

# Curcumin-Induced Apoptosis in Scleroderma Lung Fibroblasts

## Role of Protein Kinase C $\epsilon$

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Scleroderma, a disease involving excessive collagen deposition, can be studied using fibroblasts cultured from affected tissues. We find that curcumin, the active component of the spice turmeric, causes apoptosis in scleroderma lung fibroblasts (SLF), but not in normal lung fibroblasts (NLF). This effect is likely to be linked to the fact that although curcumin induces the expression of the phase 2 detoxification enzymes heme oxygenase 1 and glutathione S-transferase P1 (GST P1) in NLF, SLF are deficient in these enzymes, particularly after curcumin treatment. The sensitivity of cells to curcumin-induced apoptosis and the expression of GST P1 (but not heme oxygenase 1) are regulated by the  $\epsilon$  isoform of protein kinase C (PKC $\epsilon$ ). SLF, which contain less PKC $\epsilon$  and less GST P1 than NLF, become less sensitive to curcumin-induced apoptosis and express higher levels of GST P1 when transfected with wild-type PKC $\epsilon$ , but not with dominant-negative PKC $\epsilon$ . Conversely, NLF become sensitive to curcumin-induced apoptosis and express lower levels of GST P1 when PKC $\epsilon$  expression or function is inhibited. The subcellular distribution of PKC $\epsilon$  also differs in NLF and SLF. PKC $\epsilon$  is predominantly nuclear or perinuclear in NLF but is associated with stress fibers in SLF. Just as PKC $\epsilon$  levels are lower in SLF than in NLF *in vitro*, PKC $\epsilon$  expression is decreased in fibrotic lung tissue *in vivo*. In summary, our results suggest that a signaling pathway involving PKC $\epsilon$  and phase 2 detoxification enzymes provides protection against curcumin-induced apoptosis in NLF and is defective in SLF. These observations suggest that curcumin may have therapeutic value in treating scleroderma, just as it has already been shown to protect rats from lung fibrosis induced by a variety of agents.

Scleroderma is a debilitating autoimmune disease that affects over 100,000 people in the United States, mostly women. The hallmark of scleroderma is dermal fibrosis. When accompanied by visceral organ fibrosis, significant morbidity and mortality results (1). Despite improved symptomatic treatment, there is no definitive therapy for the underlying disease process.

Protein kinase C (PKC) isoforms comprise a family of serine-threonine kinases that forms three subfamilies based on structural homologies and sensitivity to activators: the conventional PKCs ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ) are Ca<sup>++</sup>-dependent and require phosphatidylerine, diacylglycerol, or phorbol esters for activation; the novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\nu$ ,  $\theta$ ) are Ca<sup>++</sup>-independent but can be activated by phorbol esters; the atypical PKCs ( $\zeta$ ,  $\lambda$ ) are Ca<sup>++</sup>-independent and are insensitive to diacylglycerol and phorbol

esters (2). Our previous studies (3) highlight the  $\epsilon$  isoform of PKC (PKC $\epsilon$ ) as a signaling molecule that functions aberrantly in scleroderma lung fibroblasts (SLF). In particular, using PKC $\epsilon$  antisense oligodeoxynucleotides (AS ODN) to eliminate PKC $\epsilon$  protein inhibits expression of the ECM protein tenascin-C in normal lung fibroblasts (NLF), but promotes tenascin-C expression in SLF. PKC $\delta$  has also been suggested to play a role in fibrosis in scleroderma because it promotes collagen expression and because it is present at higher levels in scleroderma dermal fibroblasts than in normal dermal fibroblasts (4).

Curcumin (diferuloylmethane) is a major component of the spice turmeric. Turmeric has been used in Chinese and Indian herbal medicine to treat a wide range of conditions. The efficacy of turmeric in these folk applications has not been proven (5). Practitioners of alternative medicine recommend curcumin as a treatment for autoimmune diseases. The scientific literature has reported an equally diverse set of biological activities for curcumin. It has been reported to act as an anti-inflammatory agent (6), to inhibit pulmonary fibrosis (7, 8), to inhibit tumor promotion, tumor cell proliferation, and metastasis (9, 10), to be a powerful antioxidant (11), and to be an effective topical microbicide (12). The list of molecular mechanisms that may be involved in mediating the effects of curcumin is equally bewildering. It inhibits enzymes including nitric oxide synthase (13) and protein tyrosine kinase (14). It affects arachidonic acid metabolism (15) and is an inhibitor of transcription factors nuclear factor- $\kappa$ B and c-Jun/AP-1 (16, 17). Nevertheless, many biological functions of curcumin result from its effects on PKC. For example, curcumin opposes the ability of phorbol esters to activate PKC in 3T3 cells (18), to promote tumor growth (9), and to activate the transcription factors nuclear factor- $\kappa$ B and c-jun/AP-1 (19). Moreover, the effects of curcumin can be similar to the effects of PKC inhibitors: both curcumin and staurosporine inhibit the function of c-jun/AP-1 (19) and induce apoptosis in transformed cell lines (20).

Curcumin has been described as a natural chemoprotective agent because it induces the expression of phase 2 detoxification enzymes such as heme oxygenase 1 (HO-1) and glutathione S-transferase (GST) (21, 22). However, given that curcumin can induce apoptosis (20), it is also reasonable to view curcumin as a toxic xenobiotic that cells attempt to inactivate by producing phase 2 detoxification enzymes. The expression of many phase 2 detoxification enzymes is under the control of the antioxidant response element. Enzyme expression is induced when the transcription factor Nrf2 is released from its interaction with its cytoplasmic chaperone Keap1 and translocates to the nucleus, where it interacts with the antioxidant response element (23). Curcumin induces the expression of phase 2 detoxification enzymes by promoting the inactivation of Nrf2-Keap1 complexes and thereby promoting the translocation of Nrf2 to the nucleus (22). Interestingly, the phosphorylation of Nrf2 by PKC also promotes its dissociation from Keap1 and thereby promotes the expression of phase 2 detoxification enzymes (24). Thus the interrelationship between curcumin and PKC is complex because in some cases (9, 18–20) the effects of curcumin oppose the

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Abbreviations: antisense, AS;  $\alpha$ -smooth muscle actin, ASMA; bronchoalveolar lavage, BAL; constitutively active, CA; dominant-negative, DN; glutathione S-transferase, GST; heme oxygenase 1, HO-1; normal lung fibroblasts, NLF; oligodeoxynucleotides, ODN; phosphate-buffered saline, PBS; protein kinase C, PKC; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE; scleroderma lung fibroblasts, SLF; sense, S; wild type, WT.

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effects of PKC whereas curcumin and PKC may synergize in inducing phase 2 detoxification enzymes.

Because of our previous observations on altered PKC $\epsilon$  signaling in SLF and the reported ability of curcumin to affect PKC signaling, we compared the effects of curcumin on SLF and NLF. Strikingly, although curcumin had little or no effect on NLF, it caused SLF to retract from the culture vessel and undergo apoptosis. This effect is clearly related to PKC $\epsilon$  signaling, given that SLF ordinarily are deficient in PKC $\epsilon$ , that enhancing PKC $\epsilon$  expression protects SLF from curcumin-induced apoptosis, and that inhibiting PKC $\epsilon$  expression or function makes NLF sensitive to curcumin. The importance of PKC $\epsilon$  in lung fibrosis is further supported by the observations that the subcellular distribution of PKC $\epsilon$  is very different in NLF and SLF, and that PKC $\epsilon$  expression is also severely decreased in fibrotic lung tissue *in vivo*. It is likely that SLF are sensitive to curcumin-induced apoptosis because curcumin-treated SLF, unlike curcumin-treated NLF, contain extremely low levels of the phase 2 detoxification enzymes HO-1 and GST P1. The role of GST P1 in protecting cells against curcumin-induced apoptosis may be central, given that just as PKC $\epsilon$  regulates curcumin-induced apoptosis, it also regulates both the basal and curcumin-induced expression of GST P1 in both NLF and SLF. In summary, these results support the idea that curcumin may be useful in treating lung fibrosis in general and scleroderma in particular.

## Materials and Methods

### Cell Culture

Cells were cultured at 37°C in a 10% CO<sub>2</sub> incubator. Fibroblasts were derived from lung tissue obtained at autopsy from scleroderma patients and from age-, race-, and sex-matched normal subjects and used between passages two and four (3). The harvesting of tissue was approved by the MUSC IRB for Human Research. Fibroblasts were also derived from bronchoalveolar lavage (BAL) fluid from patients with scleroderma as previously described (25). For each experiment, cells were harvested by trypsinization and grown in 6-well plates in "serum-containing medium" (Dulbecco's modified Eagle's medium/10% fetal calf serum/2 mM L-glutamine/50  $\mu$ g per ml gentamicin sulfate). When the cell layer was nearly confluent, the medium was replaced with "serum-free medium" (Dulbecco's modified Eagle's medium/50  $\mu$ M ascorbic acid/2 mM L-glutamine/50  $\mu$ g per ml gentamicin sulfate). The next morning the medium was again replaced with serum-free medium supplemented with curcumin (Sigma, St. Louis, MO) (from 1,000 $\times$  stocks dissolved in dimethyl sulfoxide) or with a similar dilution of dimethyl sulfoxide without curcumin. Unless otherwise indicated, these incubations were for 6 h and the final concentration of curcumin was 10  $\mu$ M.

Experiments involving pretreatment with PKC $\epsilon$  sense oligodeoxynucleotides (S ODN) and AS ODN were performed as previously described (3). Phosphorothioated ODN were synthesized in the Oligonucleotide Synthesis Facility at the Medical University of South Carolina. Sequences were: antisense 5'-GCCATTGAACACTACCAT-3', sense 5'-ATGGTAGTGTTC AATGGC-3'. For experiments involving transfection, cells were incubated with FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions using 1  $\mu$ g of plasmid DNA per well in 6-well plates. Following a 2-d incubation in serum-containing medium, transfected cells were further incubated in serum-free medium, then treated with curcumin as described above. Constructs used were: wild-type (WT) PKC $\epsilon$  in the pMT2 vector (26) (a kind gift from Dr. Peter J. Parker, Imperial Cancer Research Fund, London, UK) and dominant-negative (DN) and constitutively active (CA) PKC $\epsilon$  in the SRD vector (27) (kind gifts from Dr. Shigeo Ohno, Yokohama City University School of Medicine, Yokohama, Japan).

### Analyses of Apoptosis

Whether cells had undergone apoptosis was evaluated by three independent methods: morphology, TUNEL labeling of DNA strand breaks,

and FACS analysis of the DNA content of cells. Morphology was evaluated in cells stained using crystal violet (28). For TUNEL (29), cells were fixed, permeabilized, and labeled using the *in situ* Cell Death Detection kit as recommended by the manufacturer (Roche Diagnostics). For FACS analysis of apoptosis, single cell suspensions were prepared by trypsinization, fixed in 70% ethanol, and stained with propidium iodide to detect DNA. Fluorescence was measured at > 620 nm. Cells that had not been treated with curcumin were used to set gates that would exclude subcellular particles and multicellular aggregates. Cells are defined as apoptotic if they contain less than the G0 level of DNA. A minimum of 10,000 events were collected per sample. Mean values from three independent experiments are presented.

### Western Blot Analyses

Cell layers were extracted with boiling sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Aliquots representing material derived from the same number of cells were probed using rabbit polyclonal antibodies against PKC $\alpha$  (sc-208) and PKC $\epsilon$  (sc-214) from Santa Cruz Biotechnology (Santa Cruz, CA), an anti-actin monoclonal antibody (MAB1501; Chemicon International, Temecula, CA), and appropriate secondary antibodies.

### Statistical Significance

All experiments were repeated using at least three independent strains of SLF and NLF with similar results. *P* values were calculated using the two-tailed *t* test.

### Polyclonal Anti-PKC $\epsilon$ for Immunohistochemistry

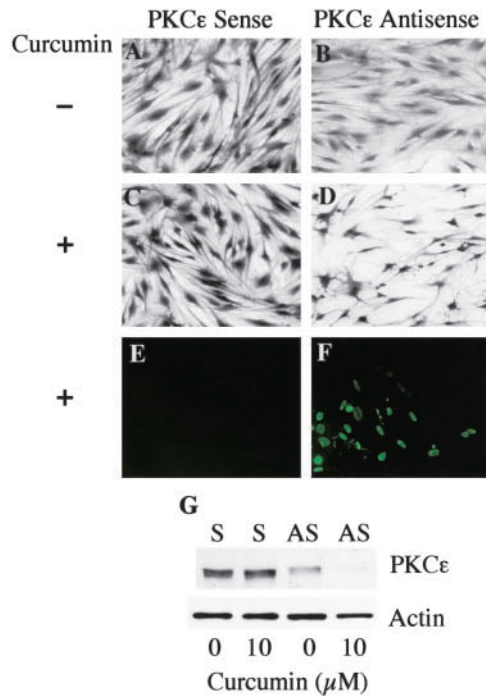
Commercially available antibodies against PKC $\epsilon$  did not provide us with acceptable staining in immunohistochemistry experiments. Therefore, we prepared a rabbit polyclonal antibody against a bacterial fusion protein containing amino acids 323–407 of human PKC $\epsilon$  linked to GST. Total IgG was isolated from immune rabbit serum. Immune IgG was affinity purified using immobilized PKC $\epsilon$ -GST fusion protein and depleted of anti-GST antibodies using immobilized GST.

### Immunocytochemistry

NLF and SLF ( $5 \times 10^4$  in 1 ml serum-containing medium per well) were incubated overnight in 4-well glass chamber slides (Nalge Nunc International, Naperville, IL). The medium was replaced with serum-free medium for 24 h. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) adjusted to pH 7.5 for 10 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 5 min, blocked with 3% bovine serum albumin in PBS, and sequentially incubated with primary antibodies (polyclonal anti-PKC $\epsilon$  for immunohistochemistry [see above]; polyclonal anti-PKC $\alpha$  sc-208, Santa Cruz Biotechnology; monoclonal anti- $\alpha$ -smooth muscle actin [ASMA], Sigma) and appropriate fluorescent secondary antibodies. The slides were mounted in a 1:1 mixture of glycerol and PBS and examined by fluorescent microscopy.

### Bleomycin-Induced Lung Fibrosis

The following procedure was approved by the MUSC Institutional Animal Care and Use Committee. Ten-week-old, male CD1 mice (Charles River, Boston, MA) anesthetized by inhalation of an initial dose of 4% isoflurane (Baxter, Deerfield, IL) followed by a continuing 1.5% received a single intratracheal administration of bleomycin (0.045 U in 45  $\mu$ l of sterile saline; Calbiochem, La Jolla, CA) or of saline alone. After 14 d, mice were anesthetized with isoflurane and killed by cervical dislocation. Lungs were removed and fixed overnight in 0.1 M Tris (pH 7.4)/0.05% calcium acetate/0.5% zinc acetate/0.5% zinc chloride. The tissue was then dehydrated through an alcohol series and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, and endogenous peroxidase activity quenched using 3% hydrogen peroxide. Sections were blocked using 3% bovine serum albumin/1% goat serum/0.1% Triton X-100/PBS, incubated overnight with blocking buffer containing polyclonal anti-PKC $\epsilon$  for immunohistochemistry, then for 1 h with blocking buffer containing goat anti-rabbit IgG conjugated to horseradish peroxidase. Color was developed using DAB/Metal concentrate (Pierce, Rockford, IL). Sections were counterstained with hematoxylin, dehydrated, coverslipped, and observed. For Western



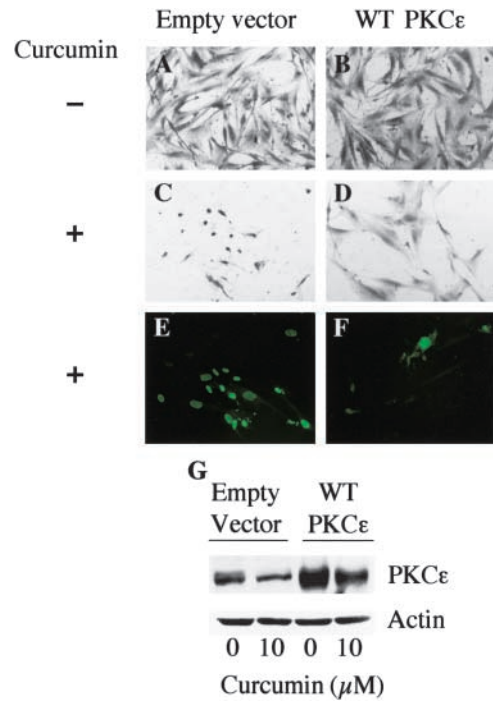
**Figure 1.** PKC $\epsilon$  AS treatment makes NLF sensitive to curcumin-induced apoptosis. As indicated, cells were treated with PKC $\epsilon$  S ODN (A, C, E) or PKC $\epsilon$  AS ODN (B, D, F) in the absence (A, B) or presence (C–F) of 10  $\mu\text{M}$  curcumin. Cells were stained using crystal violet (A–D) or fluorescently labeled by TUNEL (E, F). (G) PKC $\epsilon$  and actin levels were determined by Western blotting of the cell layer from cultures treated with PKC $\epsilon$  AS ODN (AS) or PKC $\epsilon$  S ODN (S), then incubated for 6 h in the presence or absence of curcumin. Gel samples were normalized to include extract from the same number of cells.

blotting, a set of lungs from a saline or a bleomycin-treated mouse was homogenized using a Tissue Tearor in 1.5 ml of 25 mM Tris (pH 8.0) containing a cocktail of protease inhibitors (N-ethylmaleimide [10 mM], benzamide [5 mM], leupeptin [50  $\mu\text{g}/\text{ml}$ ], pepstatin A [5  $\mu\text{g}/\text{ml}$ ], PMSF [2 mM]). Ten micrograms of total protein from each homogenate was resolved by SDS-PAGE and Western blotted using anti-PKC $\epsilon$  as described above.

## Results

### Curcumin Causes Apoptosis in SLF, but Not in NLF

The morphology of NLF is not affected by 10  $\mu\text{M}$  curcumin (compare Figures 1A and 1C). Even after a 36 h exposure (not shown), curcumin has no effect. In contrast, within 6 h 10  $\mu\text{M}$  curcumin treatment causes SLF to retract and become little more than nuclei with a minimal amount of surrounding cytoplasm and thin, scraggly processes reaching out to the culture substratum (compare Figures 2A and 2C). In accord with their appearance, these cells are easily detached. When the dose dependence of the curcumin-induced change in cell shape was examined (Table 1), we found that no strains of SLF were sensitive to 2.5  $\mu\text{M}$  curcumin, some strains were sensitive to 5  $\mu\text{M}$  curcumin, and almost all strains were sensitive to 10  $\mu\text{M}$  curcumin. Consequently, further experiments were performed using 10  $\mu\text{M}$  curcumin except as indicated. Fibroblasts can be harvested from BAL fluid from patients with scleroderma, but not from BAL fluid from normal volunteers. Interestingly, fibroblasts harvested from BAL fluid are even more sensitive to curcumin than are SLF grown from lung tissue explants. In particular, all three strains of SLF harvested from BAL fluid were sensitive to 2.5  $\mu\text{M}$  curcumin (Table 1).



**Figure 2.** Transfection with WT PKC $\epsilon$  makes SLF partially resistant to curcumin-induced apoptosis. As indicated, cells were transfected with empty vector (A, C, E) or WT PKC $\epsilon$  (B, D, F) and incubated in the absence (A, B) or presence (C–F) of 10  $\mu\text{M}$  curcumin. Cells were stained using crystal violet (A–D) or fluorescently labeled by TUNEL (E, F). (G) PKC $\epsilon$  and actin levels were determined by Western blotting of the cell layer from cultures transfected with Empty Vector or WT PKC $\epsilon$ , then incubated for 6 h in the presence or absence of curcumin. Gel samples were normalized to include extract from the same number of cells.

Two methods were used to confirm that the change in shape of SLF treated with curcumin represents apoptosis. DNA strand breaks were detected by TUNEL labeling. SLF cultured in the presence of curcumin were TUNEL-positive (Figure 2E), whereas NLF cultured in the presence of curcumin were TUNEL-negative (Figure 1E). Both SLF and NLF cultured in the absence of curcumin were TUNEL-negative (data not shown). Apoptosis was also quantified by FACS analysis of the loss of DNA from cells. Only  $2.7 \pm 0.2\%$  of NLF contained less than the typical G0 level of DNA. This parameter was slightly increased in untreated SLF and in NLF treated with curcumin. However, a massive increase in the number of cells that had lost DNA ( $53.1 \pm 4.1\%$ ,

**TABLE 1.** Concentration dependence of curcumin-induced changes in cell shape

Cell Type	Number of Strains Sensitive to the Indicated Concentration of Curcumin		
	2.5 $\mu\text{M}$	5 $\mu\text{M}$	10 $\mu\text{M}$
NLF	0 of 4	0 of 4	0 of 4
SLF	0 of 7	4 of 7	6 of 7
SLF from BAL fluid	3 of 3	3 of 3	3 of 3

The indicated cells were treated with the indicated concentration of curcumin. After a 6-h incubation, the cultures were examined to determine whether curcumin had caused the cells to retract and become only loosely attached to the culture substrate.

*Definition of abbreviations:* BAL, bronchoalveolar lavage; NLF, normal lung fibroblasts; SLF, scleroderma lung fibroblasts.

$P < 0.001$ ) was observed when SLF were treated with curcumin (Table 2, *Experiment A*). Together these results strongly support the idea that curcumin promotes apoptosis in SLF, but not in NLF.

The ability of curcumin to promote apoptosis was observed in six of seven SLF strains examined; none of four NLF strains showed sensitivity (data not shown). This reproducibility is better than that observed with most fibroblast phenotypes associated with scleroderma; many such phenotypes are present in  $< 50\%$  of strains examined (30).

#### PKC $\epsilon$ Regulates the Sensitivity of Cells to Curcumin-Induced Apoptosis

Given the relationship between curcumin treatment and PKC activity (9, 18–20) and the fact that depletion of PKC $\epsilon$  in NLF and SLF has opposite effects on the expression of the ECM protein tenascin (3), PKC $\epsilon$  was a prime candidate to be a regulator of the sensitivity of cells to curcumin-induced apoptosis. When PKC $\epsilon$  levels were compared, we found that on average SLF contained 60% less PKC $\epsilon$  than did NLF ( $P < 0.001$ ) (Figure 3A). In contrast, PKC $\alpha$  levels were similar in SLF and NLF (Figure 3A). Curcumin treatment caused a further, dramatic increase in the disparity in PKC $\epsilon$  levels between NLF and SLF. Although curcumin had little or no effect on PKC $\epsilon$  levels in NLF, curcumin treatment further decreased PKC $\epsilon$  levels in SLF in a dose-dependent manner (Figures 3B and 3C). In particular, the higher molecular weight member of the doublet of PKC $\epsilon$  polypeptides decreased  $\sim 90\%$  in concentration, whereas the level of the lower member of the doublet decreased only 30–40% (Figure 3B). The fact that curcumin did not affect the level of PKC $\alpha$  present in either NLF or SLF (Figure 3B) indicates that curcumin does not affect all PKC isoforms.

The facts that SLF contain less PKC $\epsilon$  than NLF and that this level is further decreased by curcumin treatment raised the possibility that inhibiting the expression or function of PKC $\epsilon$  in NLF might make them sensitive to curcumin-induced apoptosis. Therefore, NLF were treated with PKC $\epsilon$  AS ODN to inhibit the expression of PKC $\epsilon$  or were transfected with DN PKC $\epsilon$  to inhibit its function. As expected, PKC $\epsilon$  AS ODN decreased the level of PKC $\epsilon$  as compared with the PKC $\epsilon$  S ODN control (Figure 1G). Interestingly, curcumin co-treatment further decreased the level of PKC $\epsilon$  in PKC $\epsilon$  AS ODN-treated cells but had no effect on PKC $\epsilon$  levels in PKC $\epsilon$  S ODN-treated cells. Transfection

experiments resulted in about a 3-fold overexpression of DN PKC $\epsilon$ , WT PKC $\epsilon$ , or CA PKC $\epsilon$  as compared with endogenous PKC $\epsilon$  (data not shown). When these cells were examined for apoptosis by FACS analysis, we found that indeed NLF treated with PKC $\epsilon$  AS ODN (Table 2, *Experiment B*) or transfected with DN PKC $\epsilon$  (Table 2, *Experiment C*) are as sensitive as SLF to curcumin-induced apoptosis. In control experiments, NLF treated with PKC $\epsilon$  S ODN (Table 2, *Experiment B*) or transfected with WT PKC $\epsilon$ , CA PKC $\epsilon$ , or empty vector (Table 2, *Experiment C*) were not sensitive to curcumin-induced apoptosis. Consistent with the FACS analysis data, NLF treated with PKC $\epsilon$  AS ODN showed the apoptotic phenotype (Figure 1D) and were TUNEL-positive (Figure 1F) in the presence of curcumin. In control experiments, NLF treated with PKC $\epsilon$  S ODN (Figure 1) or transfected with WT PKC $\epsilon$ , CA PKC $\epsilon$ , or empty vector (not shown) did not show the apoptotic phenotype (Figure 1C) and were TUNEL-negative (Figure 1E) in the presence of curcumin.

If decreasing the level of PKC $\epsilon$  in NLF makes them sensitive to the effects of curcumin, then, conversely, increasing the level of PKC $\epsilon$  in SLF may protect them from the effects of curcumin. To test this idea, SLF were transfected with WT PKC $\epsilon$  resulting in an approximate 2.5-fold increase in the level of PKC $\epsilon$  in the cells (Figure 2G). This treatment partially protected SLF against curcumin-induced apoptosis. In particular, transfection with WT PKC $\epsilon$  decreased the number of apoptotic cells from 60% to 18% as estimated by FACS analysis (Table 2, *Experiment D*), caused many cells to not assume the apoptotic phenotype (compare Figures 2D and 2C), and decreased the number of TUNEL-positive cells (compare Figures 2F and 2E). As expected, transfection with CA PKC $\epsilon$  also protected SLF against curcumin-induced apoptosis while transfection with DN PKC $\epsilon$  or empty vector provided no protection (Table 2, *Experiment D*). Consistent with the fact that transfection with WT PKC $\epsilon$  did not completely protect SLF from curcumin-induced apoptosis, PKC $\epsilon$  levels were decreased by curcumin treatment in SLF transfected with either empty vector or with WT PKC $\epsilon$  (Figure 2G).

#### Induction of Phase 2 Detoxification Enzymes by Curcumin

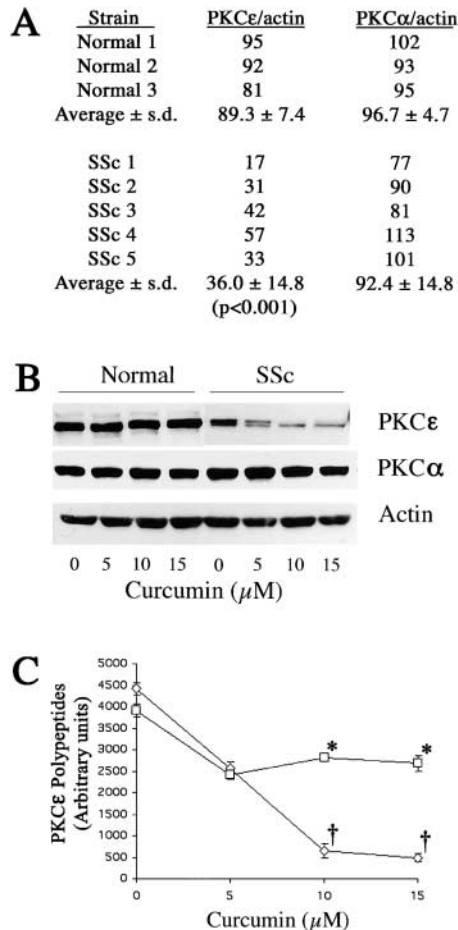
Curcumin has been reported to induce the expression of phase 2 detoxification enzymes (21, 22). This fact raises the possibility that SLF, but not NLF, are sensitive to curcumin-induced apoptosis because phase 2 detoxification enzymes are induced by

TABLE 2. FACS analysis of apoptosis

Experiment	Cell Type	Treatment	Apoptotic Cells (% of total population)	
			No Curcumin	10 $\mu$ M Curcumin
A	NLF	None	2.7 $\pm$ 0.2	3.8 $\pm$ 0.5
	SLF	None	4.5 $\pm$ 0.8	53.1 $\pm$ 4.1
B	NLF	PKC $\epsilon$ S ODN	3.8 $\pm$ 0.2	4.8 $\pm$ 0.4
	NLF	PKC $\epsilon$ AS ODN	4.3 $\pm$ 0.1	64.5 $\pm$ 1.8
C	NLF	Empty vector	4.8 $\pm$ 0.2	5.9 $\pm$ 1.1
	NLF	WT PKC $\epsilon$	3.6 $\pm$ 0.5	3.7 $\pm$ 0.8
	NLF	CA PKC $\epsilon$	3.8 $\pm$ 0.7	3.9 $\pm$ 0.5
	NLF	DN PKC $\epsilon$	6.5 $\pm$ 1.1	54.4 $\pm$ 1.2
D	SLF	Empty vector	4.4 $\pm$ 0.3	60.1 $\pm$ 2.5
	SLF	WT PKC $\epsilon$	4.5 $\pm$ 0.2	18.0 $\pm$ 1.2
	SLF	CA PKC $\epsilon$	5.2 $\pm$ 1.3	23.9 $\pm$ 2.4
	SLF	DN PKC $\epsilon$	4.8 $\pm$ 0.4	55.8 $\pm$ 1.7

Cells are defined as apoptotic if they contain less than the G0 level of DNA. DNA levels are quantified in terms of fluorescent labeling of the DNA using propidium iodide. Cells that had not been treated with curcumin were used to set gates that would exclude subcellular particles and multicellular aggregates. A minimum of 10,000 events were collected per sample. Mean values from three independent experiments are presented.

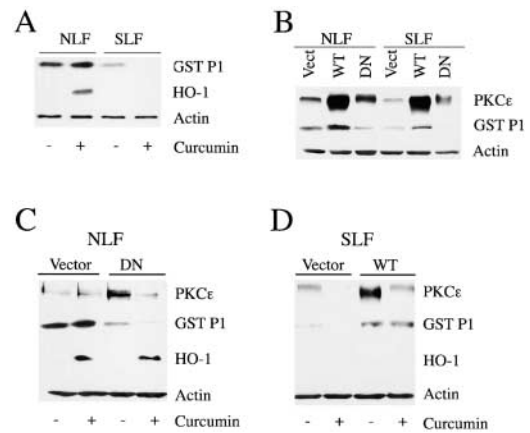
*Definition of abbreviations:* CA, constitutively active; DN, dominant-negative; NLF, normal lung fibroblasts; PKC, protein kinase C; SLF, scleroderma lung fibroblasts.



**Figure 3.** Altered PKC $\epsilon$  levels in SLF in the absence and presence of curcumin. (A) PKC $\epsilon$ , PKC $\alpha$ , and actin levels were quantified by densitometric analyses of Western blots of cells from three strains of NLF and five strains of SLF cultured in the absence of curcumin. The data presented are PKC levels divided by actin levels in arbitrary units. (B) The indicated cells (Normal or SSc) were incubated for 6 h in the presence of the indicated concentrations of curcumin. PKC $\epsilon$ , PKC $\alpha$ , and actin levels were determined by Western blotting of the cell layer. Gel samples were normalized to include extract from the same number of cells. (C) Densitometric quantification of the effect of curcumin on the relative levels of the two PKC $\epsilon$  polypeptides in SLF. These data represent the average  $\pm$  SD from three independent experiments similar to the experiment shown in B. *Diamonds*, upper band; *squares*, lower band. \* $P < 0.005$  versus 0  $\mu$ M curcumin level, † $P < 0.001$  versus 0  $\mu$ M curcumin level.

curcumin in NLF, but not in SLF. To evaluate this possibility, we examined the expression of HO-1 and several isoforms of GST by Western blotting. Particularly striking results were obtained with HO-1 and GST P1. In the absence of curcumin, HO-1 expression was below detection in both NLF and SLF; in the presence of curcumin HO-1 was prominently expressed by NLF but was still below detection in SLF (Figure 4A). In the absence of curcumin, GST P1 was expressed at higher levels by NLF than by SLF (Figure 4A). Curcumin treatment increased GST P1 expression by NLF while inhibiting the expression of GST P1 by SLF (Figure 4A). Thus both HO-1 and GST P1 are expressed at far higher levels in curcumin-treated NLF than in curcumin-treated SLF.

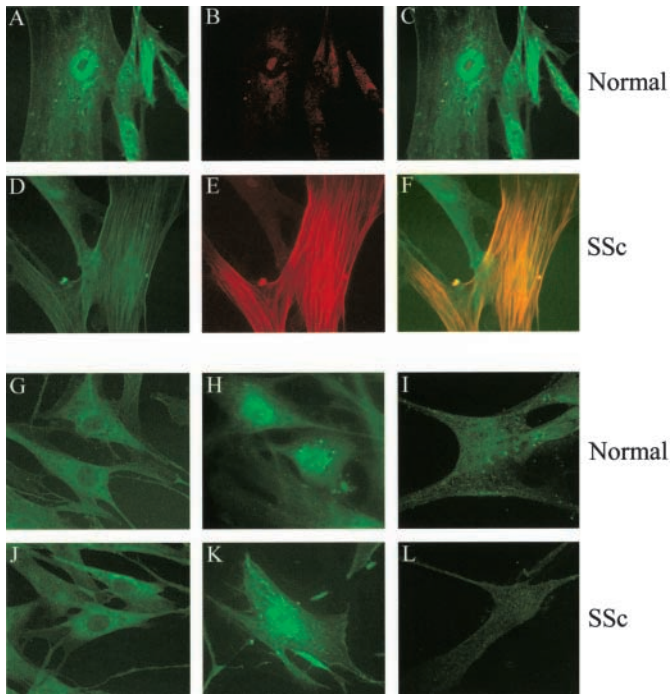
Given that NLF contain more PKC $\epsilon$  (and more GST P1) than do SLF, we next examined the effect of overexpressing WT



**Figure 4.** Regulation of HO-1 and GST P1 expression in NLF and SLF by curcumin and PKC $\epsilon$ . (A) GST P1, HO-1, and actin levels were determined by Western blotting of extracts of NLF and SLF incubated for 6 h in the absence (–) or presence (+) of curcumin. (B) PKC $\epsilon$ , GST P1, and actin levels were determined by Western blotting of extracts of NLF and SLF transfected with empty vector (Vect), WT PKC $\epsilon$  (WT), or DN PKC $\epsilon$  (DN). The high levels of PKC $\epsilon$  detected under WT and DN demonstrate the success of the transfections. (C, D) PKC $\epsilon$ , GST P1, HO-1, and actin levels were determined by Western blotting of NLF (C) and SLF (D) transfected with empty vector (Vector), DN PKC $\epsilon$  (DN), or WT PKC $\epsilon$  (WT) and incubated for six hours in the absence (–) or presence (+) of curcumin. The high levels of PKC $\epsilon$  detected under DN and WT (in the absence of curcumin) demonstrate the success of the transfections.

PKC $\epsilon$  and DN PKC $\epsilon$  in NLF and SLF on HO-1 and GST P1 expression. In all cases HO-1 expression was below detection (data not shown). Interestingly, WT PKC $\epsilon$  increased GST P1 expression in both NLF and SLF, whereas DN PKC $\epsilon$  decreased GST P1 expression in both NLF and SLF (Figure 4B). These observations support the idea that the level of GST P1 expressed by lung fibroblasts is regulated by the level of PKC $\epsilon$  activity in the cells.

Because transfection with DN PKC $\epsilon$  causes NLF to become sensitive to curcumin-induced apoptosis and because transfection with WT PKC $\epsilon$  protects SLF from curcumin-induced apoptosis, we also examined the effect of curcumin on HO-1 and GST P1 expression in NLF transfected with DN PKC $\epsilon$  (Figure 4C) and in SLF transfected with WT PKC $\epsilon$  (Figure 4D). Neither treatment affected HO-1 expression, i.e., curcumin strongly induced HO-1 expression in both NLF transfected with an empty vector and NLF transfected with DN PKC $\epsilon$  (Figure 4C), HO-1 expression remained below detection in curcumin-treated SLF even if the cells were transfected with WT PKC $\epsilon$  (Figure 4D). In contrast, just as transfection with DN PKC $\epsilon$  caused NLF to behave like SLF in terms of curcumin-induced apoptosis, transfection with DN PKC $\epsilon$  also caused NLF to behave like SLF in terms of GST P1 expression. In particular, transfection of NLF with DN PKC $\epsilon$  decreased baseline GST P1 expression (in the absence of curcumin) and GST P1 expression in these cells was further decreased in the presence of curcumin (Figure 4C). Conversely, just as transfection with WT PKC $\epsilon$  caused SLF to behave like NLF in terms of curcumin-induced apoptosis, transfection with WT PKC $\epsilon$  also caused SLF to behave like NLF in terms of GST P1 expression. In particular, transfection of SLF with WT PKC $\epsilon$  greatly increased baseline GST P1 expression (Figure 4D). In contrast to empty vector-transfected SLF in which curcumin treatment decreased the already low level of GST P1 expression, curcumin treatment of WT PKC $\epsilon$ -transfected SLF



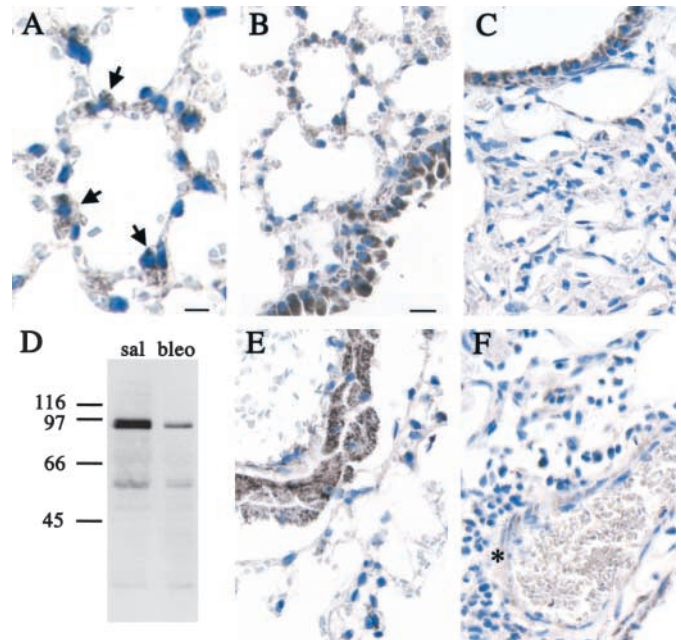
**Figure 5.** Immunohistochemical detection of PKC $\epsilon$  in NLF and SLF. NLF (A–C, G–I) and SLF (D–F, J–L) were cultured on 4-well glass chamber slides. WT PKC $\epsilon$  was overexpressed in NLF (H) and SLF (K); AS ODN treatment inhibited PKC $\epsilon$  expression in NLF (I) and SLF (L). Cells were then fixed, permeabilized, and incubated with rabbit anti-PKC $\epsilon$  (A, D, H, I, K, L), mouse monoclonal anti-ASMA (B, E), rabbit anti-PKC $\alpha$  (G, J), or both rabbit anti-PKC $\epsilon$  and mouse monoclonal anti-ASMA (C, F). Following incubation with Cy2-labeled goat anti-rabbit IgG antibodies and/or Cy3-labeled goat anti-mouse IgG antibodies, staining patterns were detected by fluorescent microscopy.

did not decrease the high level of GST P1 expression in these cells (Figure 4D). In summary, these results demonstrate that HO-1 expression is induced by curcumin in NLF but not in SLF, and that this induction in NLF and this failed induction in SLF do not involve PKC $\epsilon$ . In contrast, PKC $\epsilon$  does regulate the higher baseline level of GST P1 in NLF than in SLF, the further induction of GST P1 in NLF treated with curcumin, and the inhibition of expression of GST P1 in SLF treated with curcumin.

#### The Distribution of PKC $\epsilon$ Is Abnormal in SLF Cultures and in Fibrotic Lung Tissue

Because we have demonstrated here and in previous studies (3) that the function of PKC $\epsilon$  is aberrant in SLF, we also examined the possibility that the distribution of PKC $\epsilon$  might be abnormal in SLF cultures (Figure 5) and in fibrotic lung tissue *in vivo* (Figure 6). In NLF, the predominant localization of PKC $\epsilon$  was nuclear and perinuclear (Figure 5A). In contrast, in SLF PKC $\epsilon$  was present in stress fibers where it colocalized with ASMA (Figures 5D–5F). PKC $\epsilon$  and ASMA did not colocalize in NLF cultures (Figures 5A–5C). Just as PKC $\alpha$  was expressed at similar levels in NLF and SLF, it had a similar distribution in the cytoplasm (and not in the nucleus) of both NLF and SLF (compare Figures 5G and 5J).

Because enhancing PKC $\epsilon$  expression protects SLF from curcumin-induced apoptosis, whereas inhibiting PKC $\epsilon$  expression makes NLF sensitive to curcumin, we also examined the distribution of PKC $\epsilon$  in cells in which PKC $\epsilon$  levels had been enhanced or inhibited. Consistent with the possibility that nuclear and



**Figure 6.** PKC $\epsilon$  in control and fibrotic mouse lungs. Control mice received saline vehicle intratracheally, fibrosis was induced by the addition of bleomycin to the vehicle. Paraffin sections of lung tissue were incubated with rabbit anti-PKC $\epsilon$  (A–C, E, F). Following incubation with HRP-conjugated goat anti-rabbit IgG antibodies, staining was detected as brown reaction product. (A) Control lung tissue. High-magnification image showing staining of cells identified as type II pneumocytes (arrows). Scale bar = 5  $\mu$ m. B, C, E, and F are all at the same, lower magnification. Scale bar in B = 10  $\mu$ m. (B) Control lung tissue. Strong staining of cells lining a large bronchiole and noticeable staining of type II pneumocytes. (C) Fibrotic lung tissue. Lesser staining of cells lining a large bronchiole and little or no staining of any cell types in fibrotic alveoli. (E) Control lung tissue. Strong staining of vascular smooth muscle. (F) Fibrotic lung tissue. Little or no staining of vascular smooth muscle (asterisk). (D) Lung extracts from saline (sal) and bleomycin-treated mice (bleo) were analyzed by Western blotting with anti-PKC $\epsilon$ . The same amount of total protein was loaded in each lane. The migration of standard proteins is indicated by their molecular weight divided by 1,000.

perinuclear PKC $\epsilon$  protect cells from curcumin-induced apoptosis, when WT PKC $\epsilon$  was overexpressed in SLF, its localization was primarily nuclear and perinuclear (Figure 5K). Overexpressed WT PKC $\epsilon$  also had a primarily nuclear and perinuclear localization pattern in NLF (Figure 5H). Interestingly, the enhanced expression of WT PKC $\epsilon$  in SLF caused the association of endogenous PKC $\epsilon$  with stress fibers to be lost. Further supporting the idea that nuclear and perinuclear PKC $\epsilon$  protect cells from curcumin-induced apoptosis, when PKC $\epsilon$  expression in NLF was inhibited using AS ODN (making the cells sensitive to curcumin), nuclear and perinuclear PKC $\epsilon$  staining was lost (Figure 5I). As expected, AS ODN also decreased PKC $\epsilon$  staining in SLF (Figure 5L).

*In vivo* in normal mouse lung tissue, PKC $\epsilon$  is expressed at high levels in vascular smooth muscle (Figure 6E) and in cells lining large bronchioles (Figure 6B) (both round Clara cells and ciliated cells). PKC $\epsilon$  is also expressed in alveoli (Figure 6A), at high levels in cells that we have identified as type II pneumocytes based on their staining (data not shown) with antibodies against prosurfactant C (Research Diagnostics, Inc., Flanders, NJ) and at lower levels in other cell types. Both Western blotting and immunohistochemical experiments demonstrate a major decrease

in PKC $\epsilon$  in mice in which lung fibrosis has been induced by treatment with bleomycin. Densitometric analyses of Western blots (Figure 6D) showed a 77% decrease in PKC $\epsilon$  levels in bleomycin-treated lung tissue. Staining is detectable in the linings of the bronchioles (Figure 6C), but at a much lower level than in normal mice. Little or no PKC $\epsilon$  staining is observed in vascular smooth muscle (Figure 6F) or in alveoli (Figure 6C), even when the alveoli have a relatively normal morphology. Although PKC $\epsilon$  cannot be detected in the malformed, fibrotic alveoli of bleomycin-treated mice, prosurfactant C-positive cells (i.e., type II pneumocytes) are present in increased numbers (data not shown). Thus the loss of PKC $\epsilon$  from the alveoli of bleomycin-treated mice is not simply due to the loss of type II pneumocytes. In summary, both *in vitro* (Figure 5) and *in vivo* (Figure 6) studies support the idea that alterations in the subcellular and cellular distribution and levels of expression of PKC $\epsilon$  play a role in the progression of lung fibrosis.

## Discussion

Our results demonstrate that curcumin induces apoptosis in SLF, but not in NLF, due to aberrant PKC $\epsilon$  signaling in SLF and due to the failure of SLF to upregulate the expression of the phase 2 detoxification enzymes HO-1 and GST P1 in response to curcumin. The failure of SLF to upregulate HO-1 in response to curcumin is independent of PKC $\epsilon$ , whereas the failure to upregulate GST P1 is a direct consequence of altered PKC $\epsilon$  signaling. At present, we have not yet determined whether HO-1 and GST P1 protect NLF (but not SLF) against curcumin-induced apoptosis by metabolizing curcumin, by metabolizing other small molecules, or via their participation in signaling cascades (31). Protection against apoptosis of lung fibroblasts is not the only homeostatic activity of PKC $\epsilon$ . For example, activation of PKC $\epsilon$  protects cardiomyocytes, perfused hearts, and the hearts of living mice against ischemia-induced damage (32). We speculate that the ability of PKC $\epsilon$  to protect heart tissue against ischemia-induced damage may also involve the regulation of the expression of phase 2 detoxification enzymes (in particular GST P1) by PKC $\epsilon$ .

Our results suggest that the ability of PKC $\epsilon$  to promote cellular homeostasis depends not only on the concentration of the protein in cells, but also on its subcellular distribution and molecular form. The distribution of PKC $\epsilon$  is very different in SLF and NLF; in SLF it is primarily associated with stress fibers, in NLF its localization is primarily nuclear and perinuclear. When PKC $\epsilon$  is overexpressed in SLF, thereby protecting the cells against curcumin-induced apoptosis, PKC $\epsilon$  staining is found in a nuclear and perinuclear pattern and not in association with stress fibers. Thus, in order for PKC $\epsilon$  to protect cells against curcumin-induced apoptosis, it must be present in a nuclear and perinuclear pattern and not in association with stress fibers. Figures 3 and 6 reveal that PKC $\epsilon$  migrates as a closely spaced doublet on SDS-PAGE. Our recent observations indicate that the higher molecular weight form is phosphorylated, whereas the smaller form is not phosphorylated (data not shown). The higher molecular weight form is lost in SLF treated with curcumin and in the lung tissue of mice in which fibrosis is induced by bleomycin treatment. Although the details remain to be worked out, it is likely that the loss of phosphorylated PKC $\epsilon$  plays a key role in the curcumin-induced apoptosis of SLF and in the progression of lung fibrosis *in vivo*.

NLF and SLF are not the only set of normal and pathologic cells in which curcumin causes apoptosis in the cells of pathologic origin, but not in the normal cells. Curcumin also can cause the apoptosis of transformed cell lines, but not of their normal counterparts (20). Normal cells are protected from apoptosis by

survival signals initiated by cell-substrate adhesion (anchorage). Given that PKC $\epsilon$  interacts with  $\beta$ 1 integrins and regulates cell adhesion and migration (33), it is possible that anchorage-induced survival signals involve PKC $\epsilon$ . It is also possible that curcumin promotes apoptosis in SLF and in transformed cells by inducing the expression of proapoptotic proteins. For example, curcumin has been shown to increase cellular levels of p53 in human basal cell carcinoma cells resulting in apoptosis (34).

HPLC chromatograms provided by the vendors indicate that 70–85% of the material in the commercial preparations used in this study is authentic curcumin and that the structurally related compounds demethoxycurcumin and bisdemethoxycurcumin make up almost all of the rest of the preparations. Only the final 1% of the preparation is unidentified. The functions of curcumin, dimethoxycurcumin, and bisdemethoxycurcumin were compared in one study (35). Curcumin and demethoxycurcumin showed similar activity in three assays, whereas bisdemethoxycurcumin was somewhat less active. These results suggest that the major components in commercial curcumin are similar to each other in both structure and function. Recently we obtained a > 99% pure preparation of authentic curcumin from Alexis Biochemicals (San Diego, CA). This preparation caused SLF to undergo apoptosis while having no effect on NLF. Thus, authentic curcumin does have the biological activity described in our experiments. Whether it is the most active component or the only active component in the preparations that we used remains to be determined.

Curcumin has been reported to protect rats against lung fibrosis induced by bleomycin, by cyclophosphamide, and by whole-body irradiation (7, 8, 36). Recently, we have reproduced this effect in mice treated with bleomycin (data not shown). How might our observations on the ability of curcumin to induce apoptosis in SLF be related to the ability of curcumin to block the progression of lung fibrosis in rats and mice? Prosurfactant C is a marker for type II pneumocytes, and we observe that prosurfactant C-positive type II pneumocytes are also PKC $\epsilon$ -positive in normal lung tissue. The fact that we observe an increased number of prosurfactant C-positive cells in the lungs of bleomycin-treated mice but that these cells have little PKC $\epsilon$  suggests that one step in the progression of lung fibrosis is the proliferation of type II pneumocytes that have lost most of their PKC $\epsilon$ . By this reasoning, we would also expect a major portion of SLF to be type II pneumocytes that have lost PKC $\epsilon$  and have proliferated. This idea is supported by the observation that surfactant proteins are present at high levels in the serum of patients with scleroderma (37). Given that we show here that SLF are killed by curcumin, it would not be surprising for a similar population of PKC $\epsilon$ -deficient, surfactant-expressing, proliferating cells to be killed by curcumin *in vivo*, thus blocking the progression of lung fibrosis.

Given our striking observations that SLF treated with curcumin contain much lower levels of both HO-1 and GST P1 than NLF treated with curcumin, we have recently examined HO-1 and GST P1 expression in bleomycin-treated and control mouse lung tissue. HO-1 was present at much higher levels in bleomycin-treated lung tissue and was localized to macrophages. In contrast, GST P1 was present at much lower levels in bleomycin-treated lung tissue. GST P1 was present in control lung tissue in cells lining bronchioles, vascular smooth muscle, and in multiple cell types in alveoli including type II pneumocytes. Although cells lining bronchioles and vascular smooth muscle remained GST P1-positive in bleomycin-treated lung tissue, there was very little GST P1 in condensed fibrotic tissue or even in morphologically normal looking alveoli. These observations are consistent with the possibility that proliferating cells in fibrotic lung tissue

*in vivo*, like SLF, are sensitive to curcumin because they are deficient in PKCε which in turn leads to a deficiency in GST P1.

Dr. Andrew Weil, a leader in the area of alternative medicine who does not publish in mainstream scientific journals, recommends “turmeric for all inflammatory disorders, including autoimmune conditions.” Our observations raise the possibility that Dr. Weil’s suggestions have some scientifically verifiable merit and provide a molecular mechanism through which turmeric/curcumin may cause abnormal cells to undergo apoptosis. Curcumin is not the only potential treatment for lung fibrosis to come from alternative medicine. The most abundant catechin of green tea, epigallocatechin-3-gallate, has been shown to inhibit the progression of lung fibrosis in a mouse model system, possibly due to its ability to directly inhibit the activity of several enzymes that degrade extracellular matrix proteins (38).

In conclusion, our results suggest that curcumin may be a useful treatment for scleroderma lung disease in particular and lung fibrosis in general. Curcumin is already being used in clinical trials as a treatment for cancer (39) and has recently been proposed as a potential treatment for Alzheimer’s Disease (40). Curcumin would be a particularly attractive treatment for all of these diseases because the use of turmeric in traditional Indian and Chinese herbal medicine strongly suggests that curcumin would not have significant, deleterious side effects.

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