Epithelial Endoplasmic Reticulum Stress and Apoptosis in Sporadic Idiopathic Pulmonary Fibrosis

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***Rationale*: The molecular pathomechanisms underlying idiopathic pulmonary fibrosis (IPF) are elusive, but chronic epithelial injury has recently been suggested as key event.**

***Objectives*: We investigated the possible implication of endoplasmic reticulum (ER) stress–mediated apoptosis in sporadic IPF.**

***Methods*: We analyzed peripheral explanted lung tissues from patients with sporadic IPF (n = 24), chronic obstructive pulmonary disease (COPD) (n = 9), and organ donors (n = 12) for expression of major ER stress mediators and apoptosis markers by means of immunoblotting, semiquantitative reverse transcription–polymerase chain reaction, immunohistochemistry, and the TUNEL method.**

***Measurements and Main Results*: Compared with COPD and donor lungs, protein levels of ER stress mediators, such as processed p50 activating transcription factor (ATF)-6 and ATF-4 and the apoptosis-inductor CHOP (C/EBP-homologous protein), as well as transcript levels of spliced X-box binding protein (XBP)-1, were significantly elevated in lung homogenates and type II alveolar epithelial cells (AECIIs) of IPF lungs. Proapoptotic, oligomeric forms of Bax, which play a key role in ER stress–mediated apoptosis downstream of CHOP induction, as well as caspase-3 cleavage, could be detected in IPF lungs. By means of immunohistochemistry, exclusive induction of active ATF-6, ATF-4, and CHOP in AECIIs was encountered in IPF but not in COPD or donor lungs. Immunoreactivity was most prominent in the epithelium near dense zones of fibrosis and fibroblast foci, where these ER stress markers colocalized with markers of apoptosis (TUNEL, cleaved caspase-3).**

***Conclusions*: Severe ER stress response in the AECIIs of patients with sporadic IPF may underlie the apoptosis of this cell type and development of fibrosis in this disease.**

Keywords: interstitial lung disease; lung fibrosis; type II alveolar epithelial cell; X-box binding protein-1; C/EBP-homologous protein

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

No data are available concerning the underlying reasons for apoptosis in the alveolar epithelium of patients with idiopathic pulmonary fibrosis.

What This Study Adds to the Field

Severe endoplasmic reticulum stress of type II alveolar epithelial cells represents a major reason for apoptosis of this cell type in sporadic idiopathic pulmonary fibrosis.

Sporadic idiopathic pulmonary fibrosis (IPF) belongs to the family of idiopathic interstitial pneumonias (IIPs). IPF accounts for approximately 20 to 30% of all interstitial lung diseases and probably affects 500,000 patients in the United States and the European Community (EC) (1). IPF is characterized by a progressive distortion of the alveolar architecture and replacement by fibrotic tissue, resulting in progressive dyspnea, decline in lung function, and death within 3 to 5 years after diagnosis (2). No effective pharmacologic treatment exists, with lung transplantation being the only option for affected patients.

Chronic inflammation has long been postulated to underlie this disease (3), but steroids and immunosuppressants are of limited or no help. An alternative theory of IPF, based on chronic epithelial injury and subsequent aberrant alveolar repair, has gained increasing attention (4). This hypothesis is supported by ultrastructural studies in which alveolar epithelial cell death was observed at an early stage of the disease (5, 6). Increased apoptosis of type II alveolar epithelial cells (AECIIs) was observed primarily in the hyperplastic epithelium covering the fibroblast foci (7) but also in regions of the lung with almost normal-appearing alveolar structures (8). Consistent with these results, blockade of apoptosis pathways was found to attenuate the extent of fibrosis in the bleomycin model of lung fibrosis (9).

Additional evidence suggests that mutations resulting in epithelial cell injury and apoptosis may underlie the development of lung fibrosis. Mutations in the human telomerase (*TR*) and in the telomerase reverse transcriptase (*TERT*) genes were shown to cause lung fibrosis in close to 10% of all familial forms of IIP (10). The authors proposed a model in which DNA damage results in a loss of alveolar epithelial cells, which drives the fibrotic process. Moreover, in a few kindreds with familial forms of IIP (mostly IPF or nonspecific interstitial pneumonia), mutations in the carboxyterminal domain of surfactant protein (SP)-C (*SFTPC*), a protein exclusively expressed, synthesized, and secreted by the AECII, have been shown to underlie the disease (11, 12). The mutation results in an aberrant pro–SP-C protein that cannot be correctly processed, resulting in protein misfolding, accumulation, induction of endoplasmic reticulum (ER) stress, and apoptosis of AECIIs (12–14).

In contrast to familial forms of IIP, no mutations of the *SFTPC* gene have been found in sporadic IPF or nonspecific interstitial pneumonia (15, 16). The origin of the observed AECII death in sporadic IPF therefore remains elusive. We hypothesized that, despite the absence of mutations of *SFTPC*, chronic ER stress,

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leading to apoptosis of AECII, could represent a common pathomechanistic principle in IPF. The objective of this study was to examine ER stress sensor mechanisms and ER stress–induced apoptosis pathway in sporadic IPF. Some of the results of this study have been previously reported in the form of an abstract (17).

METHODS

Patient and Control Groups

The study was approved by the local ethics committee, and informed consent was obtained from all study subjects. Explanted lungs (n = 20 for sporadic IPF [IPF_{LTX}]; n = 9 for chronic obstructive pulmonary disease [COPD]) or nonutilized donor lungs or lobes fulfilling transplantation criteria (n = 12; human donors [HDs]) were obtained from the Department of Thoracic Surgery in Vienna, Austria. Subpleural lung tissue samples were obtained from five additional patients with sporadic IPF who underwent video-assisted thoracic surgery (IPF_{VATS}) for diagnostic purposes. All IPF diagnoses were made according to the American Thoracic Society/European Respiratory Society consensus criteria (2), and a usual interstitial pneumonia pattern (*see* Figure E1 in the online supplement) was proven in all patients with IPF_{LTX} and IPF_{VATS}. Patients with IPF_{VATS} were untreated at the time of sampling. All subjects with IPF_{LTX} experienced progress under a preceding course of steroids, but six of them were not receiving steroids or immunosuppressants at the time of transplantation. The remaining 14 patients were receiving an average dose of 10.7 ± 2.4 mg prednisolone equivalent per day. Clinical information on patients and control subjects is summarized in Table 1.

A detailed description of all methods described herein is found in the online supplement.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Gene expression of SP-B, SP-C, C/EBP-homologous protein (CHOP), thyroid transcription factor-1 (TTF-1), and β-actin as well as X-box binding protein (XBP)-1 splicing was investigated with reverse transcription–polymerase chain reaction (RT-PCR). A detailed description of the methodology and the complete list of primers used are given in the online supplement (Table E1).

Western Blot Analysis

Lung homogenates or cell lysates were subjected to denaturating sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by electroblotting and immunostaining for activating transcription factor (ATF)-6α, ATF-4/cyclic AMP response element-binding protein (CREB-2), CHOP, Bax, caspase 3, Bip/Grp78, TTF-1, and β-actin. Blot membranes were developed with the ECL Plus chemiluminescent detection system (Amersham Biosciences, Uppsala, Sweden), and the band intensity of exposed film was analyzed by densitometric scanning and quantified using the AlphaEaseFC Imaging System (San Leandro, CA). The sources and dilutions of primary antibodies used are outlined in the online supplement.

Histopathology/Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue sections were routinely stained with hematoxylin and eosin. Standard methodology was used for immunohistochemical localization of p50ATF-6/ATF-6, ATF-4, cleaved caspase 3, CHOP, and pro–SP-C, as described in the online supplement.

In Situ Apoptosis Assay

For the *in situ* detection of apoptosis at the level of a single cell in lung tissue sections, the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method was performed using the *In Situ* Cell Death Detection Kit, AP (Roche Applied Science, Indianapolis, IN) according to the protocol outlined in the online supplement.

Statistical Analyses

Statistical differences between IPF, COPD, and control groups were analyzed using the nonparametric Kruskal-Wallis test. Dunn's multiple comparison test was used for posttest analysis. All statistical analyses were performed using GraphPad Prism version 4.0 software. Results were considered significant at $P < 0.05$ and are reported as $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P > 0.05$.

RESULTS

Induction of ER Stress and ER Stress–induced Apoptosis in Sporadic IPF

For analysis of the ER stress–induced apoptosis pathway, we performed comparative Western blot analysis of peripheral lung tissues from patients with IPF (IPF_{LTX}; n = 20) or COPD (n = 9) and HDs (n = 12) by use of specific antibodies against the ER stress sensor molecule ATF-6α; the ER stress transcriptional enhancers XBP-1 and ATF-4; and the ER stress–associated, proapoptotic CHOP protein. Cleavage and activation of 90-kD ATF-6α protein, resulting in the appearance of processed p50ATF6, and induced expression of ATF-4 and CHOP were detected in every IPF_{LTX} but in none of the COPD and donor lung materials (Figure 1A). Densitometric quantification of normalized immunoblots revealed an approximately two- to threefold induction in tissue contents of p50ATF-6 in IPF_{LTX} in comparison to COPD lungs and donor lungs ($P < 0.001$ vs. HD; $P < 0.01$ vs. COPD; Figure 1B), significant up-regulation of ATF-4 expression in IPF_{LTX} compared with both control groups ($P < 0.001$ vs. HD; $P < 0.05$ vs. COPD; Figure 1C), and a two- to threefold significant induction of CHOP in IPF_{LTX} compared with both control groups ($P < 0.001$ vs. HD; $P < 0.01$ vs. COPD; Figure 1D). When analyzing the apoptotic signaling pathways downstream of CHOP, we observed a prominent, approximately threefold induction of proapoptotic, dimeric Bax β-protein in lung tissue of patients with IPF_{LTX} and COPD but not in donor lungs, as determined by nonreducing electrophoresis and immunoblotting followed by densitometric quantitation ($P < 0.001$, IPF_{LTX} vs. HD; $P < 0.01$, COPD vs. HD; *see* immunoblot in Figures 1A and 1E).

All tested IPF_{LTX} and COPD lungs, but not donor lungs, showed caspase-3 activation, as evident from the detection of

TABLE 1. DEMOGRAPHIC AND CLINICAL DATA OF STUDY SUBJECTS

	Donor Lungs (*n = 12*)	Sporadic IPF_{LTX} (*n = 20*)	Sporadic IPF_{VATS} (*n = 5*)	$COPD_{LTX}$ (*n = 9*)
Mean age, yr ± SEM	45.0 ± 5.3	55.2 ± 2.9	61.0 ± 1.9	55.2 ± 1.4
Sex, n male/female	6/6	14/6	3/2	6/3
FVC, mean ± SEM	NA	49.5 ± 3.0	61.1 ± 6.7	38.4 ± 3.0
FEV_1, mean ± SEM	NA	54.6 ± 3.3	66.6 ± 6.2	17.0 ± 1.2
Smoking status, n current/former/never	NA	0/11/9	0/3/2	0/9/0
Pack-years, mean ± SEM	NA	33.0 ± 9.2	15.0 ± 7.5	51.8 ± 7.7
Histology, UIP pattern, n	0/12	20/20	5/5	0/9

Definition of abbreviations: $COPD_{LTX}$ = patients with chronic obstructive pulmonary disease who underwent lung transplantation; IPF_{LTX} = patients with IPF who underwent lung transplantation; IPF_{VATS} = patients with IPF who underwent video-assisted thoracic surgery; NA = not available; UIP = usual interstitial pneumonia.

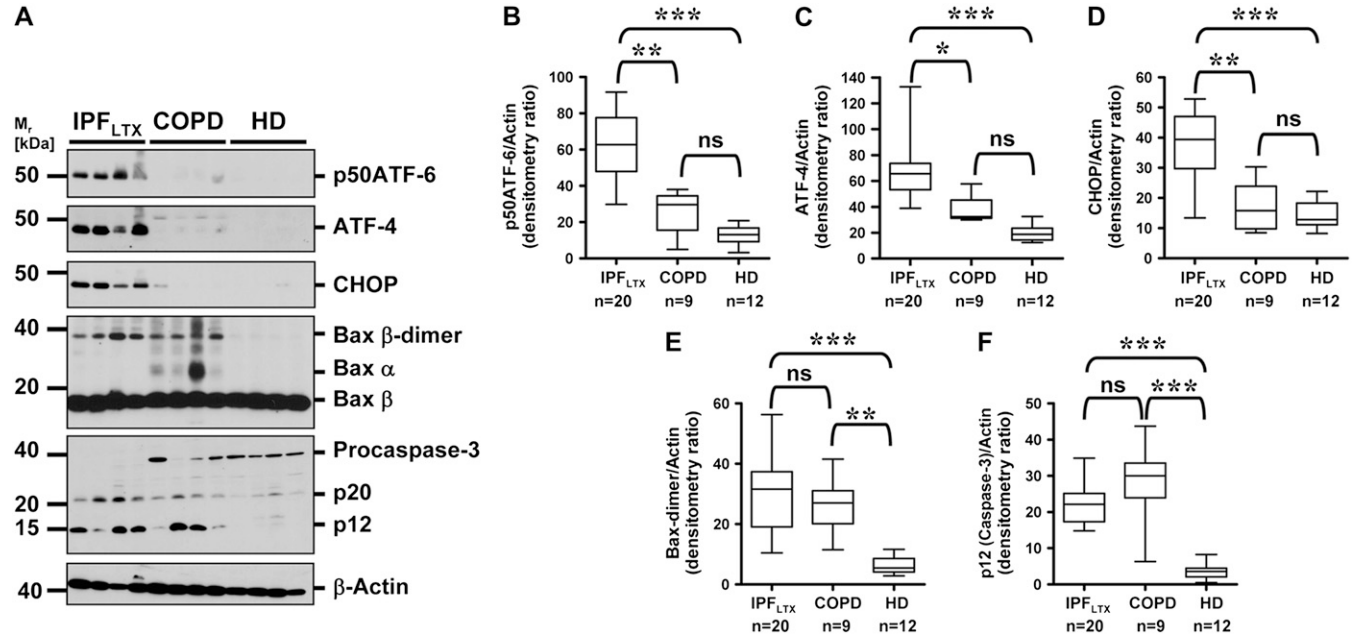

Figure 1. Induction of the endoplasmic reticulum stress–mediated apoptosis pathway in idiopathic pulmonary fibrosis (IPF). (*A*) Example immunoblots and (*B–F*) quantitative immunoblot analysis of peripheral lung tissue from patients with sporadic IPF (IPF_{LTX}) (n = 20), lung tissue from patients with chronic obstructive pulmonary disease (COPD) (n = 9), and human donor (HD) lungs (n = 12) using specific antibodies against activating transcription factor (ATF)-6α (*B*), ATF-4 (*C*), CHOP (*D*), Bax (*E*), pro–caspase 3 (*F*), and β-actin as loading control. Densitometric ratios of the respective protein to β-actin are depicted as a box-and-whisker diagram (*box* indicates 25th and 75th, *horizontal line* indicates the 50th percentile [median], and extensions above and below reflect extreme values). Measurements of individual samples were done in duplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ns = not significant.

the characteristic cleavage products p12 and p20 subunit in the immunoblots ($P < 0.001$; IPF_{LTX}, COPD vs. HD; *see* immunoblot in Figures 1A and 1F). Thus, the mitochondrial apoptosis pathway also seemed to be activated in patients with COPD. However, this was not based on an apoptotic ER stress response because induction of CHOP was not observed in COPD. This conclusion is further supported by our observation that activation of the inositol-requiring protein (IRE)-1/XBP-1 pathway, resulting in the occurrence of spliced XBP-1 mRNA, was exclusively observed in IPF_{LTX} (Figure 2). Active, spliced XBP-1 mRNA was not detected in COPD and HD lungs, but robust activation of XBP-1 was readily detected in IPF_{LTX} lung tissues (Figure 2).

To determine whether the apoptotic ER stress response could be detected at an earlier stage of the disease, we analyzed lung tissues of five patients with IPF obtained during open lung biopsy for diagnostic purposes (IPF_{VATS}). When compared with

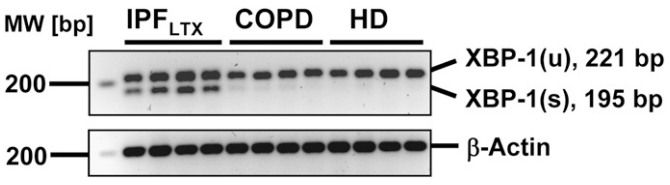

Figure 2. X-box binding protein (XBP)-1 activation in idiopathic pulmonary fibrosis (IPF) lungs. Total RNA was isolated from peripheral lung tissue of patients with IPF (IPF_{LTX}), from lung tissue of patients with chronic obstructive pulmonary disease (COPD), and human donors (HD) and subjected to reverse transcription–polymerase chain reaction analysis with primers spanning the intron of unspliced XBP-1 mRNA. XBP-1(u) denotes unspliced, inactive XBP-1; XBP-1(s) denotes spliced, active XBP-1, which was observed in all 20 IPF_{LTX} lungs, but in none of the COPD or HD lungs. Representative results are shown. MW = molecular weight.

the lung tissues obtained during transplantation, IPF_{VATS} tissues exhibited a similar extent of proapoptotic ER stress response, with activation of ATF-6 and induction of ATF-4, CHOP, Bax dimerization, and caspase-3 cleavage (Figure E2).

ER Stress and Apoptosis Occur in AECIIs

To identify the cellular source of the apoptotic ER stress response in sporadic IPF, we performed RT-PCR and immunoblotting of isolated AECII extracts. Semiquantitative RT-PCR of human AECIIs isolated from peripheral IPF_{LTX} (n = 3) and donor (n = 3) lung tissues demonstrated strong induction of CHOP expression and induced splicing of the XBP-1 transcript in IPF_{LTX}. Gene expression of the epithelial cell markers SP-B, SP-C, and TTF-1 was found to be identical in AECII preparations of IPF_{LTX} and donor lungs (Figure 3A). Consistent with ER stress, up-regulated protein expression of the ER chaperone Bip/Grp78, ATF-4, and CHOP was observed in AECII from IPF_{LTX} (n = 2) but not from donor lungs (n = 3) (Figure 3B). Due to the lack of AECII preparations, the Western blot analysis was limited to two IPF samples.

To further prove that alveolar type II cells are responsible for the observed ER stress signature in sporadic IPF, we undertook immunohistochemical analysis for p50ATF-6/ATF-6, ATF-4, CHOP, pro–SP-C, and cleaved caspase 3 in serial sections of IPF, COPD, and donor lung tissues. In the case of p50ATF-6/ATF-6, we used an antibody directed against the aminoterminal 17 amino acids of the human ATF-6 protein, which harbors a higher immunoreactivity to the processed (and thus activated) 50-kD form of ATF-6. An exclusive staining for ATF-6 (and pro–SP-C) was observed in AECIIs surrounded by fibrotic tissue (Figures 4A–4D), in AECIIs localized in thickened alveolar septa (Figures 4A, 4B, 4E, 4F and E5), and in AECIIs localized in nonfibrotic, histologically normal appearing areas of IPF lung parenchyma (Figures 4A, 4B, 4G, 4H, and E5)

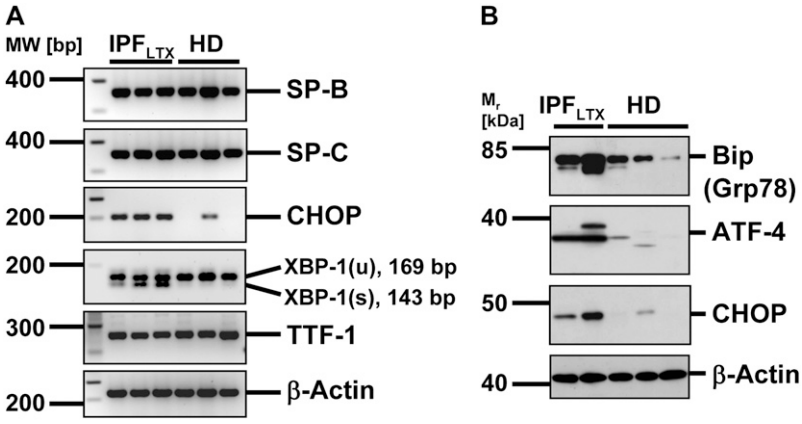

Figure 3. Apoptotic endoplasmic reticulum stress response in alveolar epithelial type II cells (AECIIs) in idiopathic pulmonary fibrosis (IPF). (*A*) Reverse transcription–polymerase chain reaction of human AECIIs isolated from peripheral explanted lung tissue (LTX) of patients with IPF (n = 3) and healthy donors (HD) (n = 3) demonstrated strong induction of C/EBP-homologous protein (CHOP) expression and exclusive splicing of the X-box binding protein (XBP)-1 transcript in IPF; in contrast, expression of surfactant protein (SP)-B, SP-C, thyroid transcription factor 1 (TTF-1) and β-actin was similar in IPF and donor lungs. (*B*) Immunoblot analysis of human AECIIs isolated from peripheral LTX lung tissue of patients with IPF (n = 2) and HD (n = 3) revealed up-regulated protein expression of Bip, ATF-4, and CHOP in IPF. Loading control: β-actin. MW = molecular weight.

but not in fibrotic tissue. In contrast, donor lungs (Figures 4I, 4J, 4K, and 4L) and COPD lungs (*see* Figure E6) did not show notable immunoreactivity for the ATF-6 antibody. AECIIs lining airspaces in areas of dense fibrosis or adjacent to underlying fibroblast foci revealed comparably stronger immunoreactivity for ATF-6 (Figures 4C, 4D, and 5A–5F) than AECIIs from histologically normal-appearing areas of IPF lungs (Figures 4A, 4B, 4G, and 4H).

As anticipated on the basis of Western blot data, immunohistochemistry for the proapoptotic transcription factor CHOP confirmed a pronounced and exclusive staining of cytoplasm and nuclei of AECIIs near dense zones of fibrosis (Figures 6A–6H) or fibroblast foci (Figures 6I–6L) but not in donor lungs (Figures 6M–6P) or COPD lungs (Fig. E7). CHOP-positive AECIIs were also detected in histologically normal-appearing areas of IPF lung parenchyma (Figures 7A–7F) but stained less intense than AECIIs in close proximity to dense fibrotic zones (Figures 7G–7J).

AECIIs in close proximity to dense fibrotic zones or fibroblast foci were immunoreactive for an antibody against

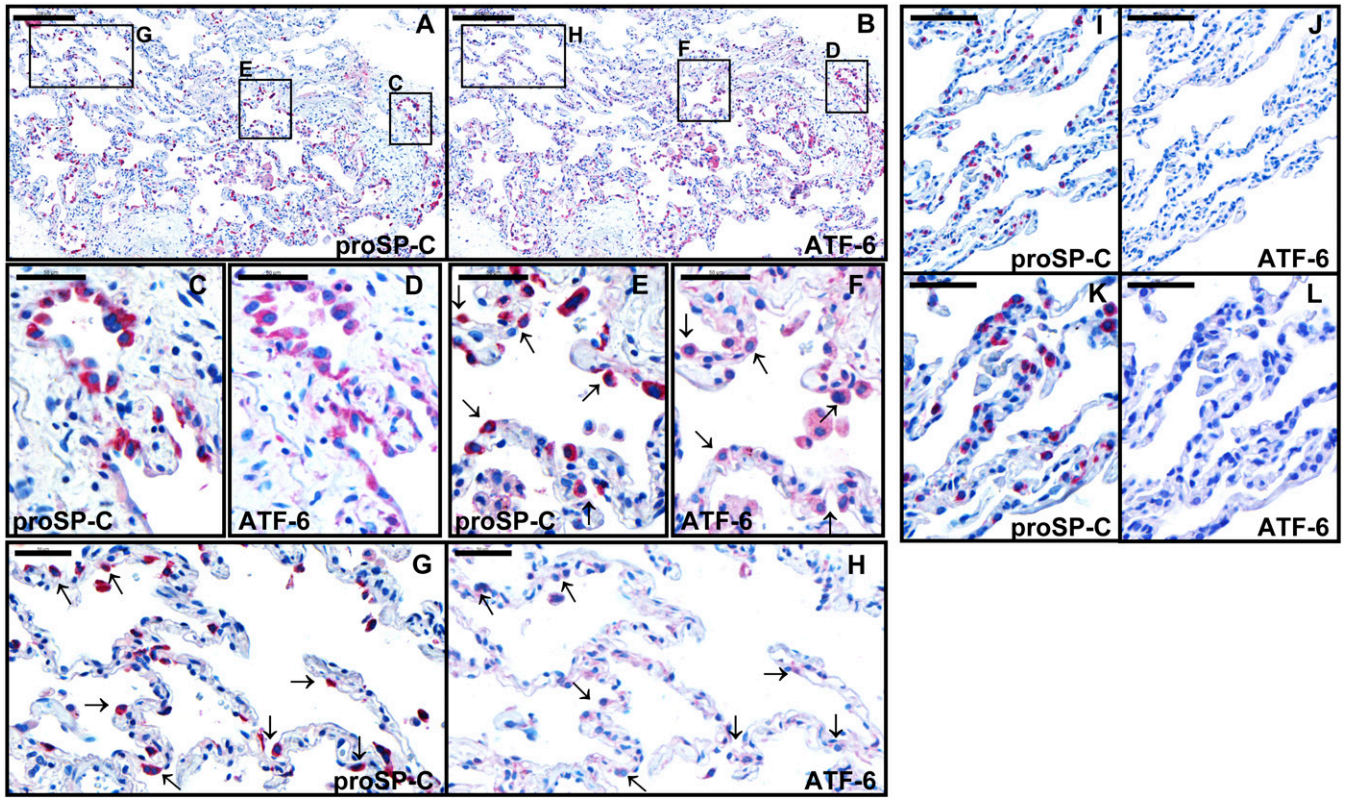

Figure 4. Up-regulation of activating transcription factor (ATF)-6 in alveolar epithelial type II cells (AECIIs) of idiopathic pulmonary fibrosis (IPF) lungs. Representative immunohistochemistry for pro–surfactant protein (SP)-C in IPF (*A*, *C*, *E*, *G*) and donor (*I*, *K*) lung tissues (*red stain* indicates AECIIs) and for ATF-6 in serial sections of IPF (*B*, *D*, *F*, *H*) and donor (*J*, *L*) lung tissues. (*B*, *D*, *F*, *H*) In IPF, localization of ATF-6 was exclusively observed in AECIIs surrounded by fibrotic tissue (*B* and *D*, *violet stain*), in AECIIs of thickened alveolar septae (*B* and *F*, *arrows*), and in AECIIs of histologically normal appearing areas of IPF lung parenchyma (*B* and *H*, *arrows*). AECIIs near dense zones of fibrosis revealed a very strong staining for ATF-6 (*B* and *D*). (*J*, *L*) No notable staining for ATF6 was observed in any cell of the donor tissues. Original magnification of photomicrographs *A* and *B*: ×100 (*bar* = 200 μm); original magnification of photomicrographs *C–H*, *K*, *L*: ×400 (*bar* = 50 μm); original magnification of photomicrographs *I* and *J*: ×200 (*bar* = 100 μm).

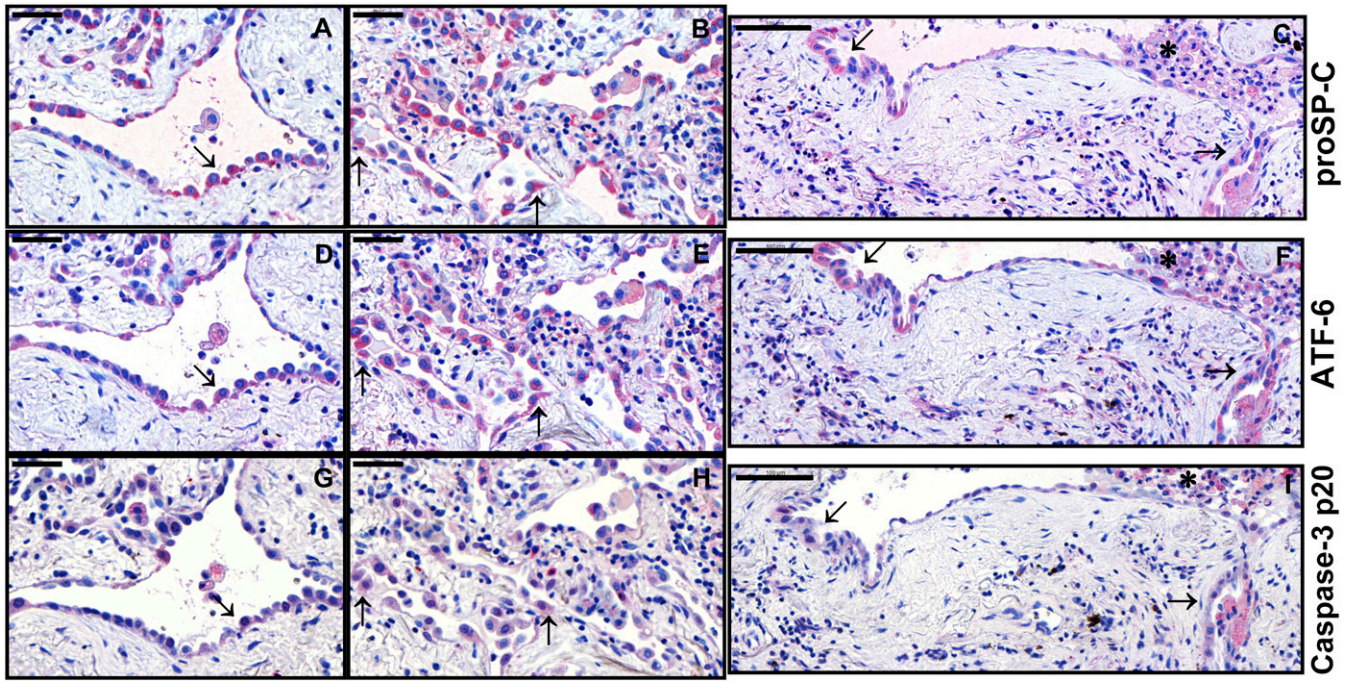

Figure 5. Colocalization of pro–surfactant protein (SP)-C, activating transcription factor (ATF)-6, and activated caspase 3 in alveolar epithelial type-II cells (AECIIs) in areas of dense fibrosis in idiopathic pulmonary fibrosis (IPF). Representative immunohistochemistry for pro–SP-C (*A–C*), ATF-6 (*D–F*), and cleaved caspase 3 (*G–I*) in serial sections of IPF lung tissues. AECIIs covering dense areas of fibrosis (*A, B*) and fibroblast foci (*C*) showed robust staining for ATF-6 (*D–F, arrows*) and caspase-3 activation (*G–I, arrows*). Some pro–SP-C, ATF-6, and caspase-3–positive cells were also detected in the airspaces, presumably representing detached apoptotic AECIIs, prone to the fate of phagocytosis by alveolar macrophages (*asterisks* in *C, F,* and *J*). Original magnification of photomicrographs *A, B, D, E, G,* and *H*: ×400 (*bar* = 50 μm); original magnification of photomicrographs *C, F,* and *I*: ×200 (*bar* = 100 μm).

cleaved (activated) caspase 3 (Figures 5H–5J). Accordingly, TUNEL-positive cells were identified as being pro–SP-C positive and thus as AECIIs (Figure E8). AECIIs in close proximity to dense fibrotic areas or fibroblast foci were found to be immunoreactive for ATF-6 and cleaved caspase 3 and showed CHOP immunoreactivity with the apoptotic TUNEL signal (Figures 5 and E8). In histologically normal appearing areas of IPF lungs and in donor lungs, apoptotic AECIIs were rarely seen (Figures E9 and E10). In COPD, apoptosis was predominantly localized to alveolar macrophages (Figure E11).

Figures 8 and E12 depict colocalization of the key markers of a proapoptotic ER stress response (ATF-6, ATF-4, CHOP) with an AECII-specific marker (pro–SP-C) and a marker of execution of apoptosis (cleaved caspase-3 p20 subunit).

DISCUSSION

IPF is a fatal disorder, with a mean survival time of 2 to 4 years (2). Its precise pathomechanism is unknown. Despite a large number of new therapeutic agents being evaluated in clinical trials, there is no effective treatment for these patients. The recurrent and frustrating observation that steroids and immunosuppressants are of limited help in IPF, coincident with new insights into genetic aberrations in familial forms of IIP, caused a paradigm shift with respect to the pathomechanism of IPF: what was once believed to be triggered by chronic inflammation is now believed to be caused by repeated alveolar epithelial cell injury (4).

In this study, we demonstrate for the first time that a severe ER stress response in the AECIIs seems to underlie the execution of the intrinsic apoptosis pathway, and thus the programmed cell death of this cell type, in patients with sporadic IPF but not in patients with COPD or in donor lungs. We observed up-regulation and cleavage/activation of ATF-6, induced expression of ATF-4 and CHOP, and exclusive activation of the IRE-1 pathway (through XBP-1 mRNA splicing) in AECIIs covering areas of dense fibrosis and fibroblast foci in IPF but not in COPD or donor lung tissues. In these areas, the ER stress signature colocalized with cleaved caspase 3 and the TUNEL signal, indicating execution of apoptosis in AECIIs, most likely as a result of the unresolved and overwhelming ER stress. In principle, the ER stress signature was also encountered in AECIIs in histologically normal appearing areas of IPF lungs but appeared less intense (CHOP, ATF-6). It may be for this reason that execution of apoptosis was rarely seen in AECIIs in histologically normal appearing areas of IPF lungs. In full accordance with previous reports, activation of the mitochondrial apoptosis pathway was also encountered in COPD (18, 19) but was unrelated to ER stress. In another recent study, activation of the unfolded protein response, as based on analysis of Bip, XBP-1, and ER degradation-enhancing alpha-mannosidase-like, has been reported in 10 patients with sporadic IPF, providing additional proof for the occurrence of this principle response in IPF (20). Overwhelming ER stress may represent an important trigger for epithelial apoptosis, which could represent a key event in the development of fibrosis in IPF. In line with this notion, the apoptotic ER stress response mediated by CHOP was similarly observed in IPF_{VATS} lung tissues, again suggesting that the apoptotic ER stress response may be an early, and not an end-stage, event in the disease (i.e., if one denotes the time of diagnosis as being early).

Multiple physiologic or pathologic conditions that affect ER homeostasis and ER capacity can trigger ER stress, including glucose starvation, impaired glycosylation, changes in the redox or ionic conditions of the ER lumen, ER lipid/glycolipid

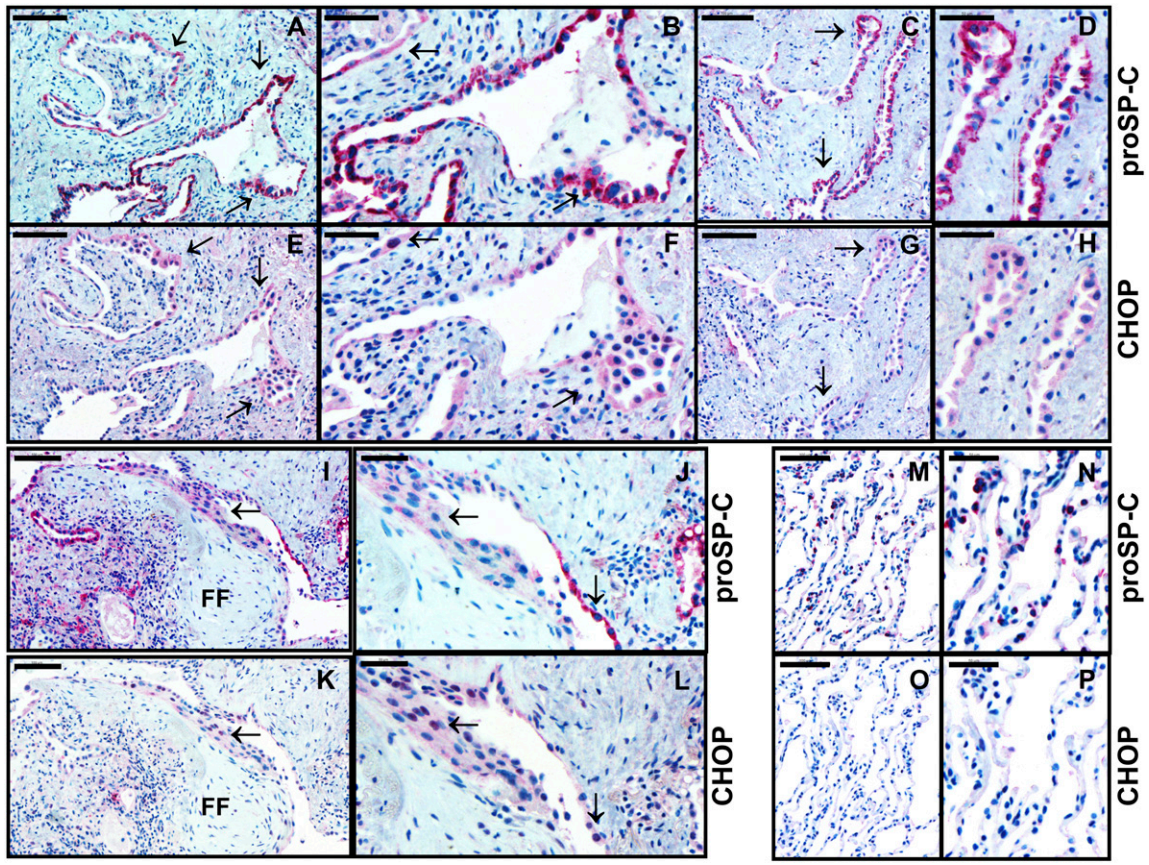

Figure 6. Induction of C/EBP-homologous protein (CHOP) in alveolar epithelial type II cells (AECIIs) of idiopathic pulmonary fibrosis (IPF) lungs. Representative immunohistochemistry for pro–surfactant protein (SP)-C in IPF (*A–D*, *I*, *J*) and donor (*M*, *N*) lung tissues (*red stain*, *arrows*) and for CHOP in serial sections of IPF (*E–H*, *K*, *L*) and donor (*O*, *P*) lung tissues (*pale pink stain*, *arrows*). In IPF, CHOP stained the nucleus and cytoplasm of hyperplastic AECIIs covering zones of overt fibrosis (*E–H*) and fibroblastic foci (*K*, *L*). Positive staining for CHOP was not seen in any cell and in any of the donor lung tissues (*O*, *P*). Original magnification of photomicrographs *A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*: ×200 (*bar* = 100 μm); original magnification of photomicrographs *B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*: ×400 (*bar* = 50 μm).

imbalances, elevated protein synthesis, and the accumulation of unfolded, misfolded, or excessive protein due to failure of protein folding, processing, transport or degradation. The unfolded protein response is an integrated response that aims to restore ER homeostasis by increasing the capacity of the ER to fold and process client proteins and to reduce the protein load in the ER (21). Translational repression, via PERK/eIF2α phosphorylation, leads to reduced protein burden and ATF-4 activation (22). Subsequent cleavage of ATF-6 to p50ATF-6 results in increased expression of ER chaperones, thus increasing the folding capacity of the ER (23). Activation of IRE-1 initiates splicing of the transcription factor XBP-1, which further up-regulates chaperone production and ERAD function, the pathway involved in the disposal of terminally misfolded proteins (24, 25). In the event that ER stress is not inhibited through implementation of these pathways, apoptosis is induced to avoid the untoward effects of cell necrosis. ER stress-mediated apoptosis has been associated with activation of c-Jun amino-terminal kinase via IRE-1 (26), activation of the ER stress–specific caspases 4 (human [27]) and 12 (mouse [28]), and induction of the proapoptotic factor CHOP (29). CHOP is a member of the C/EBP transcription factor family that heterodimerizes with other C/EBPs and mediates the induction of apoptosis through the mitochondrial pathway. It was found that enforced dimerization of Bax, a proapoptotic Bcl-2 family member, and consequent translocation of Bax-dimers from the cytosol to mitochondria play a key role in ER stress–mediated apoptosis downstream of CHOP induction (30). Hence, the profile of ER stress signaling and apoptosis disclosed herein suggest a mechanism for previously reported epithelial apoptosis in patients with IPF.

What could be the underlying reason for induction of an apoptotic ER stress response in epithelial cells from IPF lungs? Our study is limited in that we are not able to disclose the underlying reasons for the development of ER stress in sporadic IPF. However, the following aspects must be taken into consideration. First, accumulation of mutant and misfolded proteins in the ER has been shown to induce severe ER stress and to cause chronic injury to various cell types and tissues. A good example is the development of liver cirrhosis in the homozygous PI*ZZ form of α_1-antitrypsin deficiency, where the Z mutant isoform is misfolded, leading to hepatocyte injury and cirrhosis (31). In addition, conditions such as Alzheimer's, Parkinson's, and Huntington's disease; diabetes; and *SFTPC* mutations represent a large class of conformational diseases that are associated with severe ER stress and intra- and/or extracellular accumulation of abnormal, mutant protein aggregates (12, 13, 32, 33) and appearance of a novel subcellular structure called the "aggresome" (34, 35). Accordingly, *in vitro* overexpression of two distinct *SFTPC* mutations linked to familial IPF led to apoptotic ER stress accompanied by cytosolic aggregation of mutant SP-$C^{\Delta Exon4}$ and SP-C^{L188Q} (13, 14, 20).

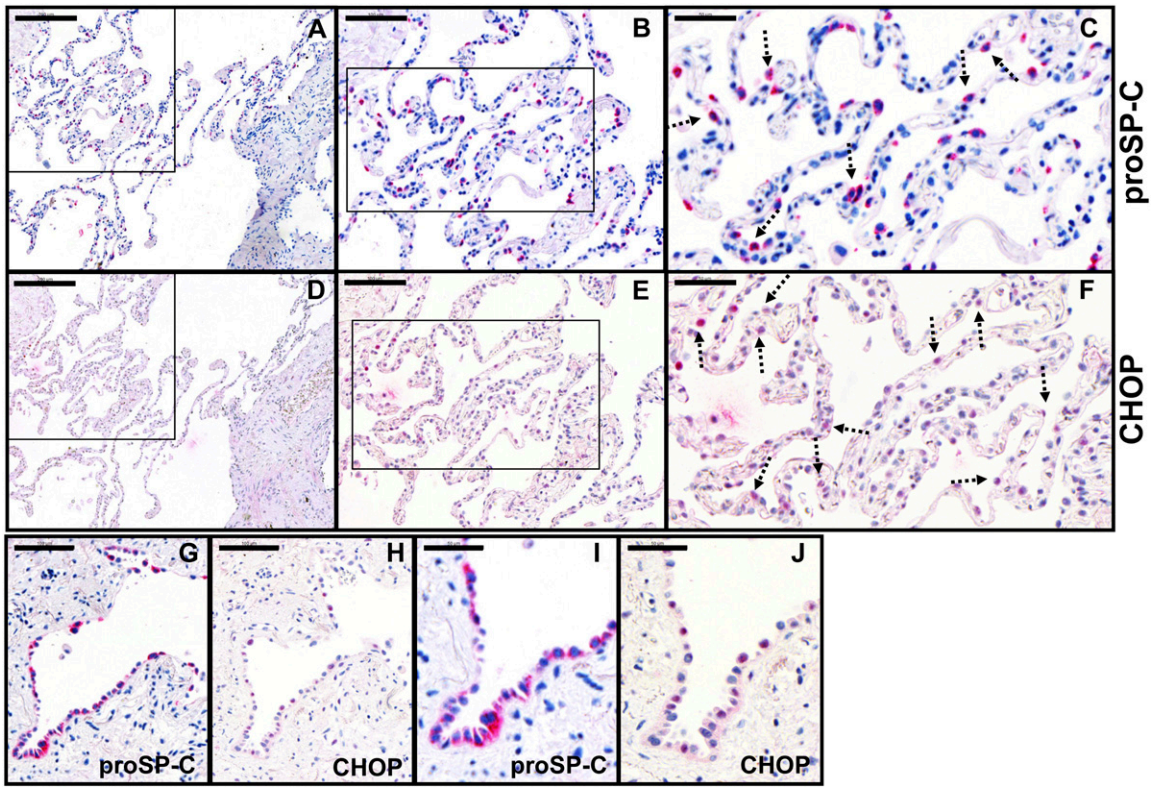

Figure 7. Induction of C/EBP-homologous protein (CHOP) in alveolar epithelial type II cells (AECIIs) of histologically normal appearing areas of idiopathic pulmonary fibrosis (IPF) lung parenchyma. Representative immunohistochemistry for pro–surfactant protein (SP)-C (*A–C, G, I*) and for CHOP (*D–F, H, J*) in serial sections of IPF lung tissue. In AECIIs of histologically normal appearing areas of IPF lung parenchyma, CHOP induction was observed to a lesser extent (*D–F*; *arrows* indicate CHOP-positive AECIIs) than in AECIIs lining airspaces near dense fibrotic zones (*H, J*). Photomicrographs *A–C, G, I, D–F, H,* and *J* are from the same IPF lung tissue section. Original magnification of photomicrographs *A* and *D*: ×100 (*bar* = 200 μm); original magnification of photomicrographs *B, E, G,* and *H*: ×200 (*bar* = 100 μm); original magnification of photomicrographs *C, F, I,* and *J*: ×400 (*bar* = 50 μm).

Second, because AECIIs are specialized secretory cells for the production and secretion of a large amount of surfactant, they may be susceptible to ER stress. Processing of hydrophobic surfactant proteins SP-B and SP-C in AECIIs is a complex process involving multiple proteolytic steps in the secretory pathway (36) and lysosomal compartment. Patients with the

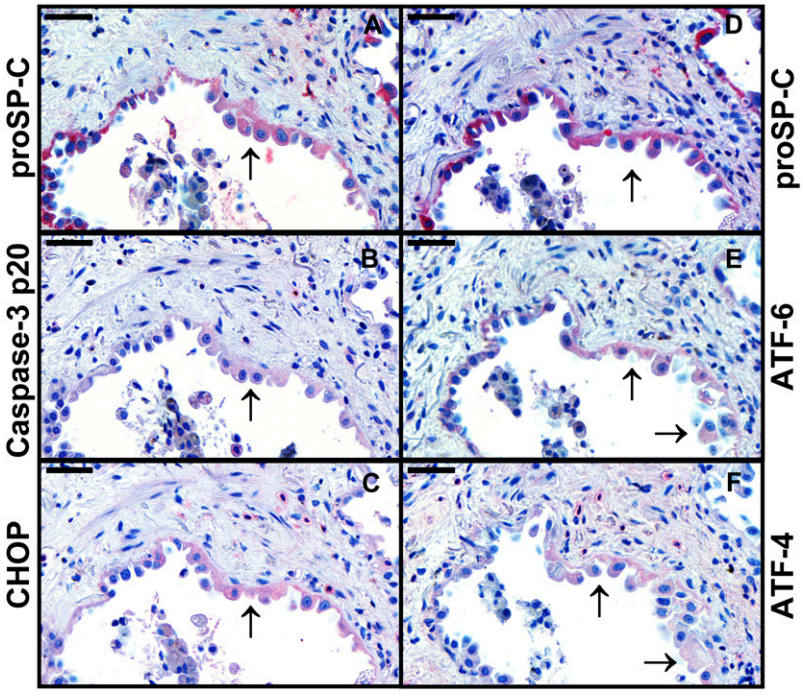

Figure 8. "Hyperplastic" alveolar epithelial type II cells (AECIIs) show severe endoplasmic reticulum stress and consecutive apoptosis. Colocalization of pro–surfactant protein (SP)-C, activated caspase 3, C/EBP-homologous protein (CHOP), activating transcription factor (ATF)-6, and ATF-4 in AECIIs covering fibroblastic foci. Representative immunohistochemistry for pro–SP-C (*A, D*), cleaved caspase 3 (*B*), CHOP (*C*), ATF-6 (*E*), and ATF-4 (*F*) in serial sections of idiopathic pulmonary fibrosis lung tissue. Original magnification of photomicrographs *A–F*: ×400 (*bar* = 50 μm).

inherited Hermansky-Pudlak syndrome, a disease affecting the lysosomal transport axis, may develop pulmonary fibrosis with similarities to IPF. Histopathologically, patients with the *HPS1* mutation reveal enlarged AECIIs with "giant" lamellar bodies due to intracellular accumulation of surfactant (37). Some lysosomal storage diseases, although primarily affecting the transport machinery for lysosomes, are associated with the induction of a severe ER stress response (38).

Third, viral replication may be one explanation for the induction of ER stress and repetitive injury to the alveolar epithelium in IPF. In addition, viral infection on top of an endogenous *SFTPC* mutation was recently shown to result in extensive epithelial apoptosis (39), thus offering a "second hit" model. Herpesvirus DNA and proteins (EBV, CMV, HHV-8) have been detected in the lungs of patients with IPF (40), with early studies showing herpesvirus infection (EBV) localized to AECII (41), although such proof of extensive herpesvirus infection was missing in other reports (42). In a recently published paper, herpesvirus proteins were localized in AECIIs of patients with sporadic IPF by immunohistochemsitry and colocalized with XBP-1 and Bip, suggesting that herpesviruses may indeed play a role in IPF disease progression (20).

Fourth, as suggested by the results of the Idiopathic Pulmonary Fibrosis International Group Exploring NAC I Annual (IFIGENIA) trial investigating high-dose *N*-acetylcysteine application versus placebo (43), increased reactive oxygen species (ROS) generation may contribute to the development of lung fibrosis (44, 45). ROS generation and ER stress response are tightly interconnected: After the induction of oxidative stress, the ER stress sensor PERK mediates phosphorylation of transcription factor NRF2, which translocates into the nucleus to up-regulate genes involved in redox maintenance (46). ROS production may cause misfolding and aggregation of oxidatively modified, abnormal proteins (47) and thus induction of the ER stress response (48). Apart from inflammatory cells that may contribute to epithelial injury and pulmonary fibrosis (44), myofibroblasts have been shown to produce high levels of H_2O_2 in response to cytokines and growth factors (49) and to cause damage to the overlying epithelium.

In conclusion, our report for the first time provides evidence that severe ER stress seems to be an important reason for the development of AECII apoptosis in IPF. The similarities between familial cases of IPF caused by misfolded SP-C and the ER stress reaction described herein suggests that the ER stress reponse may represent an important trigger mechanism of the aberrant fibrotic repair observed in IPF. This and the precise underlying reason for ER stress need to be investigated in future studies.

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