Transforming growth factor $\beta_2$ (TGF$\beta_2$) produces effective pleurodesis in sheep with no systemic complications

Y C G Lee, K B Lane, R E Parker, D S Ayo, J T Rogers, R W Diters, P J Thompson, R W Light

Abstract

Background—We have recently shown that transforming growth factor (TGF$\beta$), induces effective pleurodesis in rabbits. However, rabbits have a thin pleura while humans have a thick visceral pleura. The effect of intrapleural administration of TGF$\beta_2$, in animals with a thick pleura and its associated systemic effects have not been investigated. This study was undertaken (1) to develop a new animal model for the study of pleurodesis using sheep which have a thick pleura resembling that of humans; (2) to study the efficacy of TGF$\beta_2$, as a pleurodesis agent in the sheep model; and (3) to assess whether histological changes occur in extrapulmonary organs after intrapleural administration of TGF$\beta_2$.

Methods—Twelve sheep were divided into four groups and were given a single intrapleural injection of TGF$\beta_2$ in a concentration of 1.0 µg/kg, 0.5 µg/kg, 0.25 µg/kg or 0.125 µg/kg to the right pleural cavity via a chest tube. The left pleural cavity served as the control. Any pleural fluid that accumulated after the intrapleural TGF$\beta_2$ injection was collected and analysed. The degree of pleurodesis was graded from 1 (no adhesions) to 8 (complete symphysis >50% of chest wall) at day 14 when the sheep were killed. Biopsy specimens were taken from the lungs and extrapulmonary organs.

Results—All sheep that received $\geq 0.25$ µg/kg TGF$\beta_2$ developed excellent pleurodesis (score = 8) while those that received 0.125 µg/kg had a median score of 6. The pleurodesis score did not exceed 2 in the control (left) side of any sheep. Sheep receiving $\geq 0.50$ µg/kg TGF$\beta_2$ developed large exudative pleural effusions while those receiving a lower dose did not. The production of effusions neither hindered nor was necessary for inducing pleurodesis. There were no significant fibrotic changes in any of the extrapulmonary organs.

Conclusion—Intrapleural injection of 0.25–1.0 µg/kg TGF$\beta_2$ produces excellent pleurodesis in a new sheep model with no evidence of extrapulmonary fibrosis.

Keywords: transforming growth factor $\beta_1$; pleurodesis; sheep

Chemical pleurodesis is important in the management of malignant pleural effusions and recurrent pneumothoraces. However, currently available pleurodesing agents either have a suboptimal success rate or carry potentially serious adverse effects. Although talc is effective in inducing pleurodesis, there has been increasing concern of the risks of talc induced acute respiratory distress syndrome (ARDS) and of systemic embolisation of talc particles following its intrapleural administration. As talc and other commonly used agents produce pleurodesis by inducing injury to the pleura, pain and fever are common side effects and can occur in up to 60% of patients following talc or tetracycline pleurodesis. There is a continual search for an “ideal” agent that can safely produce effective pleurodesis.

Transforming growth factor (TGF$\beta$) is a potent stimulator of the production of extracellular matrix as well as a potent anti-inflammatory cytokine. We recently reported that TGF$\beta_2$ produced excellent pleurodesis in rabbits without inducing significant pleural inflammation. However, rabbits have a thin visceral pleura which differs from the thick visceral pleural membrane in humans. The pleura of rabbits receives its blood supply from the pulmonary circulation whereas the blood supply to the pleura of sheep and humans is derived from the systemic circulation. Sheep have a thick pleura which resembles the human pleura more closely than does the rabbit pleura. Hence, sheep have commonly been used for the study of pleural fluid dynamics. The efficacy of TGF$\beta_2$ in inducing pleurodesis in animals with a thick pleura has not been established.

TGF$\beta_2$ is a potent profibrotic cytokine and its overexpression plays a pathogenic role in fibrotic diseases, especially glomerulosclerosis and pulmonary fibrosis. The safety of the intrapleural administration of TGF$\beta_2$ has not been studied.

The purpose of this study was to determine the efficacy and safety of TGF$\beta_2$, as a novel pleurodesing agent using a new sheep model. We hypothesised that TGF$\beta_2$ administered intrapleurally would produce an effective pleurodesis and would not induce any histopathological changes in extrapulmonary organs.

Methods

TGF$\beta_2$ is a recombinant human TGF$\beta_2$ (Genzyme Corp, Framingham, MA, USA) produced in Chinese hamster ovary cells was used. TGF$\beta_2$ is a potent profibrotic cytokine and its overexpression plays a pathogenic role in fibrotic diseases, especially glomerulosclerosis and pulmonary fibrosis. The safety of the intrapleural administration of TGF$\beta_2$ has not been studied. The purpose of this study was to determine the efficacy and safety of TGF$\beta_2$, as a novel pleurodesing agent using a new sheep model. We hypothesised that TGF$\beta_2$ administered intrapleurally would produce an effective pleurodesis and would not induce any histopathological changes in extrapulmonary organs.
was formulated in a vehicle consisting of 20 mM sodium phosphate, 130 mM 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 µm filter. The TGFβ₂ concentration was determined by a sandwich enzyme linked immunosorbent assay using two monoclonal antibodies that crossreact with both TGFβ₁ and TGFβ₂. The activity of TGFβ₂ was determined using a mink lung cell (Mv1Lu) anti-proliferation assay, modified from the method described by Ogawa et al.11

ANIMAL MODEL
To determine the dose response relationship of TGFβ₂ induced pleurodesis in sheep, 12 yearling sheep of mixed breeds weighing 24–35 kg (Ligon Sheep, Nashville, TN, USA) were divided into four groups. A single intrapleural injection of TGFβ₂, was given to each sheep in the following doses: 1.0 µg/kg (group A), 0.50 µg/kg (group B), 0.25 µg/kg (group C), and 0.125 µg/kg (group D). These doses were chosen based on results of a pilot study in which sheep receiving 2 µg/kg TGFβ₂ developed complete pleural symphysis. The study was approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Each animal was anaesthetised with an intravenous injection of 2.5% sodium thiopental (Abbott, North Chicago, IL, USA) in a dose of 20 mg/kg. After shaving the chest the sheep was placed in the lateral decubitus position. The skin was sterilised with 2% chlorhexidine (DVM, Miami, FL, USA) and then with 10% povidone iodine (Baxter, Deerfield, IL, USA). A 5 cm incision was made in the lateral chest wall at the seventh intercostal space. By blunt dissection an 18 gauge Foley balloon cather with 30 ml balloon volume (Bard, Covington, GA, USA) was inserted into the pleural space under aseptic conditions and secured to the muscle layers and skin with purse string sutures. The sheep was then ventilated with a positive end expiratory pressure of 15 cm H₂O. 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 insertion of the chest tube.

A chest tube was inserted in the right pleural space of all animals through which TGFβ₂ was administered as a single intrapleural injection 24 hours after its insertion. The volume of injection was standardised at 1.0 ml/kg. In addition, a chest tube was inserted in the left pleural space of sheep in groups C and D through which an equal volume of the buffer was injected to serve as a control.

On subsequent days the chest tube was aspirated (with the catheter balloon inflated) to remove any pleural fluid produced. The volume of the fluid was recorded. The total leucocyte count was measured using an automated counter (Coulter Electronics, Luton, UK) which was calibrated daily. The first reading was discarded and the mean of the next three readings was recorded. The protein, glucose, and lactate dehydrogenase (LDH) levels were determined using a Vitros Model 950 automated analyser (Johnson & Johnson, Rochester, NY, USA).

The chest tube was removed when the pleural fluid drainage was less than 10 ml/day on two consecutive days. The sheep were killed 14 days after the intrapleural injection of TGFβ₂. At this time a consensus grade was attributed to the degree of pleurodesis using a semi-quantitative scheme (as below) by two investigators (KBL and RWL) who were blind to the treatment. The presence of haemothorax or infection was also checked and recorded if present.

PLEURODESIS SCORING SCHEME
The degree of pleurodesis was graded on the following scale of 1 to 8. 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. 1 = no adhesions between the visceral and parietal pleura; 2 = rare adhesions between the visceral and parietal pleura with no symphysis; 3 = a few scattered adhesions between the visceral and parietal pleura with no symphysis; 4 = many adhesions between the visceral and parietal pleura with symphysis involving less than 5% of the hemithorax; 5 = many adhesions between the visceral and parietal pleura with symphysis involving 5–25% of the hemithorax; 6 = many adhesions between the visceral and parietal pleura with symphysis involving 25–50% of the hemithorax; 7 = many adhesions between the visceral and parietal pleura with symphysis involving more than 50% of the hemithorax.

HISTOLOGICAL EXAMINATION
At necropsy a macroscopic examination was performed and biopsy specimens were taken from the ipsilateral and contralateral pleura and lungs, the pericardium, liver, spleen, diaphragm, kidneys, adrenal glands, ureter, urinary bladder, omentum, the small intestine, and (in female sheep) the ovaries and fallopian tubes. The tissue samples were fixed in 10%
Table 1  Results of pleurodesis score, volume of effusion produced, and analysis of pleural effusion in sheep after intrapleural TGFβ2 administration

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=3)</th>
<th>Group B (n=3)</th>
<th>Group C (n=3)</th>
<th>Group D (n=3)</th>
</tr>
</thead>
<tbody>
<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)†</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Effusion volume at 24 h (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 leucocyte count (×10³)</td>
<td>1352 (1052)</td>
<td>1938 (936)</td>
<td>3475 (108)</td>
<td>3475 (108)</td>
</tr>
</tbody>
</table>

Values are mean (SD).
†Median score.
#Insufficient fluid for analysis.

Results

PLEURODESIS

The intrapleural injection of TGFβ2 was very effective in inducing pleurodesis. Intrapleural TGFβ2 in doses of 1.0 µg/kg, 0.5 µg/kg, and 0.25 µg/kg (groups A, B and C) produced a maximum pleurodesis score of 8 in all nine sheep. The three sheep given 0.125 µg/kg TGFβ2 (group D) all had a pleurodesis score of 6. At the time of death the pleurae of the treatment side were grossly thickened in all sheep (fig 1). 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 in groups C and D given a buffer injection into the left (control) pleural space, there was essentially no pleurodesis with scores of 1–2 in all animals. One sheep in group D had evidence of a small loculated empyema in the left pleural space (control side) at the necropsic examination. There was no haemothorax in any of the sheep.

The volume of effusion produced at 24 hours and in total, the biochemical analysis, and the total leucocyte count of the pleural fluid from the treatment side of the sheep in each dosage group are summarised in table 1. None of the six sheep receiving 0.125 µg/kg or 0.25 µg/kg TGFβ2 (groups C and D) produced significant amounts of pleural fluid (<5 ml) while sheep receiving higher doses produced a large volume of effusion (mean >700 ml for both groups A and B) after intrapleural administration of TGFβ2. There was no significant drainage in these sheep 72 hours after the injection. None of the sheep developed more than 2 ml of fluid 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 in group B, and 3475/mm³ in group C).

HISTOLOGICAL EXAMINATION

The pleura of the treated side of all sheep had significant thickening with fibrovascular proliferation and a minimal inflammatory component (fig 2). There were no differences noted in the nature or intensity of the pleural reaction between animals receiving 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 consolidations. 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 TGFβ2 were examined. In the kidneys tubulointerstitial 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

neutral buffered formalin and processed by routine methods, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E). Histomorphological evaluation was performed by an experienced animal pathologist (RWD). All histological changes were graded as 0 (no abnormality), 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).
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 was present to a mild degree (median = 2) in all samples. One sheep had evidence of focal necrotising enteritis and four others had very mild coccidiosis; these findings are common in necropsy examinations of sheep and were considered incidental.

Discussion
This study has shown that TGFβ2 is effective in producing pleurodesis in sheep, an animal species with a thick pleural membrane similar to that of humans. TGFβ2 induced pleurodesis in a dose dependent fashion and was effective in creating pleural symphysis even at the low dose of 0.25 µg/kg. At this dosage there was no histological evidence of systemic complications in any of the extrapulmonary organs examined. To our knowledge this is the first study to establish the use of a sheep model to determine the effect of pleurodesing agents.

It is estimated that 20 000 patients require pleurodesis each year in the USA. 2 Currently available agents either have a low success rate (for example, bleomycin) or have potentially serious (for example, doxycycline) or fatal complications (for example, talc). 14,15 Talc is considered the most effective compound among commonly used agents. 16 However, talc induced ARDS is a growing concern and can occur in up to 9% of patients. Systemic embolisation of talc has also been identified in patients during necroscopic examination and was found in all organs in rats after talc pleurodesis. Hence, there is an ongoing search for a safe and effective agent for chemical pleurodesis.

We recently established that TGFβ2 was effective in producing pleurodesis in rabbits. 11 TGFβ2 is a multifunctional cytokine with diverse functions. In particular, it is one of the most powerful stimulators of extracellular matrix production and fibrosis in tissue repair. It also has potent anti-inflammatory properties that include downregulation of interferon induced class II MHC expression, and inhibition of T and B lymphocytes and their production of tumour necrosis factor α and interleukin 1. 10

The rabbit is the most common animal model used to study pleurodesis 17–19 although dogs 20,21 and pigs 22 have occasionally been studied. The visceral pleurae of small animals such as rabbits, dogs, and cats are thin and are different in structure from the pleurae of larger animals such as sheep, horses, and cows. The pleurae of these larger animals are more similar to the human pleura which is relatively thick. The results of pleurodesis studies using animals with thin pleurae are often different from those in humans. For example, bleomycin was completely ineffective in creating pleurodesis in rabbits 17 and a much higher dose (>5 fold) of talc is required to produce satisfactory pleurodesis in rabbits 18 than in humans. The sheep model we described can provide a useful method for future studies of pleurodesis.

We found that TGFβ2 was as effective in generating pleurodesis in sheep as it was in rabbits. All animals receiving 0.25 µg/kg or above reached our maximum pleurodesis score indicating symphysis of over 50% of the chest wall. This minimal effective dose in sheep was only 10% of that needed in rabbits (approximately 2.5 µg/kg). 11 Interestingly, intrapleural injection of a higher dose of TGFβ2 induced the production of a large volume of fluid that was dose related. At lower doses minimal amounts of pleural fluid were produced. The production of fluid neither hindered nor was necessary for the production of pleurodesis as excellent pleurodesis was generated in sheep whether or not they developed any sizeable effusion.

More importantly, the effusion induced was relatively non-inflammatory as reflected by the low leucocyte count, protein and LDH concentrations, in keeping with our observations in rabbits. The pleural biopsy samples taken at day 14 also showed no evidence of pleural inflammation. This may in part be due to the potent anti-inflammatory effects of TGFβ2. 9,10 It supports the idea that TGFβ2 produces pleural fibrosis through a novel pathway by stimulation of extracellular matrix deposition and inhibition of its degradation. This is in contrast to talc and tetracycline derivatives which produce pleurodesis by inducing acute pleural injury.

Most currently available pleurodesis agents produce the side effects of fever and pain which are probably the result of acute pleural injury and intense inflammation. Fever can occur in up to 62% of patients following talc pleurodesis 23 and may last for 72 hours. 24 Severe pain was reported in over 50% of patients who were given tetracycline pleurodesis for pneumothorax in the VA cooperative study. 24 Recent studies have also shown that levels of pro-inflammatory cytokines in the pleural fluids were significantly raised following chemical pleurodesis with talc or quinacrine. 25,26 By minimising the inflammatory process, pleurodesis with TGFβ2 may therefore produce less pain or fever, a distinct advantage as pleurodesis is mainly performed for symptomatic comfort in patients with incurable malignancies.

In sheep and humans the visceral pleurae are thick and the pleural space is drained by the lymphatic system via stroma in the parietal pleura which opens eventually into the systemic circulation. 27 The systemic absorption of TGFβ2 following its intrapleural injection has not been studied. TGFβ2 is rapidly cleared from the systemic circulation by binding to α₁-macroglobulin and by redistribution to the kidney, lung, liver, and spleen. 26,27 Hence, the serum half life of TGFβ2 is short (<5 minutes). 27 Given the short half life and the lack of information on the time course of systemic absorption (if any) of TGFβ2, from the pleural space, we did not attempt to measure the changes in serum levels of TGFβ2. Instead we
examined the lungs and extrapulmonary organs for evidence of adverse effects.

Previous studies evaluating the systemic effects of TGFβ1 have yielded different results. When TGFβ1 was applied topically to rats in doses up to 800 µg/kg there was no evidence of systemic absorption. métro No acute systemic or local reactions were seen after intravenous TGFβ1 administration in human or animal studies. Intravenous TGFβ1 was given for three weeks to four weeks to 11 patients with multiple sclerosis in doses of 0.2 µg/kg, 0.6 µg/kg, or 2.0 µg/kg. Reversible reductions in the glomerular filtration rate were observed at 2.0 µg/kg. Mild and reversible increases in hepatocellular enzymes and anaemia were also seen in some of the patients. métro

Other animal studies have shown that administration of very high doses of TGFβ1 could lead to fibrotic changes in the liver and kidneys. Rabbits given intravenous TGFβ1, in a dose of 1000 µg/kg/day developed peritoneal fibrosis and marked centrilobular degeneration in the liver. Rats treated with 800 µg/kg TGFβ1 together with volume depletion developed subcutaneous fibrous nodules and thickened vessel walls in the kidneys. These changes were not seen in rats or rabbits following chronic systemic administration of lower doses of TGFβ1. In our study none of the sheep had any of these changes. There were occasional cases of hepatic or splenic congestion and minimal renal changes in some of the sheep. Although the possibility that these effects are related to TGFβ1 cannot be entirely excluded without a control group for comparison, these findings were mild and are usually considered as incidental findings in farm sheep. Most importantly, the potential side effects of hepatic, renal, and peritoneal fibrosis were not seen in any of the sheep.

In conclusion, this study has established that the intrapleural injection of TGFβ1 is effective in producing pleuritis in sheep, a species with a thick visceral pleura similar to that of humans. The intrapleural injection of TGFβ1 stimulated significant pleural fibrosis but produced minimal pleural inflammation. High doses of TGFβ1 also induced the production of large amounts of pleural fluid. Low doses of TGFβ1 produced excellent pleurodesis without inducing pleural fluid formation. We have also shown that the intrapleural administration of TGFβ1 is safe and does not result in any systemic histological changes. A phase I clinical trial is needed to ascertain the effectiveness of TGFβ1 as a pleurodesing agent in humans.

This study was supported in part by the Genzyme Corporation (Framingham, MA, USA) and the Saint Thomas Foundation (Nashville, TN, USA). Dr Lee is a recipient of a United States-New Zealand Fulbright Graduate Scholarship. We thank Dr James Elrod for his assistance in the preparation of the illustrations.