Cancer Stem Cell Phenotype Is Supported by Secretory Phospholipase A_2 in Human Lung Cancer Cells

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Background. Lung cancer stem cells (CSCs) are a subpopulation of cells that drive growth, invasiveness, and resistance to therapy. Inflammatory eicosanoids are critical to maintain this malignant subpopulation. Secretory phospholipase A_2 group IIa ($sPLA_2$) is an important mediator of the growth and invasive potential of human lung cancer cells and regulates eicosanoid production. We hypothesized that $sPLA_2$ plays a role in the maintenance of lung CSCs.

Methods. Cancer stem cells from lung adenocarcinoma cell lines H125 and A549 were isolated using aldehyde dehydrogenase activity and flow cytometry. Protein and mRNA levels for $sPLA_2$ were compared between sorted cells using Western blotting and quantitative reverse transcriptase–polymerase chain reaction techniques. Chemical inhibition of $sPLA_2$ and short-hairpin RNA knockdown of $sPLA_2$ were used to evaluate effects on tumorsphere formation.

Results. Lung CSCs were isolated in 8.9% ± 4.1% (mean ± SD) and 4.1% ± 1.6% of H125 and A549 cells respectively. Both $sPLA_2$ protein and mRNA expression were significantly elevated in the CSC subpopulation of H125 ($p = 0.002$) and A549 ($p = 0.005$; n = 4). Knockdown of $sPLA_2$ significantly reduced tumorsphere formation in H125 ($p = 0.026$) and A549 ($p = 0.001$; n = 3). Chemical inhibition of $sPLA_2$ resulted in dose-dependent reduction in tumorsphere formation in H125 ($p = 0.003$) and A549 ($p = 0.076$; n = 3).

Conclusions. Lung CSCs express higher levels of $sPLA_2$ than the non–stem cell population. Our findings that viral knockdown and chemical inhibition of $sPLA_2$ reduce tumorsphere formation in lung cancer cells demonstrate for the first time that $sPLA_2$ plays an important role in CSCs. These findings suggest that $sPLA_2$ may be an important therapeutic target for human lung cancer.

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Lung cancer remains the leading cause of cancer-related death worldwide with an overall 5-year survival of only 16% [1]. This is a result of the advanced local and metastatic disease often present at the time of diagnosis. Clearly, there is a vital need for improved treatment options, and although some targeted therapies such as tyrosine kinase inhibitors have shown promising initial results, recurrence rates after treatment remain unacceptably high [2]. Continued work to identify other directed therapies is urgently needed.

Secretory phospholipase A_2 group IIa ($sPLA_2$) is a secreted enzyme responsible for the release of arachidonic acid in the inflammatory pathway of prostaglandin production [3]. It has been implicated as a factor in the progression of prostate, esophageal, colon, and lung cancer [4–10]. Furthermore, we have shown that this enzyme contributes to tumor growth in vivo and invasion in vitro, specifically in human lung cancer cells [8, 9].

Accruing evidence suggests that mechanisms underlying tumor recurrence and metastases are modulated by means of a self-renewing subpopulation of the overall tumor called cancer stem cells (CSCs). The CSC hypothesis states that this subpopulation of tumor cells is responsible for tumor initiation, growth, and metastases [11]. Additionally, recent findings suggest that these cells are an integral part of the ability of tumors to develop resistance to radiation and chemotherapy, leading to treatment failures caused by metastases [12, 13].

Cancer stem cells have been identified in many solid-organ tumors including breast, head and neck, colon, and lung cancer by an assay measuring aldehyde dehydrogenase (ALDH) activity [14–18]. Aldehyde dehydrogenase serves as an intracellular oxidizer responsible for production of retinoic acid, which regulates many cellular processes including cell differentiation, proliferation, and

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Abbreviations and Acronyms

ALDH	=	aldehyde dehydrogenase
ALDH-high	=	aldehyde dehydrogenase high cells
ALDH-low	=	aldehyde dehydrogenase low cells
CSC	=	cancer stem cell
FACS	=	flow-assisted cell sorting
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
RT-PCR	=	reverse transcriptase–polymerase chain reaction
shRNA	=	short-hairpin RNA
sPLA$_2$	=	secretory phospholipase A$_2$–group IIa

apoptosis [19]. A validated in vitro assay has been developed to study CSCs [20]. Culturing cancer cells in nonadherent conditions at a low cell density yields tumorspheres that represent CSCs. Maintaining tumorspheres in culture provides a functional assay to reproducibly evaluate CSC activity and allows for investigation into mechanisms that drive the CSC phenotype.

Prostaglandins, a downstream product of sPLA$_2$, have been implicated in the maintenance of CSCs [21]. Specifically, prostaglandin E$_2$ has been demonstrated to be an important driver in proliferation of the CSC subpopulation [22]. Given the role of sPLA$_2$ in prostaglandin production and our previous findings of the importance of sPLA$_2$ in tumor growth and invasion in lung cancer, we hypothesized that sPLA$_2$ would play a significant role in maintaining the CSC phenotype and influence the function of CSC in non-small cell lung cancer.

In this study we demonstrate that CSCs, identified by high levels of ALDH activity, contain an increased level of sPLA$_2$ protein and mRNA expression. Furthermore, short-hairpin RNA (shRNA) knockdown and chemical inhibition effectively decreased the CSC phenotype in non-small cell lung cancer. This is the first report implicating the importance of sPLA$_2$ in the maintenance and function of the CSC phenotype.

Material and Methods

Cell Culture and Materials

Human non-small cell lung cancer cell lines H125 and A549 were used for all experiments. H125 cells were obtained from the University of Colorado Cancer Center Tissue Culture Core (Aurora, CO), and A549 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI (Life Sciences, Grand Island, NY) or Ham's F12 (Corning, Manassas, VA), respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. An sPLA$_2$ inhibitor (S3319) was purchased from Sigma-Aldrich (St. Louis, MO). The sPLA$_2$ antibody was from Abcam (Cambridge, MA), and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Cell Signaling (Beverly, MA).

For tumorsphere culture, DMEM/F-12 (Corning) medium was supplemented with 2% B27 (Life Sciences), epidermal growth factor 20 ng/mL, and fibroblast growth factor 20 ng/mL (BD Biosciences, Franklin Lakes, NJ). Ultra-low adhesion 6-well plates (Corning) were used for plating cells in tumorsphere assays. The tumorsphere protocol was derived from a previously established method for culturing tumorspheres [20].

Aldefluor Assay

The Aldefluor assay (Stemcell Technologies, Vancouver, Canada) was used to identify CSCs by following the manufacturer's recommended protocol. Briefly, H125 and A549 cells were released from culture flasks, centrifuged at 4°C for 5 minutes at 270*g* and resuspended at 10^6 cells per vial in 1 mL of Aldefluor assay buffer. Five microliters of ALDH substrate (BODIPY [boron-dipyrromethene] aminoacetaldehyde) was added to each 1-mL vial. Half of the sample was immediately transferred to a new vial, and 5 μL of diethylaminobenzaldehyde, a potent inhibitor of ALDH, was added, which serves as the internal control. The samples were then incubated at 37°C for 30 minutes followed by centrifugation at 4°C for 5 minutes at 270*g*. The supernatant was removed, and the pellet was resuspended in 500 μL of cold assay buffer. The samples were kept on ice until evaluation by flow cytometry [15].

The assay uses an uncharged ALDH substrate, which readily crosses the cell membrane. Intracellular ALDH then converts the substrate to a charged state, which is unable to exit the cell. Cells exhibiting higher levels of ALDH activity (ALDH-high) are brighter and represent the CSC subpopulation. The diethylaminobenzaldehyde-treated sample serves as the internal control (ALDH-low). Fluorescent activity was evaluated on a MoFlo XDP 100 (Beckman Coulter, Brea, CA) flow cytometer, and ALDH-high and ALHD-low cell populations were recovered with flow-assisted cell sorting (FACS) for further analysis.

Western Blotting

The ALDH-high and ALDH-low cells recovered from FACS were centrifuged for 5 minutes at 270*g*, washed once with phosphate-buffered saline solution (PBS), and lysed in Laemmli buffer (Bio-Rad, Hercules, CA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk dissolved in 1× Tris-buffered saline (TBS), 0.1% Tween-20 (Sigma-Aldrich). Primary antibodies were dissolved in 4% bovine serum albumin (Sigma-Aldrich) in 1× TBS, 0.1% Tween-20. Secondary antibodies were prepared in 5% nonfat milk in 1× TBS, 0.1% Tween-20. Membranes were developed using Pierce ECL Chemiluminescent (Thermo Fisher Scientific, Inc, Rockford, IL). Protein quantification was performed by densitometric analysis using ImageJ Software (National Institutes of Health, Bethesda, MD). Expression is presented as sPLA$_2$ relative to GAPDH.