REVIEW

The pathogenesis of pulmonary fibrosis: a moving target

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ABSTRACT: Pulmonary fibrosis is the end stage of many diffuse parenchymal lung diseases. It is characterised by excessive matrix formation leading to destruction of the normal lung architecture and finally death. Despite an exponential increase in our understanding of potentially important mediators and mechanisms, the delineation of primary pathways has proven to be elusive.

In this review susceptibility and injurious agents, such as viruses and gastro-oesophageal reflux, and their probable role in initiating disease will be discussed. Further topics that are elaborated are candidate ancillary pathways, including immune mechanisms, oxidative and endoplasmic reticulum stress, activation of the coagulation cascade and the potential role of stem cells. This review will try to provide the reader with an integrated view on the current knowledge and attempts to provide a road map for future research.

It is important to explore robust models of overall pathogenesis, reconciling a large number of clinical and scientific observations. We believe that the integration of current data into a "big picture" overview of fibrogenesis is essential for the development of effective antifibrotic strategies. The latter will probably consist of a combination of agents targeting a number of key pathways.

Pulmonary fibrosis is the end stage of several diffuse parenchymal lung diseases (DPLDs), characterised by excessive matrix deposition and destruction of the lung architecture, finally leading to respiratory insufficiency. The most common form of pulmonary fibrosis, idiopathic pulmonary fibrosis (IPF), is a progressive disease with a 5-year survival rate of only 20%, reflecting the lack of effective therapies. In the UK, >3000 people die each year from IPF and the incidence is increasing annually by 11% [1]. The aetiology of IPF remains poorly understood, although several risk factors and predisposing factors have been proposed, including cigarette smoking, viral infections and surfactant protein polymorphisms.

Histologically, IPF lungs have alternating regions of normal lung parenchyma, interstitial inflammation, fibrosis and honeycombing. The pathophysiological basis of IPF has been the subject of much debate over the last few decades. There is now growing evidence that IPF may represent a separate disorder in which fibrogenesis results, at least in part, from multi-focal epithelial micro-injury. Repeated injury to the alveolar epithelial cell (AEC) leads to apoptosis, which might lead to disordered epithelial–fibroblastic interactions and aberrant repair processes, finally resulting in fibrosis [2].

In this review, we focus on factors that make individuals susceptible to the process of progressive fibrosis, possible agents involved in repeated injury and important elements leading to aberrant repair and pulmonary fibrosis. In addition, emerging new findings will be discussed such as immunological processes, oxidative stress, endoplasmic reticulum (ER) stress, activation of the coagulation cascade and possibly alterations in the lymphatic vessels, and finally the role of stem cells.

INITIATION OF FIBROSIS: PREDISPOSITION AND CAUSAL AGENTS

Agents responsible for the initiation of the fibrotic process remain poorly defined. It is generally accepted that repeated injury is caused

by an interaction between genetic predisposition and injurious environmental agents.

Genetic predisposition

Although most DPLDs are sporadic diseases, examples of familial occurrence exist. Familial clustering of adult idiopathic interstitial pneumonias (IIP) suggests that genetic factors might play an important role in disease development. Interesting new findings include the characterisation of the ELMOD2 gene and the role of surfactant protein (SP)-C. Interestingly, genetic alterations have also been noted in sporadic IPF. ELMOD2 mRNA expression is significantly decreased in IPF lungs in comparison with healthy control subjects. ELMOD2 has been shown to be implicated in the regulation of interferon-related antiviral responses [3].

SP-C mutations have been frequently identified in children with severe idiopathic pneumonias [4]. Interestingly, these have been identified in 50% of these children with the other 50% having sporadic disease. In both children and adults, SP-C mutations are associated with the clinical entities of nonspecific interstitial pneumonia (NSIP), desquamative interstitial pneumonia (DIP) and pulmonary alveolar proteinosis. In a recent paper, VAN MOORSEL et al. [5] showed that five out of 20 unrelated patients with familial pulmonary fibrosis had a mutation in SP-C, with this particular mutation not found in sporadic disease.

The expression of mutations, such as SP-A and SP-C, in the alveolar epithelium and the identification of mutations affecting cell stability, such as telomerase (hTERT and hTER) mutations, leads to abnormal homeostasis of the alveolar epithelium. Mutations in SP-C and SP-A lead to dysfunction in alveolar Type-II (ATII) cells; probably as a result of misfolded proteins, ER retention and activation of unfolded protein responses [6]. A recent study has also shown that genetic abnormalities involving the airways might promote pulmonary fibrosis, as recently described for MUC5 [7]. A common variant in the putative promoter of MUC5B has been identified that is associated with the development of familial interstitial pneumonia and IPF. Subjects with IPF had significantly higher expression of MUC5B in the lungs than control subjects and MUC5B was expressed in the histological lesions of IPF. However, the exact link between MUC5B hypersecretion in the airways and the presence of disturbed alveolar epithelial homeostasis in pulmonary fibrosis is not yet clear.

Injurious agents

It is currently believed that that IPF results from the association of a genetic predisposition to abnormal epithelial cell regulation with environmental triggers, leading to the formulation of the ''multiple hit hypothesis''. In fibrotic diseases with known aetiology, fibrosis is driven by persisting injury that can be mediated by immune complexes, inhaled agents such as asbestos or direct toxic injury such as radiation or drugs. In IIPs, many potential causative agents have been proposed; the best known environmental factors are viruses and gastro-oesophageal reflux (GOR).

Viruses

Viral infections have been postulated as initiators of fibrosis but are extremely difficult to study due to the variable sensitivity of respiratory sample cultures. With the development of PCR and other molecular detection methods, knowledge in this field has dramatically increased. Viruses implicated in the pathogenesis of IPF include Epstein–Barr virus (EBV), human herpes viruses 7 and 8, cytomegalovirus, hepatitis C virus, herpes simplex virus, parvovirus B19 and torque teno virus. Another issue here is whether the presence of EBV and other viruses might merely reflect alveolar epithelial damage with an increased vulnerability to infection, although there is increasing evidence for a causal role of viruses in the initiation of fibrosis.

EBV has been the infection most widely identified in IPF, providing a potential source of repetitive cell injury, a central component of the current pathogenetic hypothesis [8]. TANG et al. [9] reported the presence of EBV, detected by real time-PCR, in five (62.5%) out of eight familial cases and in 16 (64%) out of 25 sporadic cases of IPF. Although EBV usually infects the upper respiratory tract, it has also been shown to infect and replicate in the lower respiratory tract [10]. Both EBV protein and DNA expression have been identified in the lung tissue of IPF patients [11, 12]. In IPF biopsy samples, EBV gp340/220 and viral proteins, expressed during the lytic phase of EBV infection, have been localised to AEC [11]. The putative role of EBV in the development of IPF is supported by other observations. A poor prognosis in IPF patients has been associated with the expression of EBV latent membrane protein-1 in AEC [13] and with the replicative phase EBV infection. Clinical stability in two IPF patients following oral antiviral therapy has been reported [9]. Transforming growth factor (TGF)-β1, a key pro-fibrotic cytokine implicated in IPF, is a potent growth inhibitor in most cells of epithelial origin [14]. EBV-infected cells exposed to TGF-β1 show viral lytic phase activation and resistance to cell growth inhibition [15]. It has also been shown that EBV–AEC interactions might be involved in producing fibrotic scars, the pathological hallmarks of IPF. Moreover it is possible that bioactivity of EBV might induce epithelial mesenchymal transition (EMT).

Both genetic factors and virus–host interactions might be linked as demonstrated for the ELMOD2 gene. ELMOD2 is expressed by lung epithelial cells and alveolar macrophages. It regulates interferon-related antiviral responses and its expression is decreased in response to viral infection [3]. Interestingly epithelial cells and alveolar macrophages are the fundamental cell types infected by respiratory viruses. These findings strengthen the evidence for a strong correlation between virus–host interactions and genetic susceptibility factors.

Gastro-oesophageal reflux

From the 1970s onwards, an association between both GOR and hiatal herniation and DPLD has been recognised [16]. In IPF, the prevalence of GOR is estimated to be up to 90% [17]. Emerging data point to the potential role of chronic micro-aspiration due to the subclinical aspiration of small droplets, for which GOR is a risk factor. GOR and silent micro-aspiration are associated with several lung diseases, including post-transplantation rejection [18]. Another interesting study by TCHERAKIAN et al. [19] has provided evidence for a role of GOR in the pathogenesis of both IPF and acute exacerbations. These investigators studied a group of patients presenting with asymmetrical IPF. They found that GOR was present in 20

(62.5%) patients with asymmetrical IPF and in more symmetrical cases this was only present in 31.3% of patients. Patients with asymmetrical IPF were also reported to have much more acute exacerbations (46.9%) than symmetrical cases (17.2%). The authors conclude that asymmetry might highlight the role for an aetiological factor such as GOR or specific regional conditions in the development and/or progression of pulmonary fibrosis and acute exacerbation.

Other recent studies have reported on the association between acute exacerbations of IPF and GOR. Acute exacerbations are characterised by a histopathological pattern of diffuse alveolar damage superimposed on the underlying pattern of usual interstitial pneumonia (UIP). Pepsin is thought to play an important role in acute exacerbations as it has been found in the bronchoalveolar lavage (BAL) fluid of patients with acute exacerbations [20]. It was even found that elevated BAL pepsin was predictive of acute exacerbation status, mainly driven by the presence of a subgroup of cases (33%) with markedly elevated pepsin levels.

Despite the compelling data supporting a correlation between GOR and IPF, this might alternatively be explained by the fact that GOR more often occurs in fibrotic lungs, or even results from the fibrotic changes in IPF, although this seems less likely at present.

PROGRESSION OF FIBROSIS

Key cell types

Currently, pulmonary fibrosis is regarded as a disease caused by repeated subclinical injury leading to epithelial damage and subsequent destruction of the alveolar-capillary basement membrane. This process initiates the infiltration of fibrotic cells and the activation of (myo)fibroblasts. In pulmonary fibrosis the normal resolution of inflammatory and mesenchymal cells through apoptosis and phagocytosis is dysregulated. This results in the destruction of the normal lung architecture and loss of function. In IPF this process leads to death with a median time of 3 years after diagnosis. These processes are summarised in figure 1.

Alveolar epithelial cells
Current in vitro and *in vivo* evidence suggests that AEC injury is a key process in the pathogenesis of IPF. An important observation in tissue of patients with IPF, first reported by KATZENSTEIN *et al.* [8], is hyperplasia of ATII and denudation of the alveolar epithelium within fibroblastic foci [21]. Moreover, as discussed earlier, mutations in genes that affect the regenerative capacity or that cause injury/apoptosis of ATII cells have been identified in familial forms of pulmonary fibrosis. [22]. In this process injury of the ATII is crucial, as it has been shown that in the lung of IPF patients 70–80% of the ATII stained positive for markers of apoptosis [23]. As shown very recently, targeted depletion of ATII in a mouse model of pulmonary fibrosis specifically links injury of ATII to the development of lung fibrosis [24, 25]. Enhanced fibroblast differentiation and collagen production has been shown in epithelial cell/fibroblast co-cultures in the presence of injury to the epithelial cell component [24]. Moreover mesenchymal cell survival is enhanced due to increased responsiveness to growth factors and resistance to apoptosis.

As classically described, injuries to ATII result in ineffective reconstitution of a normal epithelium and drive fibrosis by inducing the resident interstitial fibroblasts to differentiate into myofibroblasts [25, 26]. These are the key effector cells in pulmonary fibrosis and are characterised by the *de novo* expression of α-smooth muscle actin, which is organised into functional stress fibres and confers contractile properties [27]. They localise to fibrotic foci and other sites of active fibrosis, and are responsible for the synthesis and deposition of extracellular matrix (ECM) and the resultant structural remodelling that leads to the loss of alveolar function. Current opinion suggests that myofibroblasts have at least three possible origins, although the relative contribution of each of these pathways to IPF is currently unknown. The most straightforward suggestion is that resident lung fibroblasts differentiate directly under the influence of the profibrotic microenvironment to form myofibroblasts. The second possibility is that epithelial cells undergo transdifferentiation to form fibroblasts/myofibroblasts by EMT. Epithelial cells lose their characteristic markers such as E-cadherin and zona occludens-1 and acquire mesenchymal markers, such as fibroblast-specific protein-1 and α-smooth muscle actin [28]. The third hypothesis suggests that myofibroblasts may be derived from circulating fibrocytes (collagen I+/CD34+/CD45RO+) or other bone marrow-derived progenitor cells [29]. Attenuation of fibrocyte trafficking in mouse models directly correlates with a reduction in pulmonary fibrosis. The number of circulating fibrocytes has been recently shown to correlate with disease activity and progression [30]. Enhanced myofibroblast transformation and proliferation along with retarded apoptosis of mesenchymal cells are a major element in the relentless progression of fibrosis [31]. Furthermore, sustained matrix deposition or lack of matrix degradation further contributes to the destruction and replacement of normal alveolar tissue with dense fibrotic lesions.

Key cytokines

Recruitment of fibroblasts, fibroblast proliferation and overproduction of ECM is driven by a complex network of inflammatory cytokines, chemokines and cell types. Key cytokines and chemokines that induce a profibrotic milieu include tumour necrosis factor (TNF)-α, TGF-β, monocyte chemotactic protein (MCP)1/CCL2, macrophage inhibitory protein (MIP)1α/CCL3 and T-helper (Th)2-chemokines such as CCL17 CCL18 and CCL22 [32, 33]. TNF-α plays a central role in the stimulation of inflammatory responses, cell–cell adhesion and trans-endothelial migration, as well as in the early events leading to the cytokine and chemokine production cascade [34]. TNF-α-transgenic mice develop a T-cell mediated alveolitis and subsequent fibrosis, while TNF-α knockout mice fail to develop fibrosis after treatment with bleomycin [35]. Despite these encouraging findings a study performed with etanercept in IPF patients showed no significant differences in efficacy end-points after treatment for 48 weeks [36].

TGF-β is the most potent profibrotic mediator characterised to date and modulates lung fibrosis through the recruitment and activation of monocytes and fibroblasts, and the induction of ECM production. Lung fibroblast proliferation is an indirect effect of TGF-β1 *via* the induction of fibroblast growth factor-2
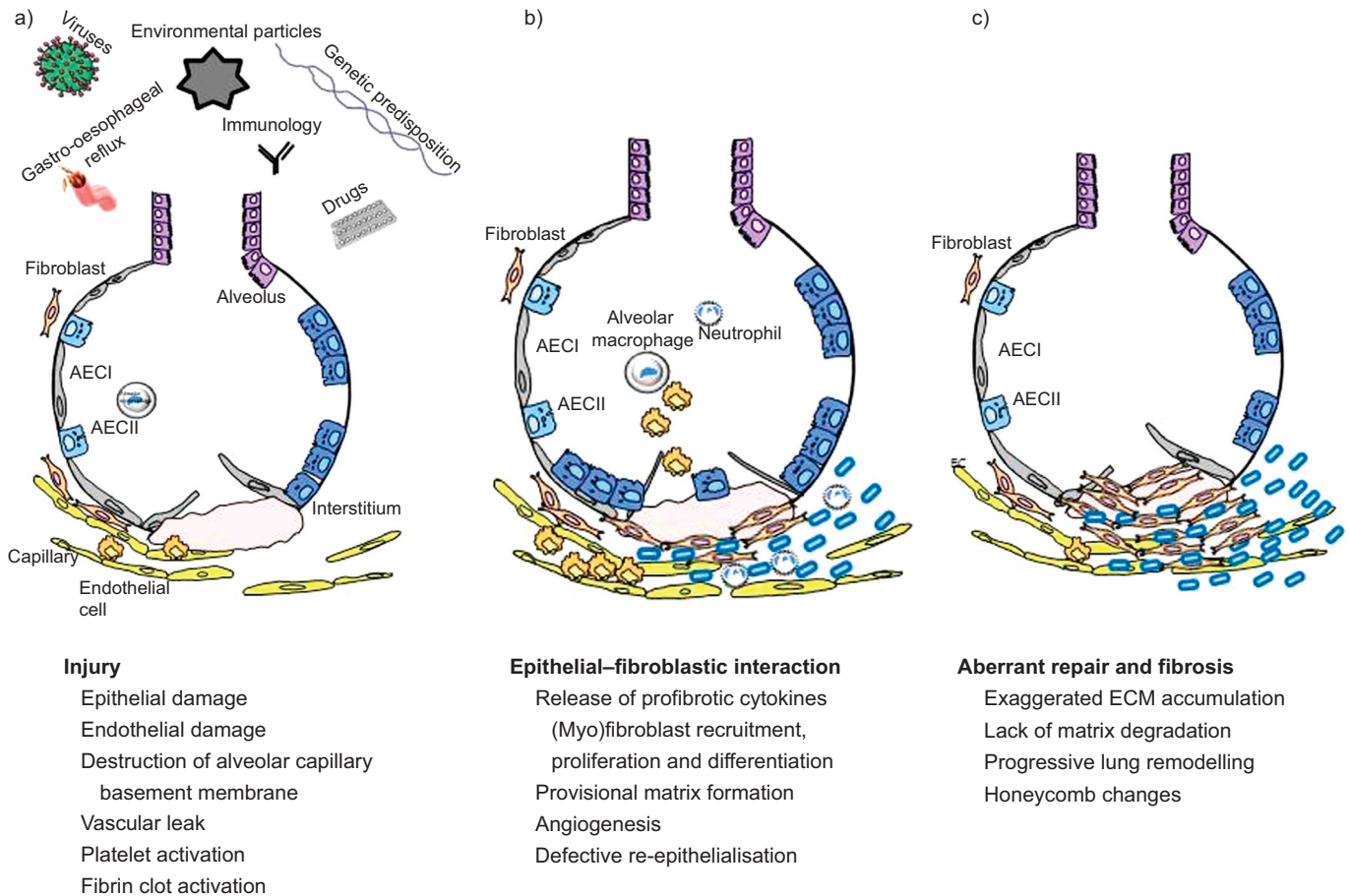
FIGURE 1. The major elements involved in induction and progression of fibrosis. a) The onset of fibrosis is characterised by both injury and susceptibility to the formation of progressive fibrosis. Many different injurious agents have been identified that lead to epithelial and endothelial damage, vascular leak and fibrin clot formation. b) This is followed by an abnormal repair process characterised by an abnormal re-epithelialisation, abundance of myofibroblasts and the formation of a collagen matrix. c) The process proceeds to excessive matrix formation leading to architectural distortion and finally death. ECM: extracellular matrix; AEC: alveolar epithelial cell.

and consequent activation of the mitogen-activated protein kinase (MAPK) pathway [37]. Furthermore, TGF-β induces fibroblasts to differentiate into myofibroblasts. TGF-β promotes ECM production by promoting ECM gene transcription, including collagen, fibronectin and proteoglycans and by suppressing the activity of matrix metalloproteinases, plasminogen activators and elastases, which results in the inhibition of collagen degradation [38].

MCP1/CCL2 and MIP1α/CCL3 are pro-inflammatory chemokines responsible for monocyte recruitment. CCL2 and CCL3 are significantly upregulated both in BAL fluid from patients with IPF and in fibrotic lungs of mice treated with bleomycin [39]. MCP1 receptor CCR2-deficient mice are protected from bleomycin-induced pulmonary fibrosis due to an impairment in macrophage recruitment and function [40], fibrogenic cytokine expression and fibroblast responsiveness to TGF-β [41].

Genomics is another field that has greatly contributed to our knowledge of fibrogenesis in recent years. Gene expression studies using lung tissue from patients with interstitial pneumonia have revealed that the molecular phenotype of IPF/UIP differs from that of NSIP, with the latter more closely resembling hypersensitivity pneumonitis [42]. Many genes

significantly increased in fibrotic lungs encode proteins associated with ECM formation and degradation and proteins expressed by smooth muscle cells, including matrilysin, matrix metalloproteinase (MMP)7 [43], which is a metalloproteinase mediator of pulmonary fibrosis. It has become clear that MMPs exert pleiotropic effects, including proteolytic degradation of the ECM and in the processing of chemokines, cytokines and growth factors [44]. It might even be possible that enhanced MMP activity is, therefore, a mechanistic driver of progressive fibrosis in IPF. The molecular expression signature distinguishing progressive IPF from relatively stable IPF includes members of the MAPK-early growth response 1-heat shock protein 70 pathway that regulate smoke-induced inflammation. However, molecular and cellular functions, such as cell proliferation, migration, invasion and cell morphology, are the most over-represented in IPF and in tissue from patients with acute exacerbations of IPF [45], with gene expression analysis identifying epithelial injury and proliferation as key molecular events [46]. Collectively, these approaches indicate that in IPF lungs, genes and mechanisms that are associated with lung development (including the Wnt/β-catenin pathway, epithelial cell plasticity and EMT) are activated, with a limited role for classical inflammation.

EMERGING NEW FINDINGS

Immunological mechanisms

The inter-relationships between inflammation and fibrosis may be considered from various perspectives. Historical data in IPF suggested that an increased percentage of lymphocytes in BAL was associated with greater responsiveness to corticosteroid therapy and better survival [47]. BAL lymphocytosis was found to be associated with moderate-to-severe alveolar septal inflammation and a relative lack of honeycombing [48]. Later, with the emergence of NSIP as a distinct entity [49], it was suspected that many patients with a BAL lymphocytosis, formerly diagnosed with IPF, did in fact have NSIP. This is supported by the fact that three major East Asian studies showed truly striking lymphocyte levels in NSIP [50–52]. It is suggested from high-resolution computed tomography scan observations that those patients might have organising pneumonia/NSIP overlap.

It is now clear that the presence of a BAL lymphocytosis does not reliably discriminate between NSIP and UIP in individual patients [53], but there are data showing that an elevated lymphocyte count in BAL can help to differentiate between IPF and other DPLD. In this regard it is important to mention the study of OHSHIMO et al. [54] who reported that a cut-off level of 30% for lymphocytes in BAL demonstrated a favourable discriminative power for the diagnosis of IPF. Six (8%) of the 74 patients with IPF based on clinical data and imaging showed a lymphocytosis of ≥30% in BAL. Their final diagnoses were idiopathic NSIP (n=3) and hypersensitivity pneumonitis (n=3).

Interstitial pneumonias other than IPF

Several studies of the reclassification of patients previously diagnosed with IPF, using current criteria, have demonstrated the impact of the histopathological pattern on long-term survival in patients with IIP. Idiopathic NSIP, DIP and cryptogenic organising pneumonia (COP) have a much better outcome than IPF [55, 56]. As COP responds dramatically to oral corticosteroids and the 5-year survival in NSIP is ~80% [57]. It can be concluded that inflammation, when indicative of COP, is clearly associated with a better reversibility and survival. COP is not considered as a fibrotic disease, although currently classified as an IIP.

The most frequent histological patterns in idiopathic disease, UIP and NSIP are frequently encountered in connective tissue disease-associated interstitial lung disease (CTD-ILD). Two key advances have been made in recent years in CTD-ILD. First, it has become clear that survival is better in CTD-ILD than in IPF [58], with the exception of lung disease in rheumatoid arthritis. Secondly, a number of histopathological features are more frequently observed in CTD than in IIP, including the pattern of NSIP and the presence of lymphoid aggregates and germinal centres in UIP (as well as more plasma cells), with a marginal relationship to survival [59]. Denser perivascular collagen deposition has also been reported in CTD.

However, it is not clear whether patients with a clinical diagnosis of IIP, in whom auto-antibodies are detected, have a different prognosis from those without antibodies. SONG et al. [59] have recently shown that the survival of patients with IPF/UIP was not altered by the presence of auto-antibodies. Earlier studies have demonstrated that antinuclear antibodies may be found in up to 10–20% of patients with so-called IPF [60], with some subsequently reclassified as NSIP [55, 56]. Antinuclear antibodies and, less frequently, anti-neutrophil cytoplasmic antibodies [61] may be found in IPF patients, although their significance is uncertain. The most frequent pathological pattern in CTD-ILD is NSIP, except for rheumatoid arthritis which is characterised by a higher frequency of UIP [62]. Moreover, it is clear that rheumatoid arthritis-associated UIP represents a possible exception to the improved survival seen in CTD-UIP compared to IPF/UIP [63]. PARK et al. [58] reported that rheumatoid arthritis-associated UIP tended to have a worse survival than non-rheumatoid arthritis CTD-associated NSIP or UIP. In a larger study of 82 patients with rheumatoid arthritis-ILD a worse survival was reported for patients with UIP pattern on high-resolution computed tomography. Moreover, they found that the estimated survival in rheumatoid arthritis-UIP did not differ significantly from that of IPF [64].

Idiopathic pulmonary fibrosis

Much information has been obtained from animal models of pulmonary fibrosis; however, it is important to bear in mind that these models are characterised by acute injury with an inflammatory response and do not truly reproduce all features central to the pathogenesis of IPF. Transfer to the lung epithelium of rodents of a variety of inflammatory cytokines and chemokines using replication-deficient adenoviral vectors resulted in marked increases in BAL inflammatory cells and tissue pneumonitis but without residual lung remodelling and fibrosis [65], with the notable exception of TGF-β1 and cytokines such as interleukin (IL)-1β that lead to overexpression of active TGF-β1. However, despite being poorly representative of human lung fibrosis, animal studies have collectively demonstrated a central role for TGF-β1 as a key profibrotic mediator, and have also shown that induction of lung fibrosis is not always dependent on the degree of the inflammatory response [65].

Although overt inflammation is absent in the lungs of patients with IPF, inflammatory cytokines and chemokines (together with the coagulation cascade and eicosanoid lipid mediators) contribute to the interplay between the lung epithelium and fibroblasts, to the polarisation toward a Type-2 helper T-cell phenotype and to lymphoid neogenesis [66, 67], as well as to angiogenesis through an imbalance between angiogenic and angiostatic CXC chemokines [66], which will be discussed further in this review. Phenotypes of M1 and M2 macrophages have been recently described that support Th1 and Th2 responses, respectively.

Th2 responses characterise a number of pulmonary diseases, many of which culminate in tissue remodelling and fibrosis. A shift towards Th2 immune responses seems to predominate in IPF and promote fibrosis primarily via the secretion of profibrotic cytokines by the injured epithelium. In murine models of lung disease, animals with a response to tissue injury predominantly of the Th2 type, are more prone to pulmonary fibrosis after lung injury than those with a predominantly Th1 response [67, 68].

It has been shown that CCL18, a CC-chemokine that is regulated by Th2 cytokines, is associated with pulmonary

fibrosis [33]. CCL17 and CCL22 and their receptor CCR4 have been found to be elevated in areas of fibrotic lung tissue as compared with normal pulmonary parenchyma [38, 69, 70]. In particular, CCR4 was found to be expressed mostly by macrophages present in fibrotic areas. Moreover it has been shown that neutralisation of CCL17 and CCL22 leads to a significant reduction of lung damage [71]. The Th2 immune response contributes to a failure of re-endothelialisation and re-epithelialisation, and leads to the release of profibrotic growth factors into the region of injury. These profibrotic cytokines initiate fibroblast migration to the site of injury and promote their proliferation and differentiation into myofibro-blasts. Inflammation and tissue remodelling with pathologic fibrosis are common consequences of Th2 responses in the lung and other organs. IL-13 and TGF-β1 are frequently co-expressed in these responses and are believed to play important roles in the pathogenesis of Th2-induced patholo-gies. This Th1/Th2 hypothesis has dominated our under-standing of immune regulation, immune pathogenesis and host defence for decades, despite its flaws and inability to explain certain data regarding T-cell mediated tissue damage.

An exciting finding is the fact that serum CCL18 concentra-tions have a predictive value in IPF [72]. CCL18 is a CC-chemokine produced by human myeloid cells and has been described as a marker of the alternative activation of macrophages. Macrophage activation by Th2 cytokines induces a special phenotype, so-called ''alternative activation''. Alternatively, activated macrophages play a role in tissue repair processes such as wound healing and fibrosis. PRASSE et al. [72] showed a significantly higher mortality in patients with serum CCL18 concentrations >150 ng·mL^{-1}. These find-ings are promising and should be implemented in the design of new clinical trials.

Recently, two novel CD4 T-cell subsets have been described that have revolutionised our understanding of immune function: the Th17 subset, which develops via different cytokine signals from those of Th1 and Th2 lineages, and the T-regulatory cells (Tregs). The Th17 subset is characterised by production of IL-17 and is involved in the pathogenesis of autoimmune tissue injury, including rheumatoid arthritis and allergen-specific responses. The central mediators generating the effector CD4+ Th17 subset are TGF-β, IL-23 and IL-17. Although Th17 cells are important in mucosal host defence, they can mediate immunopathological events. The IL-17 receptor is upregulated in the lungs of patients with hypersensitivity pneumonitis [73]. The key profibrotic cytokine in pulmonary fibrosis, TGF-β, has been found to be necessary for the differentiation of naive CD4$^+$ T-lymphocytes into Th17 cells in mice [74, 75], which could be another way TGF-β promotes fibrosis. In a comprehensive review on mechanisms of fibrosis, WYNN and RAMALINGAM [76] suggest a role for the IL-1β-IL-17-TGF-β axis.

Tregs (CD4+CD25+foxp3+) represent the first well-defined expansion of the CD4+ T-cell functional range. Tregs suppress activation of the immune system and help maintain immune homeostasis and tolerance to self-antigens [77]. In case of a shortage of Tregs, the potential amplitude of Th1 and Th2 responses is increased resulting in excessive T-cell immunity as associated with autoimmune disease, asthma and allergy.

An abundance of Tregs, however, will reduce the potential amplitude of Th1 and Th2 responses and may prevent adequate immunity to tumours and infectious diseases [77, 78]. A significant impairment of Treg suppressor function is evident in both the peripheral blood and BAL fluid in IPF patients. Identical findings were retrieved from lung tissue biopsies. Finally, an almost linear correlation of Tregs global impairment, both in functional and numerical level, with parameters of disease severity including pulmonary function tests, was demonstrated, suggesting that Tregs dysfunction may serve as a reliable prognosticator of disease progressive-ness and treatment responsiveness and may provide clinicians with a novel tool for risk stratification of patients with IPF [79]. It can be surmised that the low numbers and the systemic and local Tregs dysfunction found in IPF patients may result in inefficient control of the pre-existing over exuberant Th2 response or contribute to a Th2 skew [74].

Oxidative stress

Oxidative stress is defined as an imbalance of the generation of reactive oxygen species (ROS) in excess of the capacity to neutralise them. Overproduction of ROS might lead to oxidative stress that causes tissue injury. Oxidative stress might promote a tissue microenvironment that favours fibrosis over regeneration. This might play a role in the development of apoptosis resistance of fibroblasts in IPF [77]. It has been shown that lung myofibroblasts secrete hydrogen peroxide, which may mediate fibrogenic effects and induce epithelial apoptosis [78]. Moreover neutrophils are a major source of matrix degrading enzymes, including neutrophil elastase, and contribute to oxidative stress. Modulating oxidative stress might be an interesting way of preventing further injury in an attempt of halting the process of pulmonary fibrosis. Interesting new data show that sivestat, a neutrophil elastase inhibitor, is able to alleviate bleomycin-induced pulmonary fibrosis [79]. Moreover, deficiency of the antioxidant glu-tathione (GSH) in epithelial lining fluid is thought to play a role in the initiation and progression of fibrosis [80]. The established link between oxidative stress and IPF provided a strong rationale for clinical trials, including the evaluation of N-acetylcysteine (NAC), an antioxidant that acts as a precursor of GSH synthesis via the main metabolite cysteine. The IFIGENIA (Idiopathic pulmonary Fibrosis International Group Exploring NAC I Annual) trial examined the effect of high dose NAC in addition to corticosteroids and azathioprine. In this trial it was shown that the addition of NAC was able to slow down the progression of forced vital capacity and diffusing capacity of the lung for carbon monoxide decline in IPF [81]. However, the recent PANTHER (Prednisolone, Azathioprine and NAC: a study that evaluates response in IPF) trial has revealed that the combination of NAC with corticosteroids and azathioprine was associated with an increased risk of death and hospitalisation in comparison with placebo [82]. This has been considered new evidence against the initiation of this combination therapy for patients with IPF. One of the issues here is whether the individual characteristics of the IPF patients in this trial compare to past study populations [83], as the rate of death in the placebo arm in the PANTHER trial was only 2%, which is lower than in other placebo-controlled trials in IPF. These results certainly warrant further detailed analysis. Furthermore, it is clear that the

results of the continuing PANTHER IPF trial focussing on NAC monotherapy *versus* placebo are eagerly awaited.

Endoplasmic reticulum stress

ER stress is defined as being caused by conditions that disturb the processing and folding of proteins, which results in the accumulation of misfolded proteins in the ER and activation of the so called unfolded protein response (UPR) [84]. In the last few years, a vast amount of evidence has been provided that suggests a role for ER stress in IPF [23, 76, 85]. The initial observations were identified in AEC from families with SP-C mutation, but they have also been observed in non SP-C-related familial IPF and even sporadic IPF [4, 23]. Secreted proteins are initially delivered to the ER as unfolded polypeptide chains. Thereafter they are properly folded into functional three dimensional conformations, assembled and glycosylated. When the cell is stressed, activation of the UPR can occur, failure of the UPR response, or too severe ER stress, might lead to AEC cell death through apoptosis [85]. Other studies suggest that ER stress can lead to a fibrotic AEC phenotype *via* an EMT mechanism, as this process may be involved in the regulation of cellular phenotypes [86]. In addition, viral infection initiates the production of large amounts of viral proteins, which may activate ER stress. Interestingly, it has been shown that herpes virus proteins that are expressed in AECs lining areas of fibrosis in IPF are co-localised with ER stress markers [85].

It is clear that aberrant protein processing is a potential target for future therapy, but more research in this intriguing field is needed. In particular, it is not clear what the link is between environmental insults and ER stress, or whether cell types other than AEC might also be involved in the ER stress-UPR axis. Moreover, there might be a link between surfactant proteins and ER stress. Patients with Hermansky–Pudlak syndrome, a rare autosomal recessive disorder associated with oculocutaneous albinism and haemorrhagic diathesis, sometimes develop interstitial pneumonia. It has recently been shown that the ER stress response seen in this disorder could be due to increasing amounts of accumulating SP-B/-C. This additional ER stress further contributes to AEC apoptosis [87].

The vascular component and the coagulation cascade

Aberrant vascular remodelling is crucial for the development and progression of IPF. A number of data indicate the importance of the altered balance between pro- (CXCL8, CXCL5 and CXCL12) and anti- (CXCL9, CXCL10 and CXCL11) angiogenic CXC chemokines in promoting aberrant neoangiogenesis and lung recruitment of circulating fibrocytes with the contribution of TGF-β.

The activation of the coagulation cascade is one of the earliest events initiated following tissue injury. The primary function of the coagulation cascade is to plug damaged blood vessels and prevent blood loss. However, it is increasingly recognised that the functions of the coagulation cascade extend beyond haemostasis and that this cascade plays a central role in influencing inflammatory and tissue repair programmes. The cellular responses of the coagulation cascade are principally executed by several coagulation proteinases, acting *via* specific cell-surface receptors, the proteinase-activated receptors (PARs). This family comprises four members (PAR1 to PAR4)

but current evidence suggests a major role for the high-affinity thrombin receptor, PAR1, in influencing cellular responses in the context of lung injury. Activation of PAR1 on numerous lung cell types, including pulmonary epithelial cells, fibroblasts and macrophages, leads to the release and activation of potent pro-inflammatory and profibrotic mediators [88–90]. Moreover, PAR1 signalling in response to either thrombin or factor Xa on fibroblasts also promotes their proliferation and differentiation into matrix synthesising myofibroblasts [91–93].

Evidence that the coagulation cascade may be of pathophysiological significance has come from *in vivo* models of bleomycin-induced pulmonary fibrosis. Targeting the coagulation cascade in these models (using a variety of strategies, including direct thrombin inhibition, tissue factor pathway inhibition, intratracheal administration of activated protein C and aerosolised heparin) led to a reduction in lung collagen accumulation and the development of fibrotic lesions [94]. A central role for the cell-mediated responses of the coagulation cascade in this model was provided by evidence that PAR1-deficient mice are protected from experimental lung oedema, inflammatory cell recruitment and fibrosis [88, 95].

Current dogma assumes that coagulation zymogens are derived from the circulation and locally activated in response to injury *via* the extrinsic (tissue factor) coagulation pathway. However, it has been recently shown that coagulation factor X is locally upregulated in patients with IPF and in the murine model of bleomycin-induced lung injury, with both the bronchial and alveolar epithelium representing prominent cellular sources of this coagulation zymogen [96].

These findings herald a paradigm shift in our understanding of the tissue origin of excessive pro-coagulant signalling in lung disease and suggest that the epithelium is a major site for the initiation of such responses. In contrast to the promising preclinical studies with different anticoagulant strategies, a recent clinical trial suggests that systemic anticoagulant therapy using warfarin did not show any benefit in patients with progressive IPF. On the contrary, treatment with warfarin was associated with an increased risk of mortality in an IPF population who lacked other indications for anticoagulation [97]. It is not yet clear whether the effects of warfarin were entirely mediated *via* its anticoagulant effects or other vitamin K-dependent enzymes. It is also not known whether warfarin was effective at blocking coagulation within the intra-alveolar compartment. However, this trial raises the possibility that the coagulation cascade may also play protective roles in pulmonary fibrosis. Systemic anticoagulants may, therefore, not prove useful and future anticoagulant strategies may need to selectively target deleterious coagulation signalling responses within the intra-alveolar compartment in IPF. Novel PAR1 antagonists are currently in phase III trials in the context of cardiovascular disease and may warrant further investigation in IPF [98].

Lymphatics and pulmonary fibrosis

In normal lung tissue, lymphatic vessels exist in close proximity to the airways and major blood vessels. Due to their thin wall structure, and the lack of reliable markers the knowledge of lymphatics in normal human lung is sparse. PUSZTASZERI *et al.* [99] found that D2-40, a marker of lymphatic endothelial cells, follows bronchovascular distribution and one

study reported the absence of lymphatic vessels in the alveolar space [100]. Recently, Sozio *et al.* [101] have shown that lymphatic vessels extend within the lobule beyond respiratory bronchioles. Moreover, they provide evidence for heterogeneity of lymphatic vessels within the lobule, in comparison with lymphatics from other lung districts [101]. In pig, lung lymphatic vessels were not found in the parenchyma [102]. An interesting finding has been reported in hypoxia induced alveolar damage in a rat model [103]. The authors found perialveolar lymphatic vessels, which resolved over a 14-day period.

In IPF the data on lymphangiogenesis are sparse. It is well known that mediastinal lymphadenopathy is present in a significant amount of patients with IPF [104, 105]. In the study of EL-CHEMALY *et al.* [106], lymphatic vessels in IPF lungs were in close proximity to alveolar spaces, even in those areas with a well-preserved architecture. Moreover lymphatic vessels were present throughout the fibrotic tissue and in close proximity to the main bronchovascular tree. Within fibroblastic foci, there were no lymphatic vessels observed, while lymphatics could be seen at their periphery. In diffuse alveolar damage, intra-alveolar lymphangiogenesis is a key element of the fibrotic process [100].

However, there are also data suggesting that both the subpleural and the interlobular lymphatics in IPF lungs are significantly decreased in the severe fibroconnective lesions, with rare lymphangiogenesis [107]. Moreover, destruction of the subpleural lymphatics and apoptosis of lymphatic endothelial cells have been observed in IPF patients. The disappearance of the subpleural and interlobular lymphatics in IPF lungs, along with poor lymphangiogenesis, may significantly impair alveolar clearance and prolong exposure to damaging mediators in the interstitium, enhancing their influence on fibrogenesis. This is a particularly attractive model for acute exacerbations, in which the coagulation cascade and lymphatic disruption may synergise in a most damaging fashion.

Stem cells in pulmonary fibrosis

A broad range of cells have been shown to exhibit the capacity to differentiate into lung cells. These range from lung progenitor cells, circulating cells, mesenchymal stem cells (MSCs), induced pluripotent stem cells, placental stem cells and human embryonic stem cells (HUES). The common strategy for treatment of fibrotic lung diseases is to replace damaged epithelial and endothelial cells and restore normal repair. MSCs and placental stem cells have both been shown to express markers of lung epithelium following injection into animal models of lung diseases [108], and embryonic stem cells show features of lung epithelium *in vitro* [109].

Recently, a study from SPITALIERI *et al.* [110] has shown that HUES-3 can differentiate *in vitro* in ATII cells. Furthermore, transplantation of HUES-3-ATII into silica damaged mice significantly reduced markers of inflammation and fibrosis. In addition, lung engraftment of MSCs immediately after bleomycin-induced injury have been shown to have beneficial effects associated with decreased collagen deposition, fibrosis and MMP levels [111].

At present there are almost no clinical trials investigating this highly challenging issue of stem cells in pulmonary fibrosis. It is clear that this is an exciting new potential therapy that urgently requires further investigation.

CONCLUSION

After many years of relative standstill in pulmonary fibrosis, with treatments confined to anti-inflammatory agents, knowledge has exponentially increased in the last decade. New concepts have resulted in a major change in the understanding of this intriguing disease. In the current paradigm, pulmonary fibrosis is considered to be the result of multifactorial disease processes. Generally, it is thought that in the genetically susceptible host, exposure to injurious agents may result in phenotypic alterations of structural cells. This leads to aberrant cell interactions and finally fibrosis. Despite the unravelling of several core mechanisms of fibrosis, emerging data have generated new areas of interest.

One of the most challenging areas is the field of genetics. There is a better understanding of the factors involved in susceptibility to develop pulmonary fibrosis. Interesting new findings are ELMOD2, SP-C mutations, hTER and hTERT, and also MUC5B, although the exact linkage between MUC5B hypersecretion in the airways and the process of fibrosis is not yet clear. Also of major interest, is to further explore the link between genetic abnormalities and the pattern and severity of fibrosis in the individual patient. Furthermore, there is emerging evidence for a role of injurious agents. Promising new data have been published on the role of viruses in the initiation and propagation of the fibrotic process, especially for EBV. EBV has indeed been detected in IPF lungs, and although there are some ideas on the mechanism of EBV induced fibrosis, many questions still remain unanswered. GOR seems to be another injurious agent, although until now the effect of treatment of GOR on the formation of fibrosis was not clear at all. Whether non-acid reflux is involved, and to what extend this can be treated, is still largely unknown. In addition, it is clear that the link between injurious agents and genetic susceptibility needs more clarification.

The basic mechanisms of fibrosis have become much clearer, such as the important role of ATII cells and myofibroblasts, and also many elements of the cytokine network have been further explored. Other emerging areas are the role of immunological mechanisms on the initiation and modulation of the fibrotic process. Immunology has recently been boosted by new discoveries. One of the most exciting new findings is the fact that serum CCL18, a marker for alternative activated macrophages, has a prognostic value in IPF. This points to a possible role of macrophages in IPF. Until now these cells have been relatively neglected in IPF. Although functional CD4+ T-cell development has been dominated by the Th1–Th2 paradigm, the discovery of the Th17 pathway and its relationship with Tregs (fig. 1) opens a new and fascinating era in our understanding of adaptive immune regulation. This will certainly result in novel and more effective therapeutic approaches in IPF as in a number of autoimmune and inflammatory diseases.

Oxidative stress remains a challenging area as the role of anti-oxidative agents in the treatment of IPF is still not elucidated. The field of ER stress is much newer and many interesting findings have been published in the past few years. The relationship between ER stress and other immunological active and structural

cells, especially ATII, warrants further investigation. The knowledge of the role of the vascular compartment is rather poor, but will become increasingly important with the role of the different coagulation pathways now well established. The next step will be an attempt to come to an integrated vision on the role of those pathways and the fibrotic process and how new therapeutics can be developed based on these new findings.

A complete new area is that of pulmonary lymphatics. Further development of this intriguing field will probably lead to the discovery of new pathways in the process of fibrosis. Last but not least, the development of stem cells from different sources is undoubtedly connected with a wide range of new possibilities. *In vitro*, there are many promising observations, but the usefulness of the different types of stem cells to stop the relentless process of fibrosis and to restore appropriate repair still needs to be explored.

Although seemingly the major steps and key players in the formation of fibrosis have been identified, it remains nevertheless important to explore robust models of overall pathogenesis, reconciling a large number of clinical and scientific observations. We believe that the integration of current data into a ''big picture'' overview of fibrogenesis is essential for the development of effective anti-fibrotic strategies.

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