ORIGINAL RESEARCH

Thrombin Increases Lung Fibroblast Survival while Promoting Alveolar Epithelial Cell Apoptosis via the Endoplasmic Reticulum Stress Marker, CCAAT Enhancer–Binding Homologous Protein

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Abstract

Apoptosis of alveolar epithelial cells (AECs) and survival of lung fibroblasts are critical events in the pathogenesis of pulmonary fibrosis; however, mechanisms underlying the apoptosis of AECs and the resistance of lung fibroblasts to apoptosis remain obscure. Herein, we demonstrate that the fate of these two cell types depends on the expression of CCAAT enhancer–binding homologous protein (CHOP). We observed that thrombin, which is overexpressed in scleroderma (SSc; systemic sclerosis) and other interstitial lung diseases (ILDs), increases the expression of CHOP in primary AECs and in A549 cells via an Ets1-dependent pathway. In addition, thrombin activates caspase-3 in AECs and induces apoptosis of these cells in a CHOP-dependent manner. In contrast, thrombin decreases endoplasmic reticulum stress–induced CHOP in lung fibroblasts through Myc-dependent mechanisms and protects such cells from apoptosis. Furthermore, when lung fibroblasts are transfected with recombinant CHOP, they then undergo apoptosis, even in the presence of thrombin, suggesting that CHOP signaling pathways are downstream of thrombin. In accordance with the differential effects of thrombin on AECs and lung fibroblasts, we observed strong expression of CHOP in AECs in fibrotic lung tissue isolated from patients with SSc-associated ILD (SSc-ILD), but not in lung myofibroblasts nor in normal lung tissue. Expression of CHOP in SSc lung is accompanied by positive staining for the thrombin receptor, protease-activated receptor-1, and for terminal deoxynucleotidyl transferase dUTP nick end labeling, suggesting roles for both thrombin and CHOP in AEC apoptosis in SSc-ILD. We conclude that regulation of CHOP by thrombin directs AECs toward apoptosis while promoting survival of lung fibroblasts, ultimately contributing to the persistent fibroproliferation seen in SSc-ILD and other fibrosing lung diseases.

Keywords: thrombin; CCAAT enhancer–binding homologous protein; scleroderma; interstitial lung disease; apoptosis

Clinical Relevance

Pulmonary fibrosis is an irreversible and progressive disease process, often leading to respiratory failure and death with unresolved pathogenesis and unresponsiveness to currently available therapies. This study provides a novel insight into thrombin-driven mechanism of pulmonary fibrosis.

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ER stress has been implicated in the development of heart, kidney, and liver fibrosis (4–7). ER stress is also a prominent feature of idiopathic pulmonary fibrosis (IPF) (8), wherein ER stress induces epithelial–mesenchymal transition in fibrotic lung together with apoptosis of alveolar epithelial cells (AECs) (9). Mouse studies have revealed that induction of ER stress in the alveolar epithelium predisposes to enhanced lung fibrosis after treatment with bleomycin, which is mediated at least in part by AEC apoptosis (10). Knockout of CHOP has been reported to attenuate various forms of fibrosis (e.g., cholestasis-induced liver injury, cardiac and renal fibrosis) (4–7).

Systemic sclerosis (SSc; scleroderma) is a multisystem fibrotic disorder that affects the skin and internal organs. Interstitial lung disease (ILD) or pulmonary fibrosis occurs in up to 60% of patients with SSc, and is a leading cause of mortality and morbidity in SSc (11, 12). Although the pathogenesis of SSc-associated ILD (SSC-ILD) remains unclear, several studies have suggested that apoptosis of AECs and survival of lung fibroblasts represent two prominent features of this and other fibrotic lung diseases (reviewed in Ref. 13). Apoptosis of AECs could interfere with synthesis of surfactant and cause a marked disruption in the integrity of the alveolar epithelium, which has been assumed to be one of the first events in the initiation of fibrogenesis, whereas lung fibroblasts, another key player in the pathogenesis of fibrosis, may actually develop resistance to apoptosis (14–17). The increased survival and proliferation of fibroblasts is responsible for excessive collagen production and ECM deposition. Recent studies have revealed that the induction of ER stress facilitates the progression of lung disease, at least in part by increasing apoptosis of AECs, in either IPF or experimental models of lung fibrosis (8, 10, 18). However, the precise mechanisms underlying the regulation of increased apoptosis of AECs and resistance to apoptosis of fibroblasts in SSc-ILD remain unclear.

A number of reports have demonstrated that thrombin activity is increased in bronchoalveolar lavage fluid obtained from patients with SSc-ILD, as well as in experimental models of bleomycin-induced pulmonary fibrosis (19, 20). Thrombin, mostly through protease-activated receptor (PAR)-1, can modulate tissue repair by stimulating differentiation of normal fibroblasts to a myofibroblast phenotype, promoting adhesion and proliferation of endothelial cells and inducing secretion of several profibrotic factors, such as transforming growth factor-β and connective tissue growth factor (21–23). Despite the evidence of thrombin's role in the pathogenesis of SSc-ILD, there has been no previous report specifically addressing whether thrombin regulates AECs and fibroblasts in this disorder, subsequently changing the fate of each of these important cell types. The present investigation was initiated to examine this possibility.

Materials and Methods

Cell Culture

Lung tissues were collected postmortem from five control subjects and from five patients with SSc who fulfilled the American College of Rheumatology preliminary criteria for SSc and had evidence of lung involvement according to guidelines of the Institutional Review Board of the Medical University of South Carolina. The diagnosis of SSc-ILD was confirmed by histological examination of postmortem lung tissue. In addition, lung tissue from mice described in our earlier publication (22) was obtained in agreement with guidelines of the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Lung fibroblasts were isolated from human and mouse lung tissue as previously described (21) and used between second and fourth passages in all experiments. Primary AECs were isolated from mouse lung tissue using a protocol of dispase digestion–agar instillation adapted from DeMaio and colleagues (24) (*see* the online supplement and Figures E1 and E2 in the online supplement for details). Human lung adenocarcinoma epithelial cells A549 were purchased from Lonza (Walkersville, MD) (*see* the online supplement and Figure E3 for details).

Immunohistochemistry

Lung tissues were washed with PBS, fixed with 4% paraformaldehyde, and embedded in paraffin blocks. The 7-μm paraffin sections were immunostained with different antibodies, as described in the online supplement. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed using *In Situ* Cell Death Detection Kit from Roche Diagnostics (Indianapolis, IN) in accordance to the manufacturer's instructions. Fluorescence signals were visualized with a Leica DMI4000B fluorescence microscope (Leica, Buffalo Grove, IL) equipped with a Hamamatsu Camera Controller (ORCA-ER; Hamamatsu, Shizuoka, Japan) and quantified using Adobe Photoshop CS3 software (Adobe, San Francisco, CA).

Cell Death Detection Assay

Cell Death Detection ELISA Kit from Roche Diagnostics was used to detect apoptosis in cultured cells. The cells were plated on 12-well plates and treated with thrombin, Fas ligand (FasL), and PAR-1 antagonist, SCH79797, for 24 hours. In one part of the experiments, A549 cells were transfected with CHOP or control small interfering RNA (siRNA) from Santa Cruz Biotechnology (Santa Cruz, CA). Cell lysates were collected in accordance with the manufacturer's instructions, transferred to a streptavidin-coated ELISA plate, and incubated with anti-histone and anti-DNA antibodies. A peroxidase substrate was applied, and the plates were read at 405 nm on a spectrophotometer.

Preparation of Cell Extracts and Immunoblotting

Cells were collected and analyzed by immunoblotting as previously described (21, 22). In some experiments, cells were incubated with and without various commercially available inhibitors and/or transfected with siRNAs from Santa Cruz Biotechnology in accordance with manufacturer's instructions. The nuclear proteins were extracted as previously described (25).

Luciferase Assay

Cells were cultured in 24-well plates and transfected with CHOP promoter luciferase reporter construct (generously provided by Dr. Pierre Fafournoux, Institut National de la Recherche Agronomique de Theix, France) using Effectene Transfection Reagent (Qiagen, Germantown, MD). In all experiments, green fluorescent protein plasmid was cotransfected to standardize for transfection efficiency. The cells were incubated with thrombin, tunicamycin, or a combination of thrombin and tunicamycin for 24 hours and lysed in Passive Lysis Buffer according to the

Promega luciferase assay system protocol (Promega, Madison, WI). The luciferase activity of the cell lysates was measured with luciferase substrate using a luminometer. Data are expressed as relative firefly luciferase signal normalized by the green fluorescent protein signal for each individual analysis. Each sample was analyzed in triplicate.

Statistical Analysis

Statistical analyses were performed with KaleidaGraph 4.0 (Synergy Software, Reading, PA). All data were analyzed using ANOVA with *post hoc* testing. The results were considered significant if P was less than 0.05.

Results

Effect of Thrombin on Expression of ER Stress Markers in Different Cell Lines

Increased ER stress has recently been reported in patients with pulmonary fibrosis (8). To determine whether thrombin is involved in ER stress in fibrotic lung tissue, we investigated effects of thrombin on ER stress markers in lung fibroblasts and in AECs. We first studied the effects of thrombin on expression of the ER chaperone, binding immunoglobulin protein (BiP) (Grp78), which is always up-regulated in response to ER stress (26), as well on the expression of activating transcription factor (ATF)-4, and the proapoptotic ER stress marker, CHOP. We observed that thrombin increases the expression of CHOP in primary mouse AECs and in A549 cells; however, thrombin has no observable effect on CHOP expression in lung fibroblasts. Surprisingly, we found that thrombin does not affect BiP and ATF-4 expression in any of the cell lines tested, suggesting that thrombin induces expression of CHOP in AECs via a novel non–ER stress–dependent pathway (Figure 1A). To induce ER stress, we used tunicamycin, which is known to inhibit protein N–linked glycosylation, leading to accumulation of unfolded proteins and consequent unfolded protein response (UPR)/ER stress. We observed that tunicamycin readily elevates the expression of BiP, ATF-4, and CHOP in primary mouse AECs and in A549 cells, as well as in primary lung fibroblasts. Thrombin treatment of AECs in combination with tunicamycin results in increased expression of CHOP, whereas combinatory treatment of lung fibroblasts with thrombin and tunicamycin leads to decreased amounts of CHOP, suggesting that thrombin may be a negative regulator of CHOP expression in both normal and SSc lung fibroblasts (Figures 1A and 1B).

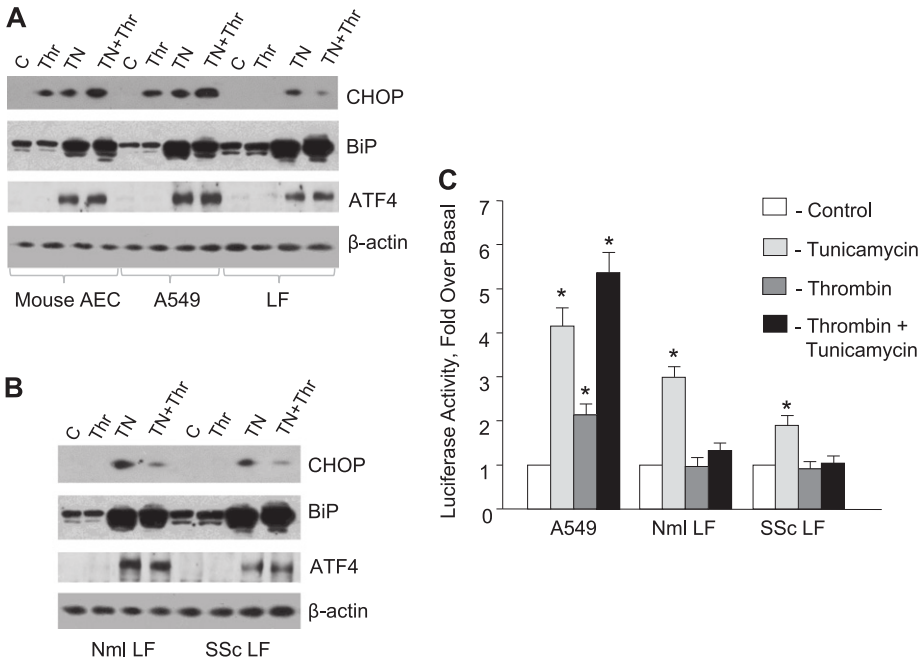

Figure 1. (*A*) Different effects of thrombin (Thr) on expression of CCAAT enhancer–binding homologous protein (CHOP), binding immunoglobulin protein (BiP), and activating transcription factor (ATF)-4 in alveolar epithelial cell (AEC) and lung fibroblast (LF) cell lines. Confluent cultures of primary mouse AECs, A549 cells, and LFs were incubated in serum-free medium as control (C), with Thr (1 U/ml), with tunicamycin (TN; 5 μg/ml), and with combination of tunicamycin and Thr for 24 hours. Cells were collected with lysis buffer, subjected to SDS–polyacrylamide gels, and analyzed by immunoblotting using anti-CHOP, anti-BiP, and anti–ATF-4 antibodies from Cell Signaling Technology. The experiment was performed four times using four different primary AEC and LF cell lines; representative immunoblots are presented. Expression of β-actin is shown to confirm the equal loading of protein. (*B*) Comparative effect of Thr on CHOP expression in normal (Nml) and scleroderma (SSc; systemic sclerosis) LFs. Cell extracts from three Nml and three SSc samples were analyzed by immunoblotting; representative blots are presented. (*C*) Transcriptional activity of CHOP promoter in AEC and LF cell lines. The experiments were performed using A549 cell line, five different Nml, and five SSc LF cell lines. Each sample was analyzed in triplicate. Data are expressed as relative luciferase activity (mean ± SEM), normalized as described in MATERIALS AND METHODS. *Significantly different from control ($P < 0.05$).

To further investigate the regulation of CHOP expression in lung fibroblasts and epithelial cells, we performed a luciferase reporter assay. We observed that tunicamycin increases the transcriptional activity of CHOP promoter 4.16-fold in A549 cells, whereas this increase was only 2.99- and 1.89-fold in normal and SSc lung fibroblasts, respectively. Thrombin stimulates transcriptional activity of CHOP in A549 cells alone and in combination with tunicamycin, but does not affect the transcriptional activity of CHOP in lung fibroblasts. Moreover, thrombin diminishes tunicamycin-induced luciferase activity in normal and SSc lung fibroblasts (Figure 1C).

Regulation of CHOP by Thrombin in AECs and Lung Fibroblasts

To examine the pathways involved in thrombin-induced regulation of CHOP, we used commercially available inhibitors and siRNAs. We found that inhibition of protein kinase C and NF-κB with calphostin C and (2E)-3-[(4-Methylphenyl)sulfonyl] acrylonitrile 11-7082, respectively, has no effect on thrombin-dependent CHOP regulation (data not shown). We observed that depletion of the transcription factor, Ets1, with siRNA dramatically reduces thrombin-induced CHOP in A549 cells;

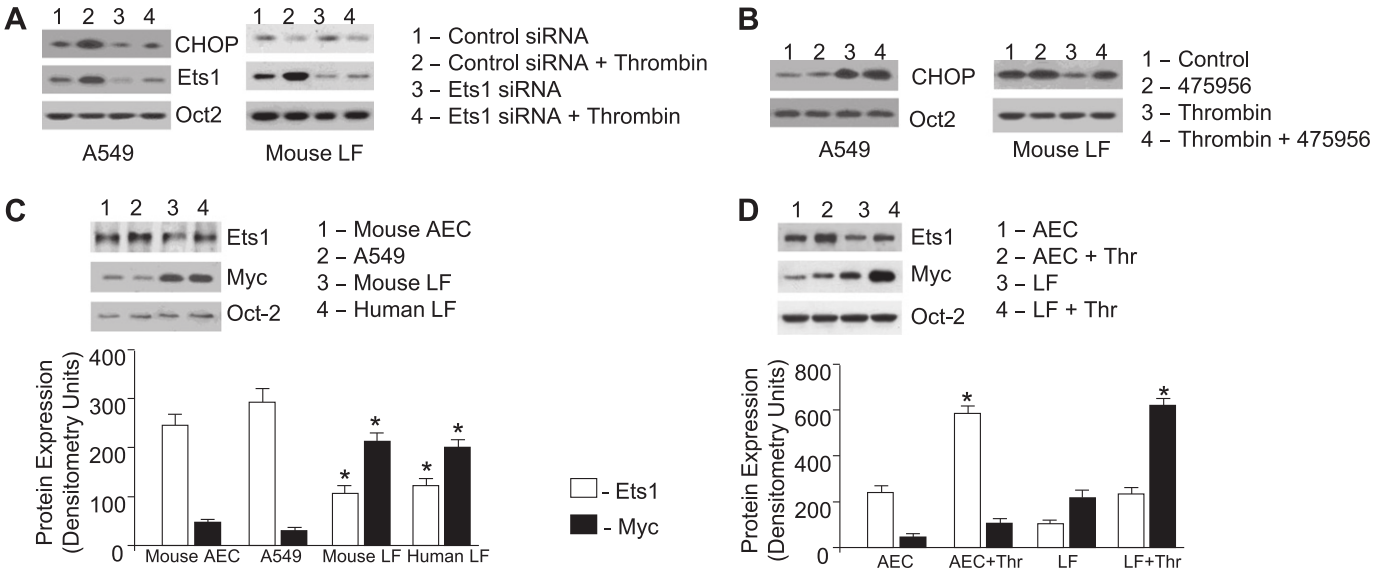

Figure 2. Regulation of CHOP by Thr in AECs and LFs. Cells were cultured on 100-mm plates followed by incubation with or without Thr. In a part of the experiments, cells were transfected with small interfering RNA (siRNA) or treated with commercially available inhibitors. Nuclear proteins were extracted using the NE-PER Kit from Pierce (Rockford, IL) and analyzed by immunoblotting. Expression of homeodomain protein Oct-2, which is known to be unaffected by Thr (9), is shown to confirm equal loading of proteins. (*A*) Effects of Thr and Ets1 siRNA on CHOP expression in LFs and A549 cells. (*B*) Effects of Thr and Myc inhibitor 475,956 on CHOP expression in A549 cells and in tunicamycin-induced LFs. Western blot images presented in (*A*) and (*B*) are representative of three independent experiments. (*C*) Expression of Ets1 and Myc in different cell lines. *$P < 0.05$ LFs versus AECs. (*D*) Effect of Thr on Ets1 and Myc expression in primary mouse AECs and LFs. *$P < 0.01$versus Thr-unstimulated cells. Images in (*C*) and (*D*) were analyzed using ImageJ software (Bethesda, MD). Densitometric analysis of immunoblots from five independent experiments is presented. Values are means ± SEM.

however, it has no effect on CHOP in lung fibroblasts (Figure 2A). Myc inhibitor 475956, in contrast, has no effect on thrombin-induced CHOP expression in A549 cells, but diminishes the effect of thrombin on CHOP in tunicamycin-treated lung fibroblasts (Figure 2B).

Ets1 is a critical regulator of growth-related responses, and Myc is a well recognized negative regulator of CHOP expression (27, 28). We observed that primary mouse AECs and A549 cells contain significantly less Myc protein, but more Ets1 protein, compared with lung fibroblasts (Figure 2C). Thrombin further increases expression of Ets1 in primary AECs and further increases expression of Myc in lung fibroblasts (Figure 2D).

Thrombin Induces Apoptosis of AECs in a CHOP-Dependent Manner

Next, we investigated effects of thrombin on caspase-3 in lung epithelial cells and lung fibroblasts. We incubated human A549 cells, mouse primary AECs, and mouse lung fibroblasts with thrombin for 24 hours and observed that thrombin activates caspase-3 in AECs, but not in lung fibroblasts (Figure 3A). To determine where CHOP is involved in thrombin-induced caspase-3 activation, we knocked down CHOP expression in AECs by CHOP siRNA. We observed that depletion of CHOP expression by siRNA prevents thrombin-induced cleavage and activation of caspase-3 in both human A549 AECs and in mouse primary AECs (Figure 3B).

Activation of the thrombin PAR-1 receptor is known to mediate apoptosis of intestinal epithelial and lung epithelial cells (29, 30). We observed that thrombin up-regulates the expression of proapoptotic protein CHOP in both human A549 AECs and mouse primary AECs, and that CHOP siRNA does not interfere with PAR-1 expression (Figure 3B). To define whether PAR-1 is involved in thrombin-induced expression of CHOP, we used the PAR-1 antagonist, SCH79797. We found that SCH79797 does not affect endogenous levels of CHOP and Ets1 in primary mouse AECs, but decreases thrombin-induced expression of these proteins (Figure 3C), suggesting that thrombin up-regulates Ets1 and CHOP in AECs via its receptor, PAR-1.

To measure the level of apoptosis in lung epithelial cells, we employed a Cell Death Detection ELISA that detects cytoplasmic histone-associated DNA fragments from apoptotic cells. After 24 hours of incubation with thrombin, A549 cells demonstrated 3.85-fold and primary mouse AECs demonstrated 4.29-fold more DNA fragments compared with control cells (Figure 4A). The PAR-1 antagonist, SCH79797, alone had no effect on cell apoptosis, but it diminished thrombin-induced apoptosis in A549 cells and in primary AECs (Figure 4A).

To investigate whether CHOP mediates thrombin-induced apoptosis of AECs, we used CHOP siRNA. Transfection of A549 cells with CHOP siRNA results in significant reduction of thrombin-induced CHOP (Figure 4B). We further observed that depletion of CHOP decreases thrombin-induced apoptosis 2.6-fold, suggesting that thrombin induces apoptosis of AECs in a CHOP-dependent manner. Next, we transfected A549 cells with CHOP and measured the level of apoptosis again in the presence and absence of thrombin. We found that thrombin induces apoptosis of A549 cells transfected with vector alone in a manner similar to nontransfected cells; however, cells transfected with CHOP-pcDNA3 undergo apoptosis without any

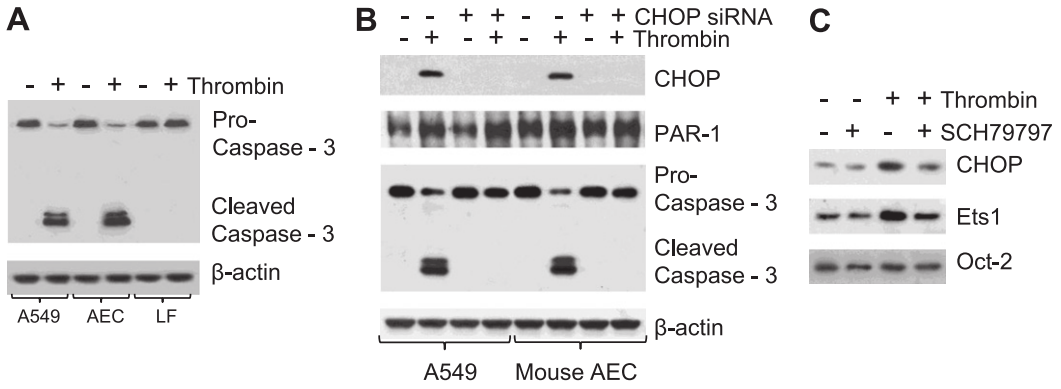

Figure 3. CHOP mediates Thr-induced cleavage of caspase-3 in AECs. (*A*) Effect of Thr on caspase-3 in AECs and LFs. Confluent cultures of A549 cells, primary mouse AECs, and mouse LFs were incubated with or without Thr for 24 hours and analyzed by Western blot using anti–caspase-3 antibody. Expression of β-actin is shown to confirm the equal loading of protein. Note that caspase-3 antibody recognizes full-length pro–caspase-3 (35 kD) and the fragment (17 kD) from caspase-3 resulting from cleavage. (*B*) Effect of CHOP on protease-activated receptor (PAR)-1 expression and Thr-induced cleavage of caspase-3 in AECs. A549 and primary mouse AECs were transfected with control siRNA or CHOP siRNA and incubated with or without Thr for 24 hours. Cells were collected with lysis buffer, subjected to SDS–polyacrylamide gels, and analyzed by immunoblotting using indicated antibodies. (*C*) PAR-1 mediates Thr-induced expression of Ets1 and CHOP in AECs. Primary mouse AECs were incubated with and without Thr and PAR-1 antagonist SCH79797 for 24 hours, followed by nuclear protein extraction and immunoblotting. Representative blots from three independent experiments are presented.

additional stimulus (Figure 4C). The amount of DNA fragments in cells transfected with CHOP-pcDNA3 increases 5.13-fold in 24 hours. Thrombin further enhances apoptosis of CHOP-transfected A549 cells, increasing DNA fragments to 6.17-fold (Figure 4C).

CHOP-Induced Apoptosis of Lung Fibroblasts

Our previous studies demonstrated that thrombin leads to normal lung fibroblast differentiation to myofibroblasts that are resistant to apoptosis (21, 25, 31). To determine whether CHOP would interfere with thrombin-promoted lung fibroblast resistance to apoptosis, we transfected lung fibroblasts with CHOP-pcDNA3.1. Lung fibroblasts transfected with vector only were used as controls. After 48 hours of transfection, cells were incubated with or without thrombin and FasL for 24 hours and analyzed for apoptosis. We observed that lung fibroblasts transfected with CHOP become apoptotic without any additional treatment (5.01 ± 0.9 optical density (O.D.) vs. vector-transfected 0.86 ± 0.48 O.D.; $P < 0.001$; Figure 4D). FasL further increases apoptosis of CHOP-transfected lung fibroblasts (8.53 ± 1.47 O.D. vs. 5.01 ± 0.9 O.D.; $P < 0.05$). Thrombin diminishes FasL-induced apoptosis of vector-transfected lung fibroblasts; however, thrombin has no such effect on CHOP-transfected cells, suggesting that CHOP exerts proapoptotic effects downstream of thrombin.

Studies of CHOP in SSc-ILD Lung Tissue

Increased expression of CHOP has recently been demonstrated in the alveolar epithelium of patients with IPF (8). To establish the existence and localization of CHOP within lung tissue of patients with SSc-ILD, we undertook immunofluorescent analysis for CHOP, surfactant protein C, and smooth muscle actin (SMA) in serial sections of lung tissue from patients with end-stage SSc-ILD and from healthy control subjects. SPC and SMA were used as markers for alveolar epithelial type II cells and myofibroblasts, respectively.

In normal lung, we observed thin layers of alveolar structures composed of septa, vascular components, and connective tissues (Figure 5A). Alveolar septa were thin, allowing maximum air to occupy the lung. Normal alveoli randomly contained type II AECs characterized by positive expression of SPC (Figures 5B and 5C). SMA was expressed predominantly in smooth muscle cells restricted to blood vessels and airways (Figures 5C and 5D). We did not observe any positive staining for CHOP in normal lung tissue (Figures 5E and 5F).

All five samples of lung tissue representing end-stage SSc-ILD were characterized by severe disarrangement of lung architecture. In areas of diffuse fibrosis, the alveolar septa were markedly thickened and the residual air spaces were either very small or star shaped, reflecting the honeycombing of end-stage SSc-ILD (Figure 5G). Quantitative analysis demonstrated that CHOP was expressed in the majority of AECs that also positively stained for pro-SPC (Figures 5J, 5K, 5M, and 5O). In contrast, myofibroblasts stained positively for α-SMA did not show notable immunoreactivity for CHOP (Figures 5I, 5L, and 5N).

We previously demonstrated that thrombin PAR-1 receptor is abundantly expressed in lung tissue from patients with SSc-ILD (25). To determine whether CHOP-positive cells also express PAR-1, we performed double immunofluorescence staining for CHOP and PAR-1 expression. We observed that more than 40% of total cells in SSc-ILD lung express PAR-1; importantly, nearly all CHOP-positive cells also expressed PAR-1 (Figure 6).

To establish whether expression of CHOP in AECs is accompanied by apoptosis, DNA fragmentation in SSc-ILD lung sections was studied by terminal deoxynucleotidyl transferase dUTP nick end labeling reaction in combination with immunofluorescence staining for CHOP. We found that most of the apoptotic cells in SSc-ILD tissue are characterized by the expression of CHOP. After quantification,

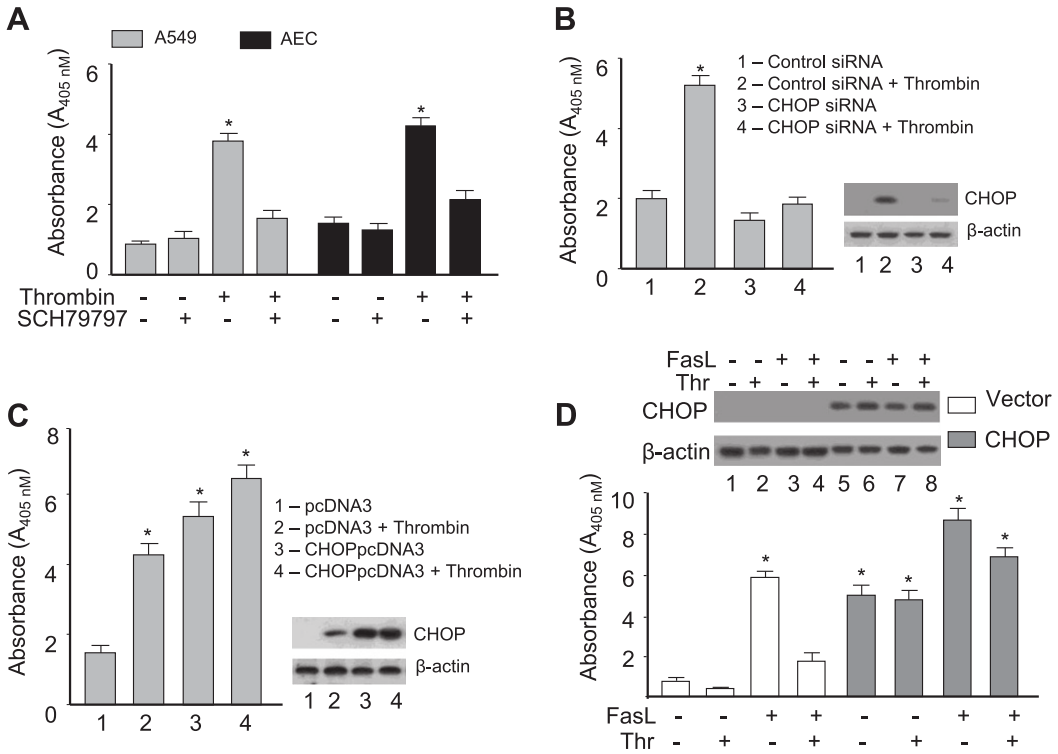

Figure 4. Measurement of apoptosis in AECs and LFs. (*A*) Thr induces apoptosis of A549 cells and primary mouse AECs via PAR-1. Cells were plated on 12-well plates, treated with and without Thr and PAR-1 antagonist, SCH79797, for 24 hours, and processed for Cell Death Detection ELISA. (*B*) Depletion of CHOP by siRNA decreases the Thr-induced apoptosis in A549 cells. Cells were transfected with CHOP siRNA or control siRNA followed by incubation with Thr for 24 hours, and processed for Cell Death Detection ELISA. (*C*) Transfection of A549 with recombinant CHOP results in cell apoptosis with or without Thr. Cells were transfected with recombinant CHOP or empty vector followed by incubation with Thr for 24 hours. Cell Death Detection ELISA was performed according to manufacturer's instructions. Each *bar* represents the mean ± SEM of duplicate determinations in three experiments. *Statistically significant differences between cells stimulated with Thr versus control ($P < 0.05$). (*D*) CHOP-induced apoptosis of LFs. Vector and CHOP-transfected cells were stimulated with Thr or Fas ligand (FasL) for 24 hours followed by measurement of apoptosis using Cell Death Detection ELISA. Each *bar* represents the mean ± SEM of duplicate determinations in three experiments. *Statistically significant differences versus pcDNA3.1-transfected cells incubated in serum-free medium. Immunoblot demonstrates transfection efficiency CHOP-transfected (*lanes 5–8*) LFs versus vector-transfected (*lanes 1–4*).

10.4% of total cells demonstrated evidence of apoptosis; 95.2% of those were also CHOP positive (Figure 7).

Discussion

Although the pathogenesis of pulmonary fibrosis is unclear, a large body of evidence suggests that resistance to apoptosis in myofibroblasts and increased apoptosis in AECs are fundamental processes (13, 32, 33). The factors responsible for these processes are not well established, although an important role for one of the central fibrogenic mediators, transforming growth factor-β, has been demonstrated (13, 32, 33). Thrombin, one of the first factors activated on tissue injury, has been similarly shown to stimulate apoptosis of AECs and to induce a myofibroblast phenotype with resistance to apoptosis (25, 30). Apoptosis of AECs can be also induced by CHOP (8, 34), a proapoptotic marker of ER stress and UPR. Recently, emerging studies have confirmed that ER stress plays an important role in the pathogenesis of ILD (8, 10). However, an association of ER stress and the distinct fate of AECs and lung fibroblasts has not yet been elucidated.

Herein, we report that the expression of CHOP, a proapoptotic transcription factor implicated in the UPR to ER stress, is increased in primary AECs and in A549 cells, but not in lung fibroblasts when exposed to the same external stimuli. In addition, immunohistochemical staining identifies the presence of CHOP in AECs surrounded by fibrotic tissue in patients with SSc-ILD, but not in myofibroblasts in the same tissue samples. Importantly, we observed that nearly all apoptotic cells in SSc-ILD lung tissue are also CHOP-positive, suggesting an important contribution of CHOP to the increased apoptosis of AECs in pulmonary fibrosis.

A second important observation in this study concerns the differential regulation of CHOP by thrombin in lung fibroblasts and epithelial cells. Our data suggest that thrombin up-regulates CHOP via a novel ER stress–independent, but Ets1-dependent, pathway in AECs, and that thrombin down-regulates CHOP via cMyc in lung fibroblasts (Figure E4).

Thrombin is a serine proteinase that is centrally involved in the final step of the coagulation cascade. In addition to its role in hemostasis, thrombin also mediates a number of biological responses relevant to inflammatory and tissue repair responses, predominantly via PAR-1–dependent mechanisms. Our group and others have

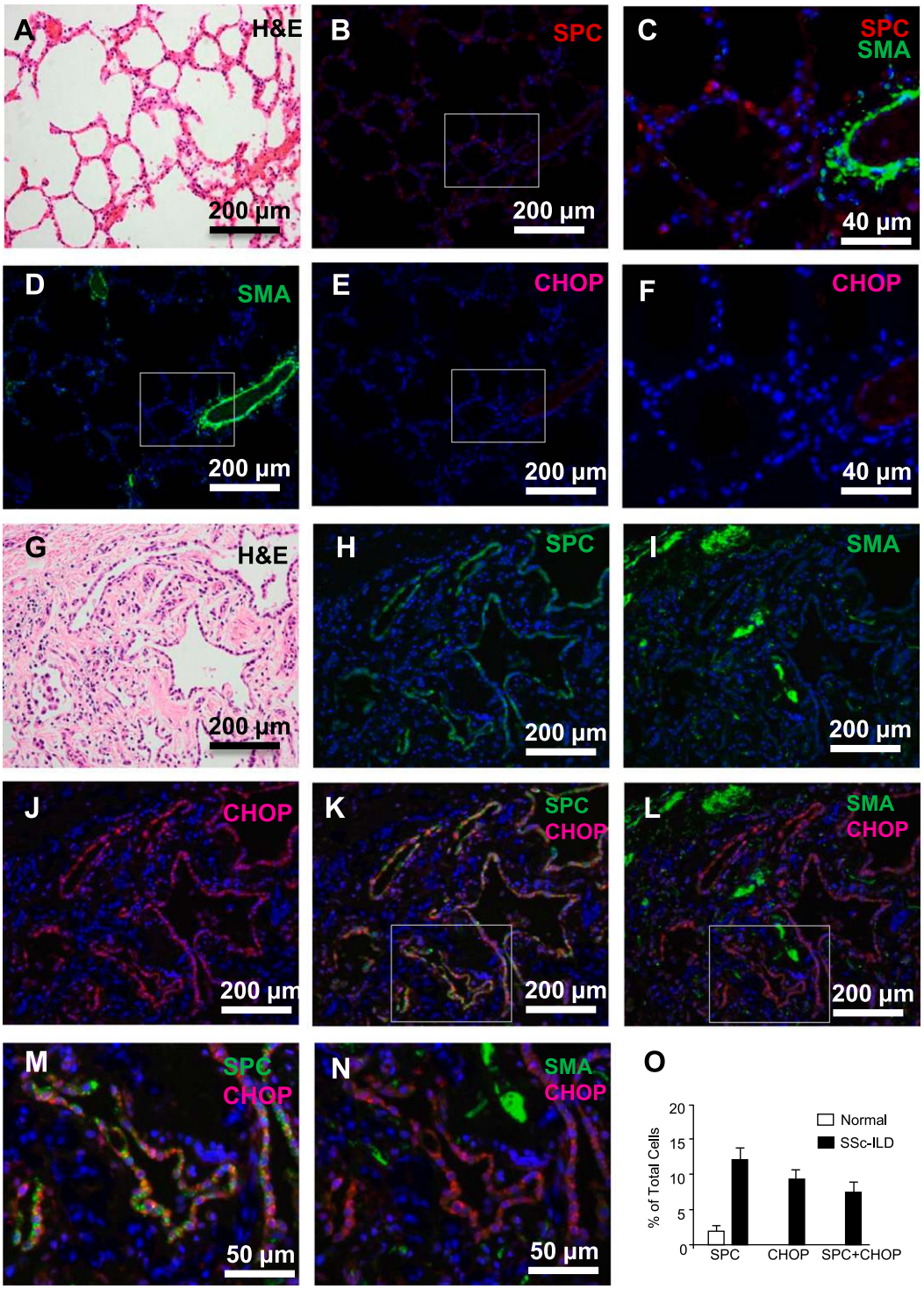

Figure 5. Expression of CHOP in AECs in areas of honeycombing and dense fibrosis in patients with SSc–interstitial lung disease (ILD). Lung sections were stained with hematoxylin and eosin (H&E) (*A* and *G*); immunofluorescent images were stained with anti–pro-surfactant protein C (SPC) (*B* and *H*), anti–smooth muscle actin (SMA) (*D* and *I*), and anti–growth arrest– and DNA damage–inducible gene (GADD) 153 CHOP (*E*, *F*, and *J*) antibodies; merged immunofluorescent image of pro-SPC with SMA (*C*), CHOP with pro-SPC (*K* and *M*), and CHOP with SMA (*L* and *N*); nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Representative images from three control subjects (normal lung tissue [*A–F*]) and five patients with SSc-ILD (*G–N*) are presented. Please note that (*C*) represents merged *insets* of (*B*) and (*D*), (*F*) is the *inset* of (*E*), (*M*) is the *inset* of (*K*), and (*N*) is the *inset* of (*L*). (*O*) Quantitative results of image analysis for SPC- and CHOP-positive cells. Cells (total, SPC positive, and CHOP positive) were counted on six randomly selected, nonoverlapping, high-power fields per sample at ×400 magnification, and are presented as means ± SEM.

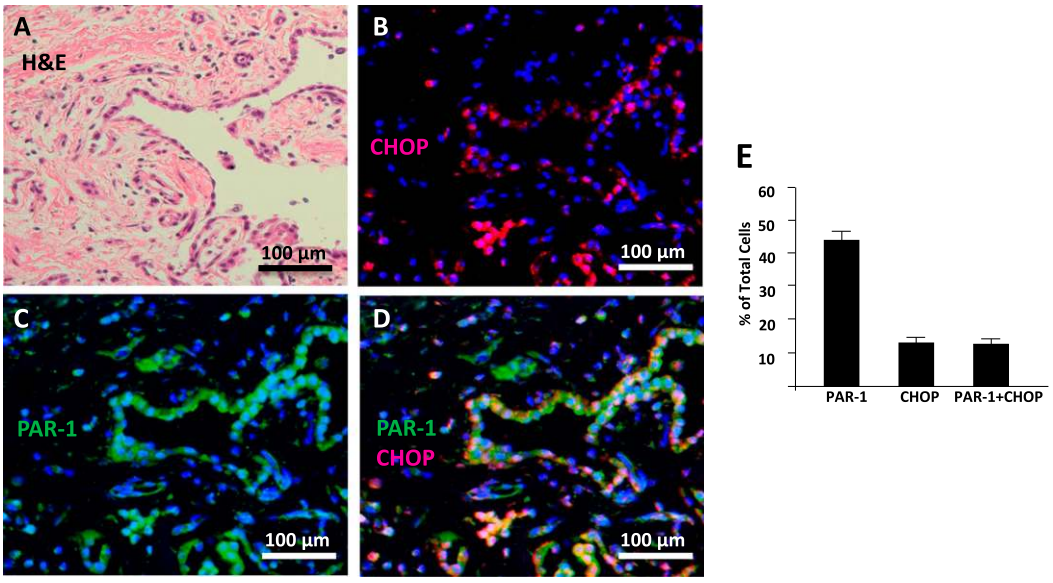

Figure 6. Colocalization of CHOP and PAR-1 in lung tissue isolated from patients with SSc-ILD. Representative images of lung sections from three patients with SSc-ILD stained with H&E (*A*), anti-GADD 153 CHOP (*B*), and anti–PAR-1 (*C*) antibodies are presented; nuclei are stained with DAPI. (*D*) Merged immunofluorescent image of CHOP with PAR-1. (*E*) Quantification of PAR-1– and CHOP-positive cells. Lung sections from three patients with SSc-ILD were analyzed at ×400 magnification. Data represent means ± SEM from six randomly selected, nonoverlapping, high-power fields.

previously shown that thrombin activity is increased in bronchoalveolar lavage fluid from patients with SSc-ILD and in bleomycin-induced pulmonary fibrosis models of ILDs (19, 20). In this study, we demonstrate that thrombin increases expression of CHOP in primary AECs and A549 cells, and that the thrombin receptor, PAR-1, colocalizes with CHOP in SSc-ILD lung. In experiments that depleted the expression of CHOP, we observed decreased apoptosis in AECs, which suggests that thrombin induces apoptosis of AECs in a CHOP-dependent manner. This is, to the best of our knowledge, the first report that thrombin induces apoptosis in AECs via a CHOP-dependent mechanism.

We previously reported that thrombin induces resistance to apoptosis in normal lung fibroblasts, and that a similar level of resistance to FasL is observed in SSc lung fibroblasts *de novo* (25). In the present study, we report that lung fibroblasts transfected with CHOP are no longer protected by thrombin from FasL-induced apoptosis. Moreover, CHOP-transfected

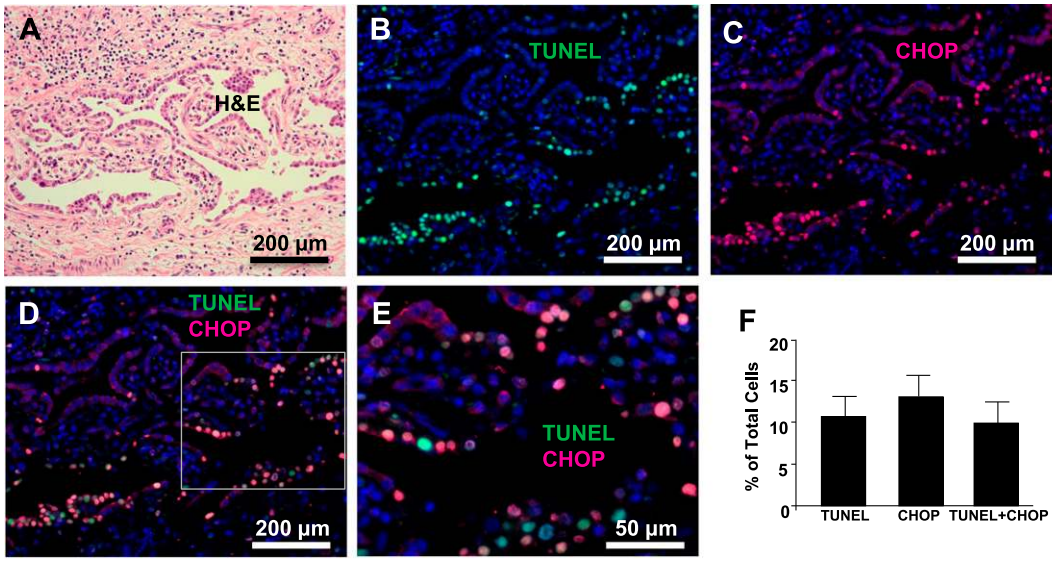

Figure 7. Analysis of apoptosis in lung tissue isolated from patients with SSc-ILD. Representative images of lung sections from three patients with SSc-ILD stained with H&E (*A*), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (*B*), and anti-GADD 153 CHOP (*C*). (*D* and *E*) Merged immunofluorescent image of TUNEL with CHOP; nuclei are stained with DAPI. Please note that (*E*) is the *inset* of (*D*). (*F*) Quantification of TUNEL- and CHOP-positive cells from three samples of SSc-ILD lung sections. Data represent means ± SEM from six randomly selected, nonoverlapping, high-power fields at ×400 magnification per sample.

lung fibroblasts became apoptotic even without FasL treatment. These data suggest that thrombin increases survival of lung fibroblasts via down-regulation of CHOP expression in these cells, which may be another potential mechanism of the increased numbers of lung fibroblasts in pulmonary fibrosis. Our results are in agreement with recent work reported by Shin and colleagues (35), demonstrating that CHOP underexpression promotes survival of synovial fibroblasts from patients with rheumatoid arthritis under ER stress.

On the other hand, we provide evidence that thrombin promotes the apoptosis of AECs in a PAR-1– and CHOP-dependent manner. This is consistent with other published work suggesting that thrombin also induces apoptosis of human lung airway and AECs through PAR-1 (30). In the context of ILD, apoptosis of epithelial cells may be harmful by increasing airway permeability and disrupting airway barrier function, thus initiating a remodeling process accompanied by fibrosis.

In conclusion, we demonstrate for the first time that the proapoptotic ER stress marker, CHOP, is expressed in AECs of patients with SSc-ILD. Moreover, CHOP appears to be involved in the regulation of apoptotic mechanisms in AECs downstream of thrombin, making CHOP a possible novel target for the treatment of SSc-ILD. Our data suggest that regulation of CHOP expression by thrombin, and perhaps by other profibrotic agents, may serve as a molecular switch directing cellular events in AECs and lung fibroblasts toward either apoptosis or survival, respectively, and that the fate of AECs and lung fibroblasts in SSc-ILD depends on the expression of CHOP in these cells. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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