Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts

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Bogatkevich, Galina S., Estella Gustilo, Jim C. Oates, Carol Feghali-Bostwick, Russell A. Harley, Richard M. Silver, and Anna Ludwicka-Bradley. Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. Am J Physiol Lung Cell Mol Physiol 288: L190–L201, 2005. First published September 24, 2004; doi:10.1152/ajplung.00448.2003.—Thrombin activates protease-activated receptor (PAR)-1 and induces a myofibroblastic phenotype in normal lung fibroblasts that resembles the phenotype of scleroderma lung fibroblasts. We now demonstrate that PAR-1 expression is dramatically increased in lung tissue from scleroderma patients, where it is associated with inflammatory and fibroproliferative foci. We also observe that thrombin induces resistance to apoptosis in normal lung fibroblasts, and this process is regulated by protein kinase C (PKC)-ε but not by PKC-α. Overexpression of a constitutively active (c-a) form of PAR-1 or PKC-ε significantly inhibits Fas ligand-induced apoptosis in lung fibroblasts, whereas scleroderma lung fibroblasts are resistant to apoptosis de novo. Thrombin translocates p21Cip1/WAF1, a signaling molecule downstream of PKC, from the nucleus to cytoplasm in normal lung fibroblasts mimicking the localization of p21Cip1/WAF1 in scleroderma lung fibroblasts. Overexpression of c-a PKC-α or PKC-ε results in accumulation of p21Cip1/WAF1 in the cytoplasm. Depletion of PKC-α or inhibition of mitogen-activated protein kinase (MAPK) blocks thrombin-induced DNA synthesis in lung fibroblasts. Inhibition of PKC by calphostin or PKC-α, but not PKC-ε, by antisense oligonucleotides prevents thrombin-induced MAPK phosphorylation and accumulation of G1 phase regulatory protein cyclin D1, suggesting that PKC-α, MAPK, and cyclin D1 mediate lung fibroblast proliferation. These data demonstrate that two distinct PKC isoforms mediate thrombin-induced resistance to apoptosis and proliferation and suggest that p21Cip1/WAF1 promotes both phenomena.

Scleroderma; apoptosis; p21Cip1/WAF1; mitogen-activated protein kinase; cyclin D1; deoxyribonucleic acid; protein kinase C

Scleroderma (systemic sclerosis, SSc) is an autoimmune connective tissue disease characterized by microvascular injury and fibrosis of skin and visceral organs (7, 50, 51). Pulmonary fibrosis is a frequent complication and major cause of death; however, the pathogenesis is unclear, and no definitive therapy exists (7, 42). Recently, the correlation between mortality and numbers of areas with fibroblastic foci has been demonstrated in interstitial pulmonary fibrosis, lending further confirmation to the importance of activated fibroblasts in the pathogenesis of pulmonary fibrosis (50). Apoptosis mediated by Fas-Fas ligand (FasL) in the lung has been implicated as an important pathway in inflammation and pulmonary fibrosis (28, 29). In many instances, resistance to apoptosis arises from constitutive or enhanced expression of signaling molecules that counteract death signals initiated by activation of the Fas receptor (53). Membrane receptor Fas (CD95) is a member of the TNF receptor family, and its natural ligand, FasL, is expressed in various tissues, including lung (29, 53). Resistance to Fas-induced apoptosis is seen in several pathological conditions, such as autoimmunity and hematological malignancy and other cancers (24, 47). Dermal scleroderma fibroblasts are specifically resistant to apoptosis induced by Fas receptor stimulation, whereas normal dermal fibroblasts are sensitive to this apoptotic stimulus (48). Transforming growth factor (TGF)-β1 has been postulated to be partially responsible for the resistance to apoptosis (24, 60), but the mechanism is not fully understood.

Activated fibroblasts or myofibroblasts that are resistant to apoptosis are major players in various fibrotic disorders, including the lung fibrosis associated with scleroderma. Previously, we showed that thrombin differentiates normal lung fibroblasts to a myofibroblastic phenotype via protease-activated receptor (PAR)-1 and a protein kinase C (PKC)-ε pathway (5, 6). Cells with a myofibroblast phenotype appear during the early stages of fibrosis and are characterized by an increase in contractile, proliferative, and cell survival capacity (5–7). Despite numerous studies, the precise signaling mechanisms that regulate lung fibroblast proliferation and their resistance to apoptosis in scleroderma lung disease remain unknown (12, 15, 24, 32, 43).

Members of the PKC family of signal transduction molecules have been widely studied in the regulation of cell growth/cell cycle progression and differentiation (18, 36, 45). PKC-dependent signal transduction pathways have been found to regulate many intracellular events in fibroblasts involved in the development of fibrosis. For example, calcium-dependent isoforms of PKC are required for mitogen-activated protein kinase (MAPK) activation by TGF-β1 (2), whereas PKC-ε is involved in cell spreading (4). Previously, we found that PKC-γ is responsible for increased synthesis of interleukin-8 by lung fibroblasts, whereas PKC-ε regulates thrombin-induced tenasin-C expression in SSc lung fibroblasts (32, 55). Recently, we demonstrated that PKC isoforms mediate the mitogenic response of lung fibroblasts to thrombin and transition of normal lung fibroblasts to myofibroblasts (5).

Several PKC isoforms mediate cell resistance to apoptosis (4, 19, 39). Evidence indicates that a major target for PKC-
regulated inhibition of cell cycle progression is the cyclindependent kinase inhibitor p21/Cip1/WAF1 (1, 25, 26, 52). Activation of PKC in NIH 3T3 cells may result in elevation of p21/Cip1/WAF1, extracellular signal-regulated kinase activation, and inhibition of DNA synthesis (3). However, recent studies have revealed that the p21/Cip1/WAF1 can also function as a positive regulator of cell cycle and protect cells from apoptotic death (1, 37, 59). It has been shown that stimulation of cells with thrombin or PAR-1-activating peptide decreases p21/Cip1/WAF1 expression and upregulates cyclin D1 (40). These results partially explain how thrombin contributes to enhanced proliferation and cell survival.

In this study, we demonstrate that abundant amounts of the thrombin receptor PAR-1 are present in scleroderma lung tissue in association with myofibroblasts. These data together with our previous findings (5, 6, 33) strongly support a role for thrombin and PAR-1 in the development of the myofibroblast phenotype and pulmonary fibrosis. We demonstrate that resistance to apoptosis in thrombin-induced myofibroblasts is regulated by PKC-α, whereas PKC-α regulates the proliferative capacity of myofibroblasts. We provide evidence that PKC-α MAPK/cyclin D1 regulates the mitogenic response of lung fibroblasts to thrombin independently from PKC-ε. However, p21Cip1/WAF1 is regulated by both PKC isoforms α and ε. Therefore, we speculate that p21Cip1/WAF1 is involved in both cell survival and DNA synthesis induced by thrombin in lung fibroblasts. These results lend additional support for the important roles of thrombin-induced signaling in the emergence, proliferation, and persistence of the myofibroblast phenotype critical for the development and progression of pulmonary fibrosis.

MATERIALS AND METHODS

Materials

Antisense and sense oligonucleotides for PKC-α, PKC-β, PKC-γ, and PKC-ε isoforms were synthesized in the Oligonucleotide Synthesis Facility at the Medical University of South Carolina. The sequences were as follows: PKC-α antisense 5′-GGTTTCCG CTGTGAGTGT-3′ and sense 5′-GGTTTTATCCACTGGTCT-3′, PKC-β1 antisense 5′-GACCGCCGCTCGACCAATT-3′ and sense 5′-AAAGATGCTGACCGCCTGCT-3′, PKC-β2 antisense 5′-AGCCGCTCGACCAAT-3′ and sense 5′-ATGGCTGACCGCCTGCT-3′, PKC-γ antisense 5′-CGGGGAAAACGTGCAGCCAT-3′ and sense 5′-ATGGCTGACCGCCTGCT-3′, PKC-ε antisense 5′-ATTGGAACACCCAT-3′ and sense 5′-ATTGGAACACCCAT-3′. Thrombin from human plasma and calphostin C was obtained from Calbiochem (La Jolla, CA). [3H]Thymidine (sp act 86.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). U0126, anti-phospho-p44/p42, and anti-p44/p42 antibodies were purchased from Cell Signaling Technology, (Beverly, MA). Anti-cyclin D1 and antibody against various PKC isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p21/Cip1/WAF1 antibody was purchased from Transduction Laboratories, and FuL from Alexis (San Diego, CA).

Determination of PAR-1 Expression in Normal and Scleroderma Lung Tissue

Tissues from three patients with SSC and three normal individuals were examined. Lung tissues were collected postmortem from SSC patients who fulfilled the American College of Rheumatology preliminary criteria for SSC and had evidence of lung involvement. The diagnosis of interstitial lung fibrosis was confirmed by histological examination of postmortem lung tissue. Tissue from patient 1 (female, age 48) was obtained at open lung biopsy, 11 yr after the onset of limited cutaneous SSC complicated by interstitial lung disease and pulmonary arterial hypertension that, subsequently, required single lung transplantation. Lung tissue was obtained at autopsy from patient 2 (male, age 61) ~20 yr after the onset of limited cutaneous SSC complicated by severe interstitial lung disease and pulmonary arterial hypertension. Lung tissue was obtained at autopsy from patient 3 (female, age 71), ~1 yr after the onset of limited cutaneous SSC complicated by severe pulmonary fibrosis. Tissues were collected from multiple parts of lung to make comparisons between the normal control and the diseased lungs from SSC with various degrees of fibrosis within the same patient.

Immunohistochemistry

Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The 4-μm paraffin sections were collected on poly-l-lysine-coated slides, deparaffinized in xylene, and rehydrated through a degrading series of ethanol before staining.

For immunostaining, tissue sections were permeated to ensure antibody infiltration by treatment in cold methanol for 10 min at −20°C. Before immunostaining, nonspecific binding sites were blocked for 30 min in PBS containing 3% BSA and 1% normal goat serum. Sections were incubated overnight with anti-PAR-1 monoclonal antibody ATAP2 raised against amino acids 42–55 of human thrombin receptor (Santa Cruz Biotechnology). Tissue sections were then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, W. Grove, PA) for 1 h at 8 μg/ml concentration. Color development was based on metal-enhanced 3,3′-diaminobenzidine (DAB) substrate (Metal Enhanced DAB Substrate Kit; Pearlce, Rockford, IL). Tissue sections were counterstained for 30 s using hematoxylin, a nucleus-sensitive dye (Harris Hematoxylin; Poly Scientific, Bay Shore, NY), mounted using cytoseal (Stephan Scientific, Kalamazoo, MI), and viewed under a Zeiss microscope (Axiovert 35) using a bright-field mode. The microscope images were obtained digitally (Spot RT Color Digital Camera; Diagnostics Instruments, Sterling Heights, MI), and they were processed to computer via software, Spot RT/version 3.2 advanced. If necessary, the collected image data were edited using Adobe Photoshop 6.0, an image-processing software.

Cell Culture

Lung fibroblasts were derived from lung tissues obtained at autopsy from scleroderma patients and from age-, race-, and sex-matched normal subjects. Lung tissue was diced (0.5 × 0.5-mm pieces) and cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin sulfate, and 5 μg/ml amphotericin B at 37°C in 10% CO2. Medium was changed every 3 days to remove dead and nonattached cells until fibroblasts reached confluence. Monolayer cultures were maintained in the same medium. Lung fibroblasts were used between second and fourth passages in all experiments. Purity of isolated lung fibroblasts was determined by crystal violet staining and by immunofluorescent staining [monoclonal antibody against human fibroblasts, as described previously (32), followed by FITC-conjugated goat anti-mouse IgG staining; Santa Cruz Biotechnology].

PKC Oligonucleotide Treatment of Normal and Scleroderma Lung Fibroblasts

Oligonucleotides were introduced in the cells as described previously (5, 6, 33). Antisense oligonucleotide for PKC isoforms and appropriate sense oligonucleotide (control) were dissolved in culture medium and sterilized by filtration through 0.2-μm cellulose acetate filters. Oligonucleotide (2 μM) and lipofectin (10 μg/ml) were mixed and preincubated for 30 min at room temperature, the mixture was...
added to the cells, and the samples were incubated for 6 h at 37°C. After cells were washed with DMEM to remove lipofectin, fresh oligonucleotides (2 μM) in DMEM with 10% FCS were added and incubation continued for 24 h at 37°C.

DNA Synthesis

Lung fibroblasts were plated on 12-well plates and allowed to grow to 80% confluence. Antisense and sense oligonucleotides were introduced to the cells as described above. Cells were synchronized with serum-free DMEM for 24 h and then treated with or without 0.5 U/ml thrombin for another 24 h. Parallel cells were pretreated for 30 min with or without mitogen/extracellular signal-regulated kinase (MEK)1/2 inhibitor U0126 (5 μM). After the incubation with thrombin, 1 μCi/ml [3H]thymidine was added to the cells. [3H]thymidine incorporation was measured as previously described (5, 34).

Preparation of Cell Extracts and Immunoblotting

Normal and SSc lung fibroblasts on 100-mm dishes were washed with ice-cold PBS and lysed with ice-cold lysis buffer (10 mM Tris, 10 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS, pH = 7.4). The protein concentration was determined by Bio-Rad protein assay in accordance with the manufacturer’s instructions. For each sample, 30 μg protein were denatured, subjected to SDS-polyacrylamide gels, and analyzed by immunoblotting as described (5, 6). The phosphorylation of p42/p44 MAPK isoforms was analyzed by Western blot using anti-phospho-p42/p44 antibody in accordance with the manufacturer’s instructions (Cell Signaling Technology). Briefly, lung fibroblasts were cultured on six-well plates (2 × 10^6 cells/well). Antisense and sense oligonucleotides were introduced to the cells as described above, and cells were synchronized with serum-free DMEM for 24 h. Parallel quiescent cells were pretreated for 30 min with or without 5 μM U0126. Next, cells were incubated with or without 0.5 U/ml thrombin for various times, rapidly washed with ice-cold PBS, and collected in 1× SDS sample buffer (100 μl/well). Sample (20 μl) was separated on 8% SDS-polyacrylamide gels and immunoblotted as described previously (58).

Plasmid Constructions

Constitutively active (c-a) PKC-ε A159E (a generous gift of Dr. S. Ohno, Yokohama City University School of Medicine) contains a substitution of glutamic acid for alanine at position 159 in the PKC-ε pseudosubstrate motif of the regulatory domain described by Ueda et al. (56). PKC-ε A159E has been subcloned into BamHI XhoI-digested pcDNA3.1/V5-His (Invitrogen) using standard procedure. The c-a form of PKC-α (a generous gift from Dr. P. J. Parker, The Imperial Cancer Research, London, UK) contains a substitution of glutamic acid at position T497 in PKC-α of a carboxyl side chain, as described by Parekh et al. (45). It has been subcloned into EcoRI/XhoI-digested pcDNA3.1. The structure of constructs was verified by nucleotide sequence analysis (Medical University of South Carolina, DNA Sequencing Core). The c-a mutant for PAR-1 (a generous gift from Dr. Shaun R. Coughlin, University of California, San Francisco, CA), generated by substitution of eight amino acids in the second extracellular loop for cognate sequence from the Xenopus thrombin (41), was subcloned to pcDNA3.1/V5-His vector. The c-a mutant for PAR-1 was amplified by PCR (forward primer 5’-CGAATTCC-CATGGGACGCAAAGG, reverse primer 5’-GCTTCGAGTATG- TAAAGAGCTTTTGT) digested with EcoRI/XhoI, and ligated into pcDNA3.1/V5-His (Invitrogen) using standard procedure. The structure of construct was verified by nucleotide sequence analysis (Medical University of South Carolina, DNA sequencing core). Lung fibroblasts were transfected with c-a PAR-1, and selected clones were tested for surface expression of c-a PAR-1 by immunoblotting with His antibody.

Cell Transfections

Normal lung fibroblasts were grown to 60–70% confluence on 10-cm plates and transfected with 1 μg of c-a PKC-α, c-a PKC-ε, dominant-negative (d-n) PKC-ε, and c-a PAR-1 forms using Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions and as previously described (5, 6).

Transfected cells were stimulated with FasL (20 ng/ml in serum-free DMEM for 24 h). Transfection with empty vectors served as a control. Apoptosis was determined by fluorescence-activated cell sorter (FACS) analysis and in adherent cells and/or by measuring soluble nuclear matrix protein (NMP) in culture media as described below.

Analysis of Cell Death

To induce apoptosis by FasL in lung fibroblasts, cells were stimulated with FasL (20 ng/ml) in the absence or presence of thrombin (0.5 U/ml) for 24 h. Apoptosis was determined by two independent methods.

Morphology. Normal lung fibroblasts were grown on cover slips, placed in serum-free medium for 24 h, and stimulated with FasL in the presence or absence of thrombin for 24 h. Cells were analyzed using a Zeiss microscope. Detection of apoptotic cells was performed on the basis of nuclear morphology and rounding up of cells that are losing adherence. Cells with 1) nuclei showing condensation, 2) blebbing membranes, and 3) a rounded morphology were classified as apoptotic.

FACS-based apoptosis assay: Propidium iodine exclusion. Lung fibroblasts were stimulated with FasL (20 ng/ml) in the presence or absence of thrombin (0.5 U/ml) for 24 h followed by fixation with ice-cold 70% ethanol for 1–7 days at −20°C. After fixation, nuclei were washed twice in PBS, resuspended in 100 μl of 20 μg/ml RNase (Sigma Chemical, St. Louis, MO), and incubated for 30 min in the dark at 37°C. Propidium iodine (10 μl of 110 μg/ml) in PBS was added to the suspension, and cells were incubated at room temperature in the dark for 10 min. Samples were kept on ice and measured within a couple of hours after staining by flow cytometry. Quantification of apoptosis was performed by FACS analysis with measurements of red fluorescence at >620 nm. A minimum of 10,000 events per each sample was measured. Detection of staining was performed using a dual-laser FACStar cell sorter (Becton-Dickinson, San Jose, CA). Multiparameter data analysis was performed with CellQuest software (Becton-Dickinson). Gated cells were analyzed using a fluorescence intensity histogram. Apoptotic cells appeared as a sub-G₁ peak (11). Data were analyzed by determining the number of apoptotic cells vs. nonapoptotic cells in the cell population and expressed as a ratio of these two cell types.

NMP 41/D Determination

Apoptosis in cells transfected with c-a, d-n form of PKC-ε and c-a PAR-1 and then stimulated with FasL was additionally measured by the determination of NMP 41/D in culture medium by an enzyme-linked immunoassay (Oncogene Research Products, San Diego, CA) according to the manufacturer’s procedure. The results were measured by reading the absorbency at 450–595 nm, which was proportional to the amount of soluble NMP 41/D present in the sample. The level of NMP 41/D detected in culture supernatant is a function of the number of dead and dying cells.

Cellular Fractionation

Lung fibroblasts were washed, collected in ice-cold PBS, and then pelleted and divided into cytosolic and nuclear fractions, as described
elsewhere (52, 62). Briefly, the cell pellet was resuspended in low-salt buffer [20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM NaVO₄, 1 mM EGTA, 1 mM EDTA, 0.2% Nonidet P-40 (NP-40), and 10% glycerol] containing protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, aprotonin, and pepstatin. After incubation on ice for 10 min, cells were centrifuged at 8,000 g for 2 min at 4°C. Supernatant was collected and considered to be the cytoplasmic fraction. The pellet was resuspended in high-salt buffer (low-salt buffer with 420 mM NaCl, 20% glycerol, and no NP-40), incubated on ice for 30 min, and centrifuged as before. Supernatant was collected as a nuclear fraction. The amounts of protein in the cytoplasmic and nuclear fractions were determined with a protein assay kit (Bio-Rad), and the fraction. The amounts of protein in the cytoplasmic and nuclear fractions were determined with a protein assay kit (Bio-Rad), and the protein was subjected to immunoblotting.

**Immunoblotting**

Lung fibroblasts were plated in four-chamber glass culture slides (Becton-Dickinson Labware, San Diego, CA) and transfected with the c-a form of PKC-α and PKC-ε AE in His/V5pcDNA3.1, as described previously (5, 6). The d-n forms of PKC-α, PKC-ε, and empty vector served as a control. After transfection, the cells were fixed in 4% paraformaldehyde (10 min, 4°C), washed with PBS followed by incubation in 100% methanol (10 min, −20°C), and then incubated with monoclonal antibody for human p21/Cip1/WAF1 for 24 h at 4°C. Subsequently, the cells were incubated with rabbit anti-PKC-α (1:200 dilution) for 1 h followed by incubation with anti-mouse IgG conjugated with 1:200 diluted FITC (green) and goat anti-rabbit antibody labeled with 1:200 diluted rhodamine (red) for 1 h. Cells transfected with PKC-ε, after staining with monoclonal antibody for p21/Cip1/WAF1, were stained for 1 h with anti-His antibody labeled with rhodamine and anti-mouse antibody labeled with FITC for 1 h and analyzed under fluorescent microscope.

**RESULTS**

**PAR-1 Expression is Enhanced in SSc Lung**

We demonstrate that PAR-1 expression is dramatically increased in lung tissue from scleroderma patients, mainly in the lung parenchyma in association with inflammatory and fibroproliferative foci. PAR-1 expression decreases in the later stages of pulmonary fibrosis (Fig. 1), suggesting its importance in the early development of pulmonary fibrosis. PAR-1, which is responsible for most cellular events induced by thrombin, colocalizes with myofibroblasts in scleroderma lung tissue, further suggesting its important role in lung fibroblast activation.

**Thrombin Promotes Resistance to Apoptosis in Lung Fibroblasts**

Thrombin induces resistance to FasL-induced apoptosis in normal lung fibroblasts, and a similar level of resistance to FasL is observed in SSc lung fibroblasts de novo (Fig. 2). Interestingly, we also found that thrombin-induced resistance to apoptosis is not specific only for FasL. We show that thrombin also induces resistance to other apoptotic factors, such as camptothecin and ceramide, and that SSc lung fibroblasts are as well resistant to apoptosis induced by these stimuli (Fig. 2A). Because of the involvement of the Fas-FasL pathway in various pulmonary disorders, we have selected this molecule for further investigations of apoptosis. We found that FasL induces apoptosis in normal lung fibroblasts in a dose-dependent manner and that this effect is inhibited by thrombin (Fig. 2B).

**Thrombin Protects Lung Fibroblasts from FasL-induced Apoptosis via PKC-ε**

Depletion of PKC-ε by antisense oligonucleotide inhibits thrombin-induced cell resistance to FasL-induced apoptosis (Fig. 3A), whereas depletion of other PKC isofoms, including PKC-α, does not exhibit significant effects (data not shown). To confirm further the specificity of PKC-ε in mediating thrombin-induced cell resistance to apoptosis, we transiently transfected cells with the c-a form of PKC-ε. Apoptosis was measured by the determination of NMP 41/7 in culture media of transfected cells, where the level of NMP 41/7 released in the media is proportional to the number of apoptotic cells. Overexpression of c-a PKC-ε in lung fibroblasts significantly inhibits release of NMP 41/7, whereas transfection with the d-n

Fig. 1. Protease-activated receptor (PAR)-1 is abundantly expressed in pulmonary fibrosis associated with scleroderma. Shown are sections of normal and systemic scleroderma (SSc) lung stained by mouse monoclonal antibody ATAP2 raised against amino acids 42–55 of human thrombin receptor PAR-1. PAR-1 was visualized as brown color by diaminobenzidine and counterstained with hematoxylin. Black arrows indicate positively stained cells and white arrow indicates negatively stained cells. A: normal lung tissue; B: lung tissue in early stage of lung involvement; C: lung tissue with the late stage of fibrosis. Note intensive staining in cellular foci in early and late stage of fibrosis and negative staining in normal lungs and in less cellular late stage of fibrosis.
form of PKC-ε has no effect (Fig. 3B). Overexpression of c-a PKC-α or d-n PKC-α did not have any effect on FasL-induced apoptosis (data not shown). The data suggest that PKC-ε is the major PKC isoform mediating thrombin-induced cell survival in lung fibroblasts. We also observed that overexpression of c-a PAR-1 inhibited FasL-induced apoptosis (Fig. 3B), confirming further that a PAR-1/PKC-ε-dependent pathway mediates thrombin-induced resistance to apoptosis in lung myofibroblasts.

Fig. 3. PAR-1 and protein kinase C (PKC)-ε mediate thrombin-induced resistance to FasL-stimulated apoptosis in lung fibroblasts. A: thrombin-induced resistance to FasL-mediated apoptosis is suppressed by PKC-ε depletion. Normal lung fibroblasts were grown to 70% confluence and then antisense (AS) oligonucleotides for PKC-ε were introduced into the cells as described earlier (5, 6). The next day, cells were stimulated with FasL (20 ng/ml) in the presence of thrombin (0.5 U/ml) for an additional 24 h. Apoptosis was determined by FACS analysis. *Statistically significant differences in apoptosis between cells stimulated with FasL and nonstimulated cells (P < 0.002). **Statistically significant differences in inhibition of FasL-induced apoptosis by thrombin (P < 0.005).

B: FasL induces apoptosis in lung fibroblasts in a dose-dependent manner, and thrombin inhibits FasL-induced apoptosis at all the FasL concentrations used. Normal lung fibroblasts were grown to 80% confluence and then stimulated with various concentrations of FasL (0–25 ng/ml) in the presence (filled bars) or absence (open bars) of thrombin (0.5 U/ml) in SFM for 24 h. For flow cytometry analysis, single cell suspensions were prepared by trypsinization. Cells were then fixed in 70% ethanol and stained with propidium iodine (120 µg/ml). Cell fluorescence was measured with a FACS scan flow cytometer. Negative control gate was set using cells incubated in SFM only. A minimum of 10,000 events was collected per sample. Measurement of fluorescence was performed at >620 nm. Apoptotic cells are expressed as a percentage of total cells in population. *Statistically significant differences in apoptosis between cells stimulated with FasL and nonstimulated cells (P < 0.002). **Statistically significant differences in inhibition of FasL-induced apoptosis by thrombin (P < 0.005).
p21/Cip1/WAF1 is Differentially Expressed in Normal and SSC Lung Fibroblasts

Evidence indicates that p21/Cip1/WAF1 is a major target for PKCs involved in cell cycling and may act as positive or negative regulator (25, 26, 37). It has been reported that localization of p21/Cip1/WAF1 within the cell is crucial for cell cycle inhibitory activity (26, 52, 59). Several cell types with cytoplasmic p21/Cip1/WAF1 have also been shown to possess anti-apoptotic function. We therefore analyzed the p21/Cip1/WAF1 expression and distribution in normal and in SSC lung fibroblasts (Fig. 4). Normal lung fibroblasts contain p21/Cip1/WAF1 mainly in the nuclear fraction, while lacking its expression in cytoplasm (Fig. 4, lanes 1 and 6, respectively). In contrast, SSC lung fibroblasts exhibit accumulation of p21/Cip1/WAF1 mainly in the cytoplasm but not in the nuclear fraction (Fig. 4, lanes 5 and 10). Stimulation of normal lung fibroblasts with thrombin results in p21/Cip1/WAF1 translocation from nucleus to cytoplasm as early as 2 h after treatment (Fig. 4, lanes 2 and 7). To determine whether PKC isoforms are involved in thrombin-induced translocation of p21/Cip1/WAF1, we employed antisense oligonucleotides for PKC-α and PKC-ε to inhibit their expression in normal lung fibroblasts. We found that depletion of either PKC-α or PKC-ε inhibits thrombin-induced translocation of p21/Cip1/WAF1 from the nucleus to cytoplasm (Fig. 4, lanes 3 and 8 and 4 and 9, respectively). SSC lung fibroblasts exhibit mainly the cytoplasmic form of p21/Cip1/WAF1, which may induce cyclin D and participate in increased proliferation. Another possible explanation is that p21/Cip1/WAF1 loses its ability to localize in the nucleus after exposure to protease.

PKC-α and PKC-ε Translocate p21/Cip1/WAF1 in Lung Fibroblasts

To further confirm that PKC-α or PKC-ε regulates p21/Cip1/WAF1 distribution within the cell, we transiently transfected normal lung fibroblasts with c-a forms of these PKC isoforms. Overexpression of PKC-α or PKC-ε in normal lung fibroblasts results in translocation of p21/Cip1/WAF1 from nucleus to cytoplasm (Fig. 5). Moreover, our data demonstrate that p21/Cip1/WAF1 is in abundance, expressed only in cytoplasm of cells overexpressing c-a PKC-α or PKC-ε, whereas in nontransfected cells p21/Cip1/WAF1 is localized only in the nucleus.

Depletion of PKC-α Abolishes the Mitogenic Effect of Thrombin in Normal and Scleroderma Lung Fibroblasts

Cells with the myofibroblast phenotype, besides resistance to apoptosis, are also characterized by an increase in proliferative capacity. Previously, we demonstrated that conventional PKC isoforms are involved in the mitogenic response of human lung fibroblasts to thrombin (5). To determine which PKC isoform is essential for DNA synthesis, we employed an antisense oligonucleotide technique. Antisense oligonucleotides were used to decrease PKC-α, PKC-β₁, PKC-β₂, PKC-γ, and PKC-ε synthesis in normal and scleroderma lung fibroblasts. Thrombin at a concentration 0.5 U/ml induced an eightfold increase in [³H]thymidine incorporation in normal lung fibroblasts (Fig. 6A). After pretreatment of cells with sense oligonucleotides, thrombin induced DNA synthesis to the same level. However, pretreatment of cells with PKC-α antisense inhibited thrombin-induced [³H]thymidine incorporation to the basal level (Fig. 6A). Similarly, thrombin induces DNA synthesis in scleroderma lung fibroblasts, however, to a lesser extent compared with normal lung fibroblasts (Fig. 6A). Note that the basal level of [³H]thymidine incorporation is twofold higher in scleroderma lung fibroblasts compared with normal lung fibroblasts. Pretreatment with PKC-β₁, PKC-β₂, or PKC-γ antisense oligonucleotides did not affect thrombin-induced [³H]thymidine incorporation, either in normal or in scleroderma lung fibroblasts (data not shown). Similarly, depletion of PKC-ε had no effect on [³H]thymidine incorporation, as previously reported (5). These results suggest that PKC-α is required for thrombin-induced DNA synthesis. Oligonucleotide treatment decreased the level of PKC proteins by 60–70%, compared with untreated cells or cells treated with sense oligonucleotide for PKC isoforms (Fig. 6B). Thrombin did not change the effect of oligonucleotides on the expression of PKC isoforms (data not shown). Antisense or sense oligonucleotide treatment did not induce cell injury or have any effect on overall protein synthesis, suggesting that inhibition of PKC protein synthesis is due directly to oligonucleotide treatment (5, 33, 55).

Effect of MEK1/2 Inhibitor on the Mitogenic Effect of Thrombin

There are several PKC-dependent pathways associated with growth in a variety of cell types (2, 4, 18, 36). To examine whether, along with PKC, the MAPK cascade is involved in thrombin-induced DNA synthesis in lung fibroblasts, we used the MEK1/2 inhibitor, U0126. U0126, at a concentration of 5
p21/WAF1

C-a PKCα

C-a PKCα - p21/WAF1 Merge

Fig. 5. p21/Cip1/WAF1 is translocated to the cytoplasm only in cells overexpressing PKC-α or PKC-ε. Normal lung fibroblasts cultured in 2-well tissue culture glass slides were transiently transfected with c-a PKC-α T497 in His/V5pcDNA3.1 (A–C) and with c-a PKC-ε A159E in His/V5pcDNA3.1 (D–F). At 48 h after transfection, cells were fixed in 4% paraformaldehyde and 100% methanol and immunostained with anti-p21/WAF1 antibody (A and B, green) and with anti-PKC-α antibody (B, red) or with anti-His antibody for PKC-ε (E, red); for details, see MATERIALS AND METHODS. Note that p21/Cip1/WAF1 is expressed in abundance only in the cytoplasm of cells overexpressing PKC-α or PKC-ε, whereas cells nonexpressing these PKC isoforms contain p21/Cip1/WAF1 only in the nucleus [A and D (green) and C and F (merged images)]. All experiments were performed 3 times, and representative images are presented.

μM, completely blocks thrombin-induced DNA synthesis in normal lung fibroblasts (Fig. 7). Interestingly, U0126 inhibited DNA synthesis even below the basal level in SSc lung fibroblasts, reaching levels observed in normal lung fibroblasts.

Thrombin Induces p44/42 MAPK Phosphorylation and Cyclin D1 Accumulation

Because the MAPK inhibitor U0126 completely blocked DNA synthesis in both normal and scleroderma lung fibroblasts, we tested the effects of thrombin on MAPK activation. A time course of thrombin-induced p44/42 MAPK phosphorylation was measured in normal lung fibroblasts seeded on six-well plates, serum starved, and treated with 0.5 U/ml thrombin for 2–120 min, followed by immunoblotting with anti-phospho-p44/42 MAPK or anti-p44/42 MAPK antibody. MAPK bands were quantified by densitometry using NIH image software (Fig. 8A.2). Phosphorylation of p44/p42 MAPK occurs within 2 min of thrombin treatment, reaching a maximum at 10 min and diminishing after 60 min (Fig. 8, A.1 and A.2). Basal levels of total p44/p42 MAPK are similar in normal and SSc lung fibroblasts; however, basal levels of phosphorylated p44/42 MAPK are 2.5-fold increased in scleroderma lung fibroblasts compared with normal cells (Fig. 8, B.1 and B.2). Thrombin induces up to a 10-fold increase in p44/42 MAPK in both normal and scleroderma lung fibroblasts. To investigate the role of PKC on p44/p42 MAPK in lung fibroblasts, we pre-treated the cells with the PKC inhibitor calphostin C. We also introduced into cells PKC-α and PKC-ε antisense and sense oligonucleotides 24 h before MAPK assay. Both calphostin C and PKC-α, but not PKC-ε, antisense oligonucleotides block thrombin-induced MAPK phosphorylation similar to the MEK1/2 inhibitor U0126 in normal and scleroderma lung fibroblasts (Fig. 8, B.1 and B.2).

Thrombin mediates the expression of the G1 phase regulatory protein cyclin D1, a downstream molecule of MAPK (16, 35, 46). Normal and scleroderma lung fibroblasts grown to confluence, serum starved, were incubated with 0.5 U/ml thrombin for 12 and 24 h. Cell extracts were analyzed by immunoblotting with anti-cyclin D1 antibody and followed by densitometric measurements (Fig. 9). Basal levels of cyclin D1 are increased in scleroderma lung fibroblasts when compared with normal cells, and thrombin increases cyclin D1 levels up to 10-fold within 24 h in both normal and SSc lung fibroblasts (Fig. 9, A.1 and A.2). Furthermore, to determine whether inhibition of MEK1/2 or PKCs affects thrombin-induced cyclin D1, normal lung fibroblasts were pretreated with 100 nM calphostin C for 30 min, PKC-α antisense, or PKC-ε antisense.
overnight, or 5 μM U0126 for 30 min before incubation with 0.5 U/ml thrombin for an additional 24 h. U0126, as well as calphostin C and PKC-α antisense oligonucleotides, abolishes thrombin-induced cyclin D1 accumulation (Fig. 9, B.1 and B.2). Preincubation of lung fibroblasts with PKC-ε antisense oligonucleotides results in only slight diminution of thrombin-induced cyclin D1 overexpression (Fig. 9, B.1 and B.2).

**DISCUSSION**

Recently, we demonstrated that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the PAR-1/PKC pathway (5, 6, 33). In the present study, we show that PAR-1 expression is dramatically increased in lung tissue from scleroderma patients, mainly in lung parenchyma and in association with inflammatory and fibroproliferative foci. Increased levels of PAR-1 have been shown in various fibrotic tissues, including lung tissue in bleomycin-induced pulmonary fibrosis (22, 23), but this is the first demonstration of PAR-1 overexpression in scleroderma lung. PAR-1, which is responsible for most cellular events induced by thrombin (8–10), colocalizes with myofibroblasts in scleroderma lung tissue and appears to decrease in later stages of pulmonary fibrosis when a decreased number of myofibroblasts is observed (32). This suggests its importance in the myofibroblast activation and, subsequently, in the development of pulmonary fibrosis.

The myofibroblast phenotype is expressed in a variety of fibrotic diseases, including scleroderma, an autoimmune connective tissue disease characterized by microvascular injury and fibrosis of skin and visceral organs (31–34, 44, 54). Cells with a myofibroblast phenotype appear in the early stages of fibrosis (31, 34) and, besides abundant expression of smooth muscle-α actin, collagens, and other extracellular matrix proteins, are characterized by an increased proliferative capacity (5, 20, 21, 33, 43, 55) and resistance to apoptosis (24). Thrombin is well known to be a mitogen for several cell types, including fibroblasts (5, 20, 43). As we reported previously, thrombin activity is significantly increased in bronchoalveolar lavage fluid from SSc patients with interstitial lung disease (43). We have also demonstrated that thrombin is a mitogen for lung fibroblasts and may be partially responsible for increased
lung fibroblast proliferation in scleroderma lung (5, 43). The present study was designed to further examine the signaling pathways regulating thrombin-induced cell proliferation and cell survival in lung fibroblasts.

Our studies indicate that lung myofibroblasts isolated from scleroderma lung are resistant to several apoptotic stimuli, including FasL, whereas normal lung fibroblasts are sensitive. Furthermore, we found that normal lung fibroblasts stimulated with thrombin became resistant to FasL-induced apoptosis similar to myofibroblasts from scleroderma lung. Transient transfection with c-a PKC-ε or c-a PAR-1 protects lung fibroblasts from FasL, suggesting that thrombin via PAR-1 and followed by activation of PKC-ε regulates cell survival. PKC-ε has been found to regulate survival of lung cancer cells (13, 48) and to induce apoptosis in other cell systems (39). Thus a protective role of PKC-ε most probably depends on the apoptotic stimuli used and may be cell specific as well. We postulate that PKC-ε is a major and specific PKC isoform regulating cell survival of lung myofibroblasts when stimulated by thrombin. Additionally, thrombin protects lung fibroblasts from apoptosis induced by different stimuli. Other factors induced by thrombin in lung fibroblasts, such as fibronectin and tenascin C (33, 55), have also been shown to protect fibroblasts from apoptosis (53), thus preserving the myofibroblast phenotype.

In the present study, we have demonstrated that p21/Cip1/WAF1 is expressed mainly in the nuclear fraction of normal lung fibroblasts. In normal lung fibroblasts treated with thrombin and in SSc lung fibroblasts, enhanced amounts of p21/Cip1/WAF1 are localized to the cytoplasm, whereas the nuclear fraction contains no or only trace amounts. The abundant expression of p21/Cip1/WAF1 in the cytoplasm in thrombin-

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**Fig. 7.** Effect of mitogen/extracellular signal-regulated kinase (MEK) 1/2 inhibitor on thrombin-induced DNA synthesis. Fibroblasts were stimulated with thrombin (0.5 U/ml) for 30 h after preincubation in the absence or presence of U0126 (5 µM) for 30 min. [3H]Thymidine incorporation was measured as described in MATERIALS AND METHODS. The experiment was performed 3 times, and mean values ± SD are presented. *Statistically significant differences between cells stimulated with thrombin vs. nonstimulated cells (P < 0.05). **Statistically significant differences between cells stimulated with thrombin vs. cells treated with U0126 and stimulated with thrombin (P < 0.001).

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**Fig. 8.** Thrombin-induced mitogen-activated protein kinase (MAPK) activation in normal and scleroderma lung fibroblasts. A.1: time course of thrombin-induced p44/42 MAPK phosphorylation was determined in normal lung fibroblasts treated with 0.5 U/ml thrombin for various time points. Cell extracts were immunoblotted with anti-phospho-p44/42 MAPK or anti-p44/42 MAPK (as a control) antibody (see details in MATERIALS AND METHODS). A.2: densitometric analysis of immunoblots from 3 independent experiments. B.1: normal and SSc lung fibroblasts were treated with 100 nM calphostin C (Calph), PKC-α AS, PKC-ε AS, or 5 µM U0126 before incubation with 0.5 U/ml thrombin (see details in MATERIALS AND METHODS). B.2: densitometric analysis of immunoblots from 3 independent experiments is presented. Note that thrombin is reaching a maximum phosphorylation of p44/42 in normal lung fibroblasts, inducing 10-fold increase in both normal and SSc lung fibroblasts. Depletion of PKC-α but not PKC-ε blocks MAPK phosphorylation induced by thrombin in both cell lines.
treated and in SSc fibroblasts suggests that p21/Cip1/WAF1 contributes to the fibrogenic qualities of myofibroblasts, such as proliferation and cell survival. We postulate that p21/Cip1/WAF1 is translocated from nucleus to cytoplasm or loses its ability to localize in the nucleus after exposure to thrombin. A similar mechanism has been proposed in other cell systems, where caspase-3 appears to be responsible for this effect (57).

We demonstrate that thrombin translocates p21/Cip1/WAF1 to the cytoplasm, thus leading to activation of cyclin D1 followed by an increase of DNA synthesis and prolonged cell survival. Interestingly, we found that both PKC isoforms, α and ε, mediate translocation of p21/Cip1/WAF1 to the cytoplasm. Because PKC-α overexpression does not protect cells from FasL-induced apoptosis as does activated PKC-ε, another pathway downstream must be involved in the regulation of resistance to apoptosis. Akt/protein kinase B kinase is known to exhibit anti-apoptotic effects, and thrombin has been shown to activate the Akt pathway (37, 60). Recently, p21/Cip1/WAF1 has been shown to act as a substrate for Akt, inducing cell survival (61). We have found that thrombin significantly phosphorylates Akt via activation of PKC-ε but not through PKC-α (data not shown). It is possible that a similar mechanism is also involved in thrombin-induced cell survival, and this could explain why only activation of PKC-ε but not PKC-α mediated resistance to apoptosis. This mechanism is currently under investigation. p21/Cip1/WAF1 may also mediate thrombin-induced proliferation, since its translocation is regulated by PKC-α, and this isoform has been shown to regulate thrombin-induced DNA synthesis. Therefore, in the second part of our study, we investigated the involvement of PKC and downstream signaling in thrombin-induced proliferation of lung fibroblasts. One of the essential signal transduction cascades for cell growth and differentiation is the MAPK pathway, linking extracellular stimuli to intracellular responses that result in gene expression and cell proliferation (35). We found that thrombin induced p44/42 MAPK phosphorylation up to 10-fold in both normal and SSc lung fibroblasts. Inhibition of thrombin-induced MAPK phosphorylation with the specific MEK1/2 inhibitor U0126 completely abolished DNA synthesis.

Fig. 9. Thrombin-induced cyclin D1 activation in normal and scleroderma lung fibroblasts. A.1: normal and SSc lung fibroblasts were incubated with 0.5 U/ml thrombin for 12 and 24 h. B.1: normal lung fibroblasts were pretreated with 100 nM calphostin C, PKC-α AS, PKC-ε AS, or 5 μM U0126 before incubation with 0.5 U/ml thrombin. Cells extracts were immunoblotted with anti-cyclin D1 antibody, and a representative blot from 3 experiments is presented. Cyclin D1 bands were quantified by densitometry using NIH Image software (A.2 and B.2). Expression of β-actin is shown to confirm normalization of the equal loading of protein in the gel lane. The experiment was performed 3 times, and representative blots are presented. Note that thrombin induces cyclin D1 in both normal and SSc lung fibroblasts. Inhibition of PKC-α abolishes thrombin-induced cyclin D1 in normal lung fibroblasts, whereas depletion of PKC-ε has only slight effect.

Fig. 10. Two distinct PKC isoforms mediate DNA synthesis and cell survival in thrombin-induced myofibroblasts. Thrombin activates PAR-1 receptor in fibroblasts (5, 46), which results in activation of two distinct α and ε PKC isoforms. PKC-α mediates thrombin-induced MAPK phosphorylation, cyclin D1 accumulation, and, subsequently, DNA synthesis, whereas PKC-ε mediates thrombin-induced resistance to apoptosis of lung myofibroblasts. Thrombin induces expression and translocation of p21/Cip1/WAF1 from nucleus to the cytoplasm via both PKC-α and -ε isoforms. The role of p21/Cip1/WAF1 in DNA synthesis and cell survival in thrombin-induced myofibroblasts is currently under investigation.
stimulated by thrombin in lung fibroblasts. PKC-α antisense oligonucleotides had the same effect as MEK1/2 inhibitor on thrombin-induced DNA synthesis and MAPK phosphorylation. We conclude that PKC-α mediates thrombin-induced MAPK phosphorylation and subsequently proliferation in lung fibroblasts. Thrombin is known to mediate the expression of the G1 phase regulatory protein cyclin D1 (16, 46). We observed that basal levels of cyclin D1 are increased in sclerodermia lung fibroblasts when compared with normal lung fibroblasts; furthermore, thrombin increased cyclin D1 levels up to 10-fold within 24 h in both normal and SSC lung fibroblasts. Thrombin-induced cyclin D1 accumulation is abolished by the MEK1/2 inhibitor U0126, and by the PKC inhibitor calphostin C and PKC-α, but not PKC-ε antisense oligonucleotides. These results suggest that thrombin-induced cyclin D1 accumulation is regulated via p44/p42 MAPK and a PKC-α-dependent pathway.

In summary, we have elucidated several signaling pathways by which thrombin mediates differentiation of normal lung fibroblasts to a myofibroblast phenotype. Our study is the first demonstration that PAR-1 is abundantly expressed in SSC lung within the fibroproliferative foci, suggesting a significant role in lung fibroblast activation. We provide compelling evidence that thrombin is an important regulator of cell survival and DNA synthesis and that these two processes are regulated via two distinct PKC isoforms. Activation of PKC-ε promotes resistance to apoptosis in thrombin-induced lung myofibroblasts, whereas activation of PKC-α is followed by activation of MAPK and cyclin D1, leading to enhanced proliferation. Induction and relocation of p21/WAF1 from the nucleus to the cytoplasm are mediated by PKC-α and PKC-ε and promotes both phenomena (Fig. 10). Taken together, these data explain how thrombin stimulates unrestricted proliferation and cell survival and initiates tissue repair in sclerodermia lung fibrosis. Our results shed light on the mechanism of thrombin-induced fibroblast activation and may have significant relevance to the pathogenesis of lung fibrosis as seen in sclerodermia and perhaps other fibrotic lung diseases as well.

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