Role of transforming growth factor and vascular endothelial growth factor and their receptors in the pathogenesis of bleomycin induced lung fibrosis

Authors
Apoorva Pandey1,2, Ritu Kulshrestha2, Himani Singh2, Shilpi Bhardwaj2, Surendra K Bansal1
1Dept of Biochemistry and 2Pathology, V.P. Chest Institute, University of Delhi, Delhi-110007, India
Corresponding Author
Dr Ritu Kulshrestha
Tel No: +91 9891334373, Email: ritukumar71@yahoo.com

Abstract
Several growth factors including transforming growth factor, vascular endothelial growth factor receptor, fibroblast growth factor etc. are known to play critical roles in the pathogenesis of pulmonary fibrosis. While these growth factors stimulate the proliferation of lung fibroblasts in vitro, their altered expression and release during angiogenesis, aberrant vascular and parenchymal remodeling in lung fibrosis remains controversial. These factors act via receptor tyrosine kinases (vascular endothelial growth factor receptor and fibroblast growth factor receptor), and serine threonine kinase receptors (transforming growth factor receptor) which in turn regulate their availability and modulate cell functions. Therefore protein kinase inhibitors are gaining popularity in the treatment of diseases due to hyperactive protein kinases (including mutant or over expressed kinases in cancer) and other chronic inflammatory diseases. Recently the tyrosine kinase inhibitors such as nintedanib have shown anti-fibrotic and anti-inflammatory effects in animal models of pulmonary fibrosis. Here, we summarise the evidence for involvement of transforming growth factor, vascular endothelial growth factor and their receptors in the pathogenesis of parenchymal and vascular remodeling in pulmonary fibrosis.

Keywords: Pulmonary fibrosis, bleomycin, Transforming growth factor-β, vascular endothelial growth factor, receptor tyrosine kinases, serine threonine kinase receptors.

Introduction
Pulmonary fibrosis is a progressive lung disease with no proven therapeutic intervention and a limited array of biomarkers. It is characterized by prominent fibroblast proliferation and amplified deposition of extracellular matrix (ECM) (Lu et al., 2010). The pathogenesis of pulmonary fibrosis is not clearly defined, with the evidence of abnormal parenchymal repair, aberrant vascular remodeling and neoangiogenesis, which are seen in both animal models and patient samples (Nobel and Norman, 2003; Steurer et al., 2007). The existence of angiogenesis in pulmonary fibrosis is also controversial. It was first described by...
Turner-Warwick, who demonstrated that neovascularization leads to anastomoses between the systemic and pulmonary microvasculature of patients with widespread pulmonary fibrosis (Turner-Warwick, 1963). Other reports also demonstrated vascular remodeling in pulmonary fibrosis and suggested that neovascularization enhances fibrosis (Cosgrove et al., 2004; Peão et al., 1994; Renzoni et al., 2003). An aberrant parenchymal and vascular remodeling is seen to progress in fibrotic lung diseases. The main cells involved include the fibroblasts, myofibroblasts, endothelial cells, vascular smooth muscle cell, alveolar epithelial cells and bronchial epithelial cells. Protein kinases play important roles in intracellular signal transduction and the functional modulation of these cells. They are classified into two broad categories based on the target amino acids of their substrates – protein tyrosine kinases and protein serine/threonine kinases (Hanks et al., 1988). These kinases function as receptors for growth factors, for products of retroviral genes or other oncogenes and regulate important cellular functions. They have been suggested to stimulate the proliferation of lung fibroblasts in vitro and play critical roles in the pathogenesis of pulmonary fibrosis. However their altered expression and release during aberrant vascular remodeling and healing in lung fibrosis remains to be evaluated. Recently, tyrosine kinase inhibitors such as nintedanib have shown anti-fibrotic and anti-inflammatory effects in animal models of pulmonary fibrosis. The new antifibrotic drugs (Gharaee-Kermani et al., 2005) are initially evaluated in animal models of bleomycin induced pulmonary fibrosis, which remains the best characterized model to date (Chua et al., 2005). Here, we summarise the evidence for involvement of vascular endothelial growth factor, transforming growth factor, receptor tyrosine kinase and receptor serine/threonine kinase in the pathogenesis of parenchymal and vascular remodeling in pulmonary fibrosis. 

**Role of receptor serine/threonine kinases (RSKs) in pulmonary fibrosis**

Receptor serine/threonine kinases (RSKs) are transmembrane proteins that have extracellular N-terminal ligand-binding domains and intracellular C-terminal kinase domains (Piek et al., 1999). Two subgroups of RSKs, (type I and II) have been identified based on their primary amino acid sequences (Piek et al., 1999). Upon ligand binding (TGF-beta and activins), the type I and type II receptors form a heteromeric complex with phosphorylation of the type II RSK followed by activation of the type I RSK. Type I RSKs then activate the downstream effectors of the signaling pathway (Piek et al., 1999) and play major roles in multiple physiological and pathological processes of early embryogenesis, adult tissue homeostasis, atherosclerosis, tissue fibrosis and cancer. TGF-β receptors are single pass serine/threonine kinase receptors. They exist in three different isoforms as TGF-β superfamily receptors (1,2,3). Of these, TGFβR1 and TGFβR2 have a high affinity for TGF-β1 and low affinity for TGF-β2. TGFβR3 has a high affinity for both TGF-β1 and TGF-β2. TGFβR2 are constitutively active and can undergo autophosphorylation (Massagué and Chen, 2000; Wrighton et al., 2009), while TGFβR1 activation requires the phosphorylation in its GS domain (TTSGSGS) by TGFβR2 (Huang and Chen, 2012).

**TGF-β1 signaling pathway in lung fibrosis**

The TGF-β1 starts with activation of TGF-β1 latent complex followed by its binding to serine/threonine receptor kinases, leading to the downstream activation of the cytoplasmic Smad proteins (Attisano and Wrana, 2002; Shi and Massagué, 2003). Among those, Smad2 and Smad 3 are receptor-activated Smad proteins that subsequently form a heteromeric complex with Smad 4. Such complexes translocate to the nucleus and regulate the transcription of mRNAs involved in apoptosis, extracellular matrix neogenesis and immune suppression (Gueders et al., 2006). Recently Smad3 has been demonstrated...
to play a role in the transcriptional regulation of type I collagen gene expression and in the development of fibrosis, both in vitro and in animal models. Therefore a targeted deletion of Smad3, is of critical importance (Wollin et al., 2015). TGF-β1/Smad signaling pathway mediates α-smooth muscle actin (α-SMA) gene expression, which is a well-known key marker of myofibroblast differentiation (Gu et al., 2007). In addition to activating Smad2/3, TGF-β can also activate mitogen-activating protein kinases (MAPKs) (ERK, p38 and JNK), phosphatidylinositol 3 kinase (PI3K)/Akt and small GTPases (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). TGF-β is a regulator of the FGF/FGFR signalling cascade, demonstrating the complex interactions among many growth factors. TGF-β induces upregulation of FGFR-1 and FGF-2 in human lung fibroblasts (Wollin et al., 2015) and proliferation of pulmonary interstitial fibroblasts. Smad3 signal transduction pathways are crucial in mediating several TGF-β1 response in fibroblasts, such as collagen synthesis (Chen et al., 2000), upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) (Verrecchia et al., 2001), and plasminogen activator inhibitor-1 (PAI-1) (Datta et al., 2000). TGFβ dysfunctions can result in various kinds of diseases, such as cancer and tissue fibrosis; therefore, the TGF-β signaling pathway is tightly regulated at different levels. Modulation of the TGF-β receptor activity is a critical step for signaling regulation and may have therapeutic potential.

**Role of transforming growth factor (TGFβ) in homeostasis of lung parenchymal cells**

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that acts as a key regulator of extracellular matrix (ECM) assembly and remodeling. It has the ability to induce the expression of ECM proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM. TGF-β signaling regulates diverse cellular processes, including cell proliferation, differentiation, apoptosis, cell plasticity and migration. Elevated TGF-β expression, and subsequent deregulation of TGF-β functions, correlates with the abnormal connective tissue deposition observed during the onset of fibrotic diseases. TGF-β signaling is finely regulated at both the extracellular and intracellular levels. Moreover the function of TGF-β signaling is not only compartment specific but also temporally specific within the epithelial, mesenchymal and immune components of the lung. While TGF-β may be central to the lung fibrotic responses, the therapeutic manipulation of TGF-β signaling remains enticing but extremely challenging. Overexpression of active TGF-β and binding of the TGF-β ligand with its receptor activates the TGF-β-Smad-3 signaling pathway. This in turn activates the SIRT1 transcription and suppresses the transcription of MMP-9 promoter by deacetylation of NF-kB eventually resulting in chronic progressive interstitial pulmonary fibrosis. Role of receptor tyrosine kinases (RTKs) in pulmonary fibrosis

The tyrosine kinases are a sub-class of protein kinase enzyme that can transfer a phosphate group from ATP to cellular proteins. Phosphorylation at tyrosine residues controls many cellular functions by regulating enzyme activity, subcellular localization, and interaction between molecules and growth factors leading to downstream cell signaling (Radha et al., 1996, Schaller et al., 1992). These cell signal transduction cascades transmit the extracellular signals through the cell membrane to the cytoplasm and to the nucleus, leading to altered gene expression (Radha et al., 1996) and ultimately altered cellular functions. If the tyrosine kinases become constitutively active, a non-stop functional state develops that may contribute to initiation or progression of cancer. The tyrosine kinases are divided into two main classes: the transmembrane receptor-linked kinases (RTK) and cytoplasmic/non-receptor PTKs. Approximately 90 Protein Tyrosine Kinases (PTKs) have been found in the human genome, so far (Alonso et al., 2004). They contain
an extracellular ligand binding domain and an intracellular catalytic domain, which mediates ATP-dependent autophosphorylation and phosphorylation of other proteins on tyrosine residues. These residues then serve as docking sites for SH2 domain (Src homology domain–2) containing downstream effectors, such as PI3 kinase. Receptor tyrosine kinases (RTKs) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. When a growth factor binds to the extracellular domain of an RTK, its dimerization is triggered with other adjacent RTKs. This dimerization leads to a rapid activation of the protein's cytoplasmic kinase domains, the first substrate for these domains being the receptor itself. The activated receptor is therefore autophosphorylated on multiple specific intracellular tyrosine residues. This local action of growth factors (e.g. bFGFs, Fibroblast growth factors) with their RTK receptors is classified as paracrine signaling.

**VEGF signaling pathway in lung fibrosis**

The receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFR) plays an important role in cellular homeostasis of the lung vascular cells. VEGF-A binds to two RTKs on the cell surface: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). These VEGF receptors have an extracellular portion, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. VEGFR-2 mediates almost all of the known cellular responses to VEGF. The function of VEGFR-1 is less well defined, and is thought to modulate VEGFR-2 signaling. VEGFR-1 acts as a dummy/decoy receptor, sequesters VEGF from VEGFR-2 binding and plays an important during vasculogenesis in the embryo. VEGFR1 regulates endothelial cell function indirectly, through macrophage recruitment (Murakami et al., 2008), and via deposition of angiogenic growth factors by these cells. Inflammatory diseases such as rheumatoid arthritis (Murakami et al., 2008), are also affected by loss of VEGFR1 kinase activity. VEGFR2 is the main VEGF receptor on endothelial cells. VEGFR2 is essential for endothelial cell biology during development and in the adult, in physiology and pathology. VEGFR2 is the main transducer of VEGFA effects on endothelial cell differentiation, proliferation, migration, and formation of the vascular tube.

VEGFA stimulates proliferation of endothelial cells through VEGFR2- induced activation of the RAS/RAF/ERK/ MAPK pathway (Meadows et al., 2001). The exact mechanism of VEGFR2-induced RAS activation is not clear; one potential pathway involves protein kinase C (PKC)-mediated activation of sphingosine kinase (SPK) (Shu et al., 2002).

Binding of VEGF to its cognate VEGF receptor in cis or trans on adjacent cells (Jakobsson et al., 2006) induces receptor homo- or heterodimerization. The consequent change in receptor conformation leads to exposure of the ATP-binding site in the intracellular kinase domain, kinase activation and auto- or transphosphorylation of tyrosine residues on the receptor dimer itself as well as on downstream signal transducers. Autophosphorylation of VEGFR leads to downstream activation of signalling pathways including Ras, phospholipase C-γ, focal adhesion kinase, p38 and PI3K (Koch et al., 2011). VEGF-A has also been shown to stimulate PDGFRs, thereby regulating mesenchymal cell migration and proliferation (Ball et al., 2007). Ultimately, these activation cascades result in the establishment of biological responses such as cell proliferation, migration and arrangement in three dimensions (3D) to form a vascular tube.

**Role of VEGF in homeostasis of lung vascular cells**

Vascular endothelial growth factor (VEGF) is a proangiogenic mediator which is involved in endothelial cell proliferation, formation of new capillary blood vessels and inducing permeability of blood vessels during the process of tissue...
VEGF has been suggested to have profibrotic and fibroproliferative effects. The main sources of VEGF in the lungs are alveolar epithelial cells, bronchial epithelial cells, airway smooth muscle cells, fibroblasts, endothelial cells and alveolar macrophages (Tuder and Yun, 2008). A significant percentage of lung VEGF is matrix bound (Tuder and Yun, 2008). VEGF is released principally by respiratory epithelial cells and is a key factor in the maintenance of endothelial cells. VEGF is produced by most parenchymal cells and acts in a paracrine manner on adjacent endothelial cells (ECs). However, autocrine VEGF has been proposed to be essential for endothelial cell survival. Migration of endothelial cells is another factor, which is critical for angiogenesis. Endothelial cells move through the protease-degraded basement membrane toward a concentration gradient of VEGFA and other growth factors (Koch et al., 2011).

VEGF transcription is mainly induced by hypoxia-inducible factor–1α, which accumulates in the cells under hypoxia, but is also increased by TGF-β1 (McMahon et al., 2006). The VEGF family is composed of five structurally related factors: VEGFA (the prototype; also denoted VEGFA165), VEGFB, VEGFC, VEGFD and placenta growth factor (PIGF). The complexity is increased further by alternative splicing (for VEGFA, VEGFB, and PIGF) and processing (VEGFA, VEGFC, and VEGFD) (Koch et al., 2011). VEGFA is alternatively spliced to generate VEGFA121, VEGFA145, VEGFA165, and VEGFA189 (numbers indicating the number of amino acid residues in each human polypeptide). While VEGFA121 is freely diffusible and binds to neither neuropilins like NRP1 nor HS (heparin sulphate), VEGFA 165 and VEGFA189 bind to both, resulting in retention on the cell surface or in the extracellular matrix (Koch et al., 2011).

VEGF increases cell survival via (PI3K)/Akt pathway activation leading to caspase-9 and Bcl-2-associated death promoter (Bad) inactivation. VEGF inhibits endothelial cell apoptosis by decreasing the expression of Bcl-2 and increases endothelial cell survival through MAPK/ERK pathway activation and SAPK/JNK pathway inhibition. VEGF also stabilizes mature vessels in the adult (Maharaj et al., 2006). Thus, decreased VEGF signaling arising as a result of oxidative stress or other causes results in endothelial cell apoptosis, migration impairment and general endothelium dysfunction. VEGF mRNA expression in pulmonary arterial endothelial cells, has been reported to decrease on 2nd and 4th week after intratracheal bleomycin (Gong et al., 2005). This was seen to correlate with the evolution of pulmonary hypertension induced by intratracheal bleomycin in immature rabbits. Therefore the cellular compartment expression of VEGF needs to be analysed. VEGF is essential for sprouting and migration of endothelial cells during angiogenesis, but also important for the maintenance of the endothelial cell survival.

Summary
In idiopathic pulmonary fibrosis, the imbalance between fibrogenic, angiogenic and angiostatic factors results in the expanding scar tissue. Binding of the TGF-β ligand with its receptor activates the TGF-β-Smad-3 signaling pathway. This in turn activates the SIRT1 transcription and suppresses the transcription of MMP-9 promoter by deacetylation of NF-kB eventually resulting in chronic progressive interstitial pulmonary fibrosis. Vascular rarefaction is seen in the scar tissue if the reduced expression of VEGF angiogenic factor, is paralleled by elevation of angiostatic molecule such as pigment epithelium derived factor and transforming growth factor (Ebina et al., 2004). Inhibition of VEGF signaling results in endothelial cell apoptosis and severe damage to the alveolar structure with the apoptosis of alveolar epithelial cells and endothelial cells. The current literature suggests that both the protein receptor kinases of the profibrotic and proangiogenic cytokines, TGF-β1 and VEGF have potential as a therapeutic target and their inhibition can be useful to limit local repair. In addition, VEGF has been suggested to have profibrotic and fibroproliferative effects.
angioproliferation and fibroproliferation. Since systemic therapy with anti-TGF-β1 and VEGF may lead to significant decrease in fibroblast proliferation, EC apoptosis, vascular permeability, edema and hemorrhage. Therefore compartment or cell-specific treatment strategies need to be evaluated to find a satisfactory way out of this dilemma.

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References


