Emerging evidence for endoplasmic reticulum stress in the pathogenesis of idiopathic pulmonary fibrosis

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ENDOPLASMIC RETICULUM (ER) stress is caused by conditions that perturb the processing and folding of proteins, resulting in the accumulation of misfolded proteins in the ER and activation of the unfolded protein response (UPR; Ref. 68). Several diseases have been linked to misfolded proteins, and over the past decade accumulating evidence suggests a role for ER stress in idiopathic pulmonary fibrosis (IPF; Refs. 29, 35). Initial observations for such an association between ER stress and interstitial lung disease came from evaluation of families with surfactant protein C (SFTPC) mutation associated familial interstitial pneumonia (FIP), the familial form of IPF, in a family with a mutation in surfactant protein C (SFTPC). Subsequent studies involving lung biopsy specimens revealed that ER stress markers are highly expressed in the alveolar epithelium in IPF and FIP. Recent mouse modeling has revealed that induction of ER stress in the alveolar epithelium predisposed to enhanced lung fibrosis after treatment with bleomycin, which is mediated at least in part by increased alveolar epithelial cell (AEC) apoptosis. Emerging data also indicate that ER stress in AECs could impact fibrotic remodeling by altering inflammatory responses and inducing epithelial-mesenchymal transition. Although the cause of ER stress in IPF remains unknown, common environmental exposures such as herpesviruses, inhaled particulates, and cigarette smoke induce ER stress and are candidates for contributing to AEC dysfunction by this mechanism. Together, investigations to date suggest that ER stress predisposes to AEC dysfunction and subsequent lung fibrosis. However, many questions remain regarding the role of ER stress in initiation and progression of lung fibrosis, including whether ER stress or the UPR could be targeted for therapeutic benefit.

familial interstitial pneumonia; herpesvirus; interstitial lung disease; surfactant protein-C; unfolded protein response

ER Stress and the UPR

The ER is involved in proper folding of membrane and secreted proteins, production of steroids, synthesis of lipids, storage and production of glycogen, and calcium homeostasis (39). Secreted proteins are initially delivered to the ER as an unfolded polypeptide chain. These polypeptides are correctly folded into functional three-dimensional conformations, assembled and glycosylated, and then proceed through the secretory pathway. In normal conditions, folding of proteins in the ER is assisted by chaperone proteins such as immunoglobulin heavy-chain-binding protein (BiP), also known as glucose regulated protein-78 (GRP78). However, when a cell is under stress due to factors such as calcium depletion, metabolic stress, reduced energy stores, elevated protein synthesis, or expression of mutant proteins, activation of the UPR can occur (46). The UPR is designed to improve protein folding, maintain cellular homeostasis, and prevent cell death from accumulation of misfolded proteins that can aggregate and interfere with...
basic cellular functions (24, 37, 68, 70). The UPR functions through mechanisms that reduce protein translation, increase expression of metabolism and redox proteins, enhance ER chaperone production, and promote protein degradation (11, 18, 19, 30, 36, 38, 44). When these UPR mechanisms fail or if ER stress is too severe, it may lead to growth arrest and cell death through apoptosis.

The UPR pathways are governed by three ER transmembrane proteins: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE-1; Refs. 24, 68) (Fig. 1). In the unstimulated state, these three proteins are bound by BiP and maintained in an inactive state (45). BiP/GRP78 is the predominant ER chaperone that belongs to the family of heat shock proteins and facilitates protein folding in the ER. Correctly assembled proteins are released from BiP and are transported to the Golgi apparatus. When abnormally folded or improperly assembled proteins remain bound to BiP, they are retained within the ER or degraded. With protein accumulation in the ER, BiP is sequestered away from the three sensors (6), allowing them to assume active conformations and initiate signaling cascades designed to protect the cell from ER stress (68).

PERK senses accumulation of misfolded proteins in the ER and, once activated, undergoes autophosphorylation and dimerization (6, 41). The activated form of PERK phosphorylates and inactivates its only identified target, the α-subunit of eukaryotic translational initiation factor 2α (eIF2α). In all eukaryotic cells, initiation of protein synthesis requires the eIF2 complex and phosphorylation of eIF2α inhibits the initiation of protein synthesis. eIF2α phosphorylation also regulates ATF4-dependent expression of ATF3 and C/EBP homologous protein (CHOP), genes involved in amino acid metabolism, and genes that promote glutathione biosynthesis (11, 18, 19, 30, 44). In states of chronic ER stress, ATF4-dependent induction of growth arrest and DNA damage inducible gene 34 (GADD34) has been shown to dephosphorylate eIF2α, allowing translational recovery (62).

When BiP is released from ATF6 during ER stress, site 1 and site 2 proteases cleave ATF6 (71, 88), releasing the cytoplasmic domain into the cytosol. The cleaved domain migrates into the nucleus where it binds to cis-acting ER stress response elements (ERSE) and activates the transcription of ER protein-folding chaperones such as BiP, GRP94, calreticulin, calnexin, and protein disulfide isomerase (PDI; Refs. 66, 83, 88, 91). ATF6 exists in two isoforms, ATF6α and ATF6β, in mammalian cells and also induces the expression of X-box binding protein 1 (XBP-1), a transcription factor activated by IRE-1 (38, 91).

IRE-1 is a transmembrane protein that has intrinsic serine/threonine kinase and endonuclease activity that is activated in response to ER stress. In mammals two homologues of IRE-1 exist, IRE1α and IRE1β, which homodimerize upon release from BiP. Dimerized IRE-1 possesses an RNase domain, which cleaves a 26-nucleotide intron sequence from the transcription factor XBP-1. Spliced XBP-1 translocates to the nucleus and binds to ERSE (different from ATF6-binding site) and promotes the transcription of ER-associated degradation (ERAD) target genes such as ER degradation enhancing α-mannosidase-like protein (EDEM; Refs. 1, 50). Interestingly, sustained IRE-1 kinase activation can cause oligomerization of RNase domains of IRE-1, resulting in relaxed specificity of the RNase (16). This change allows degradation of many ER-localized mRNAs (in addition to XBP-1) and has the potential to alter the outcome of the ER stress response.

Induction of the ERAD pathway is an important component of the ER stress response. The exact mechanism by which ERAD recognizes and degrades misfolded proteins is not clear but is divided into five steps: 1) recognition of misfolded proteins by EDEM, PDI, or BiP; 2) translocation of the misfolded proteins across the ER membrane into the cytoplasm through translocons mediated via Sec61; 3) ubiquitination of the misfolded proteins by a sequence of enzymatic reactions mediated by E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase; Refs. 28, 90);...
4) deglycosylation; and 5) transport into the proteasome for degradation (36, 38, 90).

All three UPR pathway sensors are activated to attenuate ER stress and protect the cell. However, prolonged or severe ER stress can result in cellular apoptosis through both extrinsic and intrinsic pathways involving calcium influx from the ER to the cytosol as well as signaling cascades initiated by the UPR through activation of ER bound caspase-4 (and its mouse homolog, caspase-12; Refs. 21, 63, 68, 77). Caspase-12 is activated under ER stress and mediates cell death pathways through caspase-3 and caspase-9 (92). Another transcription factor involved in ER stress induced apoptosis is CHOP, which is activated by all three UPR pathways, as ATF4, ATF6, and XBP-1 all have binding sites in the CHOP gene promoter (17, 43). c-Jun NH2-terminal kinase (JNK) has also been implicated in UPR-mediated apoptosis (58, 81). The manner by which JNK leads to apoptosis is not yet clearly determined, but a mitochondrial mechanism through the BCL2 family, release of cytochrome c into cytoplasm from mitochondria, formation of apoptotic proteasome activating factor 1 (Apaf1), and cleavage of procaspases 3, 6, and 7 have been reported (31).

Another mechanism through which cells cope with ER stress is autophagy (23), a cellular response to degrade long-lived proteins that accumulate in the ER, occurring through activation of the lysosomal pathway. Several studies (64, 68) have linked ER stress to autophagy. However, it is not clear whether ER stress-mediated autophagy protects cells from undergoing apoptosis, as some data (13, 14, 65, 76) suggest that it also induces cell death.

**ER Stress in Familial and Sporadic IPF**

ER stress and the UPR have been associated with a number of diseases, including Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, diabetes mellitus, cystic fibrosis, and more recently FIP and sporadic IPF. IPF is characterized pathologically as usual interstitial pneumonia (UIP). FIP is most commonly associated with UIP on lung biopsy, but lung pathology associated with other idiopathic interstitial pneumonias can be seen in some affected family members (72). FIP accounts for 2–20% of cases of IPF, depending on different reports (22, 42, 51, 82).

In 2001, Nogee et al. (61) first reported a mutation in the carboxy-terminal region of surfactant protein-C (SFTPC) that resulted in deletion of exon 4 and its 37 amino acids (H9004 exon4) in an infant with nonspecific interstitial pneumonia whose mother had desquamative interstitial pneumonia. Subsequently, our group (78) described a different SFTPC mutation that resulted in the substitution of glutamine for leucine at amino acid position 188 (L188Q) of pro-SP-C in a kindred

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Fig. 2. Immunohistochemistry (IHC) for components of the UPR in idiopathic pulmonary fibrosis (IPF). Lung tissue was obtained from a normal lung (A, E, and I), individuals with usual interstitial pneumonia (UIP) on biopsy from the L188Q SFTPC mutation family (B, F, and J), an individual with familial pulmonary fibrosis with UIP by biopsy without an SFTPC mutation (C, G, and K), and an individual with sporadic IPF/UIP (D, H, and L). IHC for BiP (A), EDEM (E), and XBP-1 (I) revealed little or no staining in normal lung. IHC for BiP (B), EDEM (F), and XBP-1 (J) in L188Q SFTPC-associated UIP revealed positive staining in epithelial cells lining areas of affected lung (arrows). In familial UIP in the absence of SFTPC mutations and sporadic IPF/UIP, immunostaining for BiP, EDEM, and XBP-1 was also noted in the lining epithelial cells. All images are magnification of ×200. Immunostaining controls with secondary antibody only were negative. [From Lawson et al. (35)].
with 11 adults with UIP and 3 children with nonspecific interstitial pneumonia (78). Since then, SFTPC mutations have been described in other pediatric cases of interstitial lung disease (60) and adult cases of FIP (82). SFTPC transcription and translation results in a 197 amino acid precursor protein (pro-SP-C; Ref. 86) that enters the ER where folding of the carboxy-terminal region is performed followed by processing through the secretory pathway until mature SP-C protein is packaged in the lamellar body before secretion into the alveolar space (59, 86). In vitro studies (4, 5, 26, 53) reveal that mutations in the carboxy-terminal region of SFTPC result in abnormal processing and protein misfolding, with accumulation in the ER. Modeling of the Δexon4 and L188Q mutations in vitro demonstrated that these mutant forms of pro-SP-C cause ER stress in AEC lines, including human A549 cells (35, 52, 53), mouse lung epithelial cells (MLE12; Ref. 35), and rat lung epithelial cells (RLE6TN; Ref. 74). In addition to SFTPC, mutations in SFTP2A, one of the two isoforms of surfactant protein-A, have been linked to FIP (84). In 2009, Wang et al. (84) reported two different FIP families in which rare missense mutations were identified in SFTP2A, one resulting in the substitution of valine for glycine at codon 231 (G231V) and the other in the substitution of serine for phenylalanine at codon 198 (F198S). When both of these mutations were expressed in A549 cells, the mutant surfactant protein-A forms were retained within the ER, leading to ER stress and UPR pathway activation (48, 84), suggesting a similar role for ER stress in SFTP2A mutation associated pulmonary fibrosis to that seen with SFTPC mutations.

While frequently encountered in childhood ILD, SFTPC mutations are rare in IPF (34). Nevertheless, their association with FIP highlights the importance of AECs in the initiation and early pathogenesis of IPF since surfactant proteins are produced in the lungs exclusively by type II AECs (61, 78). Investigation of the mechanisms involved in SFTPC mutation associated FIP led our group and others to evaluate the role of ER stress in other forms of IPF. In 2008, we (35) reported immunohistochemistry for markers of ER stress and UPR activation on lung tissue from FIP patients with the L188Q SFTPC mutation, FIP patients without SFTPC mutations, and sporadic IPF patients. We found that AECs lining areas of lung fibrosis were positive for BiP, EDEM, and XBP-1 expression in all three categories (Fig. 2). Similarly, Korfei et al. (29) showed that AECs lining areas of fibrosis in IPF had ER stress. Thus ER stress was identified in IPF lungs even in the absence of SFTPC mutations.

Potential Mechanisms Linking ER Stress and Lung Fibrosis

Following these important observations suggesting a role for ER stress in FIP and IPF, several studies have highlighted potential mechanisms by which ER stress may contribute to the development and progression of lung fibrosis.

AEC injury and apoptosis. When considering how ER stress may impact disease pathogenesis, most studies have focused on ER stress-induced apoptosis. In vitro experiments with expression of both the Δexon4 and L188Q SFTPC mutations have revealed that ER stress is accompanied by increased AEC death (35, 52, 53). In evaluations of the Δexon4 and L188Q SFTPC mutations, Mulugeta et al. (52, 53) noted that increased AEC death was associated with increased caspase activity, specifically through a caspase-4 (caspase-12) mechanism.

To better evaluate for potential mechanistic relationships between ER stress and lung fibrosis, mouse models have been employed. In 2003, Bridges et al. (10) reported a transgenic mouse with expression of the SFTPCTΔexon4 mutation under the human SFTP promoter during lung development. These mice had abnormal lung morphogenesis resulting in fetal death, with findings supporting protein misfolding and aberrant surfactant processing (10). Subsequently, we developed a transgenic model based on the tet-on system to conditionally express mutant L188Q SFTPCTΔexon4 exclusively in type II AECs. In the absence of doxycycline, these mice grew and developed normally. Once in adulthood, L188Q SFTPCTΔexon4 expression in these mice resulted in ER stress in type II AECs but no evidence of increased apoptosis in the lungs or fibrosis (33). However, following low-dose bleomycin, greater lung fibrosis, enhanced AEC death, and increased caspase pathway activation were observed with mutant SFTPCTΔexon4 expression. As a confirmatory model for effects of ER stress, we exposed wild-type mice to

Fig. 3. Colocalization of XBP-1 and cytomegalovirus (CMV) late antigens in alveolar epithelial cells from an individual with UIP. Confocal laser scanning microscopy with Z-stack imaging was performed on lung tissue sections. Immunofluorescence for XBP-1 (green) and CMV (red) identify expression in epithelial cells lining areas of fibrosis. On dual fluorescence imaging in a single Z-stack plane, coexpression of these proteins (yellow) is detected in alveolar epithelium. Magnification = ×200. [From Lawson et al. (35).]
tunicamycin, an agent commonly used to induce ER stress, followed by low dose bleomycin. As with L188Q SFTP C expression, tunicamycin induced ER stress in AECs but did not cause lung fibrosis. However, in combination with bleomycin, tunicamycin-treated mice developed enhanced lung fibrosis and increased AEC apoptosis.

In human IPF lung tissue, Korfei et al. (29) showed apoptotic pathway activation in the same AECs lining areas of fibrosis that prominently expressed ER stress markers. Thus these cell, animal, and human studies suggest that ER stress may impact disease pathogenesis by leading to enhanced AEC injury and death.

**Inflammation**. In some systems, ER stress and inflammation are interconnected by UPR pathways through activation of JNK, p38 mitogen-activated protein kinases, and NF-κB (67). In AECs, Maquire et al. (47) analyzed the Δexon4 SFTP C mutation in vitro and noted NF-κB pathway activation. However, their investigations of the L188Q SFTP C mutation did not find such an association. Our in vivo mouse modeling did not reveal enhanced lung inflammation with mutant L188Q SFTP C expression (33). Although interactions between the UPR and inflammatory signaling pathways could impact fibrotic remodeling, the inflammatory effects of ER stress likely vary considerably depending on the cause, severity, and duration of ER stress.

**Regulation of cell phenotype**. A variety of studies (27, 75, 87) have suggested that epithelial cells in the lungs and other organs can contribute to fibrosis by undergoing epithelial-mesenchymal transition (EMT). Ulianich et al. (80) first demonstrated that ER stress induces thyroid epithelial cells to undergo EMT. Recently, we (74) observed that inhibition of smad2 and src pathway activation, suggesting that this UPR arm was involved in ER stress-induced EMT. Furthermore, small interfering RNA silencing of IRE1α attenuated ER stress-induced EMT. Taken together, available data suggest that herpesviruses are involved in the pathogenesis of IPF and raise questions as to whether they could exert their effects through ER stress.

**Potential Causes of ER Stress in IPF**

As described above, a combination of cell culture studies, animal models, and human pathological evaluation suggests that ER stress has a role in lung fibrosis. While mutant pro-SP-C is the underlying culprit in SFTP C mutation-mediated FIP, the cause of ER stress in other families with FIP and sporadic IPF remains unknown. Certainly, aberrant protein processing (including surfactant) remains a possibility and a target for future investigations. It is also possible that AECs, in their attempt to regenerate the alveolar epithelium after injury, may inherently be under ER stress and UPR activation in their hyperplastic state secondary to intrinsic factors such as increased metabolic demand, thus perpetuating an ongoing injury-remodeling cycle. Finally, exogenous exposures could contribute to ER stress, including herpesviruses, cigarette smoke, and inhaled particulates, all of which have been shown to induce ER stress in lung epithelium (24, 25, 32, 49).

Studies from several groups have implicated herpesviruses as potentially important in the pathogenesis of IPF. Increased titers of antibodies to cytomegalovirus and Epstein Barr virus have been noted in IPF patients (89). Herpesvirus antigens can be detected in AECs lining areas of fibrosis in IPF lung biopsy samples but not in lung epithelium of controls (15, 35, 73). Interestingly, herpesviruses are known to cause ER stress and activate the UPR (24). We observed that herpesvirus proteins (Epstein Barr virus, cytomegalovirus, and Kaposi’s sarcoma herpesvirus) were expressed in AECs lining areas of fibrosis in IPF and that these viral proteins colocalized with ER stress markers (Fig. 3; Ref. 35). Taken together, available data suggest that herpesviruses are involved in the pathogenesis of IPF and raise questions as to whether they could exert their effects through ER stress.

Exposure to inhaled particulate matter has been associated with a variety of local and systemic conditions, including pulmonary fibrosis, chronic obstructive pulmonary disease, and...
cardiovascular disease (40). Inhaled environmentally derived fine particulates have been shown to activate several profibrotic intracellular signaling pathways that could contribute to lung fibrosis (8). Furthermore, recent in vitro and in vivo studies (32, 85) have demonstrated that particulate matter induces ER stress and activates all three arms of the UPR pathway in lung epithelial cells and promotes apoptosis.

Cigarette smoke has been associated with the development of both IPF (3) and FIP (72). Several studies have found that cigarette smoke exposure can activate the UPR. In vitro exposure of Swiss 3T3 cells to aqueous extracts of cigarette smoke was associated with PERK and ATF4 activation, resulting in upregulation of BiP and other ER stress associated genes (9, 20). Additional evaluations in normal and malignant lung epithelial cells similarly revealed that expression of UPR-related genes was induced by cigarette smoke exposure (25).

Together, a variety of studies have shown that environmental stimuli relevant to lung fibrosis can cause ER stress and UPR activation. Whether any or all of these environmental factors impact fibrotic remodeling through ER stress induction is an area that requires further study.

**Effects of Aging on ER Stress Responses in the Lungs**

Aging has long been considered a central player in many chronic diseases and recently has been implicated as a major cofactor in the development of IPF (12, 69), with evidence suggesting that the aged lung is at greater risk for the development of lung fibrosis following injury (57, 69). Interestingly, aging leads to both a decline in protein folding ability in cells and in impaired ability of UPR responses to maintain cellular homeostasis in the setting of ER stress (54, 55). With aging, studies have demonstrated increased proapoptotic signaling because of impaired UPR responses (56) and age-related impairments in ubiquitin-proteosome-mediated degradation pathways (7). Recently, Torres-Gonzalez et al. (79) demonstrated that infection with murine gamma herpesvirus 68 (MHV68) results in the development of lung fibrosis in aged mice, whereas young mice do not develop lung fibrosis. After MHV68 infection, aged mice developed greater ER stress in the AEC population as evidenced by increased BiP expression and increased XBP1 splicing, as well as increased AEC apoptosis, compared with young mice. The results of this study support the possibility that aging of the lung leads to diminished ability of the AEC population to maintain homeostasis in the setting of ER stressors. Taken together, these observations raise questions about the degree to which age-related impairments in UPR responses may contribute to IPF pathogenesis, consistent with speculation that IPF is a disease of aging in the lungs.

**Unanswered Questions and Future Directions**

While increasing evidence suggests a role for ER stress in IPF, the mechanisms behind such an association need to be further delineated. Given the experimental evidence to date, we speculate that ER stress leads to a vulnerable AEC population that is then highly sensitive to additional environmental insults, which together lead to aberrancies in epithelial repair processes, thus propagating fibrosis (Fig. 4). Many questions remain in determining the role for ER stress in IPF. Specifically, it will be important to determine which environmental insults lead to ER stress in human lungs and why induction of ER stress alone does not cause lung fibrosis in experimental animals. Although type II AECs appear to be the primary target for ER stress in lung fibrosis, other cells, including fibroblasts, could regulate fibrosis through UPR activation. Determining which UPR pathways are most important and which ER stress induced cellular phenotypes (inflammatory, apoptotic, or EMT) regulate fibrotic remodeling will be important for designing effective therapies to limit ER stress effects on lung fibrosis. Given that the UPR is designed to protect the cell, it is quite possible that trying to attenuate the ER stress response might have deleterious effects. Thus it may be better to develop agents that improve protein processing or block downstream signaling. To help address these and many other questions and better understand the role that ER stress plays in IPF, additional work is needed. In vitro studies have the potential to define specific targets or pathways of interest, but ultimately animal modeling will likely provide the best chance for delineating mechanistic relationships and will provide opportunities for testing new therapeutic strategies.

**Conclusions**

With this review, we provide a glimpse into the potential roles that ER stress and UPR pathway activation may play in the pathogenesis of IPF. Taken together, several lines of evidence now suggest a prominent role for ER stress in IPF, supporting the concept that dysfunctional AECs facilitate the progression of lung fibrosis. An improved understanding of factors involved in UPR pathway activation has the potential to suggest new therapeutic avenues to pursue for this devastating disease with limited therapies.

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