**TGF-β, Smad3 and the process of progressive fibrosis**

J. Gauldie*,†, P. Bonniaud‡, P. Sime‡, K. Ask* and M. Kolb§

*Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St West, Hamilton, ON, Canada L8N 3Z5; †Centre Hospitalier Universitaire du Bocage et Bourgogne, Dijon, France; ‡Department of Respiratory Medicine, University of Rochester, Rochester, NY 14627, U.S.A., and §Department of Medicine, McMaster University, 1200 Main St West, Hamilton, ON, Canada L8N 3Z5

**Abstract**

Transient adenovirus-mediated gene transfer of active TGF-β1 (transforming growth factor-β1) induces severe and progressive fibrosis in rodent lung without apparent inflammation. Alternatively, transfer of IL-1β (interleukin 1β) induces marked tissue injury and inflammation, which develops into progressive fibrosis, associated with an increase in TGF-β1 concentrations in lung fluid and tissue. Both vector treatments induce a fibrotic response involving myofibroblasts and progressive matrix deposition starting at the peri-bronchial site of expression and extending over days to involve the entire lung and pleural surface. Administration of the TGF-β1 vector to the pleural space induces progressive pleural fibrosis, which minimally extends into the lung parenchyma. The mechanisms involved in progressive fibrosis need to account for the limitation of fibrosis to specific organs (lung fibrosis and not liver fibrosis or vice versa) and the lack of effect of anti-inflammatory treatments in regulating progressive fibrosis. TGF-β1 is a key cytokine in the process of fibrogenesis, using intracellular signalling pathways involving the ALK5 receptor and signalling molecules Smad2 and Smad3. Transient gene transfer of either TGF-β1 or IL-1β to Smad3-null mouse lung provides little evidence of progressive fibrosis and no fibrogenesis-associated genes are induced. These results suggest that mechanisms of progressive fibrosis involve factors presented within the context of the matrix that define the microenvironment for progressive matrix deposition.

**Introduction**

IPF [idiopathic PF (pulmonary fibrosis)] is a chronic and progressive interstitial lung disease and is the most common idiopathic interstitial lung disease, with a median survival of approx. 3 years [1–3]. The characteristic histological features of IPF show evidence of UIP (usual interstitial pneumonitis) [1] and include: (i) exuberant extracellular matrix deposition, (ii) the presence of distinct accumulations of mesenchymal cells in ‘fibroblastic foci’ (fibroblasts and myofibroblasts), and (iii) modification of the architecture of the lung with ‘honeycomb’ features and impairment of blood–gas exchange in the later stages of the disease. Little is known about the natural history of the disease, and progression from non-specific pneumonitis to UIP is debated, as is the controversial role of inflammation in the pathogenesis of progressive PF [4–7]. What is not clear are the mechanisms, cells and mediators involved in the progressive stage of the disease.

One prominent hypothesis is that PF is caused by chronic inflammation in response to unknown aetiological agents (idiopathic), leading to tissue destruction, initiation and propagation of wound healing responses and, subsequently, to progressive fibrosis. Support for this hypothesis is provided by the presence of inflammatory cells in the parenchyma of the lung and the expression of pro-inflammatory cytokines, especially IL-1β (interleukin 1β), TGF-β (transforming growth factor-β) and TNFα (tumour necrosis factor α), in the lungs of patients with IPF [6,8]. The tissue response includes repair to parenchyma, neural and vasculature elements; thus most of the recognized growth factors in these processes, including VEGF (vascular endothelial growth factor) and NGF (nerve growth factor) are found in the tissues at some time during the response.

A more recent proposal indicates that inflammation is necessary to trigger the initiation of the fibrotic process, but subsequently plays a minor role in the progression of the disease, particularly in IPF. IPF could result from epithelial injury and abnormal wound repair and may progress through continued and aberrant epithelial mesenchymal cell interaction [7,9]. It is also recognized that patients with IPF respond poorly to anti-inflammatory therapies [10], supporting the concept that inflammation may not be a prominent aspect of pathogenesis of the progressive disease, and that intrinsic structural alterations relate more closely to pathological outcomes [11,12].

**Mediators**

There are a number of cytokines and growth factors that are found in IPF tissue; however, whether each or all play a pathogenic role in progressive fibrosis is unknown, but one growth factor in particular, TGF-β, is thought to have a
pivotal role in fibrogenesis. The profibrotic effects of TGF-β are numerous, including induction of myofibroblasts, increase in matrix synthesis, including collagens, fibronectins and proteoglycans, and inhibition of collagenolysis. Most of these effects are thought to be mediated, in the main, through the Smad signalling pathway, because Smad3-null mice do not appear to develop lung fibrosis induced by bleomycin [13], and they are also protected against radiation-induced fibrosis of the skin [14]. Smads are a family of cytoplasmic signal transducer proteins, and Smad2 and Smad3 predominantly mediate signals from activated TGF-βR (TGF-β receptor) (ALK5 in particular) [15] and interact with numerous TGF-β-responsive promoters [16]. It is of particular interest to examine the role of TGF-β, the relationship of inflammation to progressive fibrosis, and how other pro-inflammatory mediators participate in this complex disease.

We have developed animal models of PF by using transient overexpression (7–10 days) of cytokine genes in the lung of adult rodents through adenoviral-mediated gene transfer to the bronchial and bronchiolar epithelium [17,18]. Using this approach we have examined the tissue response of rodent lungs to expression of a number of pro-inflammatory cytokines, chemokines and growth factors and combined this approach with the use of the Smad3-null mouse to determine whether inflammation is crucial for progressive fibrosis and/or if inflammation could bypass the involvement of TGF-β in fibrogenesis. Moreover, these experimental models have given us some insight into the mechanisms of progressive fibrosis or scarring.

In one set of studies, overexpression of TNFα results in marked alveolar and parenchymal inflammation over a period of 7–14 days, with preservation of alveolar architecture and little evidence of tissue damage. When transgene expression declines, inflammation recedes and lung tissue returns to normal with some residual evidence of fibrosis, but this is considered minimal and not commensurate with the inflammation observed [19], suggesting that accumulation of inflammatory cells alone is not sufficient to elicit a progressive fibrotic response.

These results are in contrast with the effects of transient overexpression of IL-1β using the same adenoviral vector approach. IL-1 causes marked tissue destruction, alveolar damage and a vigorous inflammatory response over the first 14 days. Subsequently, there is excess matrix expression and accumulation and the presence of myofibroblasts and fibroblastic foci. Commensurate with this tissue response, there is evidence of increased TGF-β in the lung tissue and lavage fluid and this increase relates to enhanced collagen content of the lung as evidence of progressive fibrogenesis [9,20]. Thus tissue damage, inflammation, increased TGF-β and matrix accumulation gives evidence of progressive fibrosis, as a direct result of transient, but prolonged, overexpression of IL-1β in the lung.

A more direct evaluation of the impact of TGF-β involves the overexpression of active TGF-β in the lungs of rodents [21]. Within a few days of introducing the vector expressing spontaneously active TGF-β1, there is evidence of tissue remodelling, without any pronounced inflammation. Fibroblastic foci are induced and myofibroblast differentiation is evident [21]. These findings indicate that overexpression of a growth factor such as TGF-β alone could result in modulation of endogenous gene expression, initiation of a set of pathways, possibly autocrine in nature, and induction of progressive fibrosis, akin to human IPF, with all of the required aspects (progressive disease, fibroblastic foci, thickened pleural surface and honeycomb tissue) in the end stage of disease [21]. Recently, we have made use of a novel kinase inhibitor to block the activation of the ALK5 receptor for TGF-β, and show that this signalling cascade is critical for the development of PF, whether it is induced by TGF-β1 or bleomycin [22].

**Progressive fibrosis**

On examination of the TGF-β rat lung model, we note that the process of fibrosis begins near the tissue site where active TGF-β is being expressed, the peri-bronchial areas, and progresses to engage the whole lung, including the pleural surface, but this engagement takes time (days) to become manifest. We also note that the chest wall, adjacent to the pleural surface, is not engaged, nor is there any evidence of other organ involvement, such as liver or kidney. This is true for the human disease, as IPF patients do not develop liver fibrosis or vice versa. In a recent separate set of studies, we have transferred TGF-β to the pleural space, where the single layer of mesothelial cells is the source of overexpression, and we induced pleural fibrosis, which slowly expands into the parenchyma of the lung, but does not engage other organs (N. Décologne, M. Kolb, P. Margetts, F. Menetrier, C. Garrido, J. Gauldie, P. Camus and P. Bonniaud, unpublished work). Collectively, these results suggest that the mechanism of progression must have some limiting factor(s) and the process does not cross physical boundaries. It also suggests that in the disease (TGF-β) cannot act as a soluble, free-floating factor, but rather is presented within the context of the matrix, such that the induction of a ‘pro-fibrotic’ parenchymal cell could result in alteration to the microenvironment of that cell, with numerous growth and differentiation factors adhered to the extracellular matrix surrounding the cell, creating a new and progressive microenvironment through which the fibrogenic message is passed.

Many factors are known to have specific binding affinities for matrix proteins, such as TGF-β for the EDA (extra domain A) of cellular fibronectin [23]. Thus the message of fibrosis could be passed cell to cell in a slow progressive manner and would stop at a physical boundary, such as the pleural space. This process could engage the entire organ in a contiguous manner. We envision this cell microenvironment, with autocrine mechanisms of growth factor stimulation within the context of the matrix (Figure 1), as providing an explanation for the resistance of progressive fibrosis to therapeutic intervention with anti-inflammatory agents. Support for this concept was recently provided in a study of human IPF showing that the fibroblastic foci were connected in a contiguous manner in a ‘fibroblast reticulum’ [24].
Progression in fibrosis

The microenvironment of an activated ‘pro-fibrotic’ cell involves growth factors released by the cell and affixed to the matrix. These growth factors in turn can present to adjacent cells and stimulate growth factor-induced changes that can migrate within the context of the tissue to engage a number of contiguous cells and propagate the progressive nature of fibrosis. CTGF, connective tissue growth factor.

Inflammation, Smad pathways and fibrosis

Administration of active TGF-β to mice deficient in Smad3 blocks the ability of TGF-β1 to induce matrix gene expression, enzyme inhibitory gene expression [PAI-1 (plasminogen-activator inhibitor 1) and TIMP-1 (tissue inhibitor of metalloproteinases-1)] and matrix accumulation, and does not progress to scar formation or fibrosis [25,26]. This indicates that TGF-β and an intact Smad signalling pathway are required to initiate fibrosis and that mechanisms inducing expression of this growth factor are prominent in PF.

Since the process of inflammation is apparently linked to fibrogenesis, we administered IL-1β to the Smad3-null mouse to initiate inflammation in a direct and discrete manner to see whether acute inflammation could provide signals that would bypass the TGF-β pathway and initiate fibrosis. In both wild-type and Smad3-null mice treated with IL-1β, there is induction of marked inflammation, involving tissue damage, destruction of basement membrane, neutrophilia and mononuclear cell accumulation. However, despite the presence of marked inflammation, the Smad3-null mouse does not develop fibrosis, while the wild-type littermate exhibits a marked progressive fibrotic response within 14 days. This clearly implicates the requirement for TGF-β and the Smad3 signalling pathway in the link between inflammation and fibrosis.

Taken together, the data confirm the requirement for intact Smad pathways and the presence of active TGF-β in the progression from inflammation to fibrosis. The pathways for generation of active TGF-β from the latent and matrix-bound forms, include several proteolytic enzymes, as well as specific integrin interactions, leading to stimulation of the signalling cascade and matrix gene expression. Other data confirm the pivotal role of active TGF-β in progressive fibrosis, since bleomycin-induced inflammation can occur without progression to fibrosis in mice deficient in the epithelial cell integrin αvβ6, which activates latent TGF-β [27].

Notably, overexpression of TGF-β in experimental rodent models leads to marked up-regulation of protease inhibitors, such as TIMP and PAI-1 genes, along with excessive matrix accumulation. It may be that a ‘matrix non-degrading’ microenvironment, with enhanced presence of TIMPs, in which matrix turnover would be slowed, while matrix synthesis could progress, would lead to matrix accumulation and fibrosis.

In summary, we have shown that overexpression of TGF-β1 results in progressive fibrosis without a major inflammatory component, either in the initiation or progression of disease [21]. Overexpression of TNFα results in the opposite effect: substantial acute inflammation, with predominantly mononuclear cells and less frequent neutrophils, which resolves completely within 2 weeks, and is not followed by significant progressive fibrosis [19]. In contrast,
overexpression of IL-1β causes severe acute inflammation, associated with tissue destruction and subsequent progressive lung fibrosis [20]. The fibrotic remodelling is associated with a persistent up-regulation of endogenous TGF-β, suggesting that progressive fibrosis is more related to an impairment of the tissue repair process and not to chronic inflammation. Whether the initiation process involves injury and inflammation or not, the link to progressive fibrosis and scar formation is, at a minimum, through TGF-β and the Smad3 signalling pathway, and the progression stage of fibrosis proceeds through a tissue-restricted manner, which is likely to involve growth factor signalling within the context of the extracellular matrix.

References


Received 20 April 2007