Group IIa sPLA2 Inhibition Attenuates NF-κB Activity and Promotes Apoptosis of Lung Cancer Cells

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Abstract. Background/Aim: Group IIa secretory phospholipase A2 (sPLA2 IIa) has been implicated in the regulation of metastasis of non-small cell lung cancer (NSCLC) and the present study investigates its contribution to lung cancer growth and progression. PLA2s initiate signaling in several pathways that mediate cell survival including phosphatidylinositol 3-kinase-AKT (PI3K-AKT), p38 mitogen-activated protein kinase (p38 MAPK), extracellular-signal-regulated kinase (ERK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Materials and Methods: Human NSCLC cell lines (A549 and NCI-H358) were treated with a specific sPLA2 IIa inhibitor. Cells were assayed for apoptosis, viability, proliferation and changes in cell morphology. Effects on AKT, p38 MAPK, ERK1/2 and NF-κB signaling pathways were investigated. Results: sPLA2 IIa inhibition reduced proliferation and increased apoptosis. NF-κB activity was attenuated, whereas AKT, ERK1/2, p38 MAPK were variably affected by sPLA2 IIa inhibition. NF-κB inhibitor-associated apoptosis confirmed the dominant role of NF-κB. Conclusion: sPLA2 IIa attenuates growth and promotes apoptosis predominantly via its effects on NF-κB activity.

The activation of the phospholipase A2 (PLA2) family of enzymes plays a major role in the formation of eicosanoid lipid mediators that regulate the progression of non-small cell lung cancer (NSCLC) (1-3). Within the family of PLA2 enzymes, the isozyme group IIa secretory phospholipase A2 (sPLA2 IIa) has recently come to light as a potential biomarker and regulator of invasive potential in lung cancer (4, 5). Its specific role in lung cancer growth has yet to be examined. In fibroblasts, overexpression of sPLA2 IIa can produce an apoptosis-resistant phenotype that can be overcome by inhibition (6). In cancer cells, overexpression and exogenous administration of PLA2s can increase cell proliferation (7-9). In esophageal cancer, this stimulatory effect can be overcome by silencing sPLA2 IIa (8).

One of several potential mechanistic links between sPLA2 and proliferation is nuclear factor kappa-B (NF-κB). NF-κB transcription factors facilitate tumor survival by stimulating anti-apoptosis and cell proliferation genes (10). NF-κB target genes also include components of the tumor-promoting eicosanoid pathway including sPLA2 IIa itself (11-14). Evidence suggests that NF-κB is modulated by sPLA2 IIa inhibition since intercellular adhesion molecule-1 (ICAM-1) and sPLA2 IIa, both NF-κB target genes, are down-regulated when sPLA2 IIa is inhibited (5, 15). The documented interactions between NF-κB and sPLA2 IIa have not been previously linked to lung cancer cell survival.

Other intracellular pathways involved in tumor proliferation include phosphatidylinositol 3-kinase-AKT (PI3K-AKT) and extracellular-signal-regulated kinase (ERK) signaling cascades (16). sPLA2 ligand activity stimulates chemokine and growth factor production by signaling through PI3K-AKT, p38 mitogen-activated protein kinase (p38 MAPK), ERK1/2 and NF-κB (17-19). In prostate cancer, overexpression of sPLA2 IIa is associated with elevated AKT and NF-κB activity, although this mechanism was not examined with regard to cancer growth (13). We hypothesize a dominant link between sPLA2 IIa, NF-κB and viability of lung cancer cells. We propose that sPLA2 IIa modulates NF-κB activity in lung cancer cells and that inhibition of sPLA2 IIa reduces cell viability and promotes apoptosis through attenuation of NF-κB activity.

Materials and Methods

Cell culture and reagents. Two human NSCLC cell lines were used. A549 and NCI-H358 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown at 37°C in 5% CO2 in Ham’s F-12 (A549) or RPMI (H358) media, containing 10% fetal bovine serum (FBS). Stock and experimental solutions of tumor necrosis factor-alpha (TNF-α) (Sigma Aldrich, 3601-3608 (2012)
Figure 1. High concentrations of the group Ila secretory phospholipase A2 (sPLA2 Ila) inhibitor increase apoptosis of A549 and H358 cells after 8 h of treatment, n=3. *p=0.007, **p=0.005, compared to the control.

Figure 2. Group Ila secretory phospholipase A2 (sPLA2 Ila) inhibition reduces cell viability and proliferation of A549 and H358 cells in a concentration-dependent manner. A: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay B: 5-bromo-2′-deoxyuridine (BrdU) assay, n=3, performed in triplicate. *p<0.003, **p<0.05, compared to the control.
St. Louis, MO, USA) were prepared in phosphate-buffered saline (PBS). The sPLA2 IIa inhibitor S3319, (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO). DMSO served as the vehicle control for all experiments, with a maximum experimental concentration of 0.08%. The NF-κB inhibitor SN-50 (Enzo Life Sciences, Farmingdale, NY, USA) was dissolved in sterile water. For immunoblotting, rabbit antibodies were used against human phosphorylated and total ERK1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phosphorylated and total NF-κB p65 (Ser536), phosphorylated and total p38 MAPK, phosphorylated and total AKT (Thr308) and glyceraldehyde 3-phosphate (GAPDH) (Cell Signaling Technology, Inc., Danvers, MA, USA).

Apoptosis assay. A total of 10^5 cells per well were grown in 12-well plates for 24 h then were serum-starved (0.5% FBS) for 24 h. Cells were then treated with either the sPLA2 IIa inhibitor or NF-κB inhibitor in serum-starved media for 8 h then stained with Annexin V and propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer’s instructions and quantified by flow cytometry.

Cell viability and proliferation assays. A total of 5,000 cells per well were grown in 96-well plates and treated with the sPLA2 IIa inhibitor. Viability and proliferation were analyzed after 24 h using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-bromo-2’-deoxyuridine (BrdU) assays (Roche Applied Sciences, Indianapolis, IN, USA), according to the manufacturer’s instructions.

Immunofluorescence. A total of 5,000 cells per chamber were grown on Lab-Tek II 8-well glass chamber slides (Thermo Scientific, Rochester, NY, USA) for 24 h, then were serum-starved for 24 h. Cells were pre-treated with the sPLA2 IIa inhibitor in serum-starved medium for 1 h then stimulated with 20 ng/ml TNF-α for 8 h. Control cells were incubated with DMSO in serum-starved media and stimulated with TNF-α for 8 h. Immunofluorescence microscopy was performed to evaluate cell areas, as previously described (8).

Immunoblotting. A total of 3×10^6 cells per well were grown in 6-well culture plates for 24 h then serum-starved for 24 h prior to treatment. A time-response experiment was conducted in both cell lines to determine the time point of maximal phosphorylation of NF-κB, ERK 1/2, p38 MAPK and AKT after TNF-α stimulation. To determine the effect of sPLA2 IIa inhibition, cells were incubated with incrementally increasing concentrations of the sPLA2 IIa inhibitor in serum-starved media for 1 h, then 20 ng/ml of TNF-α was added to each well until the time of maximum phosphorylation identified in the time-response experiment. Control cells were
Figure 4. Group Ila secretory phospholipase A2 (sPLA2 Ila) inhibition reduces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) phosphorylation in A549 and H358 cells after TNF-α stimulation. Phosphorylation of extracellular-signal-regulated kinase (ERK 1/2) and p38 mitogen-activated kinase (p38 MAPK) decreased in A549 cells and increased in H358 cells after treatment. There was no difference from baseline phosphorylation of AKT when cells were treated with high concentrations of sPLA2 Ila inhibitor for either cell line, n=5.
incubated with DMSO in serum-starved media and stimulated with TNF-α at the same time points. Cells were lysed with Laemmeli buffer. Proteins were then resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes. Blocking solution contained 0.5% nonfat milk in 1× PBS with 0.1% Tween-20. Primary antibodies were dissolved in 5% bovine serum albumin containing 1× PBS with 0.1% Tween-20. Detection of bound antibodies was conducted using enhanced chemiluminescence (Pierce Protein Research Products, Thermo Scientific, Rockford, IL, USA). Image J software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis of the protein bands of interest.

Statistical analysis. Group comparisons were performed with ANOVA test. P-Values less than 0.05 were considered statistically significant. StatView V5.0 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

sPLA2 IIa inhibition in NSCLC cells promotes apoptosis and reduces proliferation. Specific sPLA2 IIa inhibition increased apoptosis of both A549 and H358 cells (Figure 1, p<0.01). This corresponded with reduced cell viability and proliferation (Figure 2, p<0.01). TNF-α slightly augmented the decrease in viability associated with sPLA2 IIa inhibition, however, the difference with and without TNF-α stimulation was not statistically significant (data not shown). Immunofluorescent staining of A549 cells with sPLA2 IIa inhibition demonstrated cells to be more contracted, with a statistically significant decrease in cell area (Figure 3, p=0.0006). These morphological changes are consistent with the phenotype of apoptosis (20). In addition, localization of sPLA2 IIa was observed predominantly around the nucleus.

sPLA2 IIa inhibition attenuates NF-κB phosphorylation in NSCLC cells. sPLA2 IIa inhibition led to a rapid and significant concentration-dependent decrease in NF-κB phosphorylation in both cell lines (Figure 4, p<0.01). There was also a significant concentration-dependent decrease in ERK1/2 and p38 MAPK phosphorylation in A549 cells. In contrast, H358 cells demonstrated an increase in p38 MAPK and ERK1/2 phosphorylation. AKT phosphorylation increased in both cell lines, with a medium concentration of sPLA2 inhibitor, but decreased to baseline levels at a higher concentration.

NF-κB inhibition promotes apoptosis of NSCLC cells. To demonstrate that the apoptosis-inducing effect of sPLA2 IIa inhibition was secondary to the down-regulation of NF-κB phosphorylation, cells were treated with the NF-κB inhibitor SN-50, which blocks NF-κB translocation to the nucleus. In both cell lines, NF-κB inhibition significantly increased apoptosis (Figure 5, p<0.001).

Discussion

The results of this study demonstrate that sPLA2 IIa plays a significant role in lung cancer cell proliferation and apoptosis, the mechanism of which is largely mediated by NF-κB activity. Although specific sPLA2 IIa inhibition influences several other major pathways associated with tumor growth and survival including ERK1/2, AKT, and p38 MAPK, it uniformly reduces NF-κB activity, thereby attenuating cell proliferation and promoting apoptosis.

The prevailing relationship between sPLA2 IIa and NF-κB is bolstered by examining two NSCLC cell lines. Both cell lines are wild-type for epidermal growth factor receptor (EGFR) and mutant for Kirsten rat sarcoma viral oncogene homolog (KRAS) but differ in functional p53 (wild-type in A549, mutant in H358). NF-κB attenuation was associated with apoptosis in both cell lines, independently of KRAS (21) and p53 status. Our results suggest that these genotypic differences have less of an influence over the dominant relationship between sPLA2 IIa, NF-κB and cell growth.

The attenuation in NF-κB activity and subsequent apoptosis seen with sPLA2 inhibition increases the attractiveness of sPLA2 IIa as a therapeutic target. RNA interference screens and mouse models of lung tumor development have identified NF-κB signaling as being essential for KRAS-driven lung tumors (22, 23). In addition, p53 mutant-lung cancer demonstrated increased NF-κB activity (24). Targets downstream of NF-κB include cellular inhibitors of apoptosis, survival caspases and anti-apoptosis members of the B-cell lymphoma 2 (BCL-2) family. In vitro and in vivo studies of NF-κB inhibitors have shown great promise as anti-lung cancer agents. However, the poor selectivity and known off-target effects of NF-κB inhibitors counteract this appeal. The existence of specific inhibitors of sPLA2 IIa (25) allows the potential for rapid translation from preclinical studies to clinical trials, particularly in a potential subset of patients with mutant KRAS lung cancer for which there are currently no targeted therapies.

The attenuation of NF-κB by sPLA2 inhibition is not only relevant for its effects on apoptosis but also because of its other target genes, which include those for sPLA2 IIa itself, and cyclo-oxygenase-2 and 5-lipoxygenase enzymes (11-14). Reducing PLA2 pathway activity can work to further dampen eicosanoid production and other inflammatory stimuli that promote tumor growth.

One critical issue with investigations of signaling cascades is that phosphorylation of ERK1/2 and other kinases may not be an appropriate measure of activity. These data indicate that although the broad pattern of pathways within a cancer cell in response to a stimulus can be markedly varied, the end result of apoptosis is the same. By looking at a cross-section of these pathways, we aimed to eliminate some ‘noise’ and identify the predominant mechanism leading to apoptosis of lung cancer cells with sPLA2 IIa inhibition.

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The specific action of sPLA2 IIa in promoting tumor growth is not fully addressed here. Immunofluorescent staining shows sPLA2 IIa localized either perinuclearly, or intranuclearly, or both. This is consistent with the location of other enzymes involved in eicosanoid production, although we can only speculate as to the interactions this location may serve to facilitate. It may be relevant for interaction with transcription factors, including NF-κB. Regarding the specific functional role of sPLA2 IIa, sPLA2s are known to have enzymatic activity and the inhibitor that we used irreversibly blocks the active site. However, sPLA2 IIa is secreted and has been reported to function as a ligand with several different receptors, including the M-type receptors to potentiate IL-6 release, and the EGFR receptor (7, 17, 18). We have reported that sPLA2 IIa inhibition reduces sPLA2 IIa mRNA (5). As NF-κB is a transcription factor for sPLA2, the results of this study suggest that the sPLA2 inhibition-associated attenuation of NF-κB is responsible for the reduced sPLA2 IIa transcription we previously described. Future studies will need to address both the ligand and enzymatic mechanisms of sPLA2 IIa in lung cancer.

In summary, we report the novel finding that selective sPLA2 IIa inhibition promotes lung cancer cell apoptosis, the mechanism of which is largely mediated by attenuation in NF-κB activity. Further investigation of the role of sPLA2 IIa in tumor growth, tumor formation, and as a potential target for therapy is warranted.

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