Original Article

Endoplasmic reticulum stress, a new wrestler, in the pathogenesis of idiopathic pulmonary fibrosis

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Abstract: Idiopathic pulmonary fibrosis (IPF) has attracted extensive attention for its unexplained progressive lung scarring, short median survival and its unresponsiveness to traditional therapies. Despite extensive studies, the mechanisms underlying IPF pathoetiologies, however, remain poorly understood. Recent advances delineated a potential function of endoplasmic reticulum (ER) stress in meeting the need of fibrotic response, which pinpointed a critical role for the unfolded protein response (UPR) pathways in IPF pathogenesis. In this review, we highlight the effect of ER stress and the activation of UPR on the survival, differentiation, function and proliferation of major profibrotic cells in lung tissues during the course of IPF, and discuss the feasibility whether targeting UPR components could be an orientation for developing effective therapeutic strategies against this devastating disorder in clinical settings.

Keywords: IPF, ER stress, UPR, profibrotic cells, pathogenesis

Introduction

Idiopathic pulmonary fibrosis (IPF) is a disease associated with severe lung dysfunction by affecting gas exchange that often bears fatal consequences [1]. The survival median range for the affected patients is about three years after the initial diagnosis [2]. The incidence of IPF has been recognized with a profound increase in elderly patients (50-70 years old), particularly in those with a history of cigarette smoking [3, 4]. There is evidence that males are more vulnerable to IPF than females [1, 5]. Despite past extensive studies, the mechanisms underlying IPF, however, remained enigmatic.

The endoplasmic reticulum (ER) is a special organelle in eukaryotic cells characterized by an interconnected network of flattened sacs or tubes encased in membranes. In general, ER is responsible for the correct folding and transport of synthesized proteins in vesicles to the Golgi apparatus. However, factors like calcium depletion, redox homeostatic alteration, nutrient deprivation and environmental insults (e.g., viral infection) can affect the folding processes of synthesized proteins, leading to an increase of unfolded proteins, a state of ER stress that triggers the activation of the unfolded protein response (UPR) [6]. To date, ER stress and UPR signaling have been recognized implicated in the pathogenesis of many complex disorders such as diabetes and neurodegenerative diseases. However its involvement in diseases associated with fibrotic remodeling of internal organs including heart, kidneys, liver, gastrointestinal tract and lungs is just recently emerging [6-11]. We, therefore, in this review, will discuss with focus on its impact on major profibrotic cells that contribute to the pathogenesis of pulmonary fibrosis. Our purpose is to deepen and expand our understanding of the mechanisms underlying the initiation and progression of IPF, thereby developing better therapeutic strategies against this devastating disorder in clinical settings.
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Figure 1. Overview of fibrogenesis in IPF. In the early phase of fibrogenesis, epithelial and/or endothelial damage caused by a variety of irritants can initiate an anti-fibrinolytic coagulation cascade, temporarily plugging the damaged vessel with platelets and fibrin-rich clots to quickly restore homeostasis. Meanwhile, thrombin and the injured epithelium can directly evoke fibroblast activation and promote fibroblast differentiation into collagen-producing myofibroblasts. After a short period of time, clot-forming responses rapidly progress into a phase, in which many inflammatory cells such as macrophages, neutrophils, and lymphocytes are recruited into the injured site, where they secrete copious amounts of cytokines to eliminate the inciting factor whilst activating the resident quiescent fibroblasts into myofibroblasts. However, once an imbalance in cytokine production coupled with dysregulated cellu-
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In general, a wound-healing response is consisted of three distinct stages: injury, inflammation and repair. In the current paradigm for IPF pathogenesis, pulmonary fibrosis progresses as a final pathological outcome of aberrant wound healing responses to persistent lung injury (Figure 1), as it is characterized by the excessive extracellular matrix (ECM) deposition in the lungs [12-15]. In the early stage, events such as ER stress, and molecular mediators such as excessive transforming growth factor β (TGF-β) activation and a variety of chemokines release, induce epithelial cell dysfunction or apoptosis, which then activate resident fibroblasts to proliferate for injury repair. Epithelial-to-mesenchymal transition (EMT) along with fibrocyte recruitment and differentiation is also considered as a pivotal feature relevant to pulmonary fibrotic remodeling [16-20]. Epithelial death would also recruit inflammatory cells such as macrophages, which then produce cytokines or chemokines to generate a microenvironment in favor of fibrosis for injury repair. As this process continues, abnormal quantities of matrix components would be produced, which then trigger an excessive deposition of scars in the lung tissues [18, 21]. Furthermore, the pathologically remodeled matrix or epigenetic changes within fibroblasts may lead to a feed-forward loop of mesenchymal cell activation and progressive fibrosis [22, 23]. Collectively, persistent irritants contribute to a cascade of abnormal regulatory mechanisms to cause vast pulmonary epithelial apoptosis, continuous fibroblast activation and increased myofibroblast differentiation, which then lead to excessive ECM deposition and distort lung tissue architecture, ultimately, resulting in pulmonary fibrosis and respiratory failure.

ER stress and the unfolded protein response (UPR)

As mentioned above, ER is an organelle responsible for proper folding of membrane and secreted proteins, lipid biosynthesis, glycogen production and storage, as well as intracellular calcium homeostasis [24, 25]. Under physiological condition, the nascent proteins enter ER lumen where they undergo a chaperone-based folding, together with intricate polypeptide modifications, including N-linked glycosylation, disulfide bond formation, proline cis-trans isomerization and so on [26-28]. The proper function of proteins requires correct folding and post-translation modification, which is assisted in part by chaperone proteins such as calnexin, calreticulin (CRT), the Hsp70 family member immunoglobulin heavy-chain-binding protein (BiP, also named as glucose regulated protein 78, GRP78). However, disturbances in redox regulation, glucose deprivation, viral infection or calcium metabolism are proved to bring about aggregation of unfolded or misfolded proteins along with ER stress [29-31]. Upon the initiation of ER stress cascade, unfolded protein response (UPR), integrated stress response (ISR) and ER-associated degradation (ERAD) are activated, which are aimed to halt protein translation, improve protein folding, sustain cellular homeostasis, and avoid cell death from accumulation of unfolded or misfolded proteins [32-35]. Nevertheless, growth arrest and cell death through apoptosis would occur once the disruption is overwhelming that these objectives could not be achieved within a certain time span [36, 37].

Generally, the UPR pathways are governed by the coordinated action of three ER transmembrane stress sensors: PKR-like ER kinase (PERK; also known as EIF2ZAK3), activating transcription factor 6α (ATF6α) and inositol-requiring enzyme 1α (IRE1α; also known as ERN1) [25, 38]. PERK and IRE-1 share similar ER luminal domain structures and a cytosolic Ser/Thr kinase domain, and are activated by autophosphorylation. In contrast, ATF6α contains a cytosolic cyclic AMP response element-binding protein (CREB)-ATF basic leucine zipper domain, and is activated by proteases [39]. Under unstressed homeostatic conditions, these three proteins maintain each in an inactive state through binding to the molecular chaperone BiP (GRP78) [40]. Once aberrant proteins accumulated in ER, more available BiP is in demand to dissociate from the ER stress sensors to interact with the exposed hydrophobic regions of these proteins [41, 42], thereby releasing the stress sensors to activate the cascade of events designed to protect the cell from ER stress (Figure 2).
Figure 2. Schematic illustration of ER stress and the activation of three UPR pathways. Under stressed condition, BiP dissociates from the ER stress sensors owing to aggregation of unfold or misfolded proteins in ER lumen, which releases the stress sensors to initiate downstream signaling. Activated PERK undergoes autophosphorylation and dimerization and subsequently inhibits ribosome assembly by phosphorylating the α-subunit of eukaryotic translational initiation factor 2 (eIF2α). Once becomes phosphorylated, eIF2α not only suppresses protein translation, but also upregulates the expression of activating transcription factor 4 (ATF4), which would induce the transcription of Golgi proteases (S1P and S2P).
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Although all three UPR pathway sensors are activated to alleviate ER stress and sustain the cellular homeostasis, cell death pathways such as caspase-4 (and its murine homolog caspase-12) and C/EBP homologous protein (CHOP), are activated by prolonged or overloaded ER stress [25, 43-45]. Caspase-4 is found in the ER membrane and activates cell death pathways through caspase-3 and caspase-9 [45]. CHOP, a major transcriptional factor for regulation of cell death under ER stress, can be activated by all three UPR pathways [46, 47], resulting in the reduction of anti-apoptotic mitochondrial protein Bcl-2, which would favor a pro-apoptotic intention at mitochondria associated with mitochondrial damage, cytochrome c release and caspase 3 activation [48].

The implication of profibrotic cellular ER stress during the course of IPF

Lung parenchyma constitutes all sorts of cell types including alveolar epithelial cells, macrophages, (myo)fibroblasts, and so forth that contributes to the progression of IPF. The past decade witnessed convincing potential links related to ER stress in these profibrotic cell types during the course of IPF [49]. In this section, we seek to bring all evidence together supporting the potential role of ER stress in those critical cells during the course of IPF development and progression.

Alveolar epithelial cells (AECs)

The alveolar surface is covered by large flat type I alveolar epithelial cells (AECIs) and small fraction of type II alveolar epithelial cells (AECIIs). The former are the main cell type to mediate lung gas exchange function based on their location in approximation to the pulmonary capillary endothelium, while the latter are responsible for the biosynthesis of pulmonary surfactant and for the maintenance of alveolar integrity due to its capability in cellular division [50-53]. Early reports suggest that ER stress-induced epithelial cell dysfunction is perceived as a considerable aspect in the pathophysiology of IPF.

AEC apoptosis

Several lines of evidence convincingly show that AECs appear to be particularly sensitive to apoptosis following lung injury in IPF [54-56]. Increasing data support that apoptosis of AECs is considered as a key incident initiating and propagating pulmonary fibrosis in the lung parenchyma [57-59]. Indeed, ER stress in AECs can be induced by numerous stimuli, among which exon4 deletion and L188Q substitution of SFTPC as well as SFTPA2 are classically identified in IPF patients [46, 60-63], and AECs are more prone to apoptosis after bleomycin treatment. In 2008, studies conducted by Mulugeta and colleagues for the first time provided evidence that a severe ER stress in AECIIs lining the areas of fibrosis was likely to underlie the execution of the intrinsic apoptosis pathway in patients with IPF [64]. Intriguingly, herpesvirus proteins (CMV, EBV, and KSHV) could be also detected in the same AECs that show evidence of ER stress and UPR activation, implicating a potential role of herpesviruses in IPF progression through induction of this pathway [65]. However, no typical IPF characteristics can be noted under conditions that AECIIs only possess the L188Q substitution of SFTPC or treat with tunicamycin alone [66]. Other than the classical bleomycin-induced pulmonary fibrosis animal model, the murine model for amiodarone (AD)-induced lung fibrosis has also revealed that intratracheal aerosol administration of AD causes an interstitial fibrosis in C57Bl/6 mice accompanied by increased AEC apoptosis, lysosomal stress and ER stress [67]. Furthermore, treatment of A549 cells, a human lung adenocarcinoma epithelial cell line, with amosite asbestos fibers induces AEC ER stress as evidenced by the increased expression of ER stress-related proteins such as ER degradation enhancing α-mannosidase-like protein (EDEM).
and CHOP), and similarly, ER Ca\(^{2+}\) release along with intrinsic apoptosis were manifested in rat primary-isolated AECIIs following the treatment [68].

As noted above, IPF is a hard-to-diagnose fatal interstitial lung disease with a poor response to traditional therapies, and therefore, early diagnostic biomarkers are urgently needed. There is evidence that circulating caspase-cleaved cytokeratin-18 (Cck-18), a cytoskeletal protein originally found in pseudostratified and simple epithelia [69], could serve as a marker of AEC apoptosis and UPR activation in IPF patients, and elevated circulating levels of Cck-18 in patients also portrays it as a useful IPF diagnostic biomarker [70]. More recently, studies revealed that ER stress-induced intrinsic apoptosis in human AECs is mediated by the generation of angiotensin (ANG) II, which can be attenuated by its counter regulatory antiapoptotic peptide ANG1-7 [71], implicating ANG1-7 possibly held therapeutic potential for the treatment of ER stress-induced pulmonary fibrosis [72]. Similarly, inhibition of CaMKII [73] and inhibitor of synoviolin (LS-102) [74] have been found to provide protection against pulmonary fibrosis through suppressing ER stress and AEC apoptosis. Moreover, DNA damage in AECIIs caused by bleomycin or doxorubicin, and ER stress caused by tunicamycin, upregulate the expression of osteopontin (OPN) in AECIIs in an ERK-dependent manner, indicating the probable function of OPN as a survival factor for AECIIs during the early stage of IPF [75]. As the massive target spots emerge, due to several critical shortages such as insufficiency in sample capacity and the lack of robust clinical trials, further studies are still required in order to confirm these therapeutic potentials.

**Epithelial-to-mesenchymal transition (EMT)**

EMT is defined as a process by which epithelial cells lose their cellular polarity and cell to cell adhesion, and then gradually convert to mesenchymal stem cells following activation by specific growth factors, of which TGF-β is the prototype [19]. Recently, EMT, in which AECs undergo transition to ECM-producing (myo) fibroblasts, has received intense attention in the pathogenesis of IPF. The response to TGF-β in EMT during pulmonary fibrosis is mediated predominantly via Smad-dependent pathways, although non-Smad signaling pathways have also been discovered under certain conditions [76]. Upon TGF-β stimulation, the complex composed of phosphorylated Smad2, Smad3 and Smad4 translocate into the nucleus, where it binds to transcriptional factors and modulate transcriptional responses. As a result, the inhibitory Smad, Smad7, can reverse fibrosis in renal and lens epithelia in a Smad-dependent manner [76-79]. In contrast, the mitogen-activated protein kinase (p38MAPK), Src family protein kinases, phosphatidylinositol 3'-OH kinase (PI3K/Akt), Rho/Rac, Wnt/β-catenin, as well as ERK have been suggested implicated in TGF-β-induced non-Smad-dependent EMT [76, 80, 81]. However, the distinction between Smad-dependent and nondependent pathways is difficult due to their significant cross talk between each other.

There is emerging evidence that accumulation of mutant SP-C protein or thapsigargin- or tunicamycin-induced ER stress in A549 and RLE-6TN cells contributes to pulmonary fibrosis through EMT at least in part dependent of Smad2/3 or Src-related pathways [82, 83]. It has also been noted that the IRE1/XBP1 pathway promotes EMT by means of mediating snail expression in pulmonary fibrosis [84]. Furthermore, Bax inhibitor-1 (BI-1) [85], an anti-apoptosis protein capable of inhibiting Bax activation [86], phenylbutyric acid (PBA), a low molecular weight fatty acid [87], and melatonin, a potent antioxidant mainly secreted by pineal gland [88], are also found to inhibit ER stress and EMT during bleomycin-induced pulmonary fibrosis in mice. They regulate the Ca\(^{2+}\) dynamic status and the expression of calnexin, and thereby modulating NF-κB signaling, and attenuating the activation of ER stress-related proteins. Collectively, ER stress is likely implicated in EMT during IPF development, additional studies, however, would be necessary to reach a conclusive remark.

**Macrophages**

Macrophages are present in almost all tissues of the body and play a critical role in innate and adaptive immunity in response to the change of microenvironment [89]. Generally, pulmonary macrophages can be divided into alveolar macrophages (AMs) that strategically distributed in the airways, and interstitial macrophages (IMs) that positioned in the lung parenchymal tissues [90, 91]. Macrophages that mainly produce...
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Pro-inflammatory cytokines are called classically activated macrophages (M1), which can be activated either by IFN-γ or LPS [92], whereas those activated by IL-4 or IL-13 associated with attenuation of inflammation and enhancement of tissue repair are referred as alternatively activated macrophages (M2) [93, 94]. During activated immune responses of IPF, different classes of macrophages with distinct functions are now recognized.

Over past few years, our understanding of the mechanisms underlying pulmonary fibrosis has been further expanded by the discovery of ER stress in pulmonary macrophages [95]. It was initially noted that mesenchymal stem cells (MSCs) hold promise as a novel treatment in IPF through the secretion of stanniocalcin-1 (STC1). Specifically, STC1 regulates oxidative and ER stress along with reduced TGF-β production in pulmonary macrophages to attenuate fibrosis in the lung [96]. Indeed, it has been well recognized that predominant infiltration of M2 macrophages in the areas of lung fibrosis acts as a vital regulator of fibrogenesis during IPF development and progression [97-99]. Similarly, the role of IL-4 and IL-13-mediated signaling in M2 macrophage polarization has been well established both in vitro and in vivo [100]. IL-4 receptor-α (IL-4Rα) signals through a JAK-STAT6 pathway to regulate the expression of numerous genes essential for M2 polarization such as arginase 1 (Arg1), macrophage mannose receptor 1 (also known as Cd206), resistin-like-α (also known as Fizz1) and chitinase 3-like 3 (also known as Ym1) [101, 102]. IL-4 may also induce the activation of phosphoinositide 3-kinase (PI3K), as evidenced by that phosphatidylinositol-3,4,5-trisphosphate, a product of PI3K, can be dephosphorylated by the phosphatase SHIP, and mice deficient in Ship manifest impaired M2 polarization [103].

Interestingly, recent evidence indicates that ER stress probably modulates the activation of M2 macrophages [104]. During the past few years, our laboratory has been focused on the effect of ER stress on fibrogenesis. In a model of unilateral ureteral obstruction (UUO)-induced renal fibrosis, we first noted that mice deficient in Chop were protected from UUO-induced renal fibrosis [105]. We demonstrated evidence that Chop deficiency provides protection for tubular cells against UUO-induced apoptosis and secondary necrosis along with attenuated Hmgb1 passive release and active secretion. As a result, loss of Chop repressed Hmgb1/TLR4/NF-kB signaling, thereby inhibiting UUO-induced IL-1β production. Subsequently, the IL-1β downstream Erk1/2 activity and its related c-Jun activity were reduced, which led to attenuated production of TGF-β1 along with repressed renal fibrosis following UUO insult. Consistent results were reported by Tanaka and colleagues [106]. We, therefore, next expanded our discoveries into the pathogenesis of IPF [107]. We first demonstrated evidence that pulmonary fibrosis manifests altered Chop expression and ER stress in both IPF patients and animals with bleomycin-induced pulmonary fibrosis. In consistent with these observations, mice deficient in Chop were protected from bleomycin-induced lung injury and fibrosis. Specifically, loss of Chop significantly attenuated TGF-β production along with reduced M2 macrophage infiltration in the lung following bleomycin induction. Mechanistic studies revealed that Chop deficiency suppressed M2 program in macrophages, which then attenuated TGF-β secretion. Loss of Chop enhanced the expression of SOCS1 and SOCS3, thereby inhibiting STAT6/PPARγ signaling that is essential for macrophage M2 program [107]. In sharp contrast to the above reported data, Ayauband colleagues held the viewpoint that Chop plays a role in bleomycin triggered macrophage apoptosis, which then protects Grp78−/− mice from bleomycin-induced lung injury and fibrosis [108]. Nevertheless, no matter what point of view is more persuasive, both of which suggested the implication of ER stress in modulating macrophage polarization, which contributes to the pathogenesis of pulmonary fibrosis.

(Myo)fibroblasts

New discoveries in rodent model have revealed that mesenchymal fibroblasts are essential for forming vascular network, sensing damage, recruiting inflammatory cells, as well as remodeling the extracellular matrix of body organs, which are beneficial by maintaining physiological tissue homeostasis. However, injuries, infections and cellular damage trigger differentiation of fibroblasts into activated myofibroblasts that drive pathological inflammation and excessive extracellular matrix deposition, ultimately leading to tissue fibrosis with progressive scarring [109].
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The pathologic hallmarks of IPF are the activation and proliferation of fibroblast-like cells and differentiation of myofibroblasts in the lung tissues [110], which not only arise from lung resident fibroblasts, but also derive from circulating fibrocytes and bone marrow-derived progenitor cells [111-113]. Fibroblast migration, proliferation, myofibroblast differentiation and ECM accumulation, are triggered by epithelial cell dysfunction and aberrant epithelial-mesenchymal signaling, and regulated by various cytokines, especially TGF-β [112]. Although advances have been made in understanding of the pathogenesis in (myo)fibroblasts during IPF, many crucial mechanisms underlying disease etiologies remain unclear.

In 2012, Baek and colleagues reported that altered GRP78 expression was noted not only in AECs but also in fibroblasts in lung tissues from IPF patients [114], implying ER stress may be also critical in fibroblastic differentiation over the evolution of pulmonary fibrosis. Indeed, studies both in mouse and human fibroblasts revealed that TGF-β1 substantially facilitated the production of ER stress-associated proteins (GRP78, XBP-1, and ATF6α) along with high levels of α-SMA and collagen type I expression. In line with these crosslinks, the 150-kDa oxygen-regulated protein (ORP150), one of ER chaperones, could promote bleomycin-induced pulmonary fibrosis via augmenting pulmonary levels of TGF-β1 and myofibroblasts [115]. In support of this notion, mouse embryonic fibroblasts (MEFs) isolated from Crt−/− mouse and human IPF lung fibroblasts with knockdown of CRT by siRNA impaired TGF-β-induced collagen trafficking and matrix assembly in a Ca2+-dependent manner [116]. Together, those data establish a novel mechanism in which ER stress modulates fibroblast proliferation and myofibroblast differentiation contributing to the development of pulmonary fibrosis.

Summary and perspectives

We reviewed recent studies relevant to major profibrotic cells in lung tissues and collected evidence that ER stress may act as a critical player during the initiation and progression of pulmonary fibrosis (Figure 3). Those studies demonstrated feasibility that targeting ER...
stress could be a viable therapeutic strategy against IPF in clinical settings. While these discoveries are exciting, many questions remain unsolved regarding the exact mechanisms of ER stress and UPR pathway in IPF pathogenesis. However, the development of therapeutic agents that interfere with specific components of the UPR pathway would be useful tools to fully establish the role of ER stress in IPF pathotiology, which would ultimately pave new therapeutic avenues for this devastating disorder.

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Disclosure of conflict of interest

None.

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