Transforming growth factor β_2 (TGF β_2) produces effective pleurodesis in sheep with no systemic complications

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Abstract

Background—We have recently shown that transforming growth factor (TGF) β_2 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 β_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 β_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 β_2 .

Methods—Twelve sheep were divided into four groups and were given a single intrapleural injection of TGF β_2 in a concentration of 1.0 $\mu\text{g/kg}$, 0.5 $\mu\text{g/kg}$, 0.25 $\mu\text{g/kg}$ or 0.125 $\mu\text{g/kg}$ 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 β_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 $\geq 0.25 \mu\text{g/kg}$ TGF β_2 developed excellent pleurodesis (score = 8) while those that received 0.125 $\mu\text{g/kg}$ had a median score of 6. The pleurodesis score did not exceed 2 in the control (left) side of any sheep. Sheep receiving $\geq 0.50 \mu\text{g/kg}$ TGF β_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 $\mu\text{g/kg}$ TGF β_2 produces excellent pleurodesis in a new sheep model with no evidence of extrapulmonary fibrosis.

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Keywords: transforming growth factor β ; pleurodesis; sheep

Chemical pleurodesis is important in the management of malignant pleural effusions and recurrent pneumothoraces.¹ However, currently available pleurodesing agents either have a suboptimal success rate or carry potentially serious adverse effects.² Although talc is effective in inducing pleurodesis, there has been increasing concern of the risks of talc induced acute respiratory distress syndrome (ARDS)^{3,4} and of systemic embolisation of talc particles following its intrapleural administration.^{3,5,6} 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.^{2,7} There is a continual search for an “ideal” agent that can safely produce effective pleurodesis.^{7,8}

Transforming growth factor (TGF) β is a potent stimulator of the production of extracellular matrix^{9,10} as well as a potent anti-inflammatory cytokine. We recently reported that TGF β_2 produced excellent pleurodesis in rabbits without inducing significant pleural inflammation.¹¹ 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.¹² 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.^{13,14} The efficacy of TGF β_2 in inducing pleurodesis in animals with a thick pleura has not been established.

TGF β 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 β_2 has not been studied.

The purpose of this study was to determine the efficacy and safety of TGF β_2 as a novel pleurodesing agent using a new sheep model. We hypothesised that TGF β_2 administered intrapleurally would produce an effective pleurodesis and would not induce any histopathological changes in extrapulmonary organs.

Methods

TGF β_2

A recombinant human TGF β_2 (Genzyme Corp, Framingham, MA, USA) produced in Chinese hamster ovary cells was used. TGF β_2

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Figure 1 The visceral pleura (lifted with forceps) is significantly thickened and can only be separated from the underlying lung after intense blunt dissection.

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 μ m filter. The TGF β_2 concentration was determined by a sandwich enzyme linked immunosorbent assay using two monoclonal antibodies that crossreact with both TGF β_2 and TGF β_3 . The activity of TGF β_2 was determined using a mink lung cell (Mv1Lu) anti-proliferation assay, modified from the method described by Ogawa *et al.*¹⁵

ANIMAL MODEL

To determine the dose response relationship of TGF β 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 TGF β_2 was given to each sheep in the following doses: 1.0 μ g/kg (group A), 0.50 μ g/kg (group B), 0.25 μ g/kg (group C), and 0.125 μ g/kg (group D). These doses were chosen based on results of a pilot study in which sheep receiving 2 μ g/kg TGF β_2 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 ballooned catheter 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 H $_2$ O. 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 TGF β_2 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 TGF β_2 . 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 no symphysis; 5 = many adhesions between the visceral and parietal pleura with symphysis involving less than 5% of the hemithorax; 6 = many adhesions between the visceral and parietal pleura with symphysis involving 5–25% of the hemithorax; 7 = many adhesions between the visceral and parietal pleura with symphysis involving 25–50% of the hemithorax; 8 = 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 TGF β_2 administration

	Group A (n=3)	Group B (n=3)	Group C (n=3)	Group D (n=3)
TGF β_2 dose (μ g/kg)	1.0	0.5	0.25	0.125
Pleurodesis score (1–8)†	8	8	8	6
Effusion volume at 24 h (ml)	600 (521)	643 (127)	0.3 (0.6)	0.0 (0.0)
Total effusion volume drained (ml)	715 (341)	965 (383)	0.7 (0.6)	0.0 (0.0)
Protein (mg/dl)	3.9 (0.2)	3.6 (0.3)	#	#
LDH (IU/l)	958 (80)	896 (156)	#	#
Glucose (mg/dl)	72.7 (5.9)	62.0 (1.0)	#	#
Total leucocyte count (/mm ³)	1352 (1052)	1938 (936)	3475 (108)	#

Values are mean (SD).

†Median score.

#Insufficient fluid for analysis.

Figure 2 The pleura of the treatment side (A) was markedly thickened with a large amount of collagen and fibrous tissues compared with the pleura of the control side (B) of the sheep. Trichrome stain, magnification $\times 10$.

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).

Results

PLEURODESIS

The intrapleural injection of TGF β_2 was very effective in inducing pleurodesis. Intrapleural

TGF β_2 in doses of 1.0 μ g/kg, 0.5 μ g/kg, and 0.25 μ g/kg (groups A, B and C) produced a maximum pleurodesis score of 8 in all nine sheep. The three sheep given 0.125 μ g/kg TGF β_2 (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 necroscopic 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 μ g/kg or 0.25 μ g/kg TGF β_2 (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 TGF β_2 . 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 TGF β_2 (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 TGF β_2 . 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 TGF β_2 .

Specific changes that can occur with TGF β 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

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 necroscopic examinations of sheep and were considered incidental.

Discussion

This study has shown that TGF β_2 is effective in producing pleurodesis in sheep, an animal species with a thick pleural membrane similar to that of humans. TGF β_2 induced pleurodesis in a dose dependent fashion and was effective in creating pleural symphysis even at the low dose of 0.25 $\mu\text{g/kg}$. 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.¹ Currently available agents either have a low success rate (for example, bleomycin)² or have potentially serious (for example, doxycycline)² or fatal complications (for example, talc).^{3,4} Talc is considered the most effective compound among commonly used agents.^{2,16} However, talc induced ARDS is a growing concern and can occur in up to 9% of patients.⁴ Systemic embolisation of talc has also been identified in patients during necroscopic examination³ and was found in all organs in rats after talc pleurodesis.⁶ Hence, there is an ongoing search for a safe and effective agent for chemical pleurodesis.

We recently established that TGF β_2 was effective in producing pleurodesis in rabbits.¹¹ TGF β_2 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.⁹ 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^{5,17–19} although dogs^{20,21} and pigs²² 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.¹² 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 rabbits¹⁸ and a much higher dose (>5 fold) of talc is required to produce satisfactory pleurodesis in rabbits¹⁷ than in humans. The sheep

model we described can provide a useful method for future studies of pleurodesis.

We found that TGF β_2 was as effective in generating pleurodesis in sheep as it was in rabbits. All animals receiving 0.25 $\mu\text{g/kg}$ 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 $\mu\text{g/kg}$).¹¹ Interestingly, intrapleural injection of a higher dose of TGF β_2 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 TGF β .^{9,10} It supports the idea that TGF β_2 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.⁵

Most currently available pleurodesis agents produce the side effects of fever and pain² which are probably the result of acute pleural injury and intense inflammation. Fever can occur in up to 62% of patients following talc pleurodesis² and may last for 72 hours.²³ 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 TGF β_2 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 TGF β_2 following its intrapleural injection has not been studied. TGF β is rapidly cleared from the systemic circulation by binding to α_2 -macroglobulin and by redistribution to the kidney, lung, liver, and spleen.²⁸ Hence, the serum half life of TGF β is short (<5 minutes).¹⁰ Given the short half life and the lack of information on the time course of systemic absorption (if any) of TGF β_2 from the pleural space, we did not attempt to measure the changes in serum levels of TGF β_2 . Instead we

examined the lungs and extrapulmonary organs for evidence of adverse effects.

Previous studies evaluating the systemic effects of TGF β have yielded different results. When TGF β_2 was applied topically to rats in doses up to 800 $\mu\text{g/kg}$ there was no evidence of systemic absorption.²⁸ No acute systemic or local reactions were seen after intravenous TGF β_2 administration in human or animal studies.^{29,30} Intravenous TGF β_2 was given three times a week for four weeks to 11 patients with multiple sclerosis in doses of 0.2 $\mu\text{g/kg}$, 0.6 $\mu\text{g/kg}$, or 2.0 $\mu\text{g/kg}$. Reversible reductions in the glomerular filtration rate were observed at 2.0 $\mu\text{g/kg}$. Mild and reversible increases in hepatocellular enzymes and anaemia were also seen in some of the patients.²⁹

Other animal studies have shown that administration of very high doses of TGF β could lead to fibrotic changes in the liver and kidneys. Rabbits given intravenous TGF β_1 in a dose of 1000 $\mu\text{g/kg/day}$ developed periportal fibrosis and marked centrilobular degeneration in the liver.³¹ Rats treated with 800 $\mu\text{g/kg}$ TGF β_2 together with volume depletion developed subcutaneous fibrous nodules and thickened vessel walls in the kidneys.³⁰ These changes were not seen in rabbits or rats following chronic systemic administration of lower doses of TGF β .^{31,32} 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 TGF β_2 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 TGF β_2 is effective in producing pleurodesis in sheep, a species with a thick visceral pleura similar to that of humans. The intrapleural injection of TGF β_2 stimulated significant pleural fibrosis but produced minimal pleural inflammation. High doses of TGF β_2 also induced the production of large amounts of pleural fluid. Low doses of TGF β_2 produced excellent pleurodesis without inducing pleural fluid formation. We have also shown that the intrapleural administration of TGF β_2 is safe and does not result in any systemic histological changes. A phase I clinical trial is needed to ascertain the effectiveness of TGF β_2 as a pleurodesing agent in humans.

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