Decreased lung tumorigenesis in mice genetically deficient in cytosolic phospholipase \(A_2\)

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Epidemiological investigations suggest that chronic lung inflammation increases lung cancer risk. Pharmacologic and genetic evidence in mouse models indicates that lipid mediators released during pulmonary inflammation enhance lung tumor formation. Cytosolic phospholipase \(A_2\) (cPLA\(_2\)) catalyzes arachidonic acid (AA) release from membrane phospholipids. AA can then lead to the synthesis of several classes of lipid mediators, including prostaglandin (PG) biosynthesis through the cyclooxygenase (COX) pathway. We investigated a role for cPLA\(_2\) in mouse lung tumorigenesis by using mice genetically deficient in cPLA\(_2\). After multiple urethane injections into cPLA\(_2\) null mice and wild-type littermates, the number of lung tumors was determined. cPLA\(_2\) null mice developed 43% fewer tumors (from 16 ± 2 to 9 ± 2 tumors/mouse; \(P < 0.05\)) than wild-type littermates. cPLA\(_2\), COX-1, COX-2 and mPGES, and microsomal prostaglandin \(E_2\) synthase (mPGES), examined by immunohistochemistry, are present in alveolar and bronchiolar epithelia and in alveolar macrophages in lungs from naive mice and tumor-bearing mice. Tumors express higher levels of each of these four enzymes than control lungs, as determined by immunoblotting. No differences were detected in the contents of COX-1, COX-2 and mPGES between wild-type and cPLA\(_2\) null mice. Although the steady-state levels of prostaglandin \(E_2\) and prostaglandin \(I_2\) in lung tissue extracts prepared from wild-type or cPLA\(_2\) (−/−) mice were not significantly different, both prostaglandins markedly increased in tumors from wild-type mice, an increase that was significantly blunted in tumors from cPLA\(_2\) (−/−) mice. These results demonstrate a role for cPLA\(_2\) in mouse lung tumorigenesis that may be mediated by decreased prostaglandin synthesis.

Abbreviations: AA, arachidonic acid; B6, C57BL/6J mice; COX, cyclooxygenase; cPLA\(_2\), cytosolic phospholipase \(A_2\); IH, immunohistochemistry; 6-keto PGF\(_{1\alpha}\), 6-keto prostaglandin F\(_{1\alpha}\); mPGES, microsomal PGE\(_2\) synthase; PAF, platelet-activating factor; PGE\(_2\), prostaglandin \(E_2\); PG\(_{I_2}\), prostaglandin \(I_2\).

Introduction

The 5-year survival rate following diagnosis of advanced lung cancer is 15% (1), a grim statistic emphasizing the need for chemoprevention and early diagnosis. Mice develop lung tumors with molecular and histogenetic features similar to the most frequently diagnosed lung cancer subtype, adenocarcinoma (2), and are a useful model for elucidating molecular changes important to lung tumorigenesis (3). Recent genetic, pharmacologic and biochemical investigations in mice have provided evidence that inflammatory processes involving eicosanoids regulate lung tumorigenesis (4–10). Arachidonic acid (AA), the substrate used by cyclooxygenase (COX), lipoxigenase (LO) and cytochrome P450 to generate eicosanoids (Figure 1), is hydrolyzed from membrane phospholipids by cytosolic phospholipase A\(_2\) (cPLA\(_2\)). Of the multiple forms of PLA\(_2\), cPLA\(_2\) has been shown to be the major enzyme responsible for AA release and represents the rate-limiting step in eicosanoid production (11). Constitutively expressed COX-1 and inducible COX-2 each catalyze formation of prostaglandin \(H_2\), the precursor for production of prostaglandin \(E_2\) (PGE\(_2\)) and prostaglandin \(I_2\) (PGI\(_2\)) by their respective synthases, PGES and prostaglandin \(I_2\) synthase (PGIS) (12). Both prostaglandins bind to specific families of G-protein coupled receptors to exert their biological activities, and PGI\(_2\) may also activate PPAR nuclear receptors (13). Lysophospholipid formed upon scission of esterified AA from the sn-2 position of phospholipids is the precursor for platelet-activating factor (PAF) biosynthesis, a potent inflammatory mediator. Both AA and lysophospholipid exert physiological effects by themselves (14,15). Thus, numerous inflammatory mediators are produced as a consequence of cPLA\(_2\) activity (15).

A role for cPLA\(_2\) in driving tumorigenesis is rarely examined, due in part to a paucity of useful in vivo pharmacological cPLA\(_2\) antagonists. Derivation of the cPLA\(_2\) knockout mouse allows experimental assessment of the involvement of cPLA\(_2\) in pathologic processes (16,17). We herein demonstrate that genetic ablation of cPLA\(_2\) inhibits chemically induced lung tumorigenesis. PGE\(_2\) and PGI\(_2\) levels in normal and neoplastic pulmonary tissues and the cellular localization of cPLA\(_2\), COX-1, COX-2 and microsomal PGE\(_2\) synthase (mPGES) in tumor-bearing mouse lungs were examined to help clarify the basis of this requirement for cPLA\(_2\).

Materials and methods

Mice

cPLA\(_2\) null mice in a C57BL/6J-129/Sv (B6-129/Sv) chimeric background and their wild-type littermates were bred in the animal facilities at Harvard University, Denver VA Medical Center, and the University of Colorado Health Sciences Center. Since offspring produced by homozygous knockouts are non-viable (18), breeding was conducted with heterozygotes. Mice were fed Harlan Teklad 22/5 rodent chow (Harlan, Madison, WI), given water \textit{ad libitum}, and housed on hardwood bedding with a 12-h light/12-h dark cycle.
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**Fig. 1.** cPLA₂ as the starting point for generating numerous lipid mediators.

in a climate-controlled facility. Carcinogenesis was performed using 10-15-week-old mice of both genders.

**Kras allele determination**

Certain inbred strains of mice have a Kras allele that renders them sensitive to chemically induced lung tumorigenesis (e.g. A/J, 129/Sv), while others have a resistant allele and develop very few or no lung tumors (e.g. B6) (19). Mice with one copy of a 37 bp sequence in Kras intron 2 are sensitive, while strains with two copies arranged in tandem are resistant (20). Since the cPLA₂ knock-out mouse was generated as a B6/129 chimera, the Kras genotype was determined; mice bearing the resistant Kras allele were removed from experimental analysis so that only the contribution of the presence or absence of functional cPLA₂ was assessed. Genomic DNA was isolated and PCR reactions using primers for examining the Kras intron 2 polymorphism were performed as described (20).

**Lung carcinogenesis**

Because the B6/129 genetic background of the cPLA₂ --/- mice consists of resistant B6 alleles and 129/SvJ alleles that convey intermediate sensitivity (21), multiple injections of urethane were used to induce lung tumors in cPLA₂ --/- and +/- littermates; this carcinogenesis procedure induces several tumors in B6/129 chimeras (22). Mice were injected i.p. once per week for 6 weeks with 1 mg urethane (Sigma, St Louis, MO) dissolved in 0.9% NaCl/g body wt, weighed weekly, and observed daily for the duration of the study to assess their health. Nineteen weeks after the initial carcinogen injection, tumors were enumerated using fresh tissue examined under a dissecting microscope, and tumor diameters measured with digital calipers. The genetic identity of the mice (null or wild-type for cPLA₂) was not revealed until after tumor multiplicities and sizes were determined. Mice were also subjected to this carcinogenesis regimen to obtain tissue samples for immunohistochemistry and immunoblotting.

**Immunohistochemistry (IH)**

Lung tissue sections were prepared for IH as described (7). Briefly, the lungs of urethane-treated mice were perfused through the pulmonary artery with saline and fixed by inflation with 10% formalin. Lungs were dehydrated, embedded in paraffin, and cut into 4 μm sections that were rehydrated. Endogenous endoperoxide activity was inhibited by incubation with 3% H₂O₂ in methanol for 15 min, followed by antigen retrieval using warm 100 mM citrate buffer, pH 6.0. In these experiments, a mouse monoclonal cPLA₂ antibody (Santa Cruz, Santa Cruz, CA) was used at a 1:50 dilution for immunostaining after blocking endogenous mouse immunoglobulins using the Mouse-On-Mouse kit (Vector Laboratories, Burlingame, CA); goat polyclonal COX-1 and COX-2 antibodies (Santa Cruz) were used at a 1:2000 dilution for each (7). A goat polyclonal mPGES antibody (Cayman Chemical, Ann Arbor, MI) was used at a 1:500 dilution. Samples were treated with biotin-conjugated anti-mouse IgG or anti-goat IgG secondary antibody (Vector) followed by peroxidase-conjugated streptavidin tertiary antibody complex (Vector). 3,3-Diaminobenzidine (Sigma) was used as the peroxidase substrate for cPLA₂, COX-1, COX-2 or mPGES detection and hematoxylin (Sigma) was the counterstain. As a negative control for cPLA₂ staining, a tumor-bearing tissue sample from a cPLA₂ null mouse was immunostained for cPLA₂. As a negative control for mPGES staining, mPGES primary antibody was treated with a blocking peptide and then incubated with slides to demonstrate antibody specificity.

**Immunoblotting**

Lung tissue from untreated age-matched controls and tumor samples from urethane-treated B6/129 mice were homogenized in 20 mM HEPES, pH 7.5 buffer containing 10% glycerol and protease inhibitors (2 mM EDTA, 2 mM EGTA, 5 μg/ml aprotinin and 10 μM leupeptin) (7). Soluble and particulate fractions were separated by centrifugation at 16 000 g for 30 min. Particulate proteins were re-suspended in homogenization buffer with 0.1% NP-40 for immunoblotting COX-1, COX-2 and mPGES; cPLA₂ content was examined in soluble fractions. After protein concentrations were determined by the method of Lowry et al. (23), 50 μg of protein/lane applied to 10% cPLA₂, COX-1 and COX-2 or 15% (mPGES) SDS-PAGE. Separated proteins were transferred onto Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA). Primary antibodies used for immunoblotting were the same as those used for IH. For cPLA₂ detection, membranes were blocked for 30 min in 15 mM Tris, pH 7.4 buffer containing 150 mM NaCl, 3% powdered milk and 0.1% Tween-20 (Sigma), incubated with primary antibody for 1 h at a 1:300 dilution in blocking solution, and incubated for 1 h with goat anti-mouse alkaline phosphatase secondary antibody (Bio-Rad, Hercules, CA; 1:3000 dilution). Membranes were incubated with Immuno-Star chemiluminescent substrate (Bio-Rad) and exposed to CL-XPosure film (Pierce, Rockford, IL). cPLA₂ bands at 110 kDa were quantified using UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT). COX-1 and COX-2 protein immunoblotting was performed as described previously (7). Membranes were incubated with COX-1 or COX-2 primary antibody (1:800 dilution for each) or mPGES primary antibody (1:500 dilution), washed and incubated with horseradish peroxidase labeled donkey anti-goat secondary antibody (1:20 000 dilution). After treatment with ECL-reagent (Pierce), membranes were exposed to X-ray film and the resulting 72 (COX) or 14 kDa bands (mPGES) quantified as above. To confirm even protein loading of the gels, the membranes were stained with 0.1% Ponceau S (Fisher Biotech, Pittsburgh, PA) in 5% acetic acid. PGE₂ and PGI₂ estimation by enzyme immunoassay (EIA)
PGE₂ and PGI₂ are unstable (t₁/₂ = 3 min) and hydrolyzed non-enzymatically to the more stable 6-keto prostaglandin F₁α (6-keto PGF₁α) metabolite (12). PGE₂ and 6-keto PGF₁α EIA assays were performed according to manufacturer instructions (Cayman Chemical, Ann Arbor, MI). Mice were killed by lethal phenobarbital i.p. injection 19 weeks after carcinogen treatment, the lungs excised, and tumor and uninvolved lung tissue separated by dissection. Tumors dissected from a single mouse were pooled and snap frozen. Lung tissue from age-matched, untreated mice (control) were also collected. Samples were homogenized in Earl’s salt solution and an aliquot was removed for protein concentration measurement (Bio-Rad). Lipids were extracted from the remaining samples in 4 vol (w/v) methanol. Samples were centrifuged at 16 000 g for 10 min to remove tissue debris, and the supernatants used...
circumstances, the percentage of mice that had any tumors, was 100% for from two independent experiments. Tumor incidence, i.e. allele were removed from the study. Absence of cPLA2 previously (16). Since null mice did not lose body weight or have their rate of weight gain compromised, the reduced tumor multiplicity in cPLA2 (ANOVA) was used to compare more than two groups and post-hoc Newman identified using the Student’s unpaired protein.

for EIA. Calculations of prostaglandin amounts were based on measurements taken in the linear portion of the standard curve, and expressed as pg/mg

Statistical analysis
Data are presented as means ± SEM. Differences between groups were identified using the Student’s unpaired r-test. One-way analysis of variance (ANOVA) was used to compare more than two groups and post-hoc Newman-Keuls tests were used to identify differences between groups. Under all circumstances, P < 0.05 was considered to be significant.

Results
Decreased lung tumorigenesis in cPLA2 null mice
Nineteen weeks following the first of several weekly urethane injections, wild-type littermates developed 16 ± 2 tumors while cPLA2 null mice developed 9 ± 2 tumors/mouse (Figure 2), a 43% reduction (P < 0.05). These average tumor multiplicities were calculated by combining results from two independent experiments. Tumor incidence, i.e. the percentage of mice that had any tumors, was 100% for both groups of animals. Two mice homozygous for the resistant Kras allele were removed from the study. Absence of cPLA2 did not affect morbidity or mortality of mice undergoing the carcinogenesis protocol; the good health of cPLA2 null mutants not treated with carcinogens has been observed previously (16). Since null mice did not lose body weight or have their rate of weight gain compromised, the reduced tumor multiplicity in cPLA2 −/− mice was not due to caloric restriction, a condition that inhibits lung tumor formation (24). Tumor sizes were similar between groups (data not shown).

Immunohistochemical analysis of cPLA2, COX-1, COX-2 and mPGES
To determine whether tumorigenesis or the absence of cPLA2 affected immunolocalization of relevant enzymes, lungs from normal and urethane-induced, tumor-bearing B6/129 mice were examined. In lungs from untreated animals, cPLA2 was localized to bronchiolar and alveolar epithelial cells and to alveolar macrophages; staining was mainly cytoplasmic (Figure 3A). Adenomas arise from bronchiolar non-ciliated Clara cells and from alveolar Type II cells (2). In tumor-bearing animals, cPLA2 was present in epithelial cells adjacent to the tumors and throughout the tumor parenchyma. Parenchymal staining was faint while staining was more intense in tumor-associated macrophages (Figure 3B). The inset in Figure 3(B) demonstrates the lack of cPLA2 immunostaining in a tumor from a cPLA2 null mouse. Similar to cPLA2, mPGES was found in bronchiolar and alveolar epithelial cells and alveolar macrophages in normal lungs (Figure 3C); mPGES was also expressed in smooth muscle cells (data not shown). In tumor-bearing mice, mPGES localized to epithelial cells in adjacent normal tissue, within the tumor parenchyma, and in tumor-associated macrophages (Figure 3D). Pre-incubation of the primary antibody with a blocking peptide demonstrated antibody specificity (inset, Figure 3D). As has been observed previously in A/J (8), BALB (7) and other B6/129 chimeric mice (22), both COX-1 (Figure 3E) and COX-2 (Figure 3F) intensely immunostained within the tumor epithelia, as well as in surrounding uninvolved tissue and macrophages.

PGE2 and 6-keto PGF1α concentrations in lung tissue and tumors from cPLA2 null mice
To determine if decreased prostaglandin production due to diminished AA availability may be at least partly responsible for the decreased number of tumors induced in cPLA2 null mice, prostaglandin levels were measured in lungs fromagematched untreated mice (control) and tumors isolated from both wild-type and cPLA2 null mice (Figure 5). Tumors induced in wild-type mice contained more of both PGE2 and 6-keto PGF1α than control lung tissue, as normalized on a per milligram protein basis. In lung tumors isolated from cPLA2 null mice, these increases were markedly blunted. No significant differences in prostaglandin levels were observed between lungs from untreated wild-type or cPLA2 null mice.

Discussion
Individuals with chronic lung inflammatory diseases such as asthma and chronic bronchitis have increased lung cancer risk (26,27). Anti-inflammatory drugs, such as non-steroidal
Fig. 3. cPLA₂, COX-1, COX-2 and mPGES IH in lungs from untreated and urethane-treated B6/129 mice. (A) cPLA₂ and (C) mPGES immunostained in bronchiolar (B) and alveolar (A) epithelial cells as well as alveolar macrophages (M) in untreated mice. (B) cPLA₂ faintly stained in tumor parenchyma (T), with more pronounced staining in surrounding macrophages (M). Tumors from cPLA₂ null mice show no cPLA₂ staining (inset, B). (D) mPGES was present in tumor parenchyma (T) and tumor-associated macrophages (M). A blocking peptide showed specificity of the mPGES antibody (inset, D). (E) COX-1 and (F) COX-2 immunostained the tumor epithelium (T) and surrounding macrophages (M). A total of five tumors were examined from three mice with similar results. Black bar in (A) represents 50 μm.
anti-inflammatory drug (NSAIDs) that inhibit COX-1 and COX-2 activities (4,8) and lipoxygenase inhibitors that inhibit leukotriene formation (5,6), reduce the multiplicity of lung tumors in mice induced with chemical carcinogens. Transgenic mice that specifically over-express PGIS targeted to the cell types from which tumors arise develop fewer lung tumors in response to carcinogens than wild-type mice (9). A clinical trial that utilizes an inhibitor of COX-2, in addition to other chemotherapies, has shown promising preliminary results in treating early-stage lung cancer (28). The eicosanoid pathway produces potent inflammatory mediators, but pharmacological inhibitors of cPLA2 applicable for clinical studies have not been developed. Genetic ablation of cPLA2 inhibits mouse lung tumorigenesis (Figure 2), providing the first evidence that decreasing cPLA2 function protects against lung cancer.

Cultured cells derived from cPLA2-deficient animals produce very low levels of PGs compared with wild-type cells. Peritoneal macrophages isolated from cPLA2 null mice that were stimulated ex vivo with calcium ionophore or LPS produced markedly less PGE2 than cells isolated from wild-type mice (16,17,29). Steady-state tissue levels of prostaglandins, however, are not altered in these animals (Figure 5). The intracellular concentrations of COX-1, COX-2 and mPGES were also unchanged in the knockout mouse (Figure 4). These data suggest that cPLA2 is not activated in these tissues in the absence of external stimuli; prostaglandins are apparently produced basally by other forms of PLA2, such as secretory sPLA2 or calcium-independent iPLA2. In particular, iPLA2 has been implicated in phospholipid remodeling (30) and is likely to be constitutively active in those tissues in

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**Fig. 4.** cPLA2, COX-1, COX-2 and mPGES increase in tumors. Lung tissue and tumors were harvested from age-matched controls and tumor-bearing urethane-treated mice. Samples were prepared from each of three control mice and three tumor-bearing mice. (A) cPLA2, COX-1, COX-2 and mPGES in control (CON) and tumor (TUM) from cPLA2 +/+ and −/− mice. (B-E) Densitometric quantitation of protein levels from immunoblots. (*P < 0.05 versus control.)
The protective effect of cPLA2 gene ablation is mediated at least in part through decreased prostaglandin production (44), the decreased levels of lysophospholipid associated with loss of cPLA2 should decrease PAF levels.

COX inhibitors may offer more robust chemopreventive and chemotherapeutic strategies. Combination therapies of cPLA2 and COX inhibitors may additionally act on targets distinct from those inhibited by NSAIDs. Combination therapies of cPLA2 and COX inhibitors may offer more robust chemopreventive and chemotherapeutic strategies.

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