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The ECM as a driver of fibroblast senescence and disrupted epithelial repair in IPF

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DOI:
10.33612/diss.177953737

Publication date:
2021

Link to publication in University of Groningen/UMCG research database

CHAPTER 1

General introduction

Interstitial lung disease

Interstitial lung diseases (ILD) are a group of diffuse parenchymal lung disorders that are characterised by inflammation and accumulation of extracellular matrix (ECM) in the lung parenchyma. The accumulation of ECM leads to decreased gas exchange, shortness of breath, reduced exercise capability and has a devastating impact on the quality of life [1]. There are several ILD subtypes identified, including connective-tissue associated-ILD, idiopathic non-specific interstitial pneumonia (NSIP), fibrotic hypersensitivity pneumonitis, unclassifiable ILD and idiopathic pulmonary fibrosis (IPF), the latter being the most common [2]. IPF is a chronic and progressive disease of unknown aetiology with a mean survival of 3-5 years after diagnosis, which is worse than most types of cancer [3]. IPF occurs primarily in the elderly and men are twice as likely to develop fibrosis than women. The core characterisations of IPF are architectural distortion of lung tissue, including interstitial patchy areas with fibroblastic foci and honeycombing. Due to a global ageing population and regular updates on the definition of IPF, the total number of reported cases per year is on the rise. Globally the incidence of IPF is estimated to be up to 94 cases per 100,000 per year. However, data in North America and Europe combined estimates that the incidence is between 2.8 – 9.3 per 100,000 per year [4]. Up until 2014 the only definitive treatment for IPF was a lung transplantation, but with the approval of nintedanib and pirfenidone new treatment options have become available that are able to slow down disease progression, albeit at considerable cost and with significant side effect [5, 6].

Prevalence and risk factors associated with IPF

IPF accounts for 20 – 30% of patients with ILD, however, the prevalence of IPF varies depending on the population and definition used to identify IPF. Although there are guidelines available, the definition of IPF is not standardized [7]. Currently the gold standard for IPF diagnosis is a multidisciplinary team of clinicians, radiologists and histopathologists reaching a consensus after discussion. In many cases a lung biopsy is unavailable due to the procedure being too risky for patients of high age with interstitial changes being detected using high-resolution computed topography (HRCT) [8]. Despite the available guidelines, there is a large spread in reported cases around the globe, for example in the United States there are 14 to 27.9 cases per 100,000 people while European countries estimate the prevalence of IPF ranges between 1.25 to 23.4 per 100,000 people [9]. Differences in reported cases of IPF appear to be related to geographical differences in risk factors for IPF [9]. However, all studies report that the prevalence is the highest in older males of >65 years of age with no sex-related associations above 75 years of age. Interestingly, male sex is considered a risk factor.

Studies using sex-stratification determined that men who have a history of smoking and occupational exposure were more at risk to develop IPF than women [10]. Furthermore, male sex hormones have also been suggested to play a role in susceptibility to IPF, which is also observed in a mouse model of pulmonary fibrosis [11, 12].

Progression of IPF is different for each patient, with some experiencing a decline in lung function over several years while others declining much faster. To measure the progression of IPF, several criteria are used including forced vital capacity (FVC) with a decline of 5-10%, decrease in diffusing capacity of carbon monoxide (DLCO) of 10-15% and a reduction of 50m or more in the 6-minute walk distance (6MWD). Other criteria used are increased sleep dyspnoea and a reduction in the quality of life [5, 13, 14]. When symptoms worsen and lung function declines rapidly within 30 days, the patient is considered to have undergone an acute exacerbation [15].

There is a critical need for novel therapeutic options to treat IPF. However, IPF is a heterogenous disease making the development of new therapies difficult. Identification of risk factors associated with fibrosis and how they contribute to disease pathology is a good starting point. Several intrinsic risk factors have been identified, which include genetics, ageing, male gender and the composition of the lung microbiome [16]. Extrinsic risk factors include cigarette smoke, metal and wood dust exposure, and air pollution as well as comorbidities such as gastroesophageal reflux (GERD), diabetes and obstructive sleep apnoea [17]. Studies investigating genetic susceptibility have identified four groups of genes based on their role in the pathogenesis of IPF (Table 1) [18]. Mutations in the first group of genes i.e., surfactant proteins (SFTP) A and C, and ATP-binding cassette transport of the A subfamily (ABCA3) affect alveolar stability, by increasing endoplasmic reticulum stress and activation of the unfolded protein response [19-21]. Mutations in the second group of genes i.e., desmoplakin (DSP) and dipeptidyl peptidase 9 (DPP9) leads to loss of cell-cell interactions and epithelial cell integrity [22]. Mutations in the third group i.e., a telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) are associated with early senescence due to the disruption of telomerase function of . The latter leads to abnormally short telomeres that specifically affect alveolar epithelial type II cells (AEC2) [23, 24]. The last group of gene mutations in the gene encoding for mucin 5B (MUC5B) involves an altered host defence and mucociliary clearance [25].

Table 1 – Common gene mutations found in IPF.

Gene	Gene function	Reference
SFTPA2	Binds to phospholipids and contributes to lower surface tension at the air-liquid interface.	[26-28]
SFTPC	Component of pulmonary surfactant promoting alveolar patency by lowering surface tension at the air-liquid interface.	[26-28]
ABCA3	Transport of phospholipids across the membrane.	[29-31]
DSP	Desmosomal protein critical for cell-cell interactions and epithelial barrier function.	[22, 32, 33]
DPP9	Unique serine protease, involved in cell-cell adhesions.	[22, 32]
TERT	Telomerase enzyme to maintain telomere integrity.	[34-36]
TERC	Telomerase RNA template.	[35-37]
MUC5B	Mucin that contributes to the viscoelastic properties of mucous.	[33, 34, 38]

SFTPA2 = Surfactant Protein A2; SFTPC = Surfactant Protein C; ABCA3 = ATP-binding cassette transport of the A subfamily 3; DSP = Desmoplakin; DPP9 = Dipeptidyl Peptidase 9; TERT = Telomerase Reverse Transcriptase; TERC = Telomerase RNA Component; MUC5B = Mucin 5B.

Population studies have demonstrated that with age the prevalence of IPF increases with most cases being diagnosed between 50 - 70 years old. While the exact underlying mechanism(s) remains unclear, there is increasing evidence that accumulation of genetic mutations, epigenetic changes and cellular senescence, a hallmark of ageing, are implicated by their contribution to aberrant wound repair responses.

Tobacco smoking is a risk factor associated with the development of IPF. Up to 81% of patients diagnosed with IPF have a history of smoking [39]. Cigarette smoking causes injuries to the lungs that are thought to contribute to the development of IPF; but such injuries also have a role in the pathogenesis of chronic obstructive pulmonary disease (COPD), cardiovascular disorders and cancer [40]. Occupational and environmental exposures are also linked to the development of IPF [41]. Studies in the UK, USA and Japan have demonstrated a higher incidence of IPF in industrialised areas. Increased risks are associated with those who are exposed to metal and wood dust or agricultural particulates such as from farming and livestock [42-44]. In addition, particulate matter in ambient air pollution leads to epithelial damage, airway inflammation and oxidative stress, all of which are associated with higher incidences and progression of IPF [45]. Identified intrinsic risk factors that contribute to IPF are gastroesophageal reflux and lung microbiome dysbiosis [46-48]. All these risk factors have one thing in common, the ability to damage lung tissue indicating that chronic exposure is sufficient to initiate aberrant cellular responses in susceptible patients.

Pathogenesis of IPF

The lungs are subject to micro-injuries from inhalation of airborne particulate matter, noxious chemicals and pathogens but demonstrate a tremendous capacity to repair and regenerate tissue damaged from injury. However, it is theorised that IPF originates from repeated and / or non-resolving injuries to the alveolar epithelium followed by an abnormal repair response. The latter initiates a wound healing cascade but instead of wound resolution there is uncontrolled proliferation of myofibroblasts and accumulation of matrix leading to destruction of lung tissue architecture (Figure 1). As ageing is a major risk factor of IPF, the role of cellular senescence has been a major focus of IPF research . Indeed, premature senescence in both epithelial cells and fibroblasts have been increasingly implicated in IPF [49-51]. Cellular senescence is characterised by cells undergoing irreversible cell-cycle arrest, an increasing resistance to apoptosis and the development of a pro-inflammatory secretory profile known as the senescence-associated secretory phenotype (SASP) [52].

In normal wound repair processes of the lung, loss of type I AECs leads to activation of type II AECs; progenitor cells that regenerate the alveolar wall by differentiating into type I AECs. In IPF there are marked changes to the epithelium including the enlargement and elongation of AECs, cuboidal cells and an increase in AEC apoptosis that contributes to dysfunctional wound repair. Repetitive injury not only results in the depletion of type II AECs, but irreversible alterations such as DNA damage could lead to a permanent dysfunctional phenotype such as cellular senescence [53]. The injured epithelium releases a myriad of factors such as platelet derived growth factor (PDGF), transforming growth factor β1 (TGF-β1) and connective tissue growth factor (CTGF) that have a strong impact on fibroblast function. In response to injury, fibroblasts activate, migrate and differentiate into myofibroblasts and start the production of several matrix proteins including collagen, fibronectin, elastin and proteoglycans to restore homeostasis [54, 55]. Once there is sufficient tissue integrity, these myofibroblasts are cleared from the lungs. However, in IPF these cells fail to undergo apoptosis and persist at the site of injury leading to the creation of fibroblastic foci, the active site of fibrosis. It is postulated that the persistence of activated fibroblasts is due to cellular senescence contributing to fibrosis [49, 56, 57]. The senescent phenotype encompasses several changes including differential gene expression leading to the production and release of several pro-inflammatory and profibrotic cytokines, matrix metalloproteinases (MMPs) and increased expression of several extracellular matrix (ECM) proteins [58].

The ECM is a complex structure that surround cells to provide structure and support. In recent years, our understanding of the ECM has increased markedly, especially with respect to its function in providing biochemical and biomechanical cues in tissue homeostasis and injury. During normal homeostasis, cells such as fibroblasts are quiescent and maintain the ECM. Using specialised cell surface receptors, such as integrins, cells embedded within the matrix receive signals that potentially control adhesion, migration, proliferation and differentiation (REF). Mechanical forces are rapidly translated and activate signalling pathways [59]. Fibroblasts are the major source of ECM production in IPF and ECM changes in ageing have been increasingly associated with pathological conditions. Senescent cells display increased ECM production, indicated by increased expression of collagen type I and fibronectin, and higher expression of MMPs contributing to dysregulated homeostasis [58]. In addition to increased deposition, increased lung tissue stiffness leads to increased production and activation of TGF-β1 and the crosslinking enzyme family of lysyl oxidases (LOX); these changes in turn activate fibroblasts creating a positive feedback loop. These observations indicate the involvement of the ECM and the effects it may have on altering fibroblast function favouring ongoing fibrotic processes.
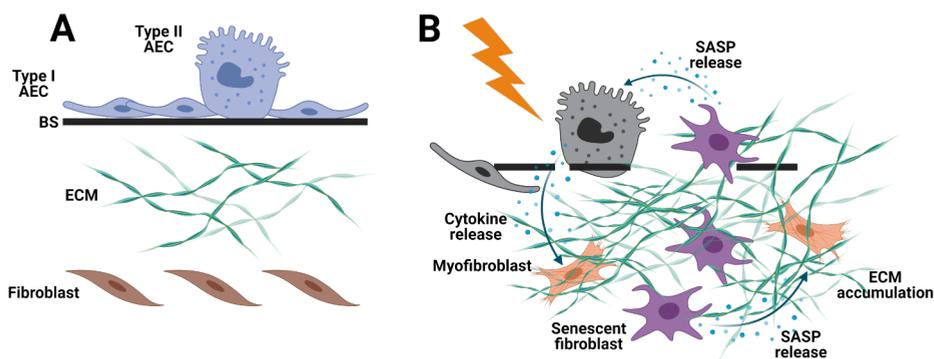

Figure 1. IPF pathology. Panel A shows the intact alveolar wall with the interstitial matrix and residing fibroblasts that maintain homeostasis. Panel B shows the proposed mechanism and the role of senescent fibroblasts in IPF. The epithelium gets damaged upon damaged epithelial cells release cytokines which leads to activation of fibroblasts into myofibroblast that produce and deposit ECM. Normally these cells are cleared from the system once homeostasis is restored. In IPF these cells remain active and continue to produce ECM which leads to fibrosis characterised by fibroblastic foci and destruction of the alveoli. The release of SASP factors by senescent fibroblasts impacts on neighbouring cells and contributes to a feedback mechanism that perpetuates fibrosis in IPF. Type I/II AEC = Type I/II alveolar epithelial cell; BS = Basement membrane; ECM = Extracellular Matrix; SASP = Senescence-associated secretory phenotype (Created with BioRender.com).

Treatment options for IPF

Treatment options for IPF are limited and can only slow down, but not halt or reverse the decline in lung function [60]. In addition to antifibrotic drugs, patients can receive supportive treatment such as oxygen therapy and medication to reduce symptoms such as couching and heartburn. In 2014 two antifibrotic drugs, nintedanib and pirfenidone, were approved for the treatment of IPF [5, 6]. Once IPF has been diagnosed, a treatment plan is devised for each individual patient as there is no standard treatment plan available. However, this remains difficult as each patient is different and not everyone reacts positive to treatment. Nintedanib is a tyrosine-kinase receptor inhibitor that acts by binding to the intracellular ATP binding pocket of platelet derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs) and vascular endothelial growth factor receptors (VEGFRs). By limiting the proliferation, migration and survival of (myo)fibroblasts and angiogenesis by endothelial cells, pericytes and vascular smooth muscle cells, nintedanib is able to slow down IPF progression [61, 62]. Analysis of data from two large clinical trials demonstrated that nintedanib reduces lung function decline, prolongs the time until the first acute exacerbation and increases the quality of life [63] While the exact mechanism of action of pirfenidone remains unclear, in vitro studies demonstrated that it is able to inhibit fibroblast secretion of SASP-related inflammatory cytokines such as TNF-α, IFN-γ and IL-6, and abrogates TGF-β1-stimulated collagen production [64]. Furthermore, pirfenidone has also been reported to have anti-oxidant properties by acting as a scavenger for hydroxyl and superoxide anions [65]. In 2018 a study was published that investigated the safety and tolerability of the combination of both drugs for the treatment of IPF. Both drugs have different working mechanisms, combining the two may result in additive benefits to slow down disease progression. They demonstrated that both drugs were tolerated without safety issues. However, more studies are needed to determine if the combination of these two drugs would provide advantages in slowing down disease progression for patients compared to either drug alone. The licensing of these two drugs have highlighted that IPF is a treatable disease, but due to their modest potency and adverse side effects, more research is needed into the pathogenesis to develop further novel therapeutic options.

The scope of this thesis

My central hypothesis is that in IPF, senescent lung fibroblasts influence the local microenvironment by increasing ECM accumulation and the secretion of SASP cytokines, which perpetuate fibroblast senescence and contribute to dysfunctional alveolar epithelial cell regeneration (figure 1). Therefore, the overall scope of this thesis is to investigate the impact of fibroblast senescence on alveolar epithelial cell regeneration

and determine if the ECM contributes to pathological senescence by creating a positive feedback loop that perpetuates disease progression.

Firstly, **chapter 2** provides a general overview on the role of cellular senescence during homeostasis, ageing and disease. We describe how the ECM can act as a modulator of cell function and summarise the available evidence about how aberrant and dysfunctional ECM can influence the senescence phenotype in chronic fibrotic diseases. Furthermore, we explore the role of ECM damage-associated molecular patterns (DAMPs), which are released during proteolytic degradation or tissue damage in IPF and the potential for targeting ECM-senescence regulatory pathways for therapeutic potential in fibrosis.

In **chapter 3** we investigate the impact of IPF-derived and senescent-induced primary lung fibroblasts on A549 alveolar epithelial cell regeneration. We investigate the influence of secreted factors of the SASP by senescent fibroblasts on alveolar epithelial cell proliferation allowing direct contact or by transfer of culture media. Lastly, we measured the wound repair response of epithelial cells in a co-culture system after mechanical injury and measured if there was altered cell-cycle inhibition in alveolar epithelial cells.

In **chapter 4** we used GelMA hydrogels to determine the impact of pathological stiffness as observed in IPF on the senescent phenotype of primary lung fibroblasts. We analysed markers of senescence in fibroblasts cultured on GelMA hydrogels mimicking healthy and fibrotic stiffness and assessed fibrosis-associated gene expression. Additionally, matrix proteins associated with senescence and fibrosis were also measured.

In the final experimental chapter (**chapter 5**) we investigated whether ECM deposition by senescent-induced, profibrotic and IPF-derived fibroblasts had impact on the senescent phenotype of primary lung fibroblasts. We measured several markers of senescence, characterised SASP secretion and assessed the expression of fibrotic response associated genes.

Finally, **chapter 6** summarises the findings of this thesis and discusses the relevance and implications of these findings, illustrating how the findings in this thesis contribute further knowledge to the field.

1

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