Increased Localization and Substrate Activation of Protein Kinase C δ in Lung Epithelial Cells following Exposure to Asbestos

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The protein kinase C (PKC) family consists of several isozymes whose substrates may be necessary for the regulation of key cellular events important in the pathogenesis of proliferative diseases. Asbestos is a carcinogen and fibroproliferative agent in lung that may cause cell signaling events through activation of PKC. Here we used a murine inhalation model of asbestos-induced inflammation and fibrosis to examine immunoreactivity of PKC δ and its substrate, phosphorylated-adducin (p-adducin), in cells of the lung. Moreover, we characterized PKC δ and p-adducin expression in a pulmonary epithelial cell line (C10) in both log *versus* confluent cells and in cells after mechanical wounding or crocidolite asbestos exposure. Both PKC δ and p-adducin were almost exclusively expressed in bronchiolar and alveolar type II (ATII) epithelial cells in lung sections and increased in these cell types after inhalation of asbestos by mice. Increases in membrane and nuclear localization of PKC δ were seen in log phase as compared to confluent C10 cells. Moreover, enhanced immunoreactivity of PKC δ was observed in epithelial cells expressing proliferating cell nuclear antigen (PCNA) after mechanical wounding or exposure to asbestos fibers. These studies show that activated PKC δ in pulmonary epithelial cells is a consequence of inhalation of asbestos and may be linked to the activation of cell proliferation. (Am J Pathol 2002; 160:1991–2000)

Asbestos is a family of naturally occurring mineral fibers associated with the development of lung cancers, mesotheliomas, and fibroproliferative diseases, ie, pleural and diffuse interstitial fibrosis or asbestosis.^{1–3} The mechanisms of asbestos-related diseases are unclear, but inhalation models have revealed that bronchiolar and alveolar type II epithelial cells (ATII), progenitor cells of bronchiolar and peripheral carcinomas, undergo early injury and proliferation and are linked to the initiation of inflammation and fibroproliferation.⁴ At low exposures,

asbestos-associated lung repair has been compared to models of wound healing where early injury by fibers to epithelial cells leads to compensatory hyperplasia.

Our laboratory has shown in pulmonary epithelial and mesothelial cells *in vitro* that asbestos activates protein kinase C (PKC) and that down-regulation or inhibition of PKC prevents asbestos-induced proto-oncogene expression.^{5,6} We have also identified the mitogen-activated protein kinase (MAPK) cascade as a signaling pathway governing initial cell injury and proliferation by asbestos.^{7–9} Recently, we have developed a murine model of asbestosis where we demonstrated increased immunoreactivity of activated (phosphorylated), extracellular signal-regulated kinases (ERKs1/2) in bronchiolar and ATII cells at sites of asbestos fiber deposition and development of fibrotic lesions.¹⁰ These studies suggest that signaling pathways such as PKC and MAPK are initiated in epithelial cells that first contact asbestos fibers after inhalation. Moreover, initial signaling events may be integral to subsequent repair processes or the pathogenesis of asbestos-related pulmonary diseases.

Several studies show that PKCs modulate MAPK pathways in a variety of cell types.^{11–13} Members of the PKC family, ubiquitous Ser/Thr kinases, regulate a wide variety of normal and pathological processes. However, identifying the mechanisms of PKC signaling and their repercussions is complicated by the large numbers of PKC isoforms expressed in individual cell types, the lack of substrate specificity among the isoforms in *in vitro* assays, and the large numbers of proteins that can serve as substrates *in vitro*.¹⁴ A group of PKC substrates named STICKs for substrates that interact with C kinase has been cloned based on their ability to bind PKC. Antisera were raised against the individual phosphopeptides corresponding to phosphorylation sequences, affinity purified, and shown to preferentially react with phosphorylated compared to unphosphorylated peptides and proteins.^{15,16} Adducin is a STICK that facilitates the bind-

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ing of actin to spectrin to form the cortical membrane cytoskeleton, a primary location for transducing extracellular signals to the cytoplasm. Preliminary studies have revealed that the phosphorylation-state selective antibody, pSer660-adducin or p-adducin, is preferentially phosphorylated by PKC δ and localizes to focal adhesions actively involved in cytoskeletal remodeling after wounding of REF2 fibroblasts *in vitro*.^{14,17}

Little is known about the PKC isoforms and substrates expressed in lung and alterations in their expression after physiologically relevant injury to pulmonary epithelial cells. In research reported here, we focused on PKC δ and p-adducin because of their links to the development of injury, proliferation, and migration.^{18–21} We hypothesized that they would be expressed in bronchiolar and ATII epithelial cells after exposure to asbestos fibers. Moreover, we used a non-transformed murine alveolar epithelial cell line (C10)²² to determine whether increased expression of PKC δ and p-adducin was observed at sites of asbestos fiber deposition or after wounding. Results show for the first time that immunoreactive PKC δ and p-adducin are expressed in pulmonary epithelial cells exposed to asbestos and localized to cell membranes of proliferating and migrating cells after wounding or damage by asbestos fibers. These studies implicate activation of PKC δ as a signaling pathway important in wounding of epithelium by asbestos fibers and the development of subsequent fibrogenesis and carcinogenesis.

Materials and Methods

Inhalation Exposures

C57/BL6 mice (8 to 12 weeks of age) were exposed to ambient air or 7 mg/m³ air (6 hours/day; 5 days/wk) of the National Institute of Environmental Health Sciences (NIEHS) reference sample of crocidolite asbestos generated as described previously.²³ Groups of mice ($n = 6–8$ per group per time point) were euthanized after 4 and 30 days of exposure, time points corresponding to initial increases in epithelial proliferation and the development of fibrotic lesions respectively. Mice were administered a lethal dose of sodium pentobarbital (Abbott Laboratories, Chicago, IL) before chest cavities were opened, a polyurethane catheter was inserted into the trachea, and the lung was instilled with phosphate-buffered saline (PBS) at a pressure of 25 cm water. Left and right lung lobes were separated by suturing and processed for immunohistochemistry and immunofluorescence, respectively.

Immunohistochemistry

Reagents were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise specified. Left lung lobes from sham- and asbestos-exposed animals were placed in a tissue cassette overnight in 4% paraformaldehyde before embedding in paraffin blocks. Lung sections were cut at a thickness of 4 μ m and some sections were stained by the Masson's trichrome technique.²³ For immunohistochemistry, lung sections were deparaffinized

in xylene 3 $\times$ 5 minutes, rehydrated through graded ethanols, and equilibrated in PBS. Antigen was retrieved by boiling slides in 0.1 mol/L citrate buffer in distilled water (pH 6.0) three $\times$ 3 minutes. Endogenous peroxidase was dampened by treatment with 3% hydrogen peroxide in methanol for 20 minutes, followed by a 10-minute wash in distilled water. Sections were encircled with a hydrophobic film (PAP PEN, Electron Microscopy Sciences, Ft. Washington, PA), and nonspecific protein was blocked with 2% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 2 hours at room temperature (RT). Excess buffer was absorbed before overlaying with primary rabbit polyclonal antibodies (PKC δ , 1 μ g/ml; pSer660-adducin, 1 μ g/ml; clone 35H- γ -adducin, 1 μ g/ml²³) in PBS containing 2% normal goat serum overnight at 4°C in a humid chamber. Immunoreactivity was then detected using the anti-rabbit IgG Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) as a chromogen according to the manufacturer's protocols. Following color development, sections were rinsed in distilled water, counterstained with hematoxylin, dehydrated, cleared, and mounted with VectaMount (Vector Laboratories).

Preparation of Lung Sections for Immunofluorescence

After suturing, right lung lobes from sham- and asbestos-treated animals were placed in OCT embedding compound (Tissue Tek, Torrance, CA) and frozen at -80°C before cryostat sectioning at 7–8 μ m onto Superfrost $+/+$ slides. Slides were placed in fresh 4% paraformaldehyde in PBS for 30 minutes at RT. After washing in PBS, sections were permeabilized in -20°C methanol for 3 minutes at RT followed by two 10-minute washes in PBS. Nonspecific antibody activity was then blocked with 5% normal goat serum (Vectastain ABC Elite kit, Vector Laboratories) in PBS for 1 hour in a humid chamber at RT. Slides were then analyzed by immunofluorescence as described below.

Co-Localization of PKC δ with p-Adducin by Immunofluorescence

To simultaneously detect PKC δ and p-adducin, mouse anti-PKC δ antibody (Transduction Laboratories, Franklin Lakes, NJ; 3 μ g/ml) and rabbit anti-pSer660-adducin antibody (1 μ g/ml) were combined and applied to pre-blocked slides overnight at 4°C in a humid chamber. Detection of p-adducin was accomplished by incubating with rabbit biotinylated IgG (Vectastain ABC Elite kit, Vector Laboratories) for 1 hour at RT followed by incubation with streptavidin-Alexa 568 (Molecular Probes, Eugene, OR) at 1:200 dilution in 1% BSA/PBS for 1 hour at RT. Secondary antibody for PKC δ (allophycocyanin-goat-anti-mouse IgG, Molecular Probes), diluted 1:100 in 1% BSA/PBS, was applied to the slides together with the streptavidin-Alexa 568 antibody. After the incubations,

Figure 1. Increased expression of PKC δ and p-adducin in lungs treated with asbestos. PKC δ (A–C) and p-adducin (D–F) were detected in lung sections using immunoperoxidase staining of lung tissue sections. PKC δ : 30-day sham, A; 30-day asbestos exposure, B; 30-day asbestos exposure, C. p-adducin: 30-day sham, D; 30-day asbestos exposure, E; 30-day asbestos exposure, F. Note localization of PKC δ and p-adducin (arrows) in bronchiolar and alveolar epithelial cells and lesions (arrowheads) after 30 days of asbestos exposure; 30-day asbestos exposure (negative staining control using isotype control antibody), G; 30-day asbestos exposure (staining control omitting primary antibody), H. Original magnification, $\times 400$ (A, B, D, E, G, H); magnification, $\times 200$ (C and F).

slides were washed in PBS, mounted with AquaPoly-Mount (Polysciences, Inc, Warrington, PA) and stored at 4°C until future examination by confocal laser scanning microscopy (BioRad MRC1024ES, Hercules, CA).

Co-Localization of PKC δ with MAC-3 and CytoKeratin7 Antibodies by Immunofluorescence

Pre-blocked slides were incubated in blocking reagent from a mouse-to-mouse kit (Vector Laboratories) for 1 hour. Primary antibodies were mixed together and applied on sections overnight at 4°C in a humid chamber. Rabbit polyclonal PKC δ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 3 μ g/ml, MAC-3 antibody (Pharmigen, San Diego, CA) was used at 20 μ g/ml, and CytoKeratin7 antibody (Dako, Carpinteria, CA) was used at 1:250 dilution. PKC δ antibody was detected using biotinylated anti-rabbit IgG (Vectastain ABC Elite kit, Vector Laboratories) for 1 hour at RT followed by

streptavidin-Alexa 568 at 1:200 dilution in 1% BSA/PBS for 1 hour at RT. MAC-3 and CytoKeratin7 were detected using allophycocyanin-goat-anti-mouse IgG diluted 1:100 in 1% BSA/PBS for 1 hour at RT. After the incubations, slides were washed in PBS, mounted with AquaPolyMount and stored at 4°C until future examination by confocal laser scanning microscopy.

Cell Culture and Treatment Conditions

A contact-inhibited, non-transformed murine alveolar type II epithelial cell line (C10) was used for *in vitro* studies.²² C10 cells were propagated in CMRL 1066 medium supplemented with L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (GIBCO BRL, Rockville, MD). Cells were grown on glass coverslips for all of the experiments. To model epithelial wounding *in vitro*, cells were grown to confluence, and a wound was created by scraping a section of the glass coverslip with a rubber policeman. The medium was removed, and fresh

Figure 2. PKC δ and p-adducin co-localize within bronchiolar epithelial cells after asbestos exposure. Representative images illustrating co-localization of PKC δ (green) and p-adducin (red) using immunofluorescence in lung tissue sections. **A:** 4-day sham exposure. **B:** 4-day asbestos exposure. **C:** 4-day asbestos exposure (staining control omitting primary antibodies). **D:** 30-day sham exposure. **E:** 30-day asbestos exposure. **F:** 30-day asbestos exposure (staining control omitting primary antibodies). Original magnification, $\times 400$.

complete medium was added. Cells were then allowed to repopulate the wounded area for 18 hours. In additional experiments, log phase cells were examined at 24 hours after plating. For all other groups, cells were grown to confluence, complete medium was removed, and serum-free medium was added 24 hours before exposure to asbestos or phorbol dibutyrate (PDBu). Crocidolite asbestos fibers (NIEHS reference sample) were suspended in Hanks' balanced salt solution (HBSS) (1 mg/ml), triturated 10 times through a 22-gauge needle to obtain a homogeneous suspension, and added directly to the medium at a final concentration of 5 $\mu\text{g}/\text{cm}^2$ -area culture dish previously shown to induce apoptosis in C10 cells.⁸ PDBu (Sigma, St. Louis, MO) was added for 10 minutes at 37°C at a final concentration of 100 ng/ml. Control cultures received medium without agents and were treated identically. All experiments were performed in triplicate.

Localization of PKC δ and Adducins in C10 Cells by Immunofluorescence

After cell monolayers grown on glass coverslips were exposed to agents as described above, culture dishes were placed on ice, the medium was aspirated, and the cells were washed twice with PBS. Cells were fixed in

3.7% paraformaldehyde in PBS for 5 minutes at RT, then washed in PBS and permeabilized in -20°C acetone for 3 minutes at RT. Cells were washed in PBS, then incubated with a blocking solution containing 1% BSA/PBS for 1 hour at RT. After aspiration of blocking solution, primary antibody (rabbit polyclonal PKC δ antibody (Santa Cruz; 3 $\mu\text{g}/\text{ml}$) diluted in 1% BSA/PBS, was added and incubated for 1 hour at RT. Cells then were washed in PBS and secondary antibody (FITC goat-anti-rabbit IgG, Jackson ImmunoResearch Laboratories; 1:200) was applied. After washing in PBS, coverslips were mounted onto slides with AquaPolyMount. For each sample, confocal images were collected in fluorescence modes, followed by electronic merging of the images.

Detection of PKC δ in Subcellular Fractions of C10 Cells by Western Blot

After treatment, cells in 100-mm dishes (2 plates per condition) were rinsed sequentially with ice-cold PBS and hypotonic lysis buffer (HLB; 25 mmol/L Tris (pH 8), 2 mmol/L MgCl_2 , 5 mmol/L KCl, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride) before collection in 200 μl of HLB. Samples were kept at

Figure 3. PKC δ is predominantly expressed in epithelial cells and not in macrophages. Representative images illustrating co-localization of PKC δ antibody (red) with MAC-3 (**A, B**) or CytoKeratin7 (**C, D**) antibodies (green) using immunofluorescence in lung tissue sections from sham control (**A, C**) or 30-day crocidolite asbestos exposed animals (**B, D**). **Arrows** in **B** show MAC-3 staining macrophages with no PKC δ reactivity. **Arrows** in **D** show co-localization of PKC δ in bronchiolar epithelial cells stained with Cytokeratin7. Original magnification, $\times 400$.

4°C for the remainder of the procedure. Cells were lysed by homogenization then centrifuged at $1000 \times g$ for 5 minutes to collect the nuclear pellet fraction. The supernatant fraction was reserved, and the nuclear pellet fraction was washed with HLB then resuspended in 100 μ l of HLB. To separate membrane from cytosol, the supernatant fraction was centrifuged at $15,000 \times g$ for 30 minutes. The supernatant served as the cytosol fraction. The resulting membrane pellet fraction was washed with HLB then resuspended in 100 μ l of HLB. All fractions were triturated through a 23-gauge needle, and equal proportions were combined with Laemmli sample buffer, boiled, and electrophoresed through a 7.5% SDS polyacrylamide gel. Separated proteins were electroblotted onto nitrocellulose and Western blotting was performed as previously described⁸ using anti-PKC δ primary antibody (Santa Cruz; 1:100 dilution) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (Vector Laboratories; 1:5000 dilution). Antibody binding was visualized by enhanced chemiluminescence according to the manufacturer's protocol (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Detection of Proliferating Cell Nuclear Antigen by Immunofluorescence

An antibody against proliferating cell nuclear antigen (PCNA) was used to identify cells undergoing DNA synthesis. Cell monolayers on coverslips were fixed in 100% methanol for 1 hour on ice, washed in PBS, and incubated in 0.1% Tween-20 in PBS for 30 minutes at RT.

After incubation in blocking solution (2% dry milk, 0.1% Tween-20 in PBS) for 30 minutes at RT, cells were incubated with a mix of mouse anti-PCNA (Pharmagen; 1:1000) and rabbit anti-PKC δ (Santa Cruz; 3 μ g/ml) for 1 hour at RT. Cells were washed twice for 20 minutes in blocking solution, and once for 10 minutes in PBS. PCNA was detected using FITC goat-anti-mouse IgG, diluted 1:200 in 10 μ g/ml BSA/PBS. PKC δ was detected using allophycocyanin-goat-anti-rabbit IgG, diluted 1:200 in 10 μ g/ml BSA/PBS. After washing in PBS, slides were mounted using AquaPolyMount, and examined using confocal scanning laser microscopy as described above.

Results

Asbestos Inhalation Evokes an Increase in PKC δ and p-Adducin Immunoreactivity in Mouse Lung

Using a mouse inhalation model of asbestosis, the expression and histological localization of PKC δ was first determined in sham and asbestos-exposed mice by immunoperoxidase staining of lung sections. In addition, as a measure of endogenous PKC δ activity, sections were also stained with an antibody specific for p-adducin (the phosphorylated form of the PKC substrates, α - and γ -adducin).¹⁷ In comparison to sham controls, asbestos exposure resulted in an empirical increase in both PKC δ and p-adducin immunoreactivity in lung sections of exposed animals (Figure 1). Expression of PKC δ and p-adducin was evident in both the bronchiolar epithelial cells and in cells lining alveolar regions. Immunoreactivity of both PKC δ and p-adducin was particularly marked in areas with peribronchiolar lesions. In addition, p-adducin was also observed in the basal lamina of blood vessels (Figure 1, D–F) as described previously in renal tubules.²⁴

To more quantitatively examine the extent of PKC δ expression and activity after asbestos exposure, lung sections from mice exposed to asbestos for 4 days and 30 days were examined by immunofluorescence. As shown in Figure 2, increases in PKC δ and p-adducin immunoreactivity were apparent by 4 days (Figure 2B) and extensive after 30 days of exposure to asbestos (Figure 2E). In addition, the immunoreactivity for PKC δ and p-adducin exhibited marked overlap, suggesting that PKC δ and p-adducin co-localize within airway cells.

Induction of PKC δ Expression by Asbestos is Specific to Epithelial Cells

To establish whether PKC δ expression was specific to bronchiolar and alveolar epithelial cells versus macrophages that infiltrate after asbestos exposure,⁴ lung sections were co-stained with antibodies recognizing macrophages (MAC-3) or the epithelial cell marker, keratin7 (CytoKeratin7). Whereas there was little overlap in staining between PKC δ antibodies and MAC-3 (Figure 3, A and B), the overlap between PKC δ antibodies and CytoKeratin7 was extensive (Figure 3, C and D). Thus, the

Figure 4. PKC δ and p-adducin are localized to the membrane and nuclei of dividing cells or confluent cells treated with PDBu. C10 cells were examined at low density (Log Phase), confluent density (Confluent) and after treatment with 100 nmol/L PDBu for 10 minutes (PDBu). **A–I:** Localization of proteins by immunofluorescence using antibodies to PKC δ (**A–C**); γ -adducin (**D–F**); and p-adducin (**G–I**). Original magnification, $\times 600$. **J:** Localization of PKC δ in subcellular fractions of C10 cells by Western blot. Tot, total extract; M, membrane fraction; C, cytosolic fraction; N, nuclear fraction.

observed increase in PKC δ expression after asbestos exposure likely originates from airway and alveolar epithelial cells.

PKC δ is Highly Expressed in Membranes and Nuclei of Wounded and/or Dividing C10 Alveolar Epithelial Cells

To further explore the etiology of the observed increase in PKC δ seen in lung epithelial cells after asbestos exposure, we used cultured alveolar type II epithelial cells (C10 cells), as a model to measure responses specific to

epithelial cells. To determine the effect of cell growth state on the localization and activity of PKC δ , immunofluorescence was used to detect PKC δ , p-adducin, and γ -adducin (unphosphorylated form) in log phase *versus* confluent cultures. As shown in Figure 4A–I, when compared with confluent cells, log phase cells exhibited PKC δ and p-adducin staining that was more localized to the cell membrane and both perinuclear and intranuclear regions. In addition, treatment of confluent cultures with the PKC activator, PDBu, resulted in a similar pattern of localization to the membrane and nucleus. The observed translocation of PKC δ to the nuclear and membrane frac-

Figure 5. PKC δ and p-adducin are localized to the membrane and nuclei of cells migrating into a wound. Confluent C10 monolayers were wounded by a rubber policeman then allowed to respond for 24 hours. Cells were then fixed and immunostained as in Figure 4 using antibodies recognizing PKC δ (A), p-adducin (B), and γ -adducin (C). D: Staining control omitting primary antibody. Original magnification, $\times 400$.

tion of log phase cells and after PDBu treatment was confirmed using Western blot analysis (Figure 4J). No changes in unphosphorylated γ -adducin were observed with addition of PDBu suggesting that the increase in p-adducin is due to an increase in the activity of PKC, not an increase in γ -adducin levels (Figure 4, G–I). These data indicate that PKC δ is activated and translocated to the membranes of proliferating cells and cells stimulated with phorbol ester.

Epithelial cell injury evoked by asbestos has been linked to wounding of epithelial cells by asbestos fibers and compensatory proliferative responses.²⁵ To determine the effects of wounding on the localization and activity of PKC δ , confluent cultures of C10 cells were wounded mechanically, allowed to recover for 18 hours, and then examined for PKC δ and p-adducin immunoreactivity. A striking accumulation of PKC δ and p-adducin was observed in the plasma membrane and nuclei of cells migrating into the wounded area (Figure 5). PKC δ immunoreactivity was also detected along intracellular fibrils extending toward the wound, possibly due to association with actin fiber projections.

Because PKC δ activity may have a role in both migration and proliferation, PKC δ localization was correlated with the proliferative status of alveolar epithelial cells. As shown in Figure 6, A–C, confluent C10 cells show diffuse PKC δ staining and no PCNA incorporation. In contrast, cells after wounding have increased PCNA staining as well as more membrane and nuclear localization of PKC δ , suggesting that PKC δ is greater in cells that are dividing (Figure 6, D–F).

Asbestos Stimulates an Increase in Both Protein Level and Membrane Localization of PKC δ and P-Adducin in C10 Alveolar Epithelial Cells

To test whether asbestos fibers stimulate patterns of membrane and nuclear localization of PKC δ similar to that observed after wounding, C10 cells were exposed to asbestos followed by immunofluorescence staining using antibodies to PKC δ and p-adducin. Asbestos fibers were detected by reflectance confocal microscopy. As shown in Figure 6, G–I, cells with direct exposure to asbestos fibers exhibited an increase in membrane and nuclear localization of PKC δ that was evident in PCNA-positive cells. Figure 7 illustrates that exposure to asbestos increased the levels of both PKC δ and p-adducin, especially in areas of direct contact of epithelial cells with the asbestos fibers. These results suggest that, like wounding, asbestos induces localization and activation of PKC δ to the membranes and nuclei of dividing lung epithelial cells.

Discussion

Asbestos is classified as a human class I carcinogen by the International Agency for Research on Cancer (IARC) and is well-recognized as a potent fibrogenic agent in occupationally exposed individuals.^{1–4} In lung cancers, which are strikingly increased in asbestos workers who smoke, asbestos fibers may act as co-carcinogens that adsorb polycyclic aromatic hydrocarbons (PAH) to their surfaces, thus increasing delivery, metabolism, and retention of chemical carcinogens in the lung.³ In addition, asbestos fibers are tumor promoters when applied to tracheal grafts after initiation of tumor development using PAH.²⁶ A feature of tumor promoters such as PDBu, 12-O-tetradecanoyl-13-phosphate (TPA), or asbestos is their ability to stimulate PKC activity. Phorbol esters such as TPA or PDBu resemble diacylglycerol in structure, thus activate PKC directly. In contrast, asbestos is an insoluble fiber that increases hydrolysis of inositol phospholipids and amounts of diacylglycerol in respiratory epithelial cells *in vitro*.²⁷ These signaling events may be linked to asbestos-induced stimulation of MAPK pathways, activation of *fos/jun* early response genes, and outcomes such as injury and/or proliferation of epithelial cells. For example, after down-modulation of PKC or use of PKC inhibitors, asbestos-induced *c-jun* and *c-fos* mRNA levels are diminished in rat pleural mesothelial cells.⁶

PKC constitutes a large family of kinases that regulates a wide variety of both normal and pathological processes. Thus, dissecting signaling pathways of distinct PKC isoforms is necessary to assess their importance in pathologies such as the fibroproliferative response to asbestos. Our findings presented here demonstrate for the first time that *in vivo* asbestos-mediated responses in lung epithelial cells include an increase in the level of PKC δ expressed specifically in bronchiolar and alveolar epithelial cells. Overexpression of dominant negative PKC δ or inhibition of PKC δ inhibits growth of mammary tumor cells in soft agar¹⁸ and prevents expression of proto-oncogenes induced by ionizing radiation in thyroid

Figure 6. Cells exhibiting increased translocation of PKC δ after wounding or asbestos exposure also exhibit an increase in PCNA. C10 cells were wounded as in Figure 5 (D–F) or exposed to asbestos ($5 \mu\text{g}/\text{cm}^2$ -area dish) for 24 hours (G–I). Cells were then co-stained by immunofluorescence using antibodies recognizing PKC δ (green) and PCNA (magenta). Asbestos fibers are shown in red. C and F represent overlay image of PKC δ and PCNA for wounded and asbestos exposed cells respectively. Original magnification, $\times 400$.

Figure 7. Increased localization of PKC δ and p-adducin in focal regions of asbestos fiber deposition. Control C10 cells (A) and cells exposed to $5 \mu\text{g}/\text{cm}^2$ area dish of asbestos for 24 hours (B, C) were examined by immunofluorescence (green) for the localization of PKC δ (A, B) and p-adducin (C). Asbestos fibers are shown in red. Original magnification, $\times 400$.

cells.²⁸ Thus, these and our results support a possible role for PKC δ in the proliferative responses of lung epithelial cells following exposure to asbestos.

The changes in PKC δ correlate with an increase in both localization and phosphorylation of adducins, a PKC δ substrate family.²¹ Adducins are central to regulation of the subcortical membrane and are necessary for cell polarity and differentiated function.²⁹ Phosphorylation of adducins by PKC δ has been associated with an increase in growth potential and dedifferentiation of tumor cells.²⁴ Taken together with the findings of others, our results support the hypothesis that PKC δ and adducin are important players in the asbestos-induced responses of lung epithelial cells.

Our *in vitro* findings show that PKC δ translocates to both the cytoplasmic and nuclear membrane of C10 lung epithelial cells after wounding or activation by PDBu. This pattern also appears in cells exhibiting focal contact with asbestos fibers. Translocation to the nucleus has been described for PKC α ^{30,31} and PKC λ ³² with functional effects on signal transduction through calcium³³ and MAPK,³⁴ respectively. Association of PKC δ with the nucleus leads to the possibility that PKC δ may also have a role in regulation of nuclear events as well as plasma membrane events. The positive correlation between PKC δ activation and expression of PCNA suggests a link between PKC δ and proliferation in cells responding to asbestos. Because, in some systems, PKC δ has opposing effects on growth compared with PKC α ,³⁵ and both isoforms translocate to the nuclear envelope after phorbol ester treatment in fibroblasts,³⁶ the two isoforms likely balance the regulation of growth promotion depending on the cellular environment.

Together our findings represent the first demonstration that PKC δ activity increases in epithelial cells of intact lung during the progression of asbestos-induced lung fibrosis. The increase in PKC δ protein level in lungs exposed to asbestos also suggests that levels of PKC δ are inducible. Thus, the response of lung epithelial cells to asbestos may include both direct activation of PKC δ and either induction of transcription or increased stabilization of PKC δ . The correlation of these findings with our localization studies, using a culture model of epithelial cell responses, leads us to the proposal that PKC δ activation and membrane translocation has an integral role in the response of epithelial cells to asbestos. Furthermore, we hypothesize that PKC δ is necessary for the migratory and proliferative responses observed in lung epithelial cells following exposure to asbestos. Further analysis of PKC isoforms activated by asbestos in lung and their functional role in cell responses will reveal specific contributions of the diverse PKC signaling pathways that lead to fibroproliferative lung diseases.

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