Role of Endoplasmic Reticulum Stress in Epithelial–Mesenchymal Transition of Alveolar Epithelial Cells

Effects of Misfolded Surfactant Protein

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Endoplasmic reticulum (ER) stress has been implicated in alveolar epithelial type II (AT2) cell apoptosis in idiopathic pulmonary fibrosis. We hypothesized that ER stress (either chemically induced or due to accumulation of misfolded proteins) is also associated with epithelial–mesenchymal transition (EMT) in alveolar epithelial cells (AECs). ER stress inducers, thapsigargin (TG) or tunicamycin (TN), increased expression of ER chaperone, Grp78, and spliced X-box binding protein 1, decreased epithelial markers, E-cadherin and zonula occludens-1 (ZO-1), increased the myofibroblast marker, α -smooth muscle actin (α -SMA), and induced fibroblast-like morphology in both primary AECs and the AT2 cell line, RLE-6TN, consistent with EMT. Overexpression of the surfactant protein (SP)–C BRICHOS mutant SP-C Δ Exon4 in A549 cells increased Grp78 and α -SMA and disrupted ZO-1 distribution, and, in primary AECs, SP-C Δ Exon4 induced fibroblastic-like morphology, decreased ZO-1 and E-cadherin and increased α -SMA, mechanistically linking ER stress associated with mutant SP to fibrosis through EMT. Whereas EMT was evident at lower concentrations of TG or TN, higher concentrations caused apoptosis. The Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4]pyrimidine (PP2), abrogated EMT associated with TN or TG in primary AECs, whereas overexpression of SP-C Δ Exon4 increased Src phosphorylation, suggesting a common mechanism. Furthermore, increased Grp78 immunoreactivity was observed in AT2 cells of mice after bleomycin injury, supporting a role for ER stress in epithelial abnormalities in fibrosis *in vivo*. These results demonstrate that ER stress induces EMT in AECs, at least in part through Src-dependent pathways, suggesting a novel role for ER stress in fibroblast accumulation in pulmonary fibrosis.

Keywords: alveolar epithelium; pulmonary fibrosis; fibroblast; myofibroblast

Idiopathic pulmonary fibrosis (IPF), one of the most common forms of chronic interstitial lung disease, is characterized by

CLINICAL RELEVANCE

This study demonstrates that endoplasmic reticulum (ER) stress due to both chemical induction and overexpression of mutant surfactant protein C that is associated with protein misfolding leads to epithelial–mesenchymal transition in alveolar epithelial cells, suggesting a novel and direct role for ER stress in the pathogenesis of pulmonary fibrosis.

fibroblast/myofibroblast accumulation and extracellular matrix (ECM) remodeling that lead to disruption of alveolar architecture and progressive fibrosis (1–3). Although chronic inflammation has traditionally been viewed as key to the pathogenesis of IPF (4), it is not a prominent feature in biopsies of patients with IPF/usual interstitial pneumonia (UIP), and anti-inflammatory and immunosuppressive therapies have been largely unsuccessful, leading to reassessment of the role of inflammation in this disorder (3–5). A resulting paradigm shift suggests a central role for alveolar epithelium in disease pathogenesis, in which IPF is believed to result from repeated episodes of alveolar epithelial cell (AEC) injury in conjunction with release of proinflammatory and profibrotic mediators that lead to fibroblast activation, exaggerated ECM deposition, and progressive fibrosis reminiscent of abnormal wound repair (3–5). In addition to the notion of dysregulated epithelial–fibroblast crosstalk and abnormal repair in promoting fibrosis, recent studies suggest that AECs themselves can give rise to fibroblasts/myofibroblasts through the process of epithelial–mesenchymal transition (EMT) (6–8).

Several reports have revealed an association between mutations in the surfactant protein (SP)–C gene (*SFTPC*) and familial pulmonary fibrosis, including cases of UIP, the pathological correlate of IPF (9–11). Overexpression of *SFTPC* constructs with C-terminal mutations in human (A549) and mouse lung epithelial cells indicates that disruption of intracellular processing of mutant SP-C precursor protein leads to accumulation of misfolded protein and induction of endoplasmic reticulum (ER) stress and epithelial cell apoptosis, suggesting a role for ER stress–induced epithelial injury in the pathogenesis of pulmonary fibrosis (13, 14). Although genetic mutations in *SFTPC* are thought to be uncommon in nonfamilial forms of IPF (15), two recent studies suggest that chronic ER stress due to other causes (e.g., viral infection) may contribute to epithelial abnormalities observed in sporadic IPF (13, 16), suggesting ER stress as a common factor underlying epithelial abnormalities in IPF.

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Myofibroblasts, key effector cells in IPF (17), have been suggested to arise from resident lung fibroblasts, through differentiation of circulating bone marrow-derived progenitors, and/or directly from AECs that have undergone EMT (6, 17, 18). EMT has been increasingly implicated in fibrosis after injury in a number of organs, including kidney and lung (19, 20). Treatment of both primary AECs and lung epithelial cell lines with transforming growth factor (TGF)- β 1 induces EMT (8), and EMT has been reported to contribute to the fibroblast population in murine models of pulmonary fibrosis (7). Together with evidence for EMT in IPF lung biopsies (7, 8, 21), these studies support a role for EMT in the pathogenesis of lung fibrosis.

Association of mutant SPs with familial forms of interstitial lung disease, including pathologic UIP, demonstration that accumulation of misfolded SP-C protein in epithelial cells induces ER stress, evidence for ER stress in AECs in both familial and sporadic forms of IPF, and association of ER stress with apoptosis in IPF suggested to us that, regardless of the initiating factor(s), ER stress may serve not only as the basis for apoptosis, but may contribute to other epithelial derangements observed in IPF. In the current study, we demonstrate that both chemical induction of ER stress by thapsigargin (TG) or tunicamycin (TN) and overexpression of mutant *SFTPC* leads to EMT in AECs. Induction of EMT by overexpression of a misfolded protein serves as proof of concept that ER stress, regardless of cause, may contribute to fibrosis through EMT even in nonfamilial IPF. Furthermore, the level of ER stress to which cells are subjected appears to be a critical determinant of whether cells undergo apoptosis or EMT. These findings demonstrate that ER stress of diverse causes is associated with EMT in AECs, suggesting a novel mechanism whereby ER stress may contribute to fibroblast accumulation in pulmonary fibrosis.

MATERIALS AND METHODS

Antibodies and Reagents

Details of antibodies and reagents are provided in the online supplement.

Preparation and Treatment of Primary Rat AEC Monolayers

Alveolar epithelial type II (AT2) cells were isolated from adult male Sprague-Dawley rats by elastase disaggregation, as previously described (22). See the online supplement for details.

Measurement of Monolayer Bioelectric Properties

Transepithelial resistance (R_t [$K\Omega\text{-cm}^2$]) was measured in AECs on Days 3 and 5 after plating using a rapid screening device (MilliCell-ERS; Millipore, Bedford, MA), as previously described (23).

Culture and Treatment of RLE-6TN and A549 Cells

RLE-6TN cells and A549 cells were purchased from American Type Culture Collection (Manassas, VA). Details of cell culture are provided in the online supplement.

RNA Extraction, cDNA Synthesis, and RT-PCR

RNA was harvested using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized with random hexamers and Superscript III (Invitrogen, Carlsbad, CA). Details of PCR conditions are provided in the online supplement.

Immunofluorescence Microscopy of Primary AECs, RLE-6TN Cells, and A549 Cells

Primary AEC monolayers grown on polycarbonate filters or RLE-6TN cells and A549 cells grown on chamber slides were fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 10 minutes and processed for immunofluorescence staining, as described in the online supplement.

Western Analysis

Western analysis was performed as previously described (22), and blots were analyzed with an Alpha Ease RFC Imaging System (Alpha Innotech, San Leandro, CA). See the online supplement for detailed methods.

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling Assay

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay (Roche Molecular Biochemicals, Indianapolis, IN) was performed according to the manufacturer's instructions as detailed in the online supplement.

Vector Production and Virus Preparation

cDNA for wild-type SP-C (SP-C^{wild-type}) and BRICHOS mutant SP-C ^{Δ exon4} (deletion of exon 4) were subcloned from previously published vectors (24) and inserted into the multiple cloning sites upstream of enhanced green fluorescent protein (EGFP) (25) to generate SinhCMV-mcs(r)-pre-cppt-IG SP-C^{wildtype} and SinhCMV-mcs(r)-pre-cppt-IG SP-C ^{Δ exon4}. See the online supplement for details.

Viral transduction of A549 Cells and Primary AECs

Details of lentiviral infection are provided in the online supplement.

Bleomycin-Induced Lung Injury and Preparation of Frozen Lung Sections

Nkx2.1-Cre mice in the C57BL/6 strain were kindly provided by Dr. Stuart Anderson (Cornell University) (26). R26R- β -galactosidase (LacZ) reporter mice (27) and C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Nkx2.1-Cre;LacZ mice were generated by crossing Nkx2.1-Cre mice and R26R-LacZ reporter mice. See the online supplement for details.

Detection of LacZ Activity and Immunofluorescence Labeling of Lung Sections

LacZ activity was determined by 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside (X-gal) (Sigma, St. Louis, MO) staining. X-gal-stained sections were processed for immunofluorescence for localization of Grp78. Details are provided in the online supplement.

Statistical Analysis

Data are shown as means ($\pm$ SEM) for the indicated number of experiments (n). Proteins were normalized to an internal control and expressed as percentage of controls treated with vehicle only. We used z tests to determine if ratiometric data were statistically different from control. R_t and quantitation of TUNEL staining and α -smooth muscle actin (α -SMA) expression were analyzed by one-way ANOVA. A P value of less than 0.05 was considered statistically significant.

RESULTS

TN and TG Induce ER Stress in RLE-6TN Cells and AECs

TN elicits ER stress by inhibiting protein N-linked glycosylation, leading to accumulation of unfolded proteins and consequent unfolded protein response (UPR) activation (28). TG inhibits ER Ca^{2+} -ATPase and blocks calcium reuptake into the ER lumen, leading to disruption of protein folding and subsequent induction of ER stress (29). Effects of TN and TG on ER stress in RLE-6TN cells and primary AECs were investigated by assessing the expression profile of ER chaperone Grp78 and splicing of mRNA encoding spliced X-box binding protein (XBP)-1 (a downstream target of inositol-requiring enzyme-1). Grp78 mRNA increased 15- to 20-fold in RLE-6TN cells (Figure 1A) and 30- to 50-fold in primary AECs (Figure 1D) after treatment with TN or TG. Western analysis demonstrated significant increases in Grp78 protein in

RLE-6TN cells (Figure 1B) and primary AECs (Figure 1E) after TN or TG treatment. Consistent with induction of ER stress by these agents, an increase in the spliced form of XBP-1 mRNA (XBP-1_S, 575 bp) was observed after TN or TG treatment in RLE-6TN cells (Figure 1C) and AECs (Figure 1F). Immunofluorescence microscopy of primary AECs on Day 3 for TN and on Day 7 for TG (Figure 1G) further demonstrated intense Grp78 staining after treatment with either TN or TG on Day 2 compared with controls, consistent with UPR activation.

TN and TG Induce EMT in RLE-6TN Cells and AECs

Effects of TN and TG on expression of epithelial and mesenchymal markers were evaluated in RLE-6TN cells and primary AECs. In RLE-6TN cells treated with TN (Figure 2A) and TG (see Figure E1A in the online supplement), steady-state levels of zonula occludens (ZO)-1 and E-cadherin protein were markedly reduced to approximately 40% those of untreated cultures. To assess whether down-regulation of these epithelial markers reflected EMT, we also examined effects of TN and TG on cell morphology and α -SMA expression. After treatment of

Figure 1. Tunicamycin (TN) and thapsigargin (TG) induce endoplasmic reticulum (ER) stress in RLE-6TN cells and alveolar epithelial cells (AECs). (A) qPCR of Grp78 in RLE-6TN cells on Day 2 in culture, and (B) representative Western blot and quantitative analysis of Grp78 in RLE-6TN cells on Day 3 in culture, after treatment on Day 1 after plating with TN (2.5 $\mu\text{g/ml}$) for 24 hours, TG (0.3 μM) for 6 hours, or DMSO (vehicle control [C]). RNA and protein were harvested at 24 and 48 hours after initiation of treatment, respectively. qPCR was normalized to β -actin in (A) ($n = 5$ for TN; $n = 4$ for TG for Grp78 mRNA; $n = 5$ for Grp78 protein for both TN and TG). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as a loading control in (B). (C) PCR analysis ($n = 2$) of spliced X-box binding protein (XBP)-1 in RLE-6TN cells on Day 2 in culture treated on Day 1 after plating with TN, TG, or DMSO as in (B). Both XBP-1_U and XBP-1_S were amplified by RT-PCR and separated by 2% agarose gel electrophoresis. (D) qPCR and (E) representative Western blot and quantitative analysis of Grp78 in primary AECs treated on Day 2 after plating with TN (1 $\mu\text{g/ml}$) for 24 hours and TG (0.2 μM) for 30 minutes, or DMSO (vehicle control). RNA and protein were harvested after 24 and 48 hours, respectively. Grp78 mRNA levels shown in (D) were normalized to β -actin ($n = 4$ for Grp78 RNA for both TN and TG; $n = 5$ for TN; $n = 4$ for TG for Grp78 protein). GAPDH is shown as a loading control in (E). (F) Representative PCR analysis ($n = 3$) of spliced XBP-1 in AECs treated with TN and TG, as described in (D) and (E). XBP-1_U and XBP-1_S were amplified by RT-PCR on Day 3 in culture. (G) Immunofluorescence analysis ($n = 2$) of Grp78 (FITC, green) in AECs treated on Day 2 with TN or TG shown on Day 3 after plating for TN treatment and on Day 7 after plating for TG treatment. Nuclei (red) were stained with propidium iodide (PI). Controls were treated with DMSO (vehicle). Original magnification, $\times 400$. * $P < 0.05$ compared with control.

RLE-6TN cells with TN for 24 hours on Day 1 after plating, progressive loss of cuboidal, cobblestone appearance (Figure 2B, *i*) and acquisition of irregular spindle-shaped morphology (Figure 2B, *ii*) were observed on Day 3. Similar changes were seen after treatment of RLE-6TN cells with TG (Figure E1B, *i*, and Figure E1B, *ii*). Consistent with transition to a myofibroblast phenotype, intense staining for α -SMA in a fibril-associated pattern was observed on Day 3 in TN- (Figure 2C, *ii*) and TG- (Figure E1C*ii*), but not vehicle-treated RLE-6TN cells (Figure 2C, *i* and Figure E1C*i*). Western analysis confirmed the increase in α -SMA after treatment of RLE-6TN cells with TN (Figure 2D).

These observations were extended to primary AECs using freshly isolated rat AT2 cells grown on polycarbonate filters for 2 days, followed by treatment with TN for 24 hours, and maintained in culture for an additional 4 days. Similar to observations with RLE-6TN cells, phase contrast microscopy demonstrated a change in morphology of treated AECs from a cuboidal shape (Figure 2E, *i*) to an irregular, spindle-shaped appearance (Figure 2E, *ii*). Similar results were observed after treatment of primary AECs with TG for 30 minutes on Day 2 (Figure E1D). Intense staining for α -SMA was observed in primary AECs treated with TN (Figure 2F, *ii*) or TG (Figure E1E, *ii*), but was absent in control cells (Figure 2F, *i* and Figure E1E, *i*). Immunoreactivity for ZO-1 (Figure 2G, *ii*) and E-cadherin localized to cell borders (Figure 2G, *iv*) was concurrently reduced in TN-treated compared with untreated AECs (Figure 2G, *i* and *iii*). Similar redistribution of epithelial markers was observed in AECs treated with TG (Figure E1F). Furthermore, AEC monolayers treated with TN or TG on Day 2 failed to develop transepithelial electrical resistance on Days 3 and 5, reflecting deterioration of tight junctions accompanying EMT and consistent with loss of the epithelial phenotype (Figure 2H and Figure E1G).

Mutant SP-C Induces EMT in A549 Cells and Primary AECs

In A549 cells, overexpression of mutant SP-C leads to ER stress and α -SMA is increased in TGF- β -induced EMT (14, 30). We used both A549 cells and primary AECs to investigate the contribution of ER stress due to misfolded protein accumulation to lung fibrosis through induction of EMT. Transduction of A549 cells with mutant SP-C ^{Δ exon4} increased expression of Grp78 protein approximately 1.5-fold compared with cells transduced with either SP-C^{wild-type} or EGFP control (Figures 3A and 3B). Importantly, transduction with mutant SP-C ^{Δ exon4} increased expression of α -SMA by approximately 50% (Figures 3A and 3B) and decreased membrane localization of ZO-1 (Figure 3C), changes that were not observed with SP-C^{wild-type} compared with EGFP control. In primary AECs, overexpression of mutant SP-C ^{Δ exon4} resulted in loss of cobblestone

epithelial appearance and transition to fibroblastic-like morphology (Figure 3D), together with a decrease in ZO-1 and E-cadherin and an increase in α -SMA protein expression (Figure 3E) compared with EGFP control. The finding that overexpression of the mutated form of SP-C induces α -SMA concurrent with loss of epithelial characteristics further suggests that, in interstitial lung disease associated with *SFTPC* mutations, ER stress induced by misfolded protein may, in part, contribute to fibrosis through induction of EMT. To confirm that lentivirus constructs encoding EGFP (control), SP-C^{wild-type} (WT) and SP-C ^{Δ exon4} (Δ exon4) were equally overexpressed, we performed FACS analysis of EGFP in A549 cells and Western blotting of EGFP in primary AECs that were transduced with virus. As shown in Figures E2A and E2B, EGFP expression of all three constructs was similarly distributed in both A549 cells and primary AECs. EGFP was not detected in non-transduced cells (data not shown). When cells were transduced with lentivirus constructs encoding SP-C^{wild-type} or SP-C ^{Δ exon4}, pro-SPC (26 kD) or a truncated SP-C were observed (Figure E2B), confirming appropriate expression of the specific construct.

Effects of Pharmacological ER Stress on Apoptosis and EMT Are Dose Dependent

Induction of the UPR is designed to be protective against ER stress; however, sustained or excessive ER stress can lead to apoptosis (12). Our current studies demonstrating EMT in primary AECs in response to ER stress were performed using concentrations of 1 $\mu\text{g/ml}$ TN and 0.2 μM TG. To investigate dose-dependent effects of ER stress, AECs were treated with increasing concentrations of TN (1–10 $\mu\text{g/ml}$) or TG (0.2–0.6 μM) on Day 2 and analyzed by TUNEL staining on Day 3. Exposure to higher concentrations of TN (5–10 $\mu\text{g/ml}$) (Figure 4A) or TG (0.6 μM) (Figure E3A) induced AEC apoptosis. Concurrent reduction in EMT, as evidenced by less expression of α -SMA (Figure 4B) at higher doses, supports the notion that the extent of ER stress is critical in determining whether AECs undergo EMT or commit to apoptosis.

To further investigate cell fate decisions in response to ER stress, expression of C/EBP homologous protein (CHOP), a UPR marker that is implicated in apoptosis, was evaluated after treatment of primary AECs with TN (1 $\mu\text{g/ml}$) or TG (0.2 μM) on Day 2. Representative Western blots demonstrated CHOP induction at early time points (Day 3) at lower concentrations of TN (Figure 4C) or TG (Figure E3B) that, in the current study, induced EMT. However, up-regulation of CHOP was transient and had decreased markedly by Day 5. In contrast, increases in Grp78 evident on Day 3 were sustained through Day 5 (Figure 4C and Figure E3B). Spliced XBP-1,

Figure 2. TN induces epithelial-mesenchymal transition (EMT) in RLE-6TN cells and AECs. (A) Representative Western blot and quantitative analysis of zonula occludens (ZO)-1 and E-cadherin in RLE-6TN cells treated with TN (2.5 μ g/ml) or DMSO (vehicle control) for 24 hours on Day 1 after plating and analyzed on Day 3 ($n = 3$). $*P < 0.05$ compared with DMSO; β -actin is shown as loading control. Phase and immunofluorescence images ($n = 2$) of RLE-6TN cells on Day 3 after plating showing cell morphology (B) and α -smooth muscle actin (α -SMA) reactivity (C) after treatment with TN (2.5 μ g/ml) (ii) or DMSO (i) for 24 hours on Day 1. Original magnification for phase image, $\times 100$, and for immunofluorescence, $\times 400$. Nuclei (red) were stained with PI. (D) Representative Western blot and quantitative analysis of α -SMA in RLE-6TN cells treated with TN (2.5 μ g/ml) or DMSO (vehicle control) for 24 hours on Day 1 after plating and analyzed on Day 3 ($n = 3$). $*P < 0.05$ compared with DMSO. GAPDH is shown as loading control. Phase and immunofluorescence images ($n = 2$) of AEC monolayers show cell morphology on Day 7 (E) and α -SMA reactivity on Day 3 (F) after treatment with 1 μ g/ml TN (ii) or DMSO (i) for 24 hours on Day 2. Original magnification for phase image, $\times 100$, and for immunofluorescence, $\times 400$. Nuclei (red) were stained with PI. (G) Immunofluorescence images ($n = 2$) of AEC monolayer treated with TN or DMSO on Day 2 after plating and stained for ZO-1 (red) (i and ii) and E-cadherin (green) (iii and iv) on Day 3. Nuclei were stained with DAPI (blue) for ZO-1 and with PI (red) for E-cadherin. Original magnification, $\times 400$. (H) Transepithelial resistance (R_T) of AEC monolayers treated with TN or DMSO on Day 2 after plating was measured using a volt-ohmmeter on Days 3 and 5 after plating ($n = 4$ cell preparations). $*P < 0.05$ compared with DMSO.

a downstream target of the UPR, was also present on both Days 3 and 5, although at lower levels on Day 5 than on Day 3 (Figure 4D and Figure E3C). Despite sustained activation of the UPR under conditions of mild ER stress, these transient

increases in CHOP were insufficient to induce significant apoptosis, suggesting that the balance between different arms of the UPR determines the decision between apoptosis and survival.

Figure 3. Induction of Grp78 and α -SMA and loss of ZO-1 and E-cadherin by overexpression of mutant surfactant protein (SP)-C Δ exon4 in A549 cells and primary AECs. Representative Western blot (A) and quantitative analysis (B) of Grp78 and α -SMA on Day 3 after plating in A549 cells transduced with lentiviral vectors encoding enhanced green fluorescent protein (EGFP) (control), SP-C Δ exon4 (WT), and SP-C Δ exon4 (Δ exon4) on Day 1 ($n = 3$). (C) Immunofluorescence microscopy ($n = 2$) of ZO-1 (red) on Day 3 after plating in A549 cells transduced with lentiviral vectors (green) encoding EGFP (control), SP-C Δ exon4 (WT), and SP-C Δ exon4 (Δ exon4) on Day 1. Nuclei were counterstained with DAPI (blue). Original magnification, $\times 400$. (D) Immunofluorescence microscopy and phase images ($n = 3$) of primary AECs on Day 10 after plating showing cell morphology after transduction with lentiviral vectors (green) encoding EGFP (control), SP-C Δ exon4 (WT), and SP-C Δ exon4 (Δ exon4) on Day 2. Original magnification, $\times 200$. (E) Representative Western blot and quantitative analysis of ZO-1, E-cadherin, and α -SMA on Day 10 after plating in AECs transduced with lentiviral vectors encoding EGFP (control), SP-C Δ exon4 (WT), and SP-C Δ exon4 (Δ exon4) on Day 2 ($n = 3$). Lamin A/C and GAPDH are shown as loading controls in (A) and (E), respectively. * $P < 0.05$ compared with control.

Induction of EMT by ER Stress Is Inhibited by Src Inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine (PP2)

To elucidate mechanisms mediating ER stress-induced EMT in AECs, we performed experiments with pharmacologic inhibitors of candidate signaling pathways, including inhibitors of JNK (SP600125), phosphoinositide 3-kinase/Akt (LY294002), Akt, Rho kinase (Y27632), Raf-1 kinase (GW5074), Alk-5 (SB431542), Smad3 phosphorylation (SIS3), p38 kinase (SB202190), NF- κ B (JSH23) and Src (PP2). When assessed by immunofluorescence, PP2 abrogated effects of TN (Figure 5A) and TG (Figure 5B) on

cell shape and expression of epithelial and mesenchymal markers in primary AECs. Although induction of α -SMA was only partially inhibited by PP2, particularly after treatment with TG, expression and localization of epithelial markers was preserved, suggesting an important role for Src activation in ER stress-induced EMT. Treatment with JSH23 slightly decreased α -SMA expression without affecting cell morphology (data not shown). To exclude the possibility that reduction of α -SMA was caused by potential confounding inhibitory effects of PP2 on p38 mitogen-activated protein kinase (30), we evaluated effects of the p38 kinase inhibitor, SB202190, on TN-induced EMT. SB202190 did not affect TN-induced α -SMA expression (data

Figure 4. Effects of ER stress induced by TN are dose dependent. Immunofluorescence images and quantitative analysis of AECs treated with increasing doses of TN (1–10 $\mu\text{g/ml}$) for 24 hours on Day 2 analyzed via terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay (A) and immunostaining for α -SMA (B) on Day 3 after plating. DMSO was used as vehicle control. Percentages of TUNEL-positive cells and cells expressing α -SMA were quantified in each condition by counting cells and analyzed by one-way ANOVA. * $P < 0.05$ compared with all other conditions. (C) Representative Western blot and quantitative analysis ($n = 3$) of Grp78 and CHOP in AECs treated with TN (1 $\mu\text{g/ml}$) for 24 hours on Day 2 after plating. Protein was harvested on Days 3 and 5 after plating. GAPDH is shown as loading control. * $P < 0.05$ compared with TN on Day 3. (D) PCR analysis ($n = 2$) of spliced XBP-1 in AECs treated with TN (1 $\mu\text{g/ml}$). Both XBP-1_U and XBP-1_S were amplified by RT-PCR from RNA harvested on Days 3 and 5, and separated by 2% agarose gel electrophoresis.

not shown), suggesting that it was the inhibitory effects of PP2 on Src signaling that abrogated ER stress-induced EMT. Other inhibitors examined had no effect, and were not investigated further.

As shown in representative Western blots and quantitative analysis (Figures 5C), TN increased tyrosine phosphorylation of

c-Src, which peaked at 1 hour and, as expected, was inhibited by PP2. Similar effects on c-Src phosphorylation were observed after treatment with TG (Figure E4), confirming activation of Src as the result of chemical induction of ER stress. Interestingly, mutant SP-C Δ exon4 also induced phosphorylation of c-Src approximately 1.5-fold compared with SP-C^{wild-type} (Figure 5D),

Figure 5. Role of Src activation in ER stress-induced EMT. (A) Immunofluorescence microscopy of AECs grown on Transwell filters treated with TN (1 μ g/ml) for 24 hours, or pretreated with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) (5 μ M) for 1 hour and then treated with PP2 (5 μ M) and TN (1 μ g/ml) for 24 hours, on Day 2 after plating, and stained for α -SMA (green) (i, ii, and iii), ZO-1 (red) (iv, v, and vi), and E-cadherin (green) (vii, viii, and ix) on Day 3 ($n = 2$). Monolayers immunostained for α -SMA or E-cadherin were counterstained with PI (red); monolayers immunostained for ZO-1 were counterstained with DAPI (blue). Original magnification, $\times 400$. (B) Immunofluorescence microscopy ($n = 2$) on Day 3 of AECs grown on filters treated on Day 2 with TG (0.2 μ M) alone for 30 minutes, or pretreated with PP2 (5 μ M) for 1 hour and then treated with PP2 (5 μ M) and TG (0.2 μ M) for 30 minutes and stained for α -SMA (green) (i, ii, and iii), ZO-1 (red) (iv, v, and vi) and E-cadherin (green) (vii, viii, and ix). Monolayers immunostained for α -SMA or E-cadherin were counterstained with PI (red); monolayers immunostained for ZO-1 were counterstained with DAPI (blue). Original magnification, $\times 400$. (C) Representative Western blot and quantitative analysis of phosphorylated Src (p-Src) and total Src in AECs treated with TN (1 μ g/ml) for indicated times on Day 2 after plating ($n = 3$). To confirm inhibitory effects of PP2 on Src phosphorylation, cells were pretreated with PP2 (5 μ M) for 1 hour, followed by treatment with PP2 together with TN for an additional 1 hour. $*P < 0.05$ compared with 0 minutes. (D) Representative Western blot and quantitative analysis of p-Src and total Src ($n = 3$) on Day 2 in A549 cells transduced with lentiviral vectors encoding EGFP (control), SP-C^{wild-type} (WT), and SP-C ^{Δ exon4} (Δ exon4) on Day 1. $*P < 0.05$ compared with control.

implicating this pathway in induction of EMT in response to ER stress, regardless of the initiating factor.

Bleomycin-Induced Lung Injury Causes Up-Regulation of Grp78 in AT2 Cells

The potential contribution of ER stress to epithelial abnormalities in the bleomycin model of lung fibrosis was evaluated using Nkx2.1-Cre;LacZ mice in which AECs are permanently labeled to express β -galactosidase to allow identification of cells of epithelial origin. As shown in Figure 6A, minimal expression of Grp78 was observed in frozen lung sections from Nkx2.1-Cre;LacZ mice 12 days after intranasal administration of saline. In contrast, Grp78 protein was dramatically increased in lung sections from reporter mice 12 days after administration of

bleomycin and colocalized with X-gal-positive cells, confirming up-regulation of Grp78 in AECs (Figure 6B). These results indicate that ER stress is induced in AECs in this model of lung injury and support a role for ER stress in epithelial abnormalities observed in pulmonary fibrosis *in vivo*.

DISCUSSION

Activation of the UPR and ER stress have been demonstrated in AECs in patients with both familial and sporadic IPF, whereas, in sporadic IPF, markers of the UPR have been colocalized in AECs with markers of apoptosis (13, 16). Expression of mutant SP-C protein in AECs *in vitro* has also been shown to evoke both the UPR and apoptosis, further suggesting a role for ER stress in epithelial abnormalities

Figure 6. Increased Grp78 expression in AECs in mouse lung after bleomycin administration. Frozen lung sections from Nkx2.1-Cre;LacZ mice ($n = 2$) were stained with X-gal followed by immunocytochemistry for Grp78 (FITC, green) 12 days after intranasal administration of saline (control) (A) or bleomycin (6 U/kg) (B). Nuclei (red) were stained with PI. Original magnification for (A) and (B), $\times 400$. Arrows indicate Grp78-positive cells that are X-gal-positive of epithelial origin.

observed in IPF, and implicating ER stress in disease pathogenesis (16). We previously demonstrated colocalization of epithelial and mesenchymal markers in hyperplastic AT2 cells in IPF lung tissue, suggesting that AECs undergo EMT and contribute to fibroblast accumulation in pulmonary fibrosis (8). In the current study, we report, for the first time in the lung, that chemical induction of an ER stress response is associated with EMT in both primary AECs and an immortalized AT2 cell line, and is mediated, at least in part, in a Src kinase-dependent manner. Furthermore, induction of the UPR and acquisition of a mesenchymal phenotype through overexpression of mutant *SFTPC* in AECs *in vitro*, and up-regulation of Grp78 in AT2 cells after bleomycin-induced lung injury, support a role for this pathway in epithelial abnormalities observed *in vivo* in pulmonary fibrosis. These findings suggest that ER stress may contribute to lung fibrosis through induction of EMT.

Nascent proteins that enter the ER must be properly folded with the assistance of ER chaperones before export to the Golgi (31). Protein folding may be disrupted by a number of processes, including, for example, alterations of calcium homeostasis, viral infection, expression of mutant proteins, and altered glycosylation, leading to retention of unfolded or misfolded proteins in the ER (32). In response, the UPR becomes activated through a process involving dissociation of bound Grp78 from ER transmembrane proteins (33). The UPR is executed through the actions of three proximal ER transmembrane proteins (pancreatic ER kinase [PERK]-like ER kinase, activating transcription factor [ATF]-6, and inositol-requiring enzyme-1) (33), activation of which leads to attenuation of the effects of unfolded protein accumulation by enhancing protein folding capacity (28), attenuating global protein synthesis (34), and inducing transcription of ER chaperones and genes that promote degradation of unfolded proteins (35). If the capacity of the UPR is exceeded, ER stress results, which, if excessive or prolonged, may lead to apoptosis through both caspase-dependent and -independent pathways (36).

Activation of the UPR and ER stress have been reported in both sporadic and familial IPF in either the presence or absence of *SFTPC* mutations, whereas mutations of SP-A2 associated with pulmonary fibrosis have been shown to lead to protein instability and ER stress *in vitro* (37). In addition, in sporadic IPF, UPR markers have been colocalized with activated caspase 3 and TUNEL staining in hyperplastic AT2 cells (16), suggesting that ER stress contributes to AEC apoptosis. Our current results demonstrate that ER stress in response to either chemical induction or overexpression of mutant proteins is associated with EMT in both RLE-6TN and A549 cells, as well as primary AECs, suggesting a novel mechanism whereby ER stress might contribute directly to fibrosis. Previous studies have demonstrated that, after bleomycin-induced lung injury, up to 30% of fibroblasts may be derived via EMT (7), although a possible role for ER stress in response to bleomycin has not been evaluated. Our current results demonstrating up-regulation of Grp78 on Day 12 after bleomycin-induced injury in AECs (Figure 6) suggest a possible role for ER stress in observed epithelial abnormalities (including EMT) associated with fibrosis *in vivo*. The factors that lead to ER stress in IPF have been suggested to include viral infection, gastroesophageal reflux, and accumulation of reactive oxygen species, all of which have an increased association with IPF in sporadic disease (16, 38).

The UPR functions as an adaptive response to accumulation in the ER lumen of mis- or unfolded proteins (32). If protein accumulation is sustained and ER stress conditions do not resolve, UPR activation leads to apoptosis, although the precise mechanisms regulating transition from an adaptive, prosurvival response to a commitment to apoptosis are not fully understood (39). Rutkowski and colleagues (40) suggested that the adaptive response under conditions of mild ER stress was not the result of selective activation of specific UPR components, but rather due to early down-regulation of proapoptotic UPR components. In our studies in primary AECs under mild ER stress conditions (1–5 $\mu\text{g/ml}$ TN or 0.1–0.5 μM TG), up-regulation of CHOP was transient, increasing on Day 3 in culture after treatment on Day 2 (Figure 4C and Figure E3B) and decreasing by Day 5. In contrast, increases in Grp78 were sustained through Day 5 (Figure 4C and Figure E3B), consistent with its continued adaptive role in restoration of normal ER function (35). Although both apoptosis and EMT are features of IPF, the mechanisms that determine the choice between these two cell fates likely depends on the duration and extent of AEC injury/ER stress. Demonstration in the current study that mild ER stress (0.2 μM TG or 1–5 $\mu\text{g/ml}$ TN; Figures 4A and 4B, and Figure E3A) is associated with less apoptosis than more stringent conditions, leads us to speculate that EMT in IPF is part of an adaptive response that serves to protect cells against ER stress-induced apoptosis, while recognizing that this adaptive response to ER stress is associated with dysregulation of normal epithelial function and defective repair that may, itself, contribute to the pathogenesis of fibrosis (Figure 7).

There is increasing evidence for association of mutations of the *SFTPC* gene with chronic interstitial lung disease in both children and adults (9, 11). The C-terminal domain of SP-C is critical for normal intracellular protein processing, and mutations in this region are thought to induce cellular toxicity due to protein folding abnormalities (41). Overexpression of BRI-CHOS domain mutant forms of SP-C *in vitro* induces ER stress and apoptosis (9, 11, 13, 14, 24, 41, 42). Our studies show that activation of the UPR as a result of overexpression of the mutant protein SP-C^{Axon4} is accompanied by changes consistent with EMT in both A549 cells and primary AECs (Figure 3). These results suggest that EMT serves as a mechanism whereby mutant *SFTPC* leads to fibroblast accumulation in familial

Figure 7. Model for ER stress-induced EMT in AECs. Pharmacologic treatment of AECs (TN or TG) or transduced expression of aggregation-prone SP-C BRICHOS mutant promotes activation of the unfolded protein response (UPR). In an effort by the cell to restore proteostasis, UPR uses inositol-requiring enzyme (IRE)-1/XBP1 signaling to up-regulate folding machinery, including the chaperone Grp78. Two other UPR signaling pathways (dotted lines) that further promote folding (mediated by activating transcription factor [ATF]-6) or limit new protein synthesis (mediated by pancreatic ER kinase [PERK]-like ER kinase [PERK]) have also been shown to be activated by similar treatments (48). By definition, “ER stress” ensues when UPR cannot restore protein folding. Under conditions of “extensive” ER stress (modeled here by treatment with higher doses of TN), activation of CHOP and apoptosis can ensue, although aggregated protein conformers may also induce apoptosis by CHOP-independent (JNK, caspase 4, or mitochondrial cytochrome C/caspase 9) pathways (denoted by dashed line). Alternatively, as a prosurvival mechanism, “mild ER stress” (modeled with lower dose TN) promotes Src phosphorylation and results in EMT (demonstrated here as down-regulation of tight and adherens junction proteins [ZO-1 and E-cadherin], up-regulation of α -SMA, and alteration of cell morphology). The Src inhibitor, PP2, abrogates both ER stress-induced Src phosphorylation and EMT. Whether TN or TG directly activate Src independent of UPR activation, and mechanisms whereby ER stress lead to Src phosphorylation, are as yet undefined.

pulmonary fibrosis, and supports the notion that ER stress, regardless of cause, may be a common mediator of epithelial abnormalities in pulmonary fibrosis.

Our investigations provide evidence to support a role for activated Src kinase in mediating ER stress-induced EMT in AECs. PP2 attenuated effects of TN and TG on EMT, and Src phosphorylation was induced by TN and TG, as well as overexpression of SP-C Δ exon4. These results suggest a role for Src activation in EMT in response to both chemically induced ER stress and overexpression of misfolded SP, suggesting that Src is activated downstream of the UPR rather than due to direct effects of TN or TG. Although expression of α -SMA was not completely inhibited by PP2, especially after TG treatment, expression and localization of epithelial markers (especially E-cadherin) were preserved, suggesting an important role for Src

activation in ER stress-induced EMT. Our findings provide the first evidence in lung epithelial cells that links ER stress to EMT, at least in part through Src activation, and are consistent with a recent study in thyroid PC Cl3 cells showing that treatment with TN or TG promoted loss of differentiated phenotype and induction of changes consistent with EMT in a Src-dependent manner (43). Our current findings suggest that Src activation may be a generally applicable response to UPR activation and ER stress, particularly in cells with high protein synthetic function. However, the precise mechanism(s) that link the UPR to Src activation remain to be determined. c-Src has been shown to mediate cancer progression primarily through effects on cell adhesion, invasion, and motility, although evidence for induction of complete EMT is more limited (45–47). Src activation has also been shown to modulate cell–cell

adhesion and cell migration through effects on both focal adhesions and adherens junctions (44), and specifically to induce tyrosine phosphorylation of β -catenin that leads to disruption of its association with E-cadherin, thereby disrupting cell–cell adhesion (45–47). Whether similar mechanisms mediate EMT in response to Src activation in the context of UPR activation and ER stress remains to be determined.

In conclusion, the demonstration that ER stress is associated with EMT in AECs suggests a novel and direct role for ER stress in the pathogenesis of pulmonary fibrosis. Although ER stress in association with *SFTPC* mutations is attributed to accumulation of mutant proteins, the precise factors resulting in ER stress associated with fibrosis in nonfamilial disease remain to be determined. Differential responses of AECs to various levels of ER stress further suggest that EMT may represent an adaptive response in which the level/duration of ER stress determines the choice between cell survival and apoptosis. Modulation of ER stress responses in AECs could serve as a novel approach to ameliorate epithelial abnormalities implicated in the pathogenesis of IPF.

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