Transforming Growth Factor-β Activity in Sheep Lung Lymph during the Development of Pulmonary Hypertension

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Abstract

Chronic pulmonary hypertension is associated with extensive structural remodeling of the pulmonary arterial bed. The structural changes in the arterial walls include increased production of extracellular matrix components and smooth muscle cell hypertrophy, changes that have been similarly induced by transforming growth factor-β (TGF-β) in culture. In the present study, experiments were performed to determine whether TGF-β is present in sheep lung lymph, and whether TGF-β levels were altered in an animal model of chronic pulmonary hypertension induced by continuous air embolization. Several standard biological assays for TGF-β activity were used for these determinations including soft agar assays, inhibition of epithelial cell proliferation, and a TGF-β-specific radioreceptor assay. In each case, control lung lymph contained high concentrations of TGF-β (100 ng/ml) which required transient acidification for detection. Samples of lung lymph from hypertensive sheep showed a transient and early two- to threefold increase in concentrations of latent TGF-β. This activity could be partially blocked by TGF-β antibodies. These studies indicate that sheep lung lymph contains TGF-β and that the level of TGF-β increases early during the development of pulmonary hypertension. Thus, TGF-β may contribute to the development of the structural changes in the pulmonary arteries that occur during the onset of chronic pulmonary hypertension. (*J. Clin. Invest.* 1990. 86:1459–1464.) Key words: smooth muscle cells • pulmonary arteries

Introduction

Transforming growth factor-β (TGF-β)[1] was originally defined by its ability to transform fibroblasts in culture (1, 2), however, numerous and diverse biological effects have now been reported (3). For example, TGF-β stimulates the production of extracellular matrix components and protease inhibitors but decreases the production of proteases such that the overall effect is an increase in synthesis and deposition of extracellular matrix components (4–8). TGF-β also stimulates elastin production in cultured vascular smooth muscle cells (9), hypertrophy of smooth muscle cells (10), and depending on the culture conditions, smooth muscle cell proliferation can be stimulated or inhibited (11).

TGF-β has been found to be ubiquitous; it has been detected in a variety of cultured cells, normal and neoplastic tissues (12), and serum (13). Platelets are one of the richest sources of TGF-β (14). TGF-β is released from cells and platelets in a latent, biologically inactive form that can be activated by extremes of pH (15), although a more physiological mechanism of activation has been proposed to involve proteases (16). It is likely that activation of latent TGF-β plays an important role in the regulation of its action.

Chronic pulmonary hypertension often occurs as a secondary complication of many pulmonary disorders such as bronchopulmonary dysplasia, cystic fibrosis, chronic bronchitis, and emphysema. In some cases the hypertension becomes the predominant problem leading to death. Currently no therapy is available for the reversal of severe chronic pulmonary hypertension with the exception of heart or lung transplant. The irreversible disease is thought to result from extensive structural remodeling of the pulmonary arteries. Pathological evaluation of lung tissue from both human disease and animal models has defined a spectrum of characteristic changes that occur in the pulmonary arterial circulation. These changes include increased medial and adventitial thickness, appearance of muscle in normally nonmuscular arterial walls, and reduction in peripheral arterial volume (17). Since increased production of extracellular matrix and smooth muscle hypertrophy are features of the wall of hypertensive pulmonary arteries, and TGF-β has been shown to induce similar changes in vitro, it is possible that TGF-β contributes to the development of this disease.

TGF-β has been shown to be present in human lung lavage fluid (18), but this fluid may not be representative of findings in lung interstitium and vascular walls. In sheep, interstitial fluid can be collected as it exits the lung from the caudal mediastinal lymph node. Biochemical markers of lung damage such as increased protein flux (19), increased prostanoid release (20), and free radical production (21) have been successfully measured in lung lymph. TGF-β has not been measured in lung lymph.

This study uses control sheep to examine whether TGF-β can be detected in lung lymph. The study also uses a model of chronic pulmonary hypertension in which sheep receive continuous air embolization (22) to assess whether levels of TGF-β are altered during the development of this disease. The data indicate that latent TGF-β is a normal constituent of lung lymph and that its levels increase early during the onset of pulmonary hypertension. TGF-β may be linked to the development of the structural changes of chronic pulmonary hypertension.

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1. *Abbreviations used in this paper:* FBS, fetal bovine serum; MK, murine keratinocytes; TGF-β, transforming growth factor-β.

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Methods

Sheep model

Chronically instrumented sheep were prepared as previously described (23). Briefly, bilateral thoracotomies were performed under general anesthesia for cannulation of the efferent duct of the caudal mediastinal lymph node and for placement of catheters in the left atrium and pulmonary artery. Catheters were also placed in the jugular vein and carotid artery. The animals recovered for at least 7 d before beginning experiments. Control animals underwent identical surgery and monitoring but no air embolization.

Continuous air embolization was administered as previously described (22). After baseline physiological measurements, air was infused into the pulmonary arterial circulation through the proximal port of a Swan Ganz catheter at a rate sufficient to cause a two- to threefold increase in pulmonary vascular resistance (baseline = 3.7 Woods units; air embolization = 8.4). This elevation in pulmonary vascular resistance is associated with a 1.5-fold increase in mean pulmonary artery pressure (baseline = 19 cm H_2O; air embolization = 35). The animals were killed with an overdose of pentothal after 12 d of embolization.

Lung lymph was collected over a 30-min period and the flow rate recorded. Heparin (1 U/ml of lymph) was added to each sample. Baseline lung lymph flow averaged 4 ml/h and during air embolization it increased three- to fourfold as previously described (22). Lung lymph protein clearance was increased threefold over baseline throughout air embolization (baseline = 0.8 ml/15 min; air embolization = 2.5). Lung lymph was centrifuged and the supernatant was frozen at −70°C. Samples were obtained from 6 control sheep between 7 and 21 d after the surgery and from 6 sheep during the 12 d of continuous air embolization.

TGF-β bioassays

The presence of TGF-β was detected in control lung lymph by its ability to stimulate fibroblast (AKR-2B, clone 84A) colony formation in soft agar and to inhibit proliferation of murine keratinocytes (MK). Lung lymph was assayed for TGF-β activity in both acid treated and untreated samples. To activate latent TGF-β an aliquot of each sample, diluted with binding buffer (1:10) to prevent precipitation of proteins, was acidified to a pH < 2.0 with 12 N HCl. After 1 h, the pH was adjusted to 7.4 with 10 N NaOH.

Soft agar assay. Lung lymph was evaluated for its ability to stimulate the growth of AKR-2B (clone 84A) cells in soft agar using previously described methods (1, 13). Base layers of 0.8% agar in McCoy's 5a medium with 10% fetal bovine serum (FBS) were poured into 35-mm culture dishes. Lymph samples were added to the upper layer which contained 7.5×10^3 cells in 0.4% agar in McCoy's 5a medium with 10% FBS. Using an Ominicon Facs III image analyzer (Bausch & Lomb, Inc., Rochester, NY) the number of colonies > 50 μm in diameter that developed after 7–10 d were quantitated. Assays were performed in triplicate on samples from three control sheep.

MK inhibition assay. Mouse skin keratinocytes, a continuous epithelial cell line (24), are inhibited by picomolar concentrations of TGF-β (25) and can be used for rapid and sensitive estimates of the presence of TGF-β-like activity from a variety of sources. The effect of lung lymph on incorporation of thymidine by MK cells was determined using a previously described method (25). Rapidly growing BALB/MK cells were placed in 24-well tissue culture plates (40,000 cells/well) in minimal essential medium containing 0.05 mM calcium and supplemented with 8% dialyzed FBS and epidermal growth factor (4 ng/ml). Media was removed after 24 h and fresh media containing lymph samples were added. After 24 h incubation, [^{3}H]thymidine (2.5 μCi/well) was added to measure DNA synthesis. 2 h later, the cells were rinsed three times with ice-cold 10% trichloroacetic acid, air dried, and the DNA was extracted with 0.2 N NaOH. Radioactivity of each sample was counted with a scintillation counter. Samples from four sheep were assayed in triplicate.

Antibody neutralization. To block TGF-β activity, lung lymph samples were incubated with polyclonal antibodies to TGF-β. Anti-TGF-β antibodies were prepared as previously described (26). Lung lymph samples were incubated at 4°C overnight with 10 or 100 μg/ml of anti-TGF-β IgG or nonimmune rabbit IgG (Dako Corp., Santa Barbara, CA). The samples were then tested in the MK inhibition assay as described above.

Radioreceptor assay for TGF-β

A competitive radioreceptor assay was used to detect and quantitate TGF-β in lung lymph from control and air embolized animals. This assay is specific and not influenced by the presence of other growth factors present in lung lymph.

Lung lymph samples were diluted 10-fold with binding buffer (128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM Hepes, 2 mg/ml bovine serum albumin). Latent TGF-β was activated in an aliquot from each sample by transient acidification. The receptor assay was performed essentially as previously described (27, 28). Confluent AKR-2B (clone 84A) cells in 6-well tissue culture plates were incubated for 1 h with binding buffer. The cells were then incubated for 2 h at 22°C with binding buffer containing lymph samples and 0.25 ng of ^{125}I-labeled TGF-β in a total volume of 1 ml/well. Nonspecific binding was determined by the addition of 30 ng/ml of porcine TGF-β (Research and Diagnostic Systems, Inc. Minneapolis, MN). Results are presented as percent inhibition of binding after correction for nonspecific binding. Controls and experimental samples were assayed in triplicate.

A standard curve was constructed using porcine platelet-derived TGF-β and showed that 1 ng/ml of TGF-β resulted in 50% inhibition. The concentration of TGF-β in lung lymph was calculated by assuming that the volume of lymph resulting in 50% inhibition contained 1 ng of TGF-β.

Results

Control lung lymph

Soft agar assay. Sheep lung lymph stimulated fibroblast colony formation in soft agar (Fig. 1). Untreated lymph (30 μl) resulted in a 50% increase in colony formation compared with control. However, after transient acidification of the lymph, the same volume (30 μl) of lung lymph caused a 500% increase in the number of colonies formed compared with control, suggesting that latent TGF-β present in lung lymph had been activated. Small volumes of activated lymph (1 and 3 μl) appeared to inhibit colony formation, suggesting the presence of

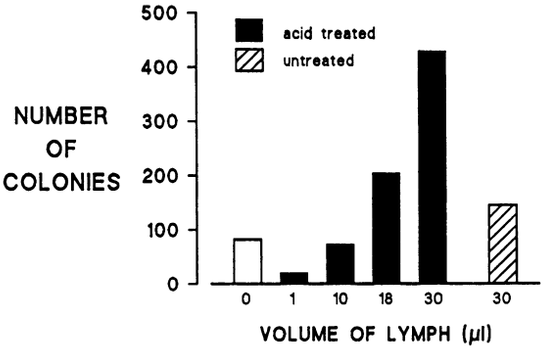

Figure 1. AKR-2B cell colony formation in soft agar was markedly stimulated by 30 μl of acid activated lymph. 30 μl of untreated lymph caused a slight stimulation. Results are from triplicate wells for each sample. Control results (cells cultured in media alone) are shown in the open bar. 3 ng of TGF-β resulted in 1,300 colonies.

other factors present in lung lymph that may inhibit the growth of AKR-2B cells in soft agar (no lymph = 82 colonies; 1 μl = 20; 3 μl = 31).

MK inhibition assay. Lung lymph inhibited the proliferation of MK cells providing additional evidence that TGF-β is present in this fluid (Fig. 2). A 30% decrease in [^{3}H]thymidine incorporation was evident when untreated lung lymph (60 μl) was assayed as compared with control cultures. After acid activation, lung lymph (60 μl) resulted in almost complete inhibition of [^{3}H]thymidine incorporation. Smaller volumes of acid activated or untreated lung lymph (20 μl) were also assayed for the ability to inhibit DNA synthesis of MK cells. This volume of untreated lung lymph has very little effect (93% of control), whereas acid-treated lymph caused a significant inhibition of [^{3}H]thymidine incorporation (71% of control). This increased inhibitory activity after acid treatment is again consistent with the presence of latent TGF-β in lung lymph.

TGF-β antibodies partially blocked lung lymph induced inhibition of MK cell proliferation (Fig. 2). Acid activated lung lymph (60 μl) that had been incubated with TGF-β antibodies did not inhibit DNA synthesis of MK cells to the same extent as acid-activated lymph alone. Incorporation of [^{3}H]thymidine was increased from 3% of control to 10% with 10 μg/ml of antibody and to 25% with 100 μg/ml of antibody. Nonimmune rabbit IgG did not alter the inhibition. Antibodies to TGF-β also blocked activity in hypertensive lymph (data not shown).

Radioreceptor assay. Acid-activated lung lymph from control sheep competed strongly for binding in the radioreceptor assay (Fig. 3 *A*). Approximately 10 μl of lymph resulted in 50% inhibition of ^{125}I-TGF-β binding (Fig. 3 *A*). The results were remarkably similar in all control samples tested and similar results were obtained with samples taken from the same animal more than 1 wk apart. No activity could be detected in an untreated lung lymph sample that was assayed immediately after collection (data not shown). Some samples that were frozen before assay showed small but detectable amounts of activity, perhaps reflecting activation of TGF-β during storage. Since most samples were frozen before the assays, it was not possible to determine whether any TGF-β was active in control lung lymph. Freezing and storage did not alter the amount of total activity detected in acid-treated samples.

As can be seen in Fig. 3 *B*, 1 ng/ml of purified TGF-β resulted in 50% inhibition of the binding of ^{125}I-TGF-β. Thus the calculated concentration of TGF-β in acid-treated control lung lymph was ~ 100 ng/ml.

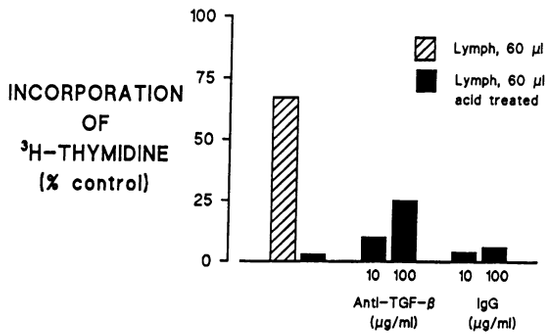

Figure 2. The incorporation of [^{3}H]thymidine into MK cells was decreased in the presence of 60 μl of untreated lymph. After acid activation this inhibitory effect was markedly increased. Preincubation of lung lymph with TGF-β antibodies partially blocked the inhibition. Results are from triplicate wells.

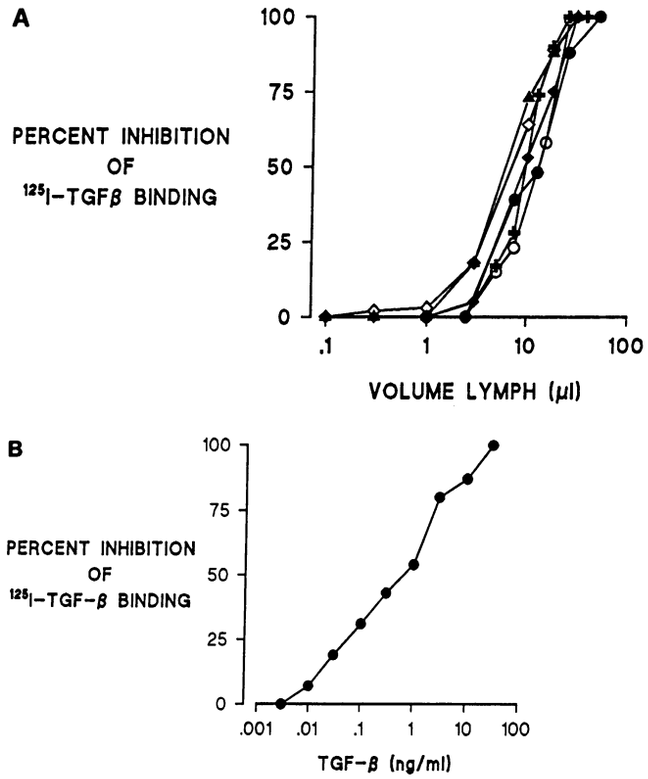

Figure 3. (*A*) Acid treated lung lymph competed with ^{125}I-TGF-β in the radioreceptor assay. Inhibition curves from six different samples of control lung lymph are shown. Results are from triplicate wells for each concentration. Approximately 10 μl of lung lymph resulted in 50% inhibition of ^{125}I-TGF-β binding. (*B*) Porcine platelet-derived TGF-β was used to construct a standard curve for the radioreceptor assay. 1 ng of TGF-β resulted in 50% inhibition of binding.

"Hypertensive" lung lymph

Radioreceptor assay. Acid-treated lung lymph collected from sheep during air embolization showed an increase in TGF-β competing activity over control lung lymph (Fig. 4). The concentrations of TGF-β in lung lymph at baseline and during air embolization are shown (Fig. 4 *A*) and the concentration was calculated from the volume of lung lymph resulting in 50% inhibition of specific binding. Lung lymph concentrations of TGF-β increased early during embolization (baseline = 77–125 ng/ml; air embolization = 154–333), but by day 5 the concentrations were similar to baseline samples. The concentration of TGF-β was lower than baseline in lung lymph collected after day 9 of embolization (27–59 ng/ml). The majority of the TGF-β present in hypertensive lymph was latent. Although some active TGF-β was present in occasional samples (data not shown), it is not yet possible to determine whether the activity was an artefact of freezing and thawing of the samples or reflects in vivo activation.

The flux of TGF-β may be a more accurate reflection of production/release of TGF-β in the lung. Flux was calculated by multiplying the concentration of TGF-β in lung lymph by its flow rate. There was a marked four- to fivefold increase in

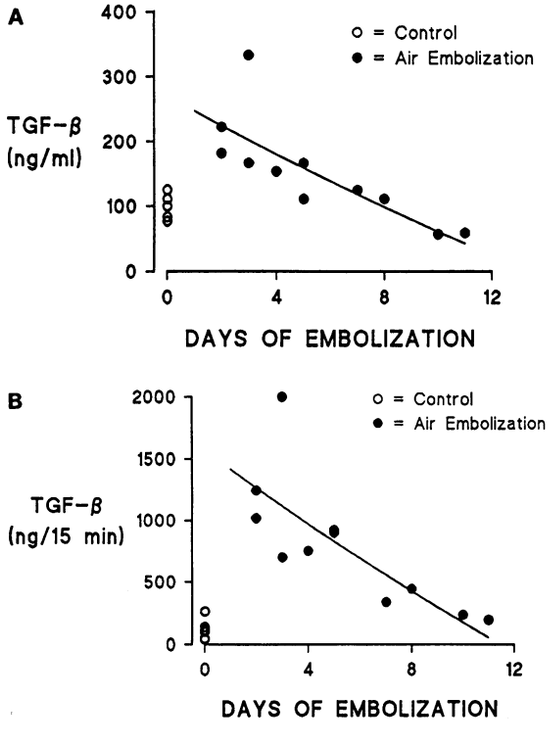

Figure 4. (*A*) TGF-β in acid treated lung lymph during 12 d of air embolization. Concentrations were calculated from the radioreceptor assay (50% inhibition = 1 ng of TGF-β). Control animals that did not receive any air embolization are shown at 0 on the *X* axis. (*B*) Flux of TGF-β from lungs during 12 d of air embolization (flux = lung lymph concentration of TGF-β times flow).

TGF-β flux during the first days of embolization which returned to control values by day 7 (Fig. 4 *B*).

Discussion

Little is known of the role of TGF-β in vivo either under normal or pathological conditions. This study demonstrates that TGF-β is a normal constituent of sheep lung lymph and that the levels increase transiently before the development of pulmonary hypertension.

The cellular origin of TGF-β in lung lymph is not known. Lung lymph is a filtrate of plasma, with the addition of factors present or produced in the interstitium and vessel walls. Normal plasma does not contain TGF-β (13), however it is possible that circulating platelets or inflammatory cells release TGF-β. A recent study of human epithelial lining fluid has suggested that airway cells, particularly alveolar macrophages, are the source of TGF-β detected in normal human epithelial lining fluid (18, 29). While it is possible that the airway cells contribute to the level of TGF-β in lung lymph, the level is tenfold greater than that found in epithelial lining fluid, suggesting that TGF-β is also produced by other cells in the interstitium and/or vascular wall. Endothelial cells (30) and lymphocytes (31, 32) have been reported to produce TGF-β in vitro and may also contribute to the TGF-β in lung lymph. Platelets are a rich source of TGF-β (13, 14) but they are not present in lung lymph.

The level of TGF-β in sheep lung lymph is higher (100 ng/ml) than that required for a maximal response in a variety of in vitro biological assays (~ 10 ng/ml). It is possible that the high levels are an artefact of surgery and lymph duct cannulation. However, this seems improbable as control values were consistent and reproducible even in samples collected up to 3 wk after surgery. Recently, high levels of TGF-β mRNA have been reported in normal murine lung (33), indicating constitutive production of TGF-β in the lung. Whether TGF-β is expressed at high levels in the lung of other species is presently not known.

The role of TGF-β in the lung, as in other tissues, is not known. TGF-β present in interstitial fluid may help maintain normal cellular homeostasis, modulating cellular proliferation and function. TGF-β has been implicated in the repair of skin wounds (34) and may have similar functions in lung injury. An interstitial reservoir of latent TGF-β in the lung would provide immediate availability of TGF-β for tissue repair in areas of injury where platelets are not normally present, and before the recruitment and/or activation of mononuclear cells.

This study has shown an increase in lung lymph TGF-β during air embolization before the onset of sustained pulmonary hypertension. In this model, the pulmonary hypertension is accompanied by several well characterized structural changes in the pulmonary arterial bed including an increase in medial thickness secondary to matrix deposition, smooth muscle hypertrophy, and perhaps hyperplasia (22). Significantly increased concentrations of elastin peptides in lung lymph have also been demonstrated (35). In culture, TGF-β has been shown to both inhibit and stimulate proliferation, depending on culture conditions (10, 11). TGF-β also causes smooth muscle cell hypertrophy (10) and stimulates elastin production (9). Such findings suggest that TGF-β could directly mediate the structural changes of chronic pulmonary hypertension. Further support comes from the work of Sarzani et al., demonstrating increased expression of TGF-β mRNA in aortae from rats with systemic hypertension (36).

The transient nature of the increase in TGF-β in lung lymph suggests that TGF-β may indirectly exert some effects by triggering a cascade of events that lead to remodeling of the pulmonary arteries. This idea is consistent with results found in a hamster bleomycin model of pulmonary fibrosis, where a burst of TGF-β mRNA expression was detected at 10 d, while the fibrosis was not present until 21 d (37). Additionally, studies have shown that TGF-β causes a delayed stimulation of fibroblast proliferation in vitro through the induction of c-*sis* production (38). Similarly, TGF-β stimulates c-*sis* (B chain of platelet-derived growth factor) in human endothelial cells (39). Platelet-derived growth factor is not only a potent smooth muscle cell mitogen (40), but also a vasoconstrictor (41). TGF-β also leads to increased production and release of another vasoconstrictor, endothelin (42). Release of such TGF-β-stimulated vasoconstrictor agents may contribute to the development of sustained pulmonary hypertension. Preliminary studies show that lung lymph from control and air embolized sheep can both stimulate and inhibit proliferation of cultured vascular smooth muscle cells depending on the concentration (43), and suggest the presence of multiple factors that modulate proliferation. The structural changes in chronic pulmonary hypertension are most likely to involve the interaction of several mediators.

The cellular source of TGF-β in lung lymph during air embolization is not known and may be multiple. Levels of constitutive production of TGF-β may change during the

pathogenesis of disease as may the contribution from other sources such as inflammatory cells. For example, inflammation has been associated with the development of chronic pulmonary hypertension both in animal models and in human disease (44) and granulocytes may well represent a source of TGF-β (45). Platelets are a rich source of TGF-β and it is possible that degranulation occurs during air embolization, releasing TGF-β into the plasma. The high baseline concentration of TGF-β in lung lymph might suggest that an increase is unnecessary to elicit pathogenic effects, but it may be that the vascular wall cannot readily utilize an interstitial pool of TGF-β and thus a local source, such as endothelial cells, inflammatory cells, or even the smooth muscle cells themselves must be generated for vascular remodeling to occur. Studies are in progress in our sheep model to determine the cellular origin of TGF-β in the lung.

Activation of latent TGF-β may be the crucial factor in controlling effects of TGF-β. Lyons et al. (16) have shown that plasmin can activate TGF-β, which doubtlessly represents a more physiological mechanism of activation than acidification. Other studies have confirmed the idea that plasmin may be involved in activation using an endothelial cell/pericyte co-culture system (46). It is possible that proteases released in areas of injury may also allow activation of latent TGF-β in the lung. The role of proteases in lung injury induced by air embolization has not been extensively studied, however a serine protease has been identified in lung lymph after 4 h of air embolization (47). Whether this protease could activate TGF-β has not been examined. One source of proteases in the lung is the granulocyte and these cells have been linked to the increased microvascular permeability that accompanies air embolization (48). Alternatively, increased production TGF-β per se might be linked to activation. Potential mechanisms for TGF-β activation need to be further explored.

In summary, we have shown that sheep lung lymph contains high levels of TGF-β activity. This activity increases during continuous air embolization before the development of structural remodeling of the pulmonary arteries. The data suggest that TGF-β could be involved in the response of the lung to the vascular changes induced by air embolization. Further studies are needed to identify the source of TGF-β and mechanisms for its activation.

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