INTRODUCTION
Introduction

Several environmental factors and circumstantial insults keep continuously challenging the structures and physiological functions of the organs of biological species. Chronic inflammation as well as oxidative stress arising from such events over a period of time can damage the normal architecture as well as the function of the tissue of any organ. So, relentlessly, the cellular mechanisms do keep fighting such threats in a bid to repair the tissue to restore normalcy of the organ/s function/s. However, when there develops an imbalance between the damage and the repair potential where the extent of damage exceeds the repair capacity, it manifests into an unsuccessful repair process leading to a highly compromised tissue repair with the deposition of fibrous tissue (e.g., collagen) which leads to an increasingly dysfunctional organs pervading the pathophysiology.

So, organ fibrosis is an intractable, progressive condition that arises in multifactorial chronic inflammatory diseases during which excessive deposition of extracellular matrix (ECM) proteins severely impairs tissue architecture, function, eventually resulting in organ failure [Kis et al., 2011]. Fibrosis affects various organs following tissue injury including lungs, liver and kidneys, and has become globally, a major cause of death. Among them, pulmonary fibrosis (PF) and kidney fibrosis have gained importance as both the organs are physiologically blood purifiers, and also, the incidence and mortalities of these diseases are on the rise [Couser et al., 2011; Kaunisto et al., 2013].

1.1. Idiopathic pulmonary fibrosis (IPF)

IPF was first identified in 1868, in a chronic pneumonitis patient with bulbous fingertips [Dempsey et al., 2006]. As various fibrosing pneumonias which are also termed as interstitial lung diseases have been described, IPF or cryptogenic fibrosing alveolitis is generally considered as the most severe form of PF. IPF is a devastating and incurable disease characterized by accumulation of fibrous material in the lung interstitium which results in loss of alveolar function, destruction of normal lung architecture causing respiratory distress [Thannickal et al., 2004; Dempsey et al., 2006]. The idiopathic pneumonias are currently divided into seven different pathologic entities; usual interstitial pneumonia or IPF is one of these and accounts for 60% of cases. The other forms include nonspecific interstitial pneumonia, respiratory bronchiolitis interstitial lung disease, desquamative interstitial pneumonia, acute interstitial
pneumonia, cryptogenic organizing pneumonia, and lymphoid interstitial pneumonia [De Vuyst et al., 2000; White et al., 2003]. The toxicants associated with the development of PF include occupational and environmental inhalation of organic and inorganic dusts, exposure to thoracic radiation and chemotherapeutic drugs such as bleomycin [Baumgartner et al., 2000; Selman et al., 2001]. In addition, patients with various rheumatologic diseases such as sarcoidosis [Steen, 2005], rheumatoid arthritis [Kim, 2006], and dermatomyositis [Fathi, 2005] also may develop pulmonary fibrosis.

1.2. Epidemiology

Few epidemiological studies have been completed in a comprehensive manner to accurately identify the prevalence and incidence of IPF. A study in Bernalillo County, New Mexico, USA reported a prevalence of 20.2 cases per 100,000 for males and 13.2 cases per 100,000 for females with IPF [Coultas et al., 1994]. It has been estimated that the incidence of IPF is 10.7 cases per 100,000 per year for males and 7.4 cases per 100,000 per year for females [Coultas et al., 1994]. The mortality rate was estimated to be 3.3 in men and 2.5 in women per 100,000 people and males have been reported more with IPF than females [Mannino et al., 1996; Johnston et al., 1997]. The majority of these data suggest that the population at highest risk are elderly men with a mean age of 67-79 years [Coultas et al., 1994]. A summary of various studies reported that the mean length of survival from the time of diagnosis varied between 3.2 and 5 years [Panos et al., 1990]. Incidence and prevalence of IPF in India is not well documented but incidences of chronic respiratory diseases are on the rise, accounting for approximately 9% of all deaths [Parekh et al., 2008].

1.3. Risk factors

Although IPF has unknown etiology, there are certain known risk factors which contribute to IPF. As environmental exposure is one of the considerable risk factors, patients with IPF have more common occupational exposure to metal dust (brass, lead, and steel), wood, stone or sand dusts, livestock, farming, stone cutting/polishing [Hubbard et al., 1996; Kitamura et al., 2007; Taskar et al., 2008]. While smoking is another important risk factor for IPF, more than 70% of IPF patients are smokers or ex-smokers. Smoking increases the risk by 1.5 to 2.5 times and it is higher in smokers of >20 packs per year [Baumgartner et al., 1997; Antoniou et al., 2008]. Several studies
have been investigated the possible role of chronic viral infection in the etiology of IPF, including Epstein-Barr virus [Egan et al., 1995], cytomegalovirus [Yonemaru et al., 1997], human herpes virus (HHV)-724, and HHV-825 and hepatitis C [Ueda et al., 1992; Irving et al., 1993]. There is a possible link between diffuse lung fibrosis and gastroesophageal reflux through its presumed association with microaspiration [Tobin et al., 1998; Ovidio et al., 2005]. Thomas et al. (2002) have identified a mutation in the prosurfactant protein-C (proSP-C) gene (leucine—glutamine) in kindred of patients with familial IPF, resulting in an improper proSP-C folding and processing in alveolar type (AT)-II cells. (Figure 1).

Figure 1: Potential risk factors of IPF. Adopted from Baumgartner et al., 1997

1.4. Symptoms and Diagnosis

Patients with IPF typically show exertional dyspnea and a non productive cough [Michaelson et al., 2000]. The physical examination reveals fine bibasilar inspiratory crackles and clubbing of digits which found in approximately 50% of patients with IPF [Turner-Warwick et al., 1980]. Impairments in gaseous exchange may be confirmed by a decrease in the carbon monoxide diffusing capacity or by hypoxemia with graded exercise testing [Marciniuk et al., 1994]. The typical chest radiograph and computed
tomography (CT) are better diagnostic methods for IPF. High-resolution CT is an advanced diagnostic method which increases spatial resolution, facilitating visualization of parenchymal details to the level of the pulmonary lobule. The typical CT features of IPF include patchy peripheral reticular abnormalities, irregular septal thickening and subpleural honeycombing which correlates with fibrosis on biopsy and with physiological impairment [Mathieson et al., 1989].

1.5. Histopathology

Characteristically, IPF has heterogeneous appearance; normal lung alternating with areas of peripheral fibrosis, interstitial inflammation and honeycomb changes, the so-called ‘temporal heterogeneity’ is a hallmark of the disease (Figure 2a). The inflammation is typically mild and consists primarily of lymphocytes but less number of neutrophils and eosinophils may be present. Dense, relatively acellular, collagen bundles with smooth muscle metaplasia can be seen on higher-power magnification. At the border between fibrotic and normal lung, a collection of fibroblasts/myofibroblasts, termed fibroblastic foci exists, represents the active lesion of IPF which is important prognostic factor for IPF (Figure 2b). Alveolar epithelial injury with hyperplastic type II pneumocytes is often seen at areas of active fibrosis [Selman et al., 2001].

1.6. Pathogenesis of PF

The pathogenesis of PF is highly intricate and not well characterized. The current accepted theory proposes that injury to the alveolar epithelium is followed by activation of pro-inflammatory and fibro-proliferative mediators invoke irresolvable tissue repair response leading to the progressive fibrosis. Repair of damaged tissues is a fundamental biological mechanism that allows the ordered replacement of dead or damaged cells after injury, a process critically important for survival. However, if this process becomes dysregulated, it can lead to the development of permanent fibrotic ‘scar’ which is characterised by the excessive accumulation of ECM components (e.g., hyaluronic acid, fibronectin, proteoglycans, and interstitial collagens) at the site of tissue injury [Wynn, 2011].
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Figure 2: Hematoxylin and Eosin stained section of lung from a patient with IPF. Histopathology image (low magnification) showed characteristic heterogeneous peripheral, subpleural location of fibrosis and honeycomb change (a). Histopathology image (high magnification) showed dense, acellular bundles of collagen with smooth muscle metaplasia (arrow) in close approximation to the fibroblastic focus (FF), a dense collection of fibroblasts and myofibroblasts. Cuboidal epithelial cells lining distorted airspaces (which may be undergoing apoptosis) are seen overlying the fibroblastic focus (b). Adopted from White et al., 2003.

Consequently, fibrogenesis is often defined as an out of control wound healing response which has four distinct stages includes a clotting/ coagulation phase, an inflammatory phase, a fibroblast migration/proliferation phase, and a final remodeling phase where normal tissue architecture is restored. In the initial stages after tissue damage, epithelial and/or endothelial cells release inflammatory mediators that stimulate a coagulation cascade that triggers clotting and development of a provisional ECM. Platelet aggregation and subsequent degranulation, in turn, promotes blood vessel dilation and increased permeability, allowing recruitment of inflammatory cells (e.g., neutrophils, macrophages, lymphocytes, and eosinophils) to the site of injury. Inflammatory cells produce a variety of cytokines and chemokines that amplify the inflammatory response and trigger fibroblast/myofibroblast proliferation and recruitment. Finally, in the wound maturation/ remodelling phase, myofibroblasts promote wound contraction, a process where the edges of the wound migrate toward the center and epithelial/endothelial cells divide and migrate over the temporary matrix to regenerate the damaged tissue. Fibrosis develops when the wound is severe, the tissue-damaging irritant persists, or when the repair process either becomes dysregulated or insufficient. Thus,
many stages in the wound repair process can go awry and contribute to scar formation, likely explaining the complex nature of PF [Wynn et al., 2011] (Figure 3).

**Figure 3**: Overview of pathogenic mechanism of PF. The figure is author’s own design and role of each mediator in pathogenesis of PF has been cited in the literature.

1.7. **Alveolar epithelial cells (AECs) targets of early lung injury**

The airway epithelium is a pseudo-stratified mucosal layer acts as a first mechanical and physical barrier of defense against various environmental insults and infections. The alveolar epithelium is composed of two morphologically distinct cell types, AT-I and AT-II cells [Johnson et al., 2002]. AT-I cells are highly attenuated and intimately involved in gaseous exchange by forming an interface with pulmonary capillaries. These cells also take part in peptide and amino acid transportation across the
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lung [Sloan et al., 2003]. AT-II cells are multifunctional that synthesize and secrete pulmonary surfactant, serve as progenitor cells for AT-I, regulate alveolar fluid balance and participate in immune response by producing molecules involved in innate host defense [Shannon et al., 2004]. Alteration of the phenotype of AECs is a typical feature in IPF, continuous damage to the epithelium and concomitant cell apoptosis are thought to contribute to the perpetuation of the fibrotic scarring [Jin and Dong, 2011]. Indeed, recent studies found that IPF patients carry increased number of apoptotic cells in alveolar and bronchial epithelia [Plataki et al., 2005]. This hypothesis is supported by bleomycin mouse model in which inhibition of epithelial cell apoptosis prevents the development of the disease [Kuwano et al., 1999]. Stimuli that trigger the apoptotic cascade in epithelial cells are still under scrutiny. Cell senescence and premature aging due to genetic factors may be one cause but environmental factors such as cigarette smoking (by self or others around), viral infections, and gastroesophageal reflux are a few of the hypotheses that are currently being investigated. Injury to the epithelium results in activation of coagulation pathway and subsequent fibrin deposition. AT-I cells, fibroblasts and alveolar macrophages express tissue factors, urokinase type plasminogen activators and plasminogen activator inhibitors which regulate the activity of fibrinolysis and the clearance of fibrin in the lung alveolus [Shetty et al., 2008]. Injury to the AECs weaken the ability of AT-I cells to clear the redundant fibrin in the lung alveolus. Hence, the amount of tissue factor which binds and activate coagulation factor-VII leads to the activation of downstream coagulation cascade. Therefore, dysregulated coagulation and fibrinolysis upon injury to the AECs and compromised repair capability of AT-I cells may lead to multiple cycles of coagulation and fibrinolysis, which may aggravate alveolar injury [Li et al., 2009]. Unlike AT-I cells, which are terminally differentiated and unable to self renewal or repair, AT-II cells are able to self-renew, repopulate and repair damaged alveolar epithelium [Reddy et al., 2004]. When the alveolar epithelium is damaged, AT-II cells start proliferating and transdifferentiate into AT-I cells to re-establish a functional alveolar epithelium. It also synthesizes some of the essential components of the basement membrane such as fibronectin, laminin and type-IV collagen that are important for maintaining the integrity of the alveolar epithelium. Injury of AT-II cells compromises their production of the components of the basement membrane, resulting in the loss of the integrity of the basement membrane. Fibroblasts and myofibroblasts can migrate into the alveolar space through the partially disrupted
and denuded basement membrane, which promotes the development of PF [Li et al., 2009].

1.8. The injured epithelium and inflammatory response

Although the anti-inflammatory agents, such as steroids having little to no effect in treatment of IPF, injury to the epithelium typically elicits immune response in the lung. Damage to epithelial and endothelial cells release a variety of chemotactic factors that recruit inflammatory monocytes and neutrophils to the site of injury. In this damaged state, circulating platelets are activated upon encountering exposed collagen [Esmon, 2005] and release growth factors such as platelet derived growth factor (PDGF), a potent chemoattractant for inflammatory cells and transforming growth factor-β (TGF-β), which stimulates ECM synthesis by local fibroblasts [Barrientos et al., 2008]. These circulating myeloid cells respond to a gradient of chemokine (C-C) ligand-2 and are recruited to damaged tissues, where they differentiate into macrophages that phagocytose the fibrin clot and cellular debris [Li et al., 2011]. Neutrophils are also recruited quickly after injury and participate in removal of tissue debris. Although the recruitment of inflammatory monocytes and neutrophils at the site of tissue injury is important for the repair process, these cells also secrete a variety of toxic mediators, including reactive oxygen and nitrogen species that are harmful to the surrounding tissues. Consequently, if the inflammatory macrophages and neutrophils are not quickly eliminated, they can further exacerbate the tissue-damaging inflammatory response that leads to scarring. The recruitment of neutrophils to the bronchoalveolar space is considered a predictor of early mortality in IPF patients [Kinder et al., 2008]. Despite of both macrophages and neutrophils have been identified as pro-fibrotic cell types in mouse models of PF, eosinophils and mast cells are other innate myeloid cells having a profibrotic role [Wynn and Ramalingam, 2012].

In adaptive immune system, CD4 positive T-helper (Th)1 and Th2 cells play an important role during inflammatory/maintenance phase of pulmonary fibrosis. Indeed, cytokines produced by these two cell types have exhibited contrasting activity in fibrogenesis. For instance, interferon (IFN)-γ, a Th1 cytokine inhibit fibrosis, where as Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 are key drivers of progressive fibrosis [Wynn et al., 1995]. Although all three Th2 cytokines have been linked to the development of fibrosis, IL-13 has emerged as a dominant mediator of fibrosis by
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stimulating the production and activation of TGF-β [Lee et al., 2001] and directly provoke the synthetic and proliferative properties of fibroblasts, epithelial cells and smooth muscle cells [Kuperman et al., 2002].

1.9. Oxidative stress and lung fibrosis

Reactive oxygen species (ROS) generated from environmental toxins exposure or inflammatory/interstitial cells mediating fibrosis have various physiological roles, their persistence can cause cellular dysfunction and death which aggravate the disease condition [Rahman et al., 2006]. The term oxidative stress encompasses all the molecular, cellular and tissue abnormalities resulting from excessive ROS production and/or depleted antioxidant defences [Jones, 2006]. In fibrosis, unremitting activation of inflammatory cells lead to production of excessive ROS which have deleterious effects to the surrounding tissue. ROS, including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) play a key role in promoting fibrosis. Although lower levels of ROS are physiologic mediate various cellular responses including proliferation, migration, differentiation, and gene expression, excess amount of ROS are toxic and can promote fibrosis [Kliment et al., 2010]. The enzymatic systems contribute to ROS production include NADPH oxidase (NOX)s, which catalyse one or two electron reductions to form O$_2^-$ and H$_2$O$_2$ [Crestani et al., 2011]. Among isoforms of NOXs enzyme, NOX4 which is induced by TGF-β plays major role in the pathogenesis of pulmonary fibrosis [Crestani et al., 2011]. ROS are also generated from mitochondria of key target cells mediating PF, as the electron transport chain uncoupled from proton pumping results in generation of ROS into the cytosol [Galluzzi et al., 2012]. Oxidative stress by NOXs and mitochondria derived ROS stimulate inflammatory signalling by ‘Nod like receptor inflammasome’ which may be crucial in driving fibrosis [Dostert et al., 2008]. Lungs express a wide variety of antioxidants to protect against oxidative stress. In fibrotic condition depletion of antioxidant defences including catalase, superoxide dismutase (SOD), glutathione, as well as nuclear factor erythroid 2-related factor 2 can result in oxidative stress and augment the fibrosis progression. In conjunction with DNA damage and p53 activation, ROS promote apoptosis of airway epithelial cells and elicit the production of cytokines and growth factors that may be important for invasive myofibroblasts differentiation and collagen accumulation [Cheresh et al., 2013]. As the interactions between oxidative stress and TGF-β are important for promoting fibrosis [Liu et al., 2010], ROS increase the TGF-β induced fibrosis by activating the latent TGF-β to active form [Pociask et al.,
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2004]. Although ROS promote activation of TGF-β gene expression, TGF-β itself increases ROS production by decreasing antioxidant defences [Kayanoki et al., 1994].

1.10. Matrix metalloproteinases (MMPs) in pathogenesis of lung fibrosis

The ultimate feature of IPF is the progressive fibrosis of lungs, resulting from dysregulated extracellular matrix control, where MMPs are believed to play an important role. MMPs are a family of neutral proteinases, well known for their degradation and remodeling of ECM proteins. MMPs play a role in cell migration, cell-cell and cell-matrix adhesion and in release and activation of extracellular matrix-bound growth factors and cytokines; all of these functions have been shown to play a role in the initiation and/or the progression of lung fibrosis [Dancer et al., 2011]. Among MMPs, MMP-2 and MMP-9 are designated as 72-kDa gelatinase A and 92-kDa gelatinase B, respectively, play a significant role in fibrosis. They facilitate inflammatory and mesenchymal cell migration by their ability of degrading basement membrane. Studies showed that the activities of MMP-2 and MMP-9 are upregulated in many pulmonary diseases including PF [Fukuda et al., 1998; Suga et al., 2000] and they are secreted by many different kinds of cells in the lungs [Ferry et al., 1997; Lemjabbar et al., 1999].

MMP-2 and MMP-9 have been reported to be widely expressed in fibrotic lungs, especially, in the areas of hyperplastic epithelial cells covering intra-alveolar fibrosis, as well as by mesenchymal and endothelial cells in the fibroblast foci [Selman et al., 2000]. MMP-2 and MMP-9 have been found to be elevated in both humans and experimental lung fibrosis [Lemjabbar et al., 1999; Perez-Ramos et al., 1999; Ruiz et al., 2003] and degrade a wide range of matrix and non-matrix substrates, particularly type-IV collagen and other basement membrane proteins [Ruiz et al., 2003]. This may be important, because the structural integrity of the alveolar wall depends on the basement membrane. A discontinuity of the basement membrane potentially allows greater access for exudative factors and interstitial cells to the alveolar space, promoting further tissue destruction and progressive fibrosis [Mckeown et al., 2009].

1.11. Inflammatory mediators that regulate PF: Role of cytokines

Over the last decade, substantial evidence has been suggesting that important stimuli to collagen deposition in PF are polypeptide mediators known as cytokines [Mc
Anutly et al., 1995. Released by resident lung cells (macrophages, AECs and endothelial cells) and recruited inflammatory cells, cytokines stimulate fibroblast proliferation and increased synthesis of ECM proteins thereby, contributing to fibrogenesis. Numerous cytokines have been implicated in the pathogenesis of PF include the following.

**1.12. Tumour necrosis factor-α (TNF-α)**

TNF-α is produced by many cells including macrophages, T-cells, mast cells and epithelial cells, but the principal source is the macrophages. The secretion of TNF-α by monocytes/macrophages is greatly enhanced by other cytokines such as IL-1, granulocyte macrophage colony stimulating factor and IFN-γ. Indirectly, via TGF-β or PDGF induction pathways, TNF-α stimulates fibroblasts proliferation, differentiation and collagen transcription [Kapanci et al., 1995]. In addition, TNF-α promotes induction of matrix-degrading gelatinases that can enhance basement membrane disruption and can facilitate fibroblasts migration [Selman et al., 2001].

**1.13. IL-1β**

Like TNF-α, IL-1β can induce acute lung injury and may contribute to progression of PF [Kolb et al., 2001]. Interestingly, IL-1β induced fibrosis is associated with increased expression of TNF-α, suggesting that IL-1β and TNF-α triggered fibrosis might be mechanistically linked. The neutrophils attracting CXC chemokines CXCL1 and CXCL2 are increased by IL-1β, illustrating how acute lung injury initiated by pro-inflammatory cytokines and neutrophils [Klob et al., 2001; Lappalainen et al., 2005].

**1.14. IL-13**

IL-13 has emerged as a dominant mediator of fibrotic tissue remodeling in several experimental and natural models of fibrosis [Chiaramonte et al., 1999]. Mechanistically, IL-13 has been hypothesized to induce fibrosis by stimulating the production and activation of TGF-β [Lee et al., 2001]. However, other studies have suggested that IL-13 can promote fibrosis independently of TGF-β [Liu et al., 2011], directly stimulating the synthetic and proliferative properties of fibroblasts, epithelial cells and smooth-muscle cells [Lee et al., 2001].
1.15. TGF-β: Significant contributor to the fibrotic response

TGF-β is one of the most potent profibrogenic cytokine evident in not only in pathogenesis of PF but also in almost all fibrotic diseases. It plays a vital role in ECM deposition by regulating crucial cellular activities such as cell proliferation, differentiation, adhesion, migration of fibroblasts/myofibroblasts and epithelial mesenchymal transition (EMT). All the other pro-fibrotic cytokines including TNF-α, IL-1β, PDGF, endothelin-1 (ET-1), connective tissue growth factor (CTGF), insulin growth factor-I (IGF-1) directly or indirectly linked to the TGF-β signalling [Kelly et al, 2003; Hetzel et al., 2005; Ingram et al., 2006]. In mammals, TGF-β exists as three isoforms; TGF-β1, 2 and 3, only TGF-β1 or TGF-β is a key contributor of PF [Bartram et al., 2004; Leask et al., 2004]. In the lung, TGF-β is produced by inflammatory cell types including macrophages, epithelial cells and fibroblasts [Bartram et al., 2004]. It is initially synthesized as a large precursor protein, which must be proteolytically cleaved before secretion from the cells. The mature TGF-β protein is secreted as an inactive homodimer, attached to the latency activated peptide (LAP). However, this LAP protein is cleaved to release active TGF-β by proteins involved in wound healing such as thrombospondin-1, the integrin αvβ6, and plasmin [Bartram et al., 2004; Leask et al., 2004]. The activated TGF-β signals via Smad’s or mitogen activated protein kinase (MAPK) pathways. TGF-β binds to TGF-β receptor (TbR)-II on the surface of target cells, results in autophosphorylation of TbR-II, and recruitment of TbR-I resulted in phosphorylation of Smad2/3 [Bartram et al., 2004]. Once Smad2/3 is phosphorylated, Smad4 is recruited and the entire complex translocates to the nucleus. Within the nucleus, the Smad2/3/4 complex binds to TGF-β responsive elements of DNA. In order for the Smad2/3/4 complex to bind to DNA, co-activator proteins such as CBP/p300 are recruited, bound and acetylated allowing access to DNA lead to activation of genes for matrix synthesis and EMT [Janknecht et al., 1998] (Figure 4). Downstream of TGF-β, several proteins are required for the enhancement of a cellular response to TGF-β for prolongation of fibrotic response. These proteins either enhance the contractile phenotype of the fibroblast or prolong the production of ECM in fibroblasts. These proteins include CTGF and fibronectin. CTGF, a member of the CCN family of matricellular proteins [Moussad et al., 2000], promotes fibroblast proliferation, matrix production, and granulation tissue formation [Shi-wen et al., 2000].
The fibroblast present in the fibrotic lungs is a specialised, highly contractile collagen producing cell termed as ‘myofibroblast’. These are hybrid cells exhibiting a phenotype between fibroblasts and smooth muscle cells. They retain expression of fibroblast cell surface markers, but also express internal markers of smooth muscle cells including α-smooth muscle actin (α-SMA) and calponin. This cell has an ability to express high levels of ECM and fibrogenic cytokines and to contribute to the altered mechanical properties of affected tissues. Interestingly, the studies indicate that lung myofibroblasts may derive from several sources including resident interstitial fibroblasts [Phan et al., 2002], circulating fibrocytes [Philips et al., 2004], bone marrow-derived fibroblast precursors [Hashimoto et al., 2004], and lung epithelial cells via EMT [Kasai et al., 2005]. Endothelin-1 and TGF-β are the key cytokines responsible for myofibroblasts differentiation. For normal healing processes, wound myofibroblasts must undergo apoptosis [Desmouliere et al., 1995]. However, in IPF, failure of apoptosis leads to myofibroblasts accumulation, exuberant ECM production, persistent tissue contraction, and pathologic scar formation [Tomasek et al., 2002].

While a wide range of factors have been identified as key mediators in fibrosis, two of such important mediators with key roles suggested in pathogenesis of fibrosis are endothelin-1 and PDGF.

1.16. Endothelin-1

Endothelins (ETs) are a family of 21 amino acid peptides, consist of three distinct highly vasoactive isoforms (ET-1, ET-2 and ET-3). ET-1 is the most abundant isoform and has been best characterized in the pathogenesis of PF. The lung has the highest levels of ET-1 secreted by endothelium, smooth muscle, airway epithelium, and a variety of other cells [Fagan et al., 2001].

1.17. Endothelin receptors

Currently, two distinct human endothelin receptors are known, ET-A and ET-B receptors belong to seven transmembrane G-protein coupled rhodopsin superfamily [Lippton et al., 1993]. ET-A has a higher affinity for ET-1 and ET-2 than ET-3, but all three have equal affinity for ET-B receptors. ET-A receptors are predominantly expressed on mesenchymal cells (fibroblasts and smooth muscle cells) and mediate cell
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proliferation and vasoconstriction. Sometimes alone, but primarily in combination with ET-A receptors, ET-B receptors can also mediate multiple detrimental effects in disease states, including cellular hypertrophy, inflammation and fibrosis [Clozel et al., 2006]. ET-1 acts in a paracrine and autocrine manner to mediate its broad range of biological activities, which include chemotactic effect on inflammatory cells, such as monocytes, neutrophils, lymphocytes, differentiation of fibroblasts and myofibroblasts. Biological actions of ET-1 involve necessarily the activation of ET-A and/or ET-B receptors which are coupled to the G-proteins (G_q, G_11, G_S, G_i2) suggesting that endothelin receptors may simultaneously stimulate multiple effectors via several types of G-proteins. This complexity together with the fact that most cells can coexpress both receptors, make rather difficult to predict the mechanism/s involved in the specific actions of ET-1 on a particular cell type. Nevertheless, the experimental evidences indicate the involvement of mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and ERK in the activation process of AP-1 transcription factor by ET-1 and also the activation of matrix-associated genetic program characterized by the expression of collagen isoforms, as well as of contractile proteins involved in enhanced myofibroblasts contraction and migration [Shi-wen et al., 2001; Xu et al., 2004; Lagares et al., 2010; Ahmedat et al., 2013]. Upstream MAPK, signaling components of the cell adhesion machinery, focal adhesion kinase (FAK) and integrin-linked kinase (ILK) have been described to participate in ET-1-mediated profibrotic actions [Lagares et al., 2012a; Shafiei and Rockey, 2012]. The activation of rac/Rho and phosphatidylinositide 3-kinase (PI3K)/Akt has been also shown to participate in the profibrotic actions of ET-1 [Shi-Wen et al., 2004; Shafiei and Rockey, 2012] (Figure 5).

1.18. ET-1 and lung fibrosis

Elevated levels of ET-1 have been reported in bronchoalveolar lavage fluid (BALF) and serum from patients with IPF [Uguccioni et al., 1995], and increased expression of ET-1 has been detected in small pulmonary blood vessels and macrophages [Uguccioni et al., 1995]. In normal lungs, moderate expression of ET-1 localised to airway epithelium, pulmonary endothelium, and vascular smooth muscle cells, indicating a role in normal lung physiology. However, in patients with IPF there is a strong and extensive expression of ET-1 in airway epithelium, proliferating type-II pneumocytes, and in endothelial and inflammatory cells which correlates with the severity of disease [Saleh et al., 1997]. Pro-inflammatory cytokines like IL-1 and TNF-α increase ET-1
mRNA expression and peptide production in human normal bronchial epithelial cells [Saleh et al., 1997]. Further evidence for a role of ET-1 in pulmonary fibrosis comes from animal models. Mice transgenic for human pre-pro-ET-1 and transcriptional regulatory elements that overexpress ET-1 develop progressive pulmonary fibrosis with recruitment of predominantly CD4-positive inflammatory cells [Hocher et al., 2000]. While inflammatory cells appear to be primed for ET-1 production, augmented ET-1 release which, intrinsically, may contribute to perpetuation of the inflammatory process [Odoux et al., 1997].

Figure 4: Overview of TGF-β signaling pathway; activated TGF-β binds to the TBR-II which results in the recruitment and phosphorylation of TBR-I. Once this occurs several pathways are activated including MAPK and Smad2/3/4. Each of these pathways has been cited in the literature for their role in fibroblast to myofibroblast differentiation. Figure is author’s design.
Figure 5: Schematic diagram showing the signaling mechanism contributing to the profibrotic actions of ET-1 in fibrosis. ET-1 acting through G-protein coupled receptors activated downstream signaling pathways resulting in enhanced production of profibrotic proteins and ECM production, remodelling and contraction. This figure is author’s design.

Several studies have demonstrated that ET-1 contributes to the development of inflammatory processes by activation of transcription factors such as NF-kB and expression of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 [Yeager et al. 2012]. These transcription factors and pro-inflammatory cytokines, in turn, stimulate ET-1 production [Virdis and Schiffrin 2003]. ET-1 also increases the synthesis of TNF-α in macrophages and monocytes which augment the inflammatory response by stimulating the chemotaxis and phagocytosis of macrophages, monocytes and neutrophils [Bellisai et
ET-1 enhances the expression of adhesion molecules on vascular endothelial cells and stimulates the aggregation of polymorphonuclear neutrophils (PMNs) contributing to inflammation and endothelial dysfunction. These intracellular PMNs may contribute to tissue damage by releasing ROS, proteases and arachidonic acid metabolites [Hansen 1995]. ET-1 stimulates the production of ROS, primarily \( \text{O}_2^- \) and consequently leading to oxidative stress. Some studies have shown the oxidative stress caused by ET-1 to be associated with augmentation of lipid peroxidation and reduction of intracellular glutathione [Scalera et al., 2002]. As per literature, both ET-A [Elmarakby et al. 2005] and ET-B receptors [Dong et al., 2005] mediate ROS generation. Since elevated levels of ET-1 induce the synthesis of \( \text{O}_2^- \) through ET-A receptors [Callera et al., 2003], inhibition of ET-A receptor signalling by ET-A antagonist not only significantly reduces production of \( \text{O}_2^- \), lipid peroxidation products, but also increases the total levels of glutathione, activities of antioxidant enzymes such as SOD and catalase in ET-1 induced oxidative stress [Ozdemir et al., 2006; Briyal et al., 2011].

1.19. Role of ET-1 in myofibroblasts differentiation and ECM production

As discussed before, myofibroblasts are key effector cells responsible for synthesis and deposition of ECM proteins. ET-1 has been able to promote induction of myofibroblasts phenotype by stimulating the expression of \( \alpha^- \)SMA contractile protein in different cell types include resident fibroblasts [Nishida et al., 2007], bone marrow derived monocytes [Binai et al., 2012], epithelial/endothelial cells [Jain et al., 2007; Piera-Velazquez et al., 2011], vascular pericytes [Fligny and Duffield, 2013] or hepatic stellate cells [Zhan and Rockey, 2011]. In normal lung fibroblasts, ET-1 promotes synthesis of collagen type-I and -III, which can be inhibited by dual endothelin receptor antagonists but not by single ET-A receptor antagonists [Shi-Wen et al., 2001]. It suggests that the mechanism is dependent upon both ET-A and ET-B receptors and implies that cross-talk between the receptors and downstream through various signaling pathways is important for collagen production [Shi-Wen et al., 2001]. In addition to fibroblasts differentiation, ET-1 interacts with other profibrotic cytokines, including TGF-\( \beta \) to promote fibrosis. Animal studies revealed the existence of cytokine hierarchy and interplay between ET-1, TGF-\( \beta \) and CTGF, which involves induction of ET-1 by TGF-\( \beta \); and both TGF-\( \beta \) and ET-1 show effect on ECM through CTGF [Clozel et al., 2005]. Studies have demonstrated that TGF-\( \beta \) induces ET-1 expression via a Smad-independent, JNK-dependent mechanism [Rodríguez-Pascual et al., 2004]. TGF-\( \beta \)
induces ET-1 in normal and fibrotic lung fibroblasts in a Smad-independent ALK5 (TBR-I)/JNK/AP-1-dependent fashion, in addition, ET-1 itself found inducing JNK. Constitutive JNK activation displayed by fibrotic lung fibroblasts can be reduced by dual ET receptor inhibition, providing evidence of an autocrine ET loop [Shi-Wen et al., 2006]. This implies ET-1 and TGF-β are likely to cooperate in the pathogenesis of pulmonary fibrosis with increased JNK activation in lung fibroblasts contributing to the persistence of the myofibroblast phenotype in pulmonary fibrosis (Figure 6).

1.20. Endothelin receptor antagonists

Taken together, these findings provide persuasive evidence of ET-1 role in the pathophysiology of PF. ET-1 mediates a wide range of biological activities which may be involved in fibrosis such as, activation of inflammation, induction of oxidative stress, myofibroblasts differentiation and deposition and turnover of ECM. Owing to pathogenic importance of ET-1 signaling in fibrosis, endothelin receptor antagonists (ERAs) gained importance as therapeutic drug candidates for treatment of fibrosis. ERAs are a new and promising class of medicines, which block the ET-A and ET-B endothelin receptors with varying degrees of selectivity. They are pharmacologically distinguished on the basis of their various affinities for the ET-A and ET-B receptors, as determined by binding studies in cell culture. They form a large group consisting of nearly 40 or more compounds, and a part of them is currently under investigation as potential therapeutic agents in clinical trials [Hynynen and Khalil 2006]. Currently, three endothelin receptor antagonists have been approved for treatment for pulmonary hypertension such as bosentan, ambrisentan and macitentan which are already in the global pharmaceutical market [Motte et al. 2006].

Bosentan a dual endothelin receptor antagonist became the first ERA to be registered in USA, the European Union and other countries by Actelion pharmaceuticals as oral medicine for patients with pulmonary hypertension under trade name of “Tracleer” [Motte et al. 2006]. As both ET-A and ET-B receptors were involved in progressive fibrosis, dual ET receptor antagonist bosentan showed promising efficacy in attenuation of various fibrotic disorders, preclinically. For instance, in bleomycin-induced PF, collagen deposition was found to be reduced following treatment with bosentan, as measured by histology and tissue morphometry [Park et al., 1997].
**Figure 6:** Schematic representation of interplay between ET-1 and TGF-β signaling in promoting fibrosis. Reproduced and modified from Shi-wen et al., 2006.

**Figure 7:** Chemical structure of bosentan (4-(1,1-Dimethylethyl) -N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy) [2,2′-bipyrimidin]-4-yl]-benzenesulfonamide monohydrate). Adopted from Pub chem, NCBI.
Bosentan inhibited the right ventricular hypertrophy by decreasing the collagen expression in rats exposed to chronic hypoxia [Choudhary et al., 2011], in combination with valsartan, bosentan ameliorates renal interstitial fibrosis by mitigating ECM synthesis promoting factors [Zhang et al., 2005]. Bosentan reduced the skin fibrosis in patients with systemic sclerosis [Kuhn et al., 2010] and also reversed the profibrotic phenotype of systemic sclerosis dermal fibroblasts [Akamata et al., 2014]. Bosentan ameliorated lung emphysema in rats by decreasing the inflammation and oxidative stress [Chen et al., 2010]. It inhibited inflammation by reducing the leukocytes adhesion [Anthoni et al., 2006]. Administration of bosentan not only reduced pulmonary hypertension, but showed beneficial hemodynamic, myocardial and anti-inflammatory effects in monocrotaline induced pulmonary hypertension [Fontoura et al., 2014]. Bosentan enhanced antioxidant enzyme activities (SOD, Catalase) and reduced oxidative stress in experimental myocardial and ischemic reperfusion [Gupta et al., 2005]. These promising experimental evidences of antifibrotic activity of bosentan paved the way to clinical trials. The first international, prospective, double-blind, clinical study was the BUILD program (BUILD-1: Bosentan Use in Interstitial Lung Disease) designed to test the efficacy of this dual ET-A/ET-B receptor blocker to treat IPF patients [King et al., 2008]. Primary end-point of this study was exercise capacity, whereas secondary objectives were time to death or disease progression. Bosentan showed superiority over placebo neither in the exercise test (6 minute walk distance), nor in time to death or disease progression. Nevertheless, a significant improvement in the progression of the disease was observed in a patient subgroup with surgical lung biopsy-proven type IPF. This observation prompted the launching of a second clinical trial (BUILD-3) restricted to patients with a confident diagnosis of IPF as they were considered most likely to respond to treatment. However, no significant difference between treatment groups was observed in the time to disease worsening (the primary end-point) [King et al., 2011].

1.21. PDGF signaling

Concomitant with ET-1, PDGF is one of the potent profibrotic growth factors contributing to fibrogenesis of lungs and other organs. PDGF is a homo or heterodimeric molecule with molecular weight of 30 kDa namely PDGF-AA, -AB, -BB, -CC, and -DD [Herdin et al., 1999], which exhibits its biological activity through two types of PDGF receptors (PDGFRs), -α and -β. Once, PDGF ligands bind to the receptors, PDGFR monomers dimerize to homo (PDGFR-αα and -ββ) or hetero (PDGFR-αβ) dimers to
induce autophosphorylation leading to activation of downstream signaling cascades. The possible PDGF-PDGFR interactions are multiple, binding of PDGF-AA or -CC to PDGFR-α, and PDGF-BB to PDGFR-β [Andrae et al., 2008]. PDGF-A/PDGFR-α and PDGF-B/PDGFR-β interactions have different biological roles. In general, PDGF-B/PDGFR-β signaling appears to be prominent in vascular remodeling, both for normal homeostasis and pathologic conditions [Olson et al., 2011]. PDGF-A/PDGFR-α signaling appears to have a broader role in tissue homeostasis and repair, in particular, in the skin, lungs, gut and kidneys. Fibroblasts and fibroblast-like cells are both major sources and targets for PDGF-A, since they express PDGFR-α on their cell surface [Yamakage et al., 1992]. Thus, paracrine and autocrine PDGF-A/PDGFR-α signaling loops can stimulate fibroblasts to synthesize extracellular matrix and release pro-fibrotic mediators. Upon ligand binding, PDGFR dimerization and autophosphorylation activate downstream signaling molecules that include Src homology 2 and pleckstrin homology domains. PDGFRs, thereby, engage several well-characterized signaling pathways such as JAK/STAT, rat sarcoma mitogen activated protein kinase (Ras-MAPK), PI3K, phospholipase C-γ pathways and others, promoting gene expression and mediating the biological functions of PDGF isoforms e.g., proliferation, migration, and survival. PDGFR also interacts with integrins inducing cell migration through activation of focal adhesion kinase [Ostendorf et al., 2012] (Figure 8).

1.22. Role of PDGF signaling in PF

An increase in mesenchymal cell number is a typical feature of fibrotic lung lesions [Crouch, 1990]. Mesenchymal cells of the lung include fibroblasts, smooth muscle cells, and myofibroblasts. Myofibroblasts are the predominant mesenchymal cell phenotype present in fibrotic lung lesions responsible for not only in the synthesis, but also in maintenance of connective tissue through activation of appropriate enzymes [Goldstein et al., 1986]. PDGF is generally regarded as a potent mitogen and chemoattractant for myofibroblasts that drives the recruitment and replication of these cells at sites of tissue injury. In human and animal models of pulmonary fibrosis, PDGF expression correlates with the expansion of the myofibroblast population that ultimately contributes to the production of ECM proteins [Bonner, 2004]. As alveolar macrophages have been identified as major source of PDGF, macrophages of IPF patients’ exhibit enhanced expression of PDGF-A & -B [Martinet et al., 1987; Homma et al., 1995].
Animal models of lung injury have been invaluable in defining a role for PDGF in the progression of fibrosis. Following the intratracheal instillation of bleomycin in rats, PDGF like peptides of two distinct classes (38 and 29 kDa) were identified in alveolar fluid, which were identified by anti-PDGF-BB and anti-PDGF-AA antibodies respectively, showed the growth-promoting activity of lung fibroblasts [Walsh et al., 1993]. The macrophage derived PDGF was chemotactic for rat lung myofibroblasts, in which, PDGF-B isoform was more potent as myofibroblasts chemoattractant as compared to PDGF-A [Osornio et al., 1990 & 1995]. While the lung myofibroblast is a major source of PDGF-AA and its receptor (PDGFR-α), this autocrine activation could be significant in stimulating myofibroblasts to proliferate and deposit collagen [Lasky et al., 1995].

The mechanisms that mediate PDGF ligand expression are poorly understood. IFN-γ and IL-1β are two major cytokines increase the expression of PDGF in macrophages and fibroblasts respectively [Raines et al., 1989; Shaw et al., 1991; Kolb et al., 2001]. Several studies demonstrated that over expression of PDGF leads to fibrosis, for instance, several experimental strategies have been used to directly increase PDGF expression in the adult rodent or murine lung, including transgenic over-expression, adenoviral-induced expression, or administration of recombinant PDGF protein. Yoshida et al. (1995) induced interstitial pneumonia and fibrosis in rats through adenoviral over-expression of PDGF-B. Yi et al. (1996) administered a single or multiple intratracheal injections of recombinant PDGF-BB in rats and observed a transient proliferation of pulmonary mesenchymal and epithelial cells accompanied by collagen deposition. In addition to increased PDGF ligand expression after lung injury, the increased expression of PDGF receptors also appears to be an important component that contributes to the hyperplastic growth of pulmonary myofibroblasts. Induction of the PDGFR-α in rat lung myofibroblasts may be exerted directly by environmental factors such as asbestos and endotoxins [Bonner et al., 1993; Coin et al., 1996]. IL-1β is the most potent endogenous factor which stimulates the PDGFR-α expression via p38 MAP kinase dependent pathway in rat lung myofibroblasts in vitro, hence, PDGFR-α up-regulation enhances the mitogenic and chemotactic responses to PDGF isoforms [Lindroos et al., 1995]. Mesenchymal mitogens including IL-1, thrombin, TGF-β and fibroblast growth factor exert their growth promoting activity through induction of PDGF-dependent pathways [Lasky et al., 2000].
Several evidences described above suggest that PDGF/PDGFR signaling is capable of mediating the fibrosis and targeting this signaling pathway might lead to show the therapeutic effects against pulmonary fibrosis. It was reported that AG1296, the inhibitor for the tyrosine kinase of PDGFR, prevented pulmonary fibrosis induced by vanadium pentoxide (V2O5) in rats [Rice et al., 1999]. Among a variety of tyrosine kinase inhibitors, imatinib mesylate (previously called STI571, Gleevec in the United States and Glivec in Europe) marketed by Novartis company is a potent and specific tyrosine kinase inhibitor against PDGFR, c-abl, bcr-abl and c-kit used in treatment of multiple cancers, especially, in chronic myeloid leukemia and gastrointestinal tumors [Druker et al., 1996].

Figure 8: Schematic overview of PDGF signaling in pathogenesis of fibrosis. Adopted from Ostendorf et al., 2012.
Imatinib showed potent antifibrotic efficacy in various preclinical animal models of fibrosis. Imatinib strongly inhibited the fibrosis of lungs induced by bleomycin via inhibiting proliferation of mesenchymal cells in vivo in mice [Aono et al., 2005]. In addition to the blocking of PDGFR signaling, imatinib inhibits TGF-β activity via inhibiting c-abl kinases [Daniels et al., 2004]. Furthermore, imatinib exhibits antifibrotic effects in a variety of fibrotic disorders including radiation-induced lung fibrosis [Abdollahi et al., 2005], kidney fibrosis [Wang et al., 2005], skin fibrosis [Horton et al., 2013], skeletal muscle dystrophy [Huang et al., 2009] and cardiac fibrosis [Ma et al., 2012]. As imatinib inhibits various fibrotic disorders in animal models, it was thought to serve as a potent antifibrotic agent for humans. However, when the clinical trials were performed in IPF patients for 96-weeks, imatinib failed to effect any survival or lung function contrary to the prognosis noticed in the experimental animal models of PF [Daniels et al., 2010].

![Structure of Imatinib](image)

**Figure 9:** Structure of imatinib (4-((4-methylpiperazin-1-yl) methyl]-N-(4-methyl-3-([4-(pyridin-3-yl) pyrimidin-2-yl]amino)phenyl)benzamide). Adopted from Pub chem, NCBI.

Primarily, for exploring therapeutic potential of antifibrotic agents’, it requires a suitable and reliable animal model which has good clinical relevance. Bleomycin induced PF is one such routinely used in vivo animal model.
1.23. *In vivo* animal model for bleomycin induced PF

Currently, bleomycin induced murine model of PF is the best characterized animal model. Ironically, this routinely used animal model for pulmonary fibrosis and impaired lung function has emerged out as the most adverse effect following the use of bleomycin both in mice and humans. Bleomycin is an anticancer agent originally isolated from *Streptomyces verticillatus* [Umezawa et al., 1967] and it has been used to treat squamous cell carcinomas and skin tumors [Umezawa et al., 1974]. The major adverse effect of bleomycin in cancer therapy is dose-dependent pulmonary toxicity resulting in fibrosis [Muggia et al., 1983]. Bleomycin has been shown to induce lung injury and fibrosis in a wide variety of experimental animals including mice, rats, hamsters, rabbits, guinea pigs, dogs, and primates over a range of doses induced *via* intraperitoneal, intravenous, subcutaneous and intratracheal delivery [Muggia et al., 1983]. Intratracheal route of administration of bleomycin (generally 1.25-4 U/kg, depending on the source) has the advantage that a single injection of the drug produces lung injury and resultant fibrosis in rodents [Phan et al., 1980]. Intratracheal delivery of the drug to rodents results in direct damage to alveolar epithelial cells and this event is followed by the development of neutrophilic and lymphocytic pan-alveolitis within the first week [Janick-Buckner et al., 1989]. Subsequently, alveolar inflammatory cells are cleared, fibroblast proliferation is noted, and extracellular matrix is synthesized [Schrier et al., 1983]. The development of fibrosis in this model can be seen biochemically and histologically on day 14, maximal responses generally noticed around days 21-28. The fibrotic response to bleomycin in mice is strain dependent, as C57Bl/6J mice are more susceptible to bleomycin-induced fibrosis than are Balb/c mice [Schrier et al., 1983]. This, very likely reflects strain-dependent differences in the expression of the bleomycin inactivating enzyme, bleomycin hydrolase. In fact, lungs are particularly sensitive to bleomycin toxicity due to the low levels of this enzyme in lung tissue compared to other body tissues [Onuma et al., 1974]. Bleomycin is thought to induce lung injury *via* its ability to cause DNA strand breakage [Lown et al., 1977] and oxidant injury [Sausville et al., 1976]. The advantages of bleomycin model are it is well characterized and has clinical relevance.
1.24. Kidney fibrosis

Kidney fibrosis, particularly tubulointerstitial fibrosis is final manifestation of almost all progressive chronic kidney diseases (CKD) [Coresh et al., 2007] and it is also a reliable predictor of prognosis and major determinant for renal insufficiency. As estimated 13% of the adult population in the USA has some degree of CKD, and in considerable proportion of cases, eventually, progresses to end-stage renal failure, a devastating condition that requires lifelong dialysis and kidney transplantation. Numerous epidemiological studies indicate that the prevalence of patients with end-stage renal disease is increasing worldwide [Coresh et al., 2007; Sharma et al., 2010]. Hypertension and diabetes are the two major global risk factors for CKD [Haroun et al., 2003]. According to the International Diabetes Federation, India stands second only to China with respect to world diabetes with 61.3 million in 2011 and the CURES cohort suggested that every fifth person in India is hypertensive [Mohan et al., 2007]. These reports indicating there is an alarming threat of CKD in Indian population. Other known risk factors for CKD include exposure to heavy metals, agrochemicals, nephrotoxic substances, use of non steroidal anti-inflammatory drugs and infectious diseases [Soderland et al., 2010].

1.25. Pathogenesis of kidney fibrosis

Similar to PF, renal fibrogenesis is considered as an aberrant wound healing process that occurs after initial insults of various injuries [Liu et al., 2006]. Variety of cells in kidneys, including fibroblasts, tubular epithelial cells, pericytes, endothelial cells, vascular smooth muscle cells, mesangial cells and podocytes, as well as the infiltrated cells such as lymphocytes, macrophages and fibrocytes, are involved in the pathogenesis of renal fibrosis, which illustrated the enormous intricacy of this disease process [Boor et al., 2010]. Major cellular events in tubulointerstitial fibrosis include: infiltration of inflammatory cells, fibroblast activation and expansion from various sources, production and deposition of a large amount of ECM components. Injured tissue incites inflammation by chemotactic cytokines which provide directional signal for the infiltration of inflammatory cells to the injured sites [Chung et al., 2011]. Although inflammation is a fundamental process of host defence mechanisms, chronic inflammation is a major driving force in the development of fibrotic diseases [Nathan et al., 2010]. Renal fibrosis is almost always preceded by the infiltration of inflammatory
cells, including lymphocytes, monocytes/macrophages, dendritic cells and mast cells in which lymphocytes and macrophages play significant role for promoting fibrogenesis [Nathan et al., 2010]. These infiltrated inflammatory cells become activated produce ROS molecules which damage the tissues and induce the secretion of fibrogenic cytokines and growth factors including chemokines, interleukins, PDGF, ET-1 and TGF-β [Vielhauer et al., 2010]. This series of events builds up sustained profibrotic cytokine pressure in the inflamed microenvironment inevitably leads to priming of fibroblasts and tubular epithelial cells to undergo phenotypic activation or transition to myofibroblasts which produce large amounts of ECM components including fibronectin and type-I and type-III collagens [Meran et al., 2011]. At least five different sources, with diverse mechanisms, have been proposed as contributors to the myofibroblast pool in diseased kidneys. These include activation of interstitial fibroblasts, differentiation of pericytes and circulating fibrocytes, phenotypic conversion of tubular epithelial cells and endothelial cells [Barnes et al., 2011]. In kidney fibrosis also, MMP-2 and MMP-9 play an important role through induction of tubular cell EMT. It has been demonstrated that MMP-2 and MMP-9 can directly induce the entire course of renal tubular cell EMT in vitro [Cheng et al., 2003; Tan et al., 2010]. MMP-2 and MMP-9, which specifically cleave type IV collagen and laminin [Lenz et al., 2000], major constituents of tubular basement membrane, contribute to tubular cell EMT via the disruption of tubular cell membrane integrity. This process has been recognized as a complementary step required for complete induction of tubular cell EMT, where it enables the newly transformed mesenchymal cells to migrate and invade the interstitial space and contribute to the development of fibrosis through ECM proteins production [Liu et al., 2004]. A broad range of factors have been identified as key mediators in renal fibrosis, like in PF, ET-1 and PGDF are the major contributors for the progression of renal fibrosis.

1.26. Role of ET-1 in kidney fibrosis

ET-1 has been described to be an important factor in renal pathophysiology. The wide range of stimuli pertinent to CKD include vasoconstrictors (Angiotensin-II, vasopressin), pro-inflammatory cytokines (TNF-α, IL-1β), hypoxia, ROS, profibrotic cytokines (TGF-β, PDGF), hyperglycaemia, acidosis and thrombin up regulates ET-1 synthesis [Attina et al., 2005]. CKD involves glomerular sclerosis and interstitial fibrosis and they occur regardless of the nature of insult. Significant protienuria, a marker for CKD, has emerged as a powerful predictor of renal disease progression [Mallick et al.,
ET system has been implicated in these processes [Kohan, 1997]. In the remnant kidney model, renal ET-1 gene expression and urinary ET-1 excretion correlate with the degree of proteinuria and extent of renal damage [Orisio et al., 1993]. Also, transgenic animals in which renal ET pathways have been upregulated, show glomerulosclerosis and renal tubulointerstitial lesions independent of changes in blood pressure [Hocher et al., 1997]. Further, infusion of ET-1 into rats over two weeks increases the permeability of isolated glomeruli to albumin and this effect was blocked by ET-A receptor antagonism [Saleh et al., 2010]. It is reported that ET-1 influences different cell types in kidneys, for example, podocytes have been shown to express ET-1 and ET receptors and enhances protein permeability [Fligny et al., 2011]. Within the glomerulus, endothelial cells are probably the main source of ET-1, which exhibit enhanced production of ET-1 in chronic kidney diseases [Lehrke et al., 2001]. Mesangial cells also produce significant amounts of ET-1 and interestingly, they synthesize and deposit more ECM upon stimulation with ET-1 [Mishra et al., 2003] than epithelial cells. ET-1 likely contributes to the development of renal inflammation; endothelial-restricted ET-1 overexpressing mice develop evidence of vascular inflammation in the absence of hypertension [Amiri et al., 2008]. Since ET-1 also mediates renal inflammation induced by Angiotensin-II, which induces hypertension and a T-cell rich renal infiltration, treatment with an ET-A receptor antagonist reduces hypertension and number of T cells in the renal cortex. ET-1 exerts a number of pro-inflammatory effects by acting as a chemoattractant for PMNs [Cui et al., 2001], which is inhibited by blockade of the ET-A receptor. ET-1 can also activate the endothelium to increase leucocyte adhesion and transmigration both [Callera et al., 2004]. It also stimulates secretion of monocyte chemoattractant protein-1 from mesangial cells, which is chemotactic to macrophages [Ishizawa et al., 2004]. As a vasoconstrictor factor, it has been long appreciated that ET-1 contributes significantly to the control of blood pressure. Nevertheless, increasing experimental evidences indicate that the actions of ET-1 linking to renal disease are mostly independent of hypertensive effects [Dhaun et al., 2012]. In the particular case of fibrosis, overexpression of ET-1 in transgenic mice has been reported to promote collagen deposition with subsequent loss of kidney function, an action not involving hemodynamical effects [Hocher et al., 1997]. Indeed, ET receptor antagonists have been shown to have beneficial effects in several models of renal disease, including hypertensive or diabetic nephropathy, and glomerulonephritis, with significant reduction in the accumulation of matrix components [Dhaun et al., 2012].
Introduction

As already mentioned, bosentan is a dual receptor antagonist showed antifibrotic efficacy in various fibroses animal models including kidney fibrosis. Bosentan ameliorates renal vascular fibrosis in mice by normalizing the collagen-I gene expression [Boffa et al., 2001] and also it showed nephroprotective activity in diabetic rats by reducing collagen-I, fibronectin, TGF-β and urinary protein excretion without reducing the blood pressure [Cosenzi et al., 2003]. Bosentan normalizes the blood pressure and reduces the deposition of ECM protiens in kidney and heart of rats with hyperinsulinemia and hypertension induced by high fructose-diet [Cosenzi et al., 2003]. A study showed that bosentan in combination with valsartan ameliorates the renal interstitial fibrosis in rats induced by aristolochic acid [Zhang et al., 2005].

1.27. PDGF and kidney fibrosis

Most of the progressive renal fibroproliferative diseases are characterized by glomerular mesangial cell proliferation and matrix accumulation [Slomowitz et al., 1988]. Renal mesangial cell growth is driven to a large extent by PDGF. The expression of PDGF ligands and PDGF receptors are low in the normal adult kidney, but are increased during renal development and during the progression of renal fibrogenesis [Abboud et al., 1995]. Despite of all four PDGF ligands identified thus far play an important role in the pathogenesis of renal fibrosis, PDGF-B likely plays a central role in mediating glomerular mesangial cell proliferation. As PDGFR-β is constitutively expressed in mesangial cells, these cells are primarily responsive to PDGF-B [Floege et al., 1998]. Hence, PDGF-B has acted as a potent mitogen and chemoattractant for renal mesangial cells and also stimulates extracellular matrix production, both of which are key events in glomerulosclerosis [Floege et al., 1993]. Administration of PDGF-BB, but not PDGF-AA, induced renal tubulointerstitial cell proliferation and fibrosis in rodents [Tang et al., 1996]. These studies demonstrated that administration of PDGF-BB alone is sufficient to cause a fibrotic response in rodent models of glomerulonephritis. The effect of PDGF-BB to increase the expression of collagen may be due to its ability to up-regulate TGF-β, which is a potent inducer of collagen production [Yamabe et al., 2000]. Other experimental strategies have also been employed to assess the importance of PDGF-BB and PDGFR-β in renal fibrosis. Antagonism of PDGF-B with neutralizing antibodies reduced mesangial cell proliferation and matrix accumulation in a rat model of mesangioproliferative nephritis [Johnson et al., 1992]. While PDGF-BB is exceedingly important in the development of renal fibrosis, other isoforms PDGF-C & -D are also
expressed during fibro-proliferative kidney disease [Eitner et al., 2003; Ostendorf et al., 2003].

As PDGFR has been contributing to renal fibrosis, imatinib (tyrosine kinase inhibitor) exhibit potent efficacy in amelioration of renal fibrosis in a variety of animal models. Imatinib reduced mesangioproliferative changes in experimental glomerulonephritis [Gilbert et al., 2001]; mildly ameliorated both renal functional and structural parameters in diabetic apolipoprotein E knockout mice [Lassila et al., 2005]; improved survival, renal function, and histology in murine lupus [Zoja et al., 2006]; and prevented chronic allograft nephropathy after rat kidney transplantation [Savikko et al., 2003]. These findings suggested that both ET-1 and PDGF signaling mechanisms extensively contribute to the progression of renal fibrosis, while the inhibitors of these signaling pathways acquired therapeutic significance in treatment of renal fibrosis. Efficacy of these therapeutic agents is determined using appropriate reliable in vivo animal models. Unilateral ureteral obstruction (UUO) induced kidney fibrosis is one such model which has potential clinical relevance.

1.28. In vivo animal model of UUO induced kidney fibrosis

UUO induced kidney fibrosis model is a suitable animal model to examine the mechanism and therapeutic interventions of tubulointerstitial fibrosis in vivo. This model can be induced in either rats or mice and shows no specific strain dependence. Complete UUO initiates a rapid sequence of events in the obstructed kidney, reduced renal blood flow and glomerular filtration rate within 24 h. The subsequent responses include interstitial inflammation (peak at day seven), fibrotic end stage by around two weeks [Chevalier et al., 2009]. Major pathways leading to the development of renal interstitial fibrosis are interstitial infiltration by macrophages, tubular cell death by apoptosis and necrosis and phenotypic transition of resident renal cells. The advantages of the complete obstruction model are good reproducibility, short time-course, easy performance and the presence of the contralateral kidney as a control.
1.29. Rationale for combination treatment in lung and kidney fibroses models

The most accumulating evidence illustrates that fibroses may have heterogenous etiology and highly intricate pathogenesis with a variety of signaling pathways or mediators involved in the progression of the disease. This may be one of the major challenges in exploring the therapeutic measures for fibrosis. Despite intensive research efforts, the etiology and pathogenesis of fibrosis have remained enigmatic and hence, a clinically endorsed drug/cure has been eluding for the dreadful disease. May be due to this reason, till today, there is no FDA approved drugs are available to treat the fibrotic diseases. Conventional treatments for managing PF are primarily based on the concept that suppression of inflammation would prevent progression of fibrosis. Although glucocorticoids with or without immunosuppressive drugs were the mainstay of therapy for IPF for more than four decades, their efficacies are unproven and toxicities are substantial [Selman, 2001]. If these treatments fail, lung transplantation is the only option but it is an expensive treatment and chances of survival are less. In the case of renal fibrosis, frequent dialysis, controlling blood pressure and diabetes are managing options for disease progression. Transplantation of kidneys, another way of treatment like in IPF, but outcome from this treatment is meager. The reason for patients’ failure to respond to anti-inflammatory therapy may be the organ is in a stage of fibrosis, rather than active inflammation at the time of diagnosis. Thus, anti-fibrotic drugs that interfere with or modulate both the processes of inflammation and fibrosis stages may have potency to retard progression of the fibrosis and improve organ function. Though both bosentan and imatinib are such drugs, monotherapy with these drugs have failed in clinical trails of IPF.

Though it is disappointing that the efficacy of bosentan and imatinib found in animal models could not be observed in clinical trials of IPF patients, the situation leaves the scope for redesigning the studies. Apart from ET-1 and PDGFR related tyrosine kinase pathways, other potential peripheral blood biomarkers are also reported associated with IPF including KL-6, surfactant proteins A and D, MMP-1 and -8, CCL-18, VEGF, YKL-40, osteopontin, circulating fibrocytes, and T cells [Vij et al., 2012]. Though it requires exploration and validation of these biomarkers for their role in IPF, most of the accumulating evidence indicates that fibrosis may have heterogeneity in the etiology with diversified signaling pathways [Vij et al., 2012]. If such were the situation with IPF, it may be possible that the failure of drugs: bosentan and imatinib in clinical trials might be
due to the fact that they were used in monotherapy, and had they been used in combination, perhaps, what would have been the result. Hence, in the backdrop of the evidence cited above, we hypothesize a drug combination might serve better for therapy of IPF than a single drug. Hence, to test this hypothesis, we proposed that simultaneous blocking of both ET-1 and PDGF signaling pathways by bosentan and imatinib combination might reduce PF more effectively than one alone. This combination efficacy was tested using bleomycin induced PF in vivo animal model and this study was extended to determine whether this combination treatment showed better efficacy than one drug alone in kidney fibrosis using UUO induced mouse model of kidney fibrosis.

1.30. Objectives

In view of the above information, present investigation has been undertaken with the following objectives.

Studying combination efficacy of bosentan with imatinib in the mice models of

1. Bleomycin induced pulmonary fibrosis and

2. UUO induced kidney fibrosis

with regards to the evaluation of

❖ The status of inflammation by,

  ✓ Inflammatory cell analysis
  ✓ Lung wet/dry weight ratio (only in PF model)
  ✓ Myleoperoxidase assay

❖ The status of oxidative stress by

  ✓ Superoxide dismutase assay
  ✓ Catalase assay

❖ The Status of fibrosis analysis by

  ✓ Hydroxyproline assay
  ✓ Gene expression of α-SMA, collagen-I and III genes
  ✓ Protein expression of α-SMA
  ✓ Histolopathological deposition of collagen in lung and kidney tissues
  ✓ Biochemical evaluation of inflammatory and fibrogenic cell migration

❖ Functional biochemical parameters (only in kidney fibrosis)

  ✓ Blood urea nitrogen
  ✓ Serum creatinine
  ✓ Proteinuria