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LUNG INJURY & FIBROSIS (CL WILSON, SECTION EDITOR)

The Vasculature in Pulmonary Fibrosis

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Abstract

Purpose of review The current paradigm of idiopathic pulmonary fibrosis (IPF) pathogenesis involves recurrent injury to a sensitive alveolar epithelium followed by impaired repair responses marked by fibroblast activation and deposition of extracellular matrix. Multiple cell types are involved in this response with potential roles suggested by advances in single-cell RNA sequencing and lung developmental biology. Notably, recent work has better characterized the cell types present in the pulmonary endothelium and identified vascular changes in patients with IPF.

Recent findings Lung tissue from patients with IPF has been examined at single-cell resolution, revealing reductions in lung capillary cells and expansion of a population of vascular cells expressing markers associated with bronchial endothelium. In addition, pre-clinical models have demonstrated a fundamental role for aging and vascular permeability in the development of pulmonary fibrosis.

Summary Mounting evidence suggests that the endothelium undergoes changes in the context of fibrosis, and these changes may contribute to the development and/or progression of pulmonary fibrosis. Additional studies will be needed to further define the functional role of these vascular changes.

Keywords Fibrosis · Endothelium · Permeability · Bronchial · Capillary · Vasculature

Introduction

Across multiple organs, fibrosis represents a state of end-organ dysfunction following severe or recurrent injury due to an inability to return to tissue homeostasis in the setting of impaired wound-healing and ineffective repair. In the lung, idiopathic pulmonary fibrosis (IPF) is a devastating lung disease marked by dysfunction of the alveolar compartment resulting in significant morbidity and mortality. The current paradigm of IPF pathophysiology involves recurrent insults to the alveolar epithelium leading to fibroblast activation corresponding to the characteristic histologic findings of spatiotemporally heterogeneous injury and fibroblastic foci [1]. This cycle of injury and pathologic tissue repair is a complex process resulting from interactions between epithelial, immune, vascular, and other mesenchymal cell populations. There is growing evidence for endothelial abnormalities in patients with pulmonary fibrosis, including recently published results demonstrating loss of capillaries and expansion of an endothelial cell population expressing markers of the bronchial circulation. There have also been recent explorations into the ability of the aged vasculature to repair from injury in the context of fibrosis and the role of sphingolipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) in vascular barrier function and development of fibrosis in pre-clinical models. Here we review recent data on endothelial heterogeneity in both health and disease, with a particular focus on recent publications exploring the endothelium in pulmonary fibrosis.

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Section 1: Normal Lung Endothelium

Adult Anatomy

The adult human lung has two distinct vascular supplies, the pulmonary circulation optimized for gas exchange, and the systemic bronchial circulation which nourishes the lung tissue

itself. The pulmonary circulation is a low-resistance vascular bed which receives the entirety of right ventricular cardiac output. Deoxygenated systemic venous blood is pumped into the pulmonary arteries. Pulmonary arterioles course alongside terminal bronchioles and transition into capillary plexuses around alveolar sacs that participate in gas exchange between the environment, alveolar epithelium, and the vascular system. Oxygen-rich blood then flows from alveolar capillaries to pulmonary veins and into the left atrium. These arteries, capillaries, and veins constitute the "pulmonary circulation."

In contrast, the bronchial circulation receives about 1% of left ventricular output, originating with 2-4 paired bronchial arteries branching directly off the thoracic aorta [2–4]. Unlike pulmonary arteries, bronchial arteries are oxygen-rich and are thought to provide nutritive blood to the conducting airways, including bronchioles, through capillary plexuses around these structures [4, 5]. Initial characterization of the bronchial veins revealed two anatomical categories: 1) "deep bronchial veins" forming rich, "lacy" networks around bronchioles, and 2) "pleuro-hilar veins", which were notable for an extensive subpleural network [6]. Bronchial veins can direct blood either back into the right atrium through the azygous vein which drains deoxygenated blood from the posterior chest into the superior vena cava, or interface with the pulmonary circulation at the post-capillary level [2, 6–8]. While bronchial vessels lack direct association with alveoli, bronchial arteries may influence alveolar function via anastomoses with pulmonary arterioles at the pre-alveolar level [4, 6–9]. Detailed reviews of the bronchial arteries, capillaries, and veins, collectively called the "bronchial circulation," can be found elsewhere [2, 8].

Insights from Single-Cell RNA-Sequencing

Alveolar Capillaries

Integration of single-cell RNA-sequencing (scRNA-seq) with histology has led to a new appreciation for lung endothelial heterogeneity [10–14]. Within the pulmonary circulation, the alveolar endothelium is composed of two intermingled capillary cell types termed aerocyte (aCap) and general capillary (gCap) [10–13]. Aerocytes are expansive, with an average per-cell volume ~5-fold greater than that of gCap cells [10], and are intimately associated with type 1 pneumocytes at alveolar entrances [10], often without intervening pericytes [15, 16]. While the number of gCap cells typically exceeds aerocytes by a ratio of 2 to 5:1 [10, 11], aerocytes likely constitute the majority of alveolar capillary surface area and are therefore pivotal for gas exchange. High expression of membrane-bound carbonic anhydrase 4 (*Ca4*), which catalyzes CO_2 production, distinguishes both aerocytes and gCap cells from the remainder of lung endothelium in humans [10]. The Antarctic icefish, which lacks red blood cells and therefore cannot use red blood cell carbonic anhydrase (CA2) for CO_2 production, uses CA4 to produce CO_2 in its gills [17], suggestive of a possible conserved role for CA4 in CO_2 metabolism in alveolar capillaries.

gCap cells are capable of both self-renewal and aerocyte differentiation, indicating that gCap cells can function as specialized progenitors that replenish capillary endothelium during maintenance and repair [10]. Normal morphogenesis of airway structures and lung vasculature requires locally coordinated signaling pathways [18]. A collection of ligand-receptor pairs are expressed by type 1 pneumocytes (AT1), pericytes, aerocytes, and gCap cells [10, 11, 16], suggestive of paracrine signaling during alveologenesis. For example, AT1-derived VEGFA is essential for aerocyte development [16], possibly via VEGFR2, which is expressed at higher levels in aerocytes than in gCap cells [10, 11, 16]. Additionally, aerocytes express the ligands Apelin (APLN) and Kit-ligand (SCF) while gCap cells express Apelin receptor (APLNR) and KIT [10, 11, 13, 16]. Considering the known pro-angiogenic/proliferative role of APLN [19–23], it is possible that an aerocyte-gCap APLN-APLNR signaling axis promotes regeneration or expansion of pulmonary capillaries, while a SCF-KIT axis maintains the gCap progenitor population by a mechanism similar to that in hematopoietic [24] and epidermal [25] homeostasis. In mice, KIT+ endothelial cells comprise ~60% of endothelium from embryonic day 16.5 to 14 days of age and this frequency is halved to ~30% at 90 days [26], consistent with the notion that stem and progenitor populations generally decline over time [27]. A more recent study that used scRNA-seq similarly observed a ~25% reduction in KIT+ gCap cells during the transition from embryonic to early postnatal life, whereas aerocytes first appear at embryonic day ~16.5 and constitute ~25% of the lung endothelium by postnatal day 3 [28]. Putative paracrine relationships among alveolar cells related to vasomotor control, leukocyte trafficking, antigen presentation, hemostasis, and lipid metabolism are discussed in Gillich et al. (2020) [10].

Bronchial Vasculature

scRNAseq of healthy human lungs has revealed a distinct cluster of endothelial cells that are enriched with transcripts encoding COL15A1, PLVAP, POSTN, as well as venous markers (e.g., ACKR1, VWF, IGFBP7) [11]. Immunolocalization revealed COL15A1+ endothelial cells in subpleural and peri-bronchial regions associated with ACKR1+ venules [11, 12]. These collective findings led to the designation of COL15A1+ endothelial cells as systemic-venous, although there is no evidence to rule out the presence of capillary-like cells within COL15A1+ scRNA-seq clusters. Notably, COL15A1+ vessels correspond to the anatomical location of bronchial veins described previously [6] (discussed above), substantiating their designation as systemic-

venous. We refer to these endothelial cells as bronchial endothelial cells.

While alveolar capillary biology appears largely conserved between mouse and human, components of bronchial vasculature are rare in mice as bronchial arterioles are primarily found in the wall of the trachea and proximal bronchi and are absent in visceral pleura [29]. scRNA-seq studies supported these early observations, reporting that endothelium of bronchial vasculature constitutes ~5-7% of human lung vasculature from explants [11], whereas these cells occur at a much lower frequency (~0.5%) in mouse lungs [11]. Rats, pigs, and dogs, on the other hand, have bronchial vasculatures more similar to that of humans [29, 30]. These differences in vascular structure likely follow differences in airway structure. For example, humans, monkeys, dogs, and cats have several orders of respiratory bronchioles that follow large tracheobronchial airways, whereas analogous respiratory bronchioles are very abbreviated or absent in rats and mice [31].

Section 2: Vasculature in Pulmonary Fibrosis

Over the years, the literature describing the vasculature in pulmonary fibrosis has demonstrated potentially conflicting findings. There have been reports of heterogeneously increased sprouting angiogenesis [32], increased capillary density and endothelial proliferation [33, 34], as well as contrasting observations of endothelial apoptosis and reduced capillary density [33–36]. These seemingly conflicting observations may be explained by the concept of alveolar capillary depletion co-occurring with expansion of a population of endothelial cells expressing bronchial markers (e.g. COL15A1) [12]. Evidence for this phenomenon was identified by scRNA-seq analysis of lung tissue from explanted end-stage IPF patients and healthy donors, which showed marked differences in endothelial composition. The proportion of aerocytes and gCap cells was reduced in IPF [12]. In contrast, the sequencing studies revealed an ~7-fold increased frequency of a population of endothelial cells in IPF expressing bronchial markers [12]. While we do not know the origin or terminus of the expanded cells, scRNA-seq analysis identified this population as belonging to a cluster of endothelial cells derived from systemic vessels within the bronchial circulation [11, 12]. Increased "bronchial" vessel density in IPF lung tissue was substantiated by immunohistochemistry for the bronchial endothelial marker COL15A1 [12].

Markers such as CD31, CD34, and VWF have traditionally been used to describe endothelium in the lung; however, the emergence of endothelial subtype-specific markers [10, 11] is an invitation to more accurately describe vasculature in pulmonary fibrosis. Moving forward, investigation of endothelial apoptosis, proliferation, permeability, association with collagen deposition, or association with specific cell types (e.g., immune, epithelial, fibroblast), should differentiate between bronchial, aCap, and gCap endothelium. Detailed imaging of bronchial, aCap, and gCap endothelial cells in IPF will be needed for insight into the spatial relationship between these cells and the cellular constituents of fibrotic and non-fibrotic microenvironments.

Alveolar Capillaries

The frequencies of AT1 cells, aerocytes, and gCap cells are markedly reduced in affected tissue of IPF patients (Figure 1). Fibroblastic foci show decreased VEGF immunoreactivity relative to surrounding non-fibrotic tissue [36], and VEGF is reduced at the mRNA level in the alveolar wall [35] and at the protein level in BALF [36, 37] in lung tissue from patients with a pathologic diagnosis of usual interstitial pneumonia (UIP). Considering the essential role of AT1-derived VEGF in aerocyte development [16], these findings suggest that the fibrotic microenvironment does not support aerocyte or gCap survival, possibly downstream of AT1 dropout. scRNA-seq data from IPF patients and healthy controls shows a marked upregulation of APLNR and APLN in gCap cells and aerocytes, respectively [12, 14]. It is possible that paracrine endothelial Apelin signaling is upregulated as a compensatory mechanism to regenerate pulmonary capillaries, consistent with evidence for gCap cells as aerocyte progenitors [10]. Selective targeting of gCap cells and aerocytes in animal models using genetic ablation or alteration of gene expression can provide insight into the roles these cells play in the pathogenesis and repair of lung injury and to evaluate the extent to which alveolar capillary dysfunction influences IPF onset and progression. Such studies may reveal targetable factors that promote differentiation, expansion, and incorporation of functional aerocytes within alveolar walls.

Bronchial Vasculature

Expansion of bronchial vasculature in pulmonary fibrosis was first described by Margaret Turner-Warwick [9], who observed numerous atypical branches from subpleural bronchial vessels extending into the lung and communicating with branches of the pulmonary artery [9]. Bleomycin-induced interstitial fibrosis in rats similarly results in expanded bronchial vasculature in both peribronchial and subpleural regions [38]. Subpleural fibrosis is a hallmark of IPF [39] and its quantification may have prognostic value [40]. These collective observations raise the possibility that bronchial vessels not only react to the fibrotic microenvironment but also contribute to disease. Interestingly, a review of recently published scRNA-seq data [12, 14, 41, 42] also suggested expansion of a population of endothelial cells which express bronchial markers (Table 1).

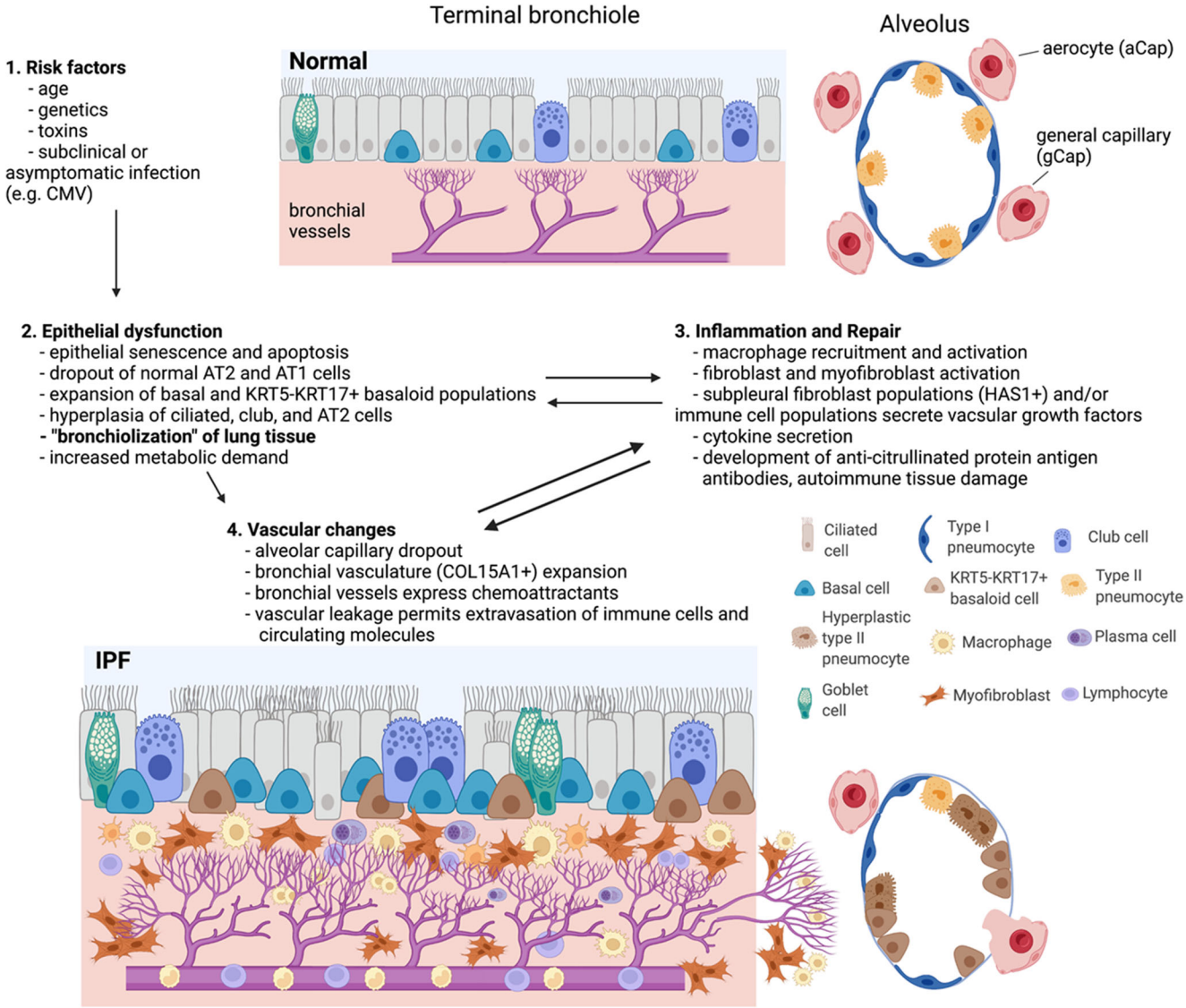

Fig. 1 IPF Pathogenesis: Current Working Model Incorporating Vascular Changes. IPF is believed to occur as a result of epithelial dysfunction, influenced by genetics and environmental factors, leading to fibroblast activation and extracellular matrix accumulation. In addition, there are contributions from the interplay between epithelial dysfunction, inflammation, repair processes and vascular changes. **1**. Risk factors for pulmonary fibrosis include age, genetics, toxins, and viral infections. **2**. Epithelial dysfunction including senescence and loss of AT2 and AT1 cells has been observed, as well as increased numbers of Krt5-Krt17+ transitional cells. **3**. Inflammatory processes, such as macrophage activation, have been identified in fibrotic lungs. In addition, fibroblast activation has been shown to play a major role in fibrosis. **4**. Vascular changes observed in pulmonary fibrosis include loss of alveolar capillaries and expansion of a systemic venous endothelial population expressing bronchial markers

Gene expression patterns observed in bronchial endothelial cells are seen in systemic extra-thoracic vasculature at both homeostasis and fibrotic disease states (Table 1). Vascular elements with specialized function (e.g., pulmonary capillaries, renal glomeruli, blood-retina barrier) acquire an organotypic [43] phenotype during development through spatiotemporal coordination of growth factor gradients and specific cell-cell interactions [16], [44–46] [47–50].

Pathologic tissue repair can fail to recapitulate developmental processes [51–58] and involve expansion of non-specialized endothelium [12, 58–64]. Expansion of non-specialized vasculature in fibrosis may occur in response to hypoxia, increased metabolic demand, and/or growth factors from infiltrating immune or fibroblastic cells [43, 61, 65–71]. In IPF, possible consequences of bronchial vessel expansion include increased vascular leak (PLVAP, ANG2), thrombogenesis (MMRN1), and recruitment of mononuclear phagocytes (POSTN, CCL14, PLVAP, SELE). These effects of bronchial endothelium have the potential to contribute to the chronic pathologic repair process that characterizes IPF (Figure 1).

Expansion of bronchial vasculature has been reported in diseases other than IPF, including lung cancer [72–74],

Table 1 Gene products enriched in bronchial endothelial cells

Gene; protein	Associations with fibrosis or tissue injury	Associations with vasculature	Immune system interactions	Other
POSTN; Periostin	- increased endothelial and subepithelial expression in PH [160] and IPF [161–163] - increased in serum of IPF patients [162] - increased expression in fibrosis of heart [164], kidney [165, 166], adipose [167], and following hypoxic kidney injury [168] - upregulated in response to hypoxia in endothelial and perivascular cells [160, 169, 170] - promotes pancreatic beta-cell regeneration [171]	- supports vascular growth [160, 172–175]	- promotes Th2-mediated allergic responses in airways [176] and skin [177] - Macrophage chemoattractant [178, 179] - promotes macrophage proliferation to increase tissue repair after I/R kidney injury [180]	- POSTN-deficient mice are resistant to bleomycin-induced pulmonary fibrosis with reduced leukocyte infiltrate in BALF [161, 163]
CCL14; C-C Motif Chemokine Ligand 14			- attracts monocytes via CCR1 [181]	
PLVAP; Plasmalemma Vesicle Associated Protein	- increased endothelial expression in pyogenic granuloma [182], cardiac fibrosis [183], and liver cirrhosis ("scar-associated" PLVAP+VWA1+ ACKR1+ cells) [59, 184]	- promotes transcytosis of albumin and macromolecules across endothelium [185, 186] - promotes angiogenesis and inflammation-induced vascular permeability [185–188]	- required for normal leukocyte trafficking in spleen, peritoneum, and lymph nodes [188, 189]- determines the size-selective entry of antigens into lymph nodes [189]	- structural component of caveolar endothelial diaphragms [190, 191]- upregulated by VEGF, positive regulator of VEGF-induced vascular leakage [185]
COL15A1; Collagen type XV Alpha 1 Chain	- cirrhotic human livers show marked elevation of vascular-associated COL15A1 [192], as does the fibrotic stroma of kidneys from patients with diabetic glomerulosclerosis or hypertensive nephrosclerosis [193]	- COL15A1+ capillaries observed in the human placenta, skin, heart, pancreas, kidney glomeruli and associated arterioles [193]. Human fetal lungs lack COL15A1 expression in alveoli, but show high expression in fibrous tissue around developing bronchioles [193]		- involved in development of skeletal muscle capillaries [193, 194]
ANGPT2; Angiopoietin-2	- Elevated serum ANGPT2 levels correlate with clinical disease progression and poor prognosis in IPF patients [195]	- an antagonist of the endothelial receptor Tie2 [196–198]	- promotes inflammation-induced increased vascular leakage through autocrine signaling [197, 198]	- reports of agonistic, endothelial-protective roles for ANGPT2 [199], particularly in lymphatic endothelium [200], have led to the belief that effects of ANGPT2 signaling are context-dependent [196–198]
APOLD1; Apolipoprotein L Domain Containing 1 (*VERGE*; Vascular Early Response Gene)	- upregulated in brain endothelium after ischemic injury and promotes tissue revascularization [201]			- expressed in endothelium of developing tissues and is inducible by multiple stimuli (ischemia, Kainic acid, TNF-α, FGF2) [202]
THBS1; Thrombospondin 1		- activates latent TGF-beta and induces endothelial apoptosis through CD36 [203], which is highly expressed by gCap and aCap cells - can inhibit endothelial nitric oxide signaling [204, 205]		- multiple domains with unique affinities for matrix components, growth factors, and membrane proteins [206] - reduces vascular nitric oxide activity [206]

Table 1 (continued)

Gene; protein	Associations with fibrosis or tissue injury	Associations with vasculature	Immune system interactions	Other
SLC2A3; Solute Carrier Family Member 3 (GLUT3)		- potentiates RhoA-mediated endothelial cell motility [207]		- facilitates cellular uptake of glucose [208]
MMRN1; Multimerin 1		- enhances platelet adhesion and thrombus formation [209]		
CYTL1; Cytokine-Like 1		- proangiogenic factor that signals via autocrine and paracrine mechanisms [210]		
MYC; MYC				- transcription factor that can regulate cell metabolism and growth [211]
HSPG2; Heparin sulfate proteoglycan 2 (Perlecan)	- upregulated in subpleural fibrotic regions in IPF [212] and dermal endothelium of patients with systemic sclerosis [62]			- has five major domains with unique affinities for matrix proteins, integrins, and growth factors [213]
SELE; E-Selectin		- positively regulates slow rolling of mononuclear phagocytes along endothelium during diapedesis [214]		

Abbreviations: *PH*, pulmonary hypertension; *IPF*, idiopathic pulmonary fibrosis; *I/R*, ischemia-reperfusion; *Th2*, T helper 2 cell

pulmonary artery occlusion [75–80], congenital cardiovascular malformations [9, 81], systemic sclerosis (SSc)-associated ILD [9], chronic lung infections (discussed in [82]), chronic thromboembolism ([83] and reviewed in [61]), idiopathic pulmonary arterial hypertension [84], cystic fibrosis [85, 86], and asthma (reviewed in [2]). These diseases share common themes of interstitial lung injury, hypoxia, and/or inflammation with subsequent angiogenesis. It is possible that expansion of bronchial vessels is a generalized and rapid response to lung injury with a resolution phase that includes regeneration of the specialized pulmonary circulation. However, resolution may be impaired in slow-progressing fibrotic diseases such as IPF and SSc-ILD.

Bronchial Vasculature in the Context of Epithelial and Immune Cells

Histopathologically, IPF is characterized by foci of myofibroblast clusters with temporal and spatial heterogeneity. The frequency of AT1 cells is markedly reduced whereas ectopic secretory ($SCGB3A2^{hi}$) and transitional AT2-like (SCGB3A2+/SFTPC+) cells occur [12, 14], consistent with reports of AT2 hyperplasia in IPF [87]. Interstitial and peribronchial regions show marked expansion of a dysplastic KRT5-/KRT17+/KRT8+/TP63+ "aberrant basaloid" population found in IPF patients but not in healthy controls [12, 14]. These dysplastic cells appear to be stalled in a transitional state between AT2 and AT1 stages of differentiation [88]. The metaplastic and dysplastic changes associated with bronchial epithelium, including basal, ciliated, club, and goblet cells [12, 14, 67, 89], is evidence for the well-described phenomenon of "bronchiolization" of the distal lung in IPF [90, 91] (Figure 1). These collective epithelial changes, including expansion of aberrant basaloid cells, are also observed in SSc-ILD [92, 93]. It remains uncertain to what extent the presence of pathologic bronchiolization in IPF is a cause or consequence of the alterations in lung vasculature [12, 14, 92]. Disease-promoting interactions between the expanded endothelial population with bronchial markers and AT1 and/or AT2 cells may occur (Table 1, discussed above). Deeper investigation in this area is warranted considering the critical role of endothelium in pulmonary physiology and the central role of AT1 and AT2 cells in alveolar regeneration and current models of IPF pathophysiology [87].

Acute exacerbations of IPF (AE-IPF) are clinically defined by short-term respiratory deterioration (< 1 month) with evidence of new alveolar infiltrates on high-resolution computed tomography that cannot be explained by infection or edema alone [94]. Despite the clear association between AE-IPF and mortality, there is a paucity of information on the pathophysiology or effective treatment [94]. There are notable similarities between acute respiratory distress syndrome and AE-IPF

including association with endothelial hyperpermeability [95–98], histologic appearance [99–101], and variable response to corticosteroids or other immunosuppressive agents [94, 102]. Consistent with the hypothesis that inflammation may be involved in AE-IPF, IPF disease progression correlates with plasma levels of B-Lymphocyte Stimulator [103], plasma IgA autoreactivity [104], and enlargement of mediastinal lymph nodes [105, 106]. In addition, lungs of a subset of IPF patients harbor ectopic lymphoid aggregates containing B cells [103, 104, 107] and plasma cells [104, 108, 109]. Notably, these lymphoid aggregates are primarily in association with bronchioles [110–112]. Thus, bronchial endothelium may contribute to AE-IPF through expression of pro-inflammatory genes (Table 1, discussed above). In addition, neovessels may be a source of the increased vascular leakage that has been observed in lungs of IPF patients [95–97]. To determine the vascular sources of endothelial hyperpermeability in IPF, future studies can aim to differentiate between bronchial and pulmonary vasculature. Such studies may improve the predictive value of vascular leakage measurements with respect to disease progression. Ongoing clinical trials are assessing Rituximab (B cell depletion), intravenous immunoglobulins, and therapeutic plasma exchange in patients with AE-IPF (NCT03584802 and NCT03286556). Considering the conjoint relationship between inflammation and endothelial hyperpermeability, the bronchial vasculature may be an unappreciated target of anti-inflammatory agents used in AE-IPF.

Novel Findings from Pre-clinical Models

It is well-established that susceptibility to IPF increases with age. Accordingly, studies have used the mouse to identify cellular changes and signaling pathways associated with pulmonary fibrosis in young and aged animals. Intratracheal administration of a single dose of bleomycin in young mice causes pulmonary fibrosis that diminishes within 3 months and is associated with a transient increase in pulmonary capillary density [113]. In contrast, bleomycin administration to aged mice causes sustained pulmonary fibrosis characterized by fibroblastic foci with reduced capillary density in both the short- and long-term [113]. Thus, a transient increase in pulmonary capillary density is associated with healing of injured tissue in mice, whereas pulmonary capillary dropout is observed in chronic fibrosis in both mice and humans [12, 33–36, 113].

Genetic and pharmacologic studies of bleomycin-treated mice have highlighted roles for several signaling pathways that directly involve endothelium and are associated with pulmonary fibrosis:

Sphingolipid Signaling

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are the two bioactive sphingolipid species with well-characterized roles in vascular development, physiology, and disease [50, 96, 114]. S1P signaling through endothelial S1P receptor 1 (S1PR1) is associated with stabilization of maturing vasculature, maintenance of the endothelial barrier and activation of endothelial nitric oxide synthase (eNOS), whereas endothelial LPA signaling increases stress fiber formation, vascular permeability, and sprouting angiogenesis [50, 96, 115]. FACS-sorted lung endothelial cells from young mice show high expression of S1PR1, LPA receptor 4 (LPAR4), and LPA receptor 6 (LPAR6) [50, 116]. The different effects of S1P and LPA on endothelium are likely attributable to differential signaling pathways activated by their respective G protein-coupled receptors; S1PR1 signals through $G_{\alpha i}$/Rac, whereas LPAR1, LPAR4 and LPAR6 signal through $G_{\alpha 12/13}$ RhoA and its target ROCK1/2 [50].

Endothelial S1PR1 expression and signaling declines with age in mice [117]. Mice with deficient endothelial S1PR1 signaling show enhanced bleomycin-induced fibrosis [118], acid-induced lung injury [117], and vascular leakage relative to wild-type animals [117–119]. Consistent with the notion that endothelial S1PR1 is protective, S1PR1 expression is reduced in pulmonary capillary endothelial cells of IPF patients relative to healthy controls [118]. In contrast to the protective effects of endothelial S1PR signaling, LPAR activation likely promotes bleomycin-induced pulmonary fibrosis and vascular leakage through ROCK1/2 [120].

Heterozygous loss of the transcription factor FOXF1 causes a rare, lethal developmental disorder called alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) characterized by respiratory failure shortly after birth [121]. On microscopic examination, alveolar capillaries are sparse and generally fail to associate with alveolar walls which, rather than displaying thin AT1 cells, are predominantly composed of hyperplastic cuboidal AT2 cells [121]. In mice, haploinsufficiency or endothelial-specific deletion of FOXF1 causes alveolar capillary dysplasia with reduced endothelial density, reduced endothelial proliferation and increased apoptosis, enhanced endothelial permeability, susceptibility to pulmonary inflammation and edema, respiratory insufficiency, lethality, and reduced S1PR1 expression [26, 122, 123]. S1PR agonist administration to endothelial FOXF1-deficient mice rescues the lung permeability deficit, prolongs survival, and reduces inflammation [122], suggesting that FOXF1-mediated S1PR1 expression is a key feature of alveologenesis.

Bronchopulmonary dysplasia (BPD) is a chronic condition that can result from use of oxygen therapy in newborns and is associated with long-term health sequelae including impaired lung function [124]. Perinatal mice exposed to hyperoxia

exhibit alveolar simplification and are used to model BPD [26]. By postnatal day 14, lungs of mice exposed to hyperoxia show a marked reduction in KIT+ endothelial cells [26], which may suggest an irreversible loss of gCap progenitor cells. Endothelial-specific deletion of FOXF1 also results in alveolar simplification and marked reduction of KIT+ endothelial cells [26], and this phenotype is exacerbated by exposure to hyperoxia. Adoptive transfer of KIT+ endothelial cells to FOXF1-haploinsufficient mice increases lung angiogenesis and prevents alveolar simplification following exposure to hyperoxia [26]. Like mice, humans showed reduced frequency of KIT+ lung endothelial cells from the perinatal period to adulthood [26]. Examination of scRNA-seq data from lungs of patients with IPF [12, 14, 41, 42] revealed reduced expression of FOXF1 in aerocytes and gCap endothelial cells relative to healthy controls (unpublished observation). Taken together, these data suggest that an endothelial FOXF1-S1PR1 axis is important for normal lung development and may be targeted to improve alveolar function.

Endothelial Nitric Oxide Synthase, Atrial Natriuretic Peptide, and cGMP

In endothelial cells, cGMP accumulates in response to binding of atrial natriuretic peptide (ANP) to guanylyl cyclase-A (GC-A) or activation of eNOS [125–129] and can influence vascular permeability [114, 130, 131]. Inhibitors of phosphodiesterase 5 (PDE5) increase intracellular [cGMP] and are efficacious in the treatment of World Health Organization group 1 pulmonary arterial hypertension [132]. Like S1PR1 expression, endothelial eNOS mRNA levels decline with aging in mouse lungs [113, 117]. Bleomycin administration to young $eNOS^{-/-}$ mice results in sustained pulmonary fibrosis [113] whereas endothelial eNOS over-expression attenuates bleomycin-induced fibrosis [133]. Similarly, administration of ANP [134], a PDE5 inhibitor [135], or over-expression of endothelial GC-A [134] each attenuates bleomycin-induced pulmonary fibrosis. PDE5 inhibition results in reduced RhoA and ROCK signaling activity in lungs of bleomycin-treated animals [135], consistent with the notation that LPAR-Rho/ROCK signaling promotes fibrosis. These data imply that eNOS and GC-A promote pulmonary capillary function in the context of injury-induced fibrosis. The extent to which perivascular cells play a role in mediating beneficial effects of nitric oxide or GC-A signaling in pulmonary capillaries (e.g., via constriction or relaxation) is not clear and warrants further investigation.

Lymphatics in Pulmonary Fibrosis

Lymphatic vasculature plays an essential role in fluid homeostasis and leukocyte trafficking. Just as blood endothelium is specialized to accomplish unique tasks [136, 137], lymphatic vessels vary with respect to morphology, permeability, mural cell coverage, intravascular pressure, and gene expression to permit vessel type-specific and organ-specific functions [138–142].

In the normal human lung, lymphatic vessels are primarily found in the pleura, subpleural, interlobular septa, and in association with bronchovascular bundles [143]. Bronchovascular bundles are also the location of adventitial fibroblasts that express VEGFD [13], a potent angiogenic and lymphangiogenic factor [144, 145].

Most studies of lung tissue from IPF patients report a direct relationship between lymphatic vessel density and fibrotic severity [146–148], with one exception that used a different anti-podoplanin antibody to identify lymphatic vessels [149], which may contribute to an alternate conclusion (reviewed in [150] and [143]). Murine models of pulmonary fibrosis consistently report increased pulmonary lymphatic vessel density [151] (reviewed in [150]). Baluk et al. (2020) found increased pulmonary lymphatic vessel density in models induced by bleomycin and telomere dysfunction.[151]. However, induction of pulmonary lymphangiogenesis using a gain-of-function approach suggested a protective role for lymphatics in pulmonary fibrosis [151].

A study of bleomycin-induced pulmonary fibrosis found induction of LYVE1, a canonical marker of lymphatic endothelium, in pulmonary blood vessels (vascular endothelium) [150]. This finding highlights the importance of marker selection when studying lymphatic vessels and underscores our incomplete understanding of pulmonary lymphatics in health and fibrosis. Integration of scRNA-seq with histology to describe lymphatic endothelial subtypes in healthy and fibrotic human lung tissue will help close this knowledge gap.

Conclusions

The lung vasculature of patients with IPF has recently been characterized at single cell resolution and shows loss of specialized pulmonary capillary endothelial cells (aerocytes, gCap cells) as well as expansion of a population of vascular cells expressing markers of bronchial endothelium. Expansion of these endothelial cells could potentially occur in response to increased metabolic demand from the bronchiolized fibrotic microenvironment, which includes airway cell dysplasia and metaplasia, fibroblast/myofibroblast activation, immune cell infiltration, as well as regional hypoxia downstream of alveolar dysfunction. Neovessels may have a hyperpermeable phenotype that, along with expression of chemoattractants, contributes to inflammation. However, the role of this expanded endothelial population in the process of fibrogenesis lacks clarity.

Loss of organ-specific vascular elements or function appears to occur not only in pulmonary fibrosis but also in a number of other disease states including liver cirrhosis, diabetic retinopathy, macular degeneration, glaucoma, and to some extent in focal segmental glomerulosclerosis and experimental nephritis [12, 58–60, 152–155] suggestive of a shared relationship among failed regenerative processes. Following IPF patients longitudinally to determine the chronologic relationship between expanded bronchial-like endothelium and fibrotic change could help determine whether these vessels have a causative role in disease progression. One such example would be evaluating for the presence of this expanded endothelial population in patient cohorts with early interstitial lung abnormalities (ILA) which can progress to clinically significant IPF [156]. In addition, these studies may reveal whether expanded abnormal vasculature can predict prognosis among patients. If so, these vessels could potentially be tracked non-invasively using MRI or PET probes [96, 157] targeting uniquely associated markers such as extracellular COL15A1.

Regardless of a causal role for vascular dysfunction in precipitating tissue injury, a lack of organ-specific vasculature may perpetuate fibrosis and impair beneficial healing responses. Several proteins with defined roles in endothelial cells (S1PR1, ROCK1/2, FOXF1, eNOS, guanylyl cyclase-A) significantly influence lung development, function, and response to injury. Aging is associated with reduced frequency of KIT+ gCap-like progenitor cells [26], as well as reduced expression of genes that promote lung healing/regeneration in alveolar capillaries.

Developmental studies have shed light on the signaling interactions between endothelial cells, epithelial cells, mesenchymal cells, and pericytes that regulate alveologenesis [15, 18, 26, 122, 123, 158, 159]. Better understanding of alveolar capillary biology, including responses to fibrosis, may lead to endothelial-targeted therapies that improve survival and function of specialized aerocytes and gCap cells, as was demonstrated in a proof-of-concept experiment that infused KIT+ endothelial cells into FOXF1$^{+/-}$ mice with hyperoxic lung injury [26]. This paradigm of endothelium-targeted therapy to enhance organotypic vascular function may be generally beneficial in diseases characterized by progressive organ fibrosis.

Declarations

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