 times higher than in the corresponding serum. In our study, the VEGF concentration in the pleural fluids was about 70 folds higher than the corresponding plasma VEGF levels, strongly suggesting that the large amount of VEGF in the effusion originated predominantly from the pleural space. Previous investigations, however, have focused on the role of infiltrating cells, such as malignant cells and inflammatory cells (Yeo et al., 1993), as the source of VEGF in the pleural fluids. This study demonstrated that the resident pleural mesothelial cells express VEGF basally, and more importantly, this expression is up-regulated by TGFβ. In humans, the pleural space is lined by an extensive monolayer of mesothelial cells of an estimated area of 2000 cm² (Sahn, 1988). While infiltrating cells (such as monocytes (Yeo et al., 1993) and macrophages (Harmey et al., 1998)) may contribute to the production of VEGF, it is likely that mesothelial cells represent an important source of VEGF in the pleura.
In this study, primary culture of murine pleural mesothelial cells were harvested and cultured to a high degree of purity. Most studies of murine mesothelial cells have employed peritoneal mesothelial cells (Gibson et al., 1998; Moalli et al., 1987; Muller and Yoshida, 1995), but it is unknown as to whether results of peritoneal mesothelial cells can be extrapolated to pleural mesothelial cells. We also established the toxic dose levels for TGFβ2, talc and doxycycline in mesothelial cells. The lethal dose of talc to murine mesothelial cells was similar to the published data for human pleural mesothelial cells and mesothelioma cells (Nasreen et al., 2000), suggesting that the behaviour of murine pleural mesothelial cells is similar to that of humans.

This is the first study to show VEGF is generated from mesothelial cells. A significant rise in VEGF was seen by 4 hours after application of TGFβ2, and the VEGF levels in the TGFβ2 group remained higher than the other groups at 24 hours. This is consistent with the results of Chua et al (Chua et al., 2000) who demonstrated that in osteoblasts, TGFβ1 stimulates an increase in the VEGF mRNA expression within one hour, and peaks by two hours.

In humans, there are three isoforms of TGFβ: -β1, -β2 and β3. While occasional functional differences have been reported, it is generally believed that TGFβ1 and -β2 have similar biological functions (Border and Noble, 1994; Kelley, 1993). TGFβ1 has been shown to induce VEGF in cultured osteoblasts (Chua et al., 2000), fibroblasts (Berse et al., 1999) and tumour cell lines (Donovan et al., 1997; Koochekpour et al., 1996). In the only other study using TGFβ2, it was shown to exert the same effect as TGFβ1, on induction of VEGF production from human glioma cells (Koochekpour et al., 1996). In a
previous study of human effusions, TGFβ₁ and -β₂ were both correlated with VEGF levels (Cheng et al., 2000a). Hence, we believe that the effect of TGFβ₁ on mesothelial VEGF production would be similar to what we have shown with TGFβ₂.

While the administration of TGFβ to mesothelial cells increased VEGF concentrations, the exact mechanism of induction and the intracellular signalling pathways will require further investigation. Based on the effect of TGFβ on other cell lines, it seems likely that TGFβ upregulates the gene transcription for VEGF, though other mechanisms that can lead to an increase in VEGF levels, such as release of intracellular stores, cannot be excluded. Also, VEGF consists of several isoforms with biologic differences. The differential expression of these isoforms by mesothelial cells after stimulation by TGFβ warrants further studies.

The TGFβ₂ (2000 pg/mL) applied to the pleural mesothelial cells was cleared rapidly. By 4 hours, the TGFβ₂ levels were below the detectable range of 50 pg/mL. In vivo TGFβ is known to have an extremely short half-life of <5 minutes, and is removed by binding to α₂-macroglobulins and by hepatic clearance (Kelley, 1993). On the other hand, the application of TGFβ₂ stimulated the production of TGFβ₁ in the mesothelial cells, which increased with time. This would be in keeping with the previously described autoinduction characteristics of TGFβ (Bascom et al., 1989; Van Obberghen-Schilling et al., 1988), see Chapter 2.
TGFβ predominantly exists in an inactive form through binding to a latent protein, and the active molecule is released only upon activation (Border and Noble, 1994). The active form is usually present in minute quantities and, together with the short half-life, is difficult to measure. Hence, most studies, including this present study, have measured the total amount of TGFβ. The total amount of TGFβ1 was only slightly higher in the TGFβ group, when compared with other treatment and control groups, Table 8.2. Such data is difficult to interpret, as the portion of activated TGFβ in each group is not known. For that reason, we applied an anti-TGF antibody, which binds to all active TGFβ isoforms but not the latent protein (Dasch et al., 1989), to the cell cultures, and showed that the VEGF production was significantly reduced in all groups. This is suggestive that the basal VEGF production from the mesothelial cells is in part due to endogenous TGFβ.

Factors other than TGFβ, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF), interleukin (IL)-1, IL-6, and IL-8 have been shown to induce VEGF production in other cell lines (Ferrara, 1999). However these factors do not consistently induce VEGF, as does TGFβ1. For example, while PDGF stimulated VEGF production in vascular smooth muscle cells (Kronemann et al., 1999) and fibroblasts (Enholm et al., 1997), it did not stimulate VEGF production in human airway epithelial cells. TGFβ, but not PDGF, EGF or KGF, was capable of inducing VEGF synthesis in transformed airway epithelial cells (Boussat et al., 2000). Furthermore, TGFβ is synergistic to PDGF (Kronemann et al., 1999) and IL-1 (Berse et al., 1999) in enhancing VEGF production.
These results have obvious clinical implications. Management of recurrent pleural effusions is a common clinical problem. The treatments frequently employed are repeated aspiration or pleurodesis. No strategy actually targets the control of increased pleural vascular permeability, which underlies the accumulation of most cases of exudative pleural effusions. Anti-VEGF antibodies have been safely used in a phase I study (Gordon et al., 1998), and its use against malignant pleural effusions is currently under clinical trial (Verheul et al., 2000). The results reported in this chapter provide further understanding of the mechanism of VEGF induction in pleural space, and may provide more target options in the pathway of increased pleural fluid formation.

In conclusion, this study showed that TGFβ is a potent stimulator for VEGF production in the pleural space in vivo. The amount of pleural fluid induced after intrapleural administration of TGFβ correlated significantly with the pleural fluid VEGF levels. In the in vitro study, pleural mesothelial cells are capable of producing VEGF, and this production was upregulated by TGFβ. Conversely, anti-TGFβ antibodies inhibited the production of VEGF by mesothelial cells. Targeting the TGFβ and VEGF pathways may provide novel treatment strategies for the management of pleural effusions.
SUMMARY

- Intrapleural administration of TGFβ induced VEGF accumulation in a dose-dependent fashion \textit{in vivo} in a validated rabbit model.

- The VEGF levels in the pleural fluids induced in turn correlated significantly with the volume of pleural effusions.

- Pleural mesothelial cells produce VEGF \textit{in vitro} and represent an important source of VEGF in the pleural space.

- The VEGF production by mesothelial cells is up-regulated by TGFβ and inhibited by anti-TGFβ antibodies.

- Targeting the TGFβ-VEGF pathway may be useful for control of pleural effusions.
CHAPTER 9

CONCLUSION
Transforming Growth Factor-β (TGFβ) has a remarkable capacity to regulate diverse vital cellular behaviors, and is one of the most complex cytokines yet discovered (Blobe et al., 2000). While investigations of TGFβ actions in the lung have focused on its action in pulmonary fibrosis, the body of work in this thesis suggests that TGFβ also plays an important role in pleural disease. In the pleural space, the resident mesothelial cells as well as the infiltrating (e.g., inflammatory and malignant) cells can produce and have receptors for TGFβ.

**Pleural Fibrosis/Pleurodesis**

TGFβ is best known for its potent pro-fibrotic properties (see Chapter 1) and these functions have both desirable and undesirable implications in pleural diseases. Pleurodesis - the obliteration of the pleural space through induction of pleural fibrosis - is a commonly employed treatment for patients with recurrent pleural effusions and pneumothoraces (Lee et al., 2001b). Currently, pleurodesis is performed by the intrapleural injection of a chemical agent or by mechanical abrasion during surgery. These processes induce acute pleural inflammation (Kennedy et al., 1995), which if intense enough, will progress to chronic inflammation and pleural fibrosis, figure 9.1. Pain and fever are common complications of pleurodesis, and are likely to be secondary to the pleural inflammatory process. Given the essential role of TGFβ in most known fibrotic processes, it is likely to be the key mediator of successful pleurodesis.
Figure 9.1. The intrapleural administration of conventional pleurodesing agents induces an acute pleural injury, and inflammation. This process is inhibited by corticosteroids. Pain and fever associated with pleurodesis are presumed to be a result of the acute pleural inflammation. The inflammation may heal with restoration of normal pleura (failed pleurodesis). However if the inflammation is sufficiently intense, it will progress to chronic inflammation and pleural fibrosis (successful pleurodesis). TGFβ is most likely the mediator of the fibrotic process.

**Mechanism of Pleurodesis**

- Injection of Pleurodesing Agent
- Pleural Injury: Inflammation
  - Inhibited by Steroid
  - Pain + Fever
    - TGFβ
- Pleural Fibrosis / Pleurodesis
In this thesis, the use of TGFβ as a fibrogenic and anti-inflammatory pleurodesing agent was assessed. Direct intrapleural administration of TGFβ2 *in vivo* induced significant pleural fibrosis/symphysis and therefore effective pleurodesis (Chapter 3). The results were consistent in rabbits (Chapter 3-5), and were reproducible in sheep, which has thick visceral pleural membranes resembling that of humans (Chapter 6). When compared with currently available pleurodesing agents, TGFβ2 induced more effective pleurodesis than talc and bleomycin (Chapter 6), and at a rate faster than talc slurry (Chapter 4). Pleurodesis was not achieved by the injection of downstream matrix proteins induced by TGFβ, such as fibronectin (Lee *et al.*, 2001a).

Histologically, the intrapleural injection of TGFβ2 stimulated a significant increase in both mature and immature collagen, resulting in marked pleural fibrosis and adhesions (Chapters 4 and 6). TGFβ is known for its ability to upregulate fibroblast production of collagen. In these studies, administration of TGFβ2 also upregulated collagen production in the pleural mesothelial cells as well (Chapter 7).

The use of low dose TGFβ2 for pleurodesis had no significant adverse effects (Chapter 6). In the animal studies assessing TGFβ2 pleurodesis, no acute physiological abnormalities were found, and the systemic levels of TGFβ1 and -β2 in sheep that received TGFβ2 pleurodesis were no different from those given talc or bleomycin. Furthermore, no histological abnormalities were detected in extra-pulmonary organs at 14 days after pleurodesis (Chapter 6). This is further supported by the results of a phase I clinical trial in multiple sclerosis patients, which demonstrated the safety of regular intravenous
infusion of TGFβ2 (Calabresi et al., 1998). Side effects were only observed in patients given the highest dosage, in whom there was a reversible reduction of renal blood flow.

As illustrated in Figure 9.1, the direct administration of TGFβ2 has the advantage of bypassing the pleural inflammatory process. Also, TGFβ2, as our results showed, has potent anti-inflammatory effects in the pleura in vivo (Chapters 3-6) and on mesothelial cells in vitro (Chapter 7). Histologically, there was no significant pleural inflammation after the intrapleural injection of TGFβ2 (Chapter 4). The pleural fluid induced after the intrapleural injection of TGFβ2 has significantly lower inflammatory markers (Chapters 3-6) and IL-8 levels (Chapter 7), when compared with those after talc or doxycycline administration. In vitro, while talc and tetracycline stimulated IL-8 release from pleural mesothelial cells, TGFβ2 did not induce IL-8 production (Chapter 7). Co-administration of systemic corticosteroids reduced the pleural inflammation and hence the efficacy of talc and doxycycline pleurodesis (Teixeira et al., 2002)(Xie et al., 1998c), but corticosteroids did not inhibit TGFβ2-induced pleurodesis (Chapter 5). These findings support the belief that TGFβ2 can induce effective pleurodesis without eliciting excessive pleural inflammation. Therefore, TGFβ2 carries a theoretical advantage of producing pleurodesis with minimal pain or fever, Figure 9.1.

Although the use of TGFβ2 in human pleurodesis has yet to be tested, the cumulative results to date challenge the traditional concept that creation of pleurodesis has to be accompanied with injury and inflammation of the pleura. It highlights the
importance of elucidating the immunological basis of pleurodesis to allow refinement of current management options.

While pleural fibrosis is essential in the setting of pleurodesis, development of pleural thickening following tuberculosis, asbestos or certain drug exposures is undesirable. Similarly, the formation of loculations in the pleural space frequently hinders complete drainage and, in empyema patients, can contribute to morbidity. While no definitive data is available, the results from this thesis raised the speculation that TGFβ is responsible for the pleural fibrotic processes in these conditions. Significant reductions in pulmonary fibrosis (Wang et al., 1999), renal fibrosis (Border et al., 1990), scar formation (Shah et al., 1992), and intra-abdominal adhesions (Lucas et al., 1996) have been achieved in animal models by neutralising TGFβ functions, or by increasing the levels of inhibitory Smad protein (Smad 7) (Nakao et al., 1999). Strategies that antagonise TGFβ activity may be a valuable and novel approach to prevent the development of pleural fibrosis.

*Critical Assessment of the Potential Shortcomings in the Experiments Presented*

While the results presented are robust, there are several important observations from these experiments that are worth noting. As the examiner pointed out there were variations in the absolute values (e.g. pleural fluid LDH) of the animal data among different experiments, although the results (in terms of effect of treatment over controls) remained the same. This observation highlighted the importance of including proper control groups for each set of experiments, as biological data can vary among different
sets of animals, reagents or with different experimental conditions. Use of historical controls should be discouraged. Ideally, if cost and resources allow, animals should be screened (with detailed physical examinations and routine blood tests) to ensure that they are in good physical conditions before the experiments. One of the sheep died before the designated end point (Chapter 6), and post-mortem examination revealed significant parasitic infestation (which is not uncommon in farm animals). Such concurrent illnesses add unnecessary ‘noise’ to the experiments. In the histological analyses (Chapters 5 and 6), the use of additional control group (animals with no treatment at all) would have allowed better interpretation of the data. It will help to determine whether some of the minor changes observed in the post-mortem examinations were within limits of normal variation. In our study we partially bypassed the problem by using very experienced animal pathologists (who were blinded to treatment groups) to interpret the histological data, though the use of a sham group would have provided valuable data.

Most of the animal pleurodesis studies examined only the visceral pleura because of the technical difficulties of obtaining adequate parietal pleura (especially in the presence of pleural symphysis). Future methods of obtaining parietal pleura in a standardized fashion would add useful information.

Further Investigations

While the results to date on the use of TGFβ as a pleurodesing agent are promising in animal studies, the long-term effect of TGFβ-induced pleurodesis need to be studied. Also, the efficacy of TGFβ pleurodesis has only been evaluated in animals with
normal pleural surfaces. Its effectiveness in abnormal (e.g. malignant or inflamed) pleural surfaces requires confirmation in a clinical trial.

As the most common indication for pleurodesis is malignant pleural effusion, questions arise on what potential effect TGFβ may have on pleural malignancies. TGFβ bears a complicated biphasic "love-hate" relationship with cancer. It functions as a potent tumour suppressor (Blobe et al., 2000) in normal human cells, including mesothelial cells (Ikubo et al., 1995), and during early stages of carcinogenesis. On the other hand, advanced tumours often become resistant to the TGFβ-mediated growth arrest (Blobe et al., 2000), thereby resulting in reduced TGFβ signalling. Once resistance develops, the more aggressive cancer cells may make use of TGFβ to facilitate their own growth, particularly through its ability to induce angiogenesis, immunosuppression (allowing cancer cells to escape immune surveillance), and matrix production (enabling cells to anchor at distant sites) (Blobe et al., 2000; Gold, 1999). Useful information on its effects on tumour growth may be gained by applying TGFβ intrapleurally to animal models of pleural malignancies.

However, TGFβ has a short half-life and there is no significant systemic absorption when administered intrapleurally at low doses. Hence, it is likely that given as a single low dose for pleurodesis, TGFβ may not have any significant impact on tumour growth or on its regression. This interesting and important question can only be adequately answered by a clinical trial with accurate measurement of patient survival and disease progression after TGFβ-induced pleurodesis.
Pleural Fluid Formation

While we showed that TGFβ produces excellent pleurodesis and prevents recurrent pleural effusions, we were most interested to find that it plays an important role in pleural fluid accumulation (Chapter 8). This apparent paradoxical concept highlights the complex nature of TGFβ, and its diverse biological functions.

Pleural effusion forms part of the common manifestation of many systemic and pulmonary diseases. Clinical studies have confirmed that TGFβ1 and -β2 are both present in pleural effusions of various aetiologies, and the pleural fluid levels of TGFβ1 and -β2 correlate with each other, as well as with that of VEGF (Cheng et al., 2000a). In the formation of exudative effusions, plasma and proteins have to move from the vascular compartment to the pleural space. During this process, the fluid has to negotiate the vascular barrier and the mesothelial monolayer. A previous investigation demonstrated that TGFβ increases the permeability of the mesothelial monolayer directly in a dose-dependent fashion (Ikubo et al., 1995).

TGFβ enhanced vascular hyperpermeability by stimulating the production of VEGF (Chapter 8). TGFβ induced a dose-dependent accumulation of VEGF in pleural fluid. The pleural fluid VEGF concentrations in turn correlated significantly with the volume of pleural effusions. We also demonstrated that while TGFβ is known to stimulate VEGF production by infiltrating inflammatory cells (Cho et al., 2001; Itaya et al., 2001), pleural mesothelial cells also produce VEGF. The mesothelial cell production
of VEGF is increased by TGFβ2 in a dose-dependent fashion, and can be inhibited by anti-TGFβ antibodies (Chapter 8). Targeting the TGFβ-VEGF pathway may provide novel treatment options in the management of recurrent pleural effusions.

**Clinical Implications**

Our current knowledge of TGFβ is likely to represent only the tip of the iceberg in its highly complex biology. The multiple functions of TGFβ can be unrelated, complimentary, or at times contradictory to each other. These functions may vary in a temporal, tissue, and/or signal transduction specific manner. Determining the factors that govern which role(s) TGFβ will adopt at a given time and site holds the crucial key to understanding its biology, as well as to allowing manipulation of this cytokine for therapeutic purposes. Attempts to modulate the TGFβ functions for disease treatment are already underway. Systemic application of TGFβ2 has been studied in a phase I clinical trial (Calabresi et al., 1998). Conversely, strategies to block TGFβ activities, including neutralising antibodies, antisense oligonucleotides, recombinant latency-associated peptide of TGFβ1 and Smad antagonists (Massague and Chen, 2000), are at various stages of development. Numerous studies are also in progress to elucidate the relationship of genetic polymorphisms of TGFβ, its receptors, and downstream signalling mediators in many pathological conditions.

Further understanding of the actions of TGFβ in the pleura will no doubt provide insight on the pathogenesis and may lead to novel therapeutic strategies for various types of pleural diseases.
SUMMARY

- Intrapleural administration of TGFβ2 can induce excellent pleurodesis effectively, rapidly and without any short-term complications.

- Three important features were observed after intrapleural injection of TGFβ2:

  - TGFβ2 stimulated significant increase in collagen deposition, resulting in marked pleural fibrosis histologically and pleural symphysis macroscopically. The results were consistent in both the rabbit and sheep models.

  - TGFβ2 stimulated fibrosis without inducing IL-8 release from mesothelial cells in vitro. In vivo, there was no significant pleural inflammation following intrapleural TGFβ2 administration. The pleural fluids induced were significantly lower in inflammatory indices and IL-8, when compared with the fluids induced by talc or doxycycline.

  - TGFβ2 stimulated VEGF production in the pleural space in vivo and in vitro, which in turn led to accumulation of large amount of pleural fluids.

- A clinical trial is required to evaluate the efficacy of TGFβ2 as a pleurodesing agent in humans. Several aspects, including long-term safety, effectiveness in abnormal pleural surfaces and effects on cancer progression, need particular attention.
REFERENCES


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FIGURES 6.1 and 6.2.
The pleura of the treatment side was markedly thickened with large amount of collagen and fibrous tissues (figure on left) when compared to the pleura of the control side (figure on right) in sheep after intrapleural TGFβ2 administration. (Trichrome stain, 40x). The black arrows indicate the thickness of the pleura; P = pleura.
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