Lung cancer cell invasion and expression of intercellular adhesion molecule-1 (ICAM-1) are attenuated by secretory phospholipase A₂ inhibition

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Objective: Invasive lung tumors are associated with intercellular adhesion molecule-1 (ICAM-1) expression. Secretory phospholipase A₂ (sPLA₂) enzymes produce inflammatory mediators that stimulate ICAM-1 expression, and upregulation of PLA₂ activity can enhance metastasis. We hypothesize a link between sPLA₂ activity, ICAM-1 expression, and tumor cell invasion. We propose that inhibition of sPLA₂ modulates ICAM-1 expression in cancer cells and attenuates their invasiveness.

Methods: Human lung adenocarcinoma cells (A549) were treated with an ICAM-1 blocking antibody and assayed for invasion. Lung cancer cells (A549 and H358) were then treated with an sPLA₂ inhibitor and evaluated by immunoblotting for ICAM-1 expression. Next cells (A549) treated with sPLA₂ inhibitor were assayed for invasion. Finally, sPLA₂ messenger RNA and protein expression were evaluated by quantitative reverse-transcriptase polymerase chain reaction and immunofluorescence microscopy, respectively. Statistical analysis was performed by the Student *t* test or analysis of variance, as appropriate.

Results: Antibody blockade of ICAM-1 decreased lung cancer cell invasion. sPLA₂ inhibition significantly reduced ICAM-1 expression and invasion. sPLA₂ inhibition also significantly decreased sPLA₂ mRNA expression and immunofluorescent staining of sPLA₂.

Conclusions: sPLA₂ plays a significant role in mediating the inflammatory signals that induce ICAM-1 expression in lung cancer cells. Inhibition of the enzyme can significantly decrease ICAM-1 expression and subsequent cancer cell invasion. This lays the groundwork for further investigation into the cellular mechanisms of sPLA₂ and its role in lung cancer. (J Thorac Cardiovasc Surg 2012;143:405-11)

Approximately 75% of patients with lung cancer have metastatic disease at the time of diagnosis, and mortality largely results from the burden of metastatic spread.¹ Improving therapy for patients with invasive disease could dramatically affect lung cancer mortality and morbidity.

Metastatic cancer cells undergo a structural alteration to invade neighboring tissue. Invasion can be facilitated by expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1).² ICAM-1 is a member of the immunoglobulin supergene family of adhesion molecules and its expression is essential for the transendothelial migration of white blood cells from capillary beds into tissues.

In malignant disease, ICAM-1 expression has been associated with more aggressive lesions in melanoma, gastric, breast, lung, and other epithelial-derived cancers.^{2,3} Serum ICAM-1 levels are increased in patients with non-small cell lung cancer,⁴ and elevated serum levels have been correlated with advanced cancer stage.⁵⁻⁷ Histologically, levels of ICAM-1 expression in lung cancer tissue are proportional to tumor stage and associated with metastatic spread to lymph nodes.^{7,8} Laboratory models have demonstrated that ICAM-1 upregulation can promote cancer cell invasion. Furthermore, decreased tumor cell invasion is associated with decreased levels of ICAM-1 expression.^{7,9}

Secretory phospholipase A₂ (sPLA₂) enzymes produce inflammatory mediators that stimulate ICAM-1 expression. These enzymes catalyze the release of arachidonic acid from cell membranes leading to the production of eicosanoids such as prostaglandins, thromboxanes, and leukotrienes.^{10,11} Recently a number of studies have looked beyond the role of phospholipase enzymes in inflammation to probe their contribution to the pathogenesis of cancer.¹²⁻¹⁷ Stimulation of phospholipase A₂ (PLA₂) activity enhances the metastatic potential of lung cancer cells in vivo.¹³ Interestingly, PLA₂ activity in tumor-associated macrophages also correlates with a more

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Disclosures: Authors have nothing to disclose with regard to commercial support.

Read at the 37th Annual Meeting of The Western Thoracic Surgical Association, Colorado Springs, Colorado, June 22-25, 2011.

Received for publication June 21, 2011; revisions received Sept 28, 2011; accepted for publication Oct 20, 2011.

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0022-5223/\$36.00

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doi:10.1016/j.jtcvs.2011.10.026

Abbreviations and Acronyms

DMSO	= dimethyl sulfoxide
ICAM-1	= intercellular adhesion molecule-1
mRNA	= messenger RNA
PBS	= phosphate-buffered saline
PLA ₂	= phospholipase A ₂
sPLA ₂	= secretory phospholipase A ₂
TNF- α	= tumor necrosis factor-alpha

invasive phenotype in vivo.¹² Regarding sPLA₂ specifically, pharmacologic inhibition of the enzyme in lung cancer cells increases apoptosis and attenuates proliferation.¹⁷ Inhibition of sPLA₂ has also been shown to attenuate total cellular and soluble ICAM-1 expression in esophageal cancer cells.¹⁶ We hypothesize a link between sPLA₂ and ICAM-1 in lung cancer cells. We propose that sPLA₂ mediates ICAM-1 expression in lung cancer cells and that inhibition of sPLA₂ decreases the invasive potential of lung cancer cells.

MATERIALS AND METHODS**Cell Culture and Reagents**

Two human non-small cell lung cancer cell lines were used. A549 cells were donated by Dr Banerjee (University of Colorado, Aurora, Colo). NCI-H358 cells were a gift from Dr O'Neill (Brigham Young University, Provo, Utah). Cells were maintained at 37°C in 5% carbon dioxide in Ham's F-12 (A549) or RMPI (H358) containing 10% fetal bovine serum. Stock and experimental solutions of tumor necrosis factor-alpha (TNF- α) (Sigma Aldrich, St Louis, Mo) were prepared in phosphate-buffered saline (PBS). The group IIa sPLA₂ inhibitor S3319 (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO). DMSO served as a vehicle control for all experiments with a maximum experimental concentration of 0.08%. DMSO was used for the same time points as the inhibitor. Rabbit anti-ICAM-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and anti-glyceraldehyde-3-phosphate antibodies (Cell Signaling Technology, Inc, Danvers, Mass) were used for Western blot. Sheep anti-ICAM-1 and a polyclonal sheep immunoglobulin G control (R&D Systems, Minneapolis, Minn) were used for ICAM-1 blockade experiments.

Matrigel Invasion Assay

A modified Boyden chamber assay was created using Transwell 8- μ m permeable inserts (Corning, Corning, NY) coated with 20- μ g/100 μ L Matrigel matrix (BD Biosciences, Franklin Lakes, NJ) placed in 24-well cell culture plates. For the neutralizing antibody experiment, A549 cells were plated in full media (media with 10% fetal bovine serum) for 24 hours and then serum-starved (media with 0.5% fetal bovine serum) for 24 hours before adding TNF- α for 8 hours. Cells were trypsinized, counted, and resuspended in serum-starved media containing ICAM-1 neutralizing antibody (25 μ g/mL) in a rotating incubator for 1 hour at 37°C. An aliquot of cells incubated with sheep immunoglobulin G at the same concentration served as an antibody control. Then, 5×10^4 cells were placed on each insert and allowed to migrate for 36 hours using full media with 10% fetal bovine serum as a chemoattractant in the bottom chamber. Inserts were fixed and stained with 0.4% crystal violet, 0.2-mol/L citrate for 1 hour, washed twice with PBS, then once with deionized water. Cells on the upper side of the insert were removed with a cotton swab. Remaining cells in the center of the insert were counted using a stereo microscope (Leica, Wetzlar,

FIGURE 1. Intercellular adhesion molecule-1 (ICAM-1) determines invasiveness of lung adenocarcinoma cells. Statistically significant reduction in invasion of A549 cells pretreated with a neutralizing ICAM-1 antibody. * $P = .003$ compared with antibody control, $n = 9$.

Germany) and SPOT basic imaging software (SPOT Imaging Solutions, Sterling Hills, Mich).

For the sPLA₂ inhibitor invasion assay, A549 cells were first grown in 25-mL flasks at a concentration of 7×10^6 cells/mL for 24 hours and then serum-starved for 24 hours. Control cells were incubated with DMSO in serum-starved media, and then TNF- α was added to the media for 8 hours. Treatment cells were incubated with 20- μ mol/L sPLA₂ inhibitor in serum-starved media for 1 hour, and then 20-ng/mL TNF- α was added to the treatment media for 8 hours. After cells were counted and resuspended in serum-starved media, 5×10^4 cells were placed on each insert and allowed to migrate for 36 hours. Cells were stained and counted as described.

Western Blot Analysis

For analysis, 3×10^6 cells were plated in 6-well culture plates in full media for 24 hours and then serum-starved for 24 hours before treatment. Cells were incubated with incrementally increasing concentrations of the sPLA₂ inhibitor in serum-starved media for 1 hour; then 20-ng/mL TNF- α was added to the treatment media for 8 hours. Control cells were incubated with DMSO in serum-starved media and stimulated with TNF- α for 8 hours. Cells were lysed with Laemmli buffer and proteins resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes and probed for ICAM-1 and glyceraldehyde-3-phosphate. Blocking solution contained 0.5% nonfat milk in 1 $\times$ PBS with 0.1% Tween-20. Antibodies were diluted in 5% bovine serum albumin and 1 $\times$ PBS with 0.1% Tween-20. After incubation with horse radish peroxidase-conjugated secondary antibody, bound antibody was detected using enhanced chemiluminescence (Pierce Protein Research Products; Thermo Scientific, Rockford, Ill). Image J software (National Institutes of Health, Bethesda, Md) was used for densitometric analysis of the protein band of interest.

Lactic Dehydrogenase Cytotoxicity Assay

Cells, 5000 per well, were plated in a 96-well culture plate and treated as described for Western blot analysis of ICAM-1 expression. Cell supernatants were assayed for lactic dehydrogenase activity per manufacturer instructions (Cayman Chemicals, Ann Arbor, Mich).

Immunofluorescence Microscopy

Cells, 5000 per chamber, were cultured on Lab-Tek II 8-well glass chamber slides (Thermo Scientific, Rochester, NY) in full media for 24 hours and then serum-starved for 24 hours. Cells were pretreated with incrementally

FIGURE 2. Decreased total cellular expression of intercellular adhesion molecule-1 (*ICAM-1*) in lung adenocarcinoma cells treated with secretory phospholipase *A*₂ (*sPLA*₂) inhibitor. Representative Western blot of A549 cells and H358 cells shows a concentration-dependent decrease in ICAM-1 expression (A). Densitometric analysis of ICAM-1 protein bands normalized to vehicle control. A statistically significant decrease in ICAM-1 expression was seen in both cell lines (B). **P* < .001 compared with control, n = 4 for each experiment. *GAPDH*, Glyceraldehyde-3-phosphate.

increasing concentrations of the sPLA₂ inhibitor in serum-starved media for 1 hour and then stimulated with 20-ng/mL TNF-α for 8 hours. Control cells were incubated with DMSO in serum-starved media and stimulated with TNF-α for 8 hours. Immunofluorescence microscopy was performed to evaluate sPLA₂ expression in A549 cells as previously described.¹⁵

Reverse-Transcriptase Polymerase Chain Reaction

Cells were grown in 12-well culture plates at a density of 1 × 10⁵ cells per well with full media and then serum-starved media for 24

hours each. Cells were incubated with 20-μmol/L sPLA₂ inhibitor for 1 hour before TNF-α was added for 2 hours, 1 hour, and 30 minutes before messenger RNA collection. Control cells were incubated with DMSO in serum-starved media and stimulated with 20-ng/mL TNF-α for the same time points. A549 cells were analyzed for mRNA expression of sPLA₂ using quantitative reverse-transcriptase polymerase chain reaction as previously described.¹⁵ Messenger RNA for sPLA₂ was normalized to mRNA for glyceraldehyde-3-phosphate in the same sample.

FIGURE 3. Secretory phospholipase *A*₂ (*sPLA*₂) inhibition of lung adenocarcinoma cells decreases invasion through a modified basement membrane matrix. Representative image of cells that have migrated through the matrix (A). Statistically significant reduction in invasion of A549 cells treated with 20-μmol/L sPLA₂ inhibitor for 8 hours (B). **P* = .019 compared with control, n = 17.

FIGURE 4. Representative immunofluorescent images of group IIa secretory phospholipase A₂ (sPLA₂) expression in A549 cells. Cell membranes appear green, nuclei appear blue, and sPLA₂ appears red. The negative control was cells in vehicle control media and tumor necrosis factor-α (TNF-α) for 8 hours stained with non-immune matched immunoglobulin G (A). In the subsequent images, cells were stained with group IIa sPLA₂ antibody, vehicle control, and TNF-α for 8 hours (B), TNF-α for 8 hours in the presence of 20-μmol/L sPLA₂ inhibitor (C), and TNF-α for 8 hours in the presence of 40-μmol/L sPLA₂ inhibitor (D). The decrease in sPLA₂ staining was significant at 40-μmol/L sPLA₂ inhibitor compared with control (E). * $P = .023$.

Statistical Analysis

All data distributions were inspected before statistical analysis. The Student t test was performed for 2 group comparisons, and an analysis of variance test was performed when comparing 3 or more groups. No multiple comparisons were conducted. SAS version 9.2 (SAS Institute, Inc, Cary, NC) was used for all statistical analysis.

RESULTS

ICAM-1 Blockade Decreases Invasion of A549 Cells

Cells treated with a neutralizing ICAM-1 antibody demonstrated significantly decreased invasion, over 70% compared with an antibody control (Figure 1, $P = .003$).

sPLA₂ Inhibition Reduces ICAM-1 Expression and Attenuates Invasion

Treatment of A549 and H358 cells with a specific group IIa sPLA₂ inhibitor led to a concentration-dependent decrease in ICAM-1 expression in both cell lines with a greater than 80% decrease at the maximum concentration of 40 μmol/L (Figure 2, A and B, $P < .001$, at 15, 20, and 40 μmol/L in both cell lines). The concentrations used did not cause cell lysis as evaluated by a lactic dehydrogenase cytotoxicity assay (data not shown). Invasion of A549 cells through a modified basement membrane matrix was significantly decreased in the presence of

FIGURE 5. Group IIa secretory phospholipase A₂ (sPLA₂) inhibition decreases group IIa sPLA₂ production in non-small cell lung cancer cells. Tumor necrosis factor- α (TNF- α) for 30 minutes increases production of sPLA₂ mRNA (A); * P = .022, n = 3 run in triplicate. A 20- μ mol/L dose of sPLA₂ inhibitor decreases sPLA₂ mRNA expression (B); * P = .044, n = 3 run in triplicate.

20- μ mol/L group IIa sPLA₂ inhibitor (Figure 3, A and B, P = .019).

sPLA₂ Inhibition Decreases sPLA₂ Expression in A549 Cells

Cells treated with sPLA₂ inhibitor show a decrease in sPLA₂ staining by immunofluorescence (Figure 4, P = .023). Quantitative reverse-transcriptase polymerase chain reaction shows a significant increase in group IIa sPLA₂ mRNA with TNF- α stimulation for 30 minutes (Figure 5, A, P = .022) and a decrease in group IIa sPLA₂ mRNA in the presence of 20 μ mol/L of inhibitor (Figure 5, B, P = .044).

DISCUSSION

Although previous investigations have linked PLA₂ activity to ICAM-1 expression, this is the first study to demonstrate that pharmacologic inhibition of group IIa sPLA₂ decreases ICAM-1 expression and invasion in lung cancer cells.^{15,18}

This effect of sPLA₂ inhibition is especially noteworthy inasmuch as the decrease in ICAM-1 expression is seen in 2 different cell lines that both harbor K-ras mutations but differ in functional p53 (wild type in A549, mutant in H358) and liver kinase B1 (mutant in A549 and wild type in H358) status. Tumor protein 53 is a widely studied tumor suppressor and liver kinase B1 is an inhibitor of mammalian target of rapamycin. Unimpeded activity of mammalian target of rapamycin can be promalignant, and mammalian target of rapamycin is often aberrantly activated in lung cancers with oncogenic K-ras mutations.¹⁹

The role of adhesion molecules in the pathogenesis, diagnosis, and prognosis of cancer has been hotly investigated. In lung cancer, ICAM-1 and E-cadherin are recognized as serum markers and, at the cellular level, as role players in metastatic transformation.^{2,4} ICAM-1 is inducible by cytokines within the inflammatory milieu of the tumor microenvironment. Its potential role in invasion is implicated by increased in vitro and in vivo invasion with ICAM-1

overexpression.⁷ Our experimental approach demonstrates that by targeting ICAM-1 through a small molecule inhibitor to sPLA₂, as well as a neutralizing antibody against cell surface ICAM-1, invasion was decreased.

Antibody blockade of ICAM-1 resulted in a greater inhibition of invasion than the sPLA₂ inhibitor, suggesting a therapeutic application for an ICAM-1 antibody. Antibody therapy targeting adhesion molecules is not ideal because adhesion proteins are essential for normal leukocyte function and the risk may outweigh the benefit. Solid tumors are particularly challenging targets for antibody therapy secondary to poor tumor penetration compared with small molecules.²⁰ Despite decades of research, the Food and Drug Administration has not yet approved any monoclonal antibody available for lung cancer. Importantly, small molecule inhibitors like the sPLA₂ inhibitor can achieve the same goal of reducing ICAM-1 expression, in addition to exhibiting other antitumor effects, for less immune reactivity and expense.

PLA₂ enzymes produce arachidonic acid, a precursor to products of cyclooxygenase enzymes such as prostaglandins. Prostaglandins have been implicated in the pathogenesis of epithelial-derived tumors and prostaglandin production is elevated in tumor specimens of patients with lung cancer.²¹ In vivo studies of cyclooxygenase-2 inhibitors have shown therapeutic trends. A clinical trial of celecoxib in non-small cell lung cancer is associated with decreased biomarkers associated with metastatic potential.²² In addition to its enzymatic activity, sPLA₂ also functions as a ligand with the ability to interact with cell surface receptors like CD40, of the TNF- α receptor family, to enhance monocyte response to inflammatory states and possibly potentiate PLA₂ pathways.¹¹ In this way, sPLA₂ secreted by cancer cells or tumor-associated cells may act as a ligand to further potentiate inflammatory cell signals associated with ICAM-1 expression.²³ Effective sPLA₂ inhibition may have an added advantage over cyclooxygenase-2 inhibitors by decreasing this potentiation.

A striking result of this study is the finding that cellular inhibition of group IIa sPLA₂ in lung cancer cells decreases the production of the enzyme itself. Potential mechanisms could be through decreased transcriptional regulation either by the enzyme itself or through downstream products. In addition, lung cancer cell apoptosis increases with prolonged exposure to sPLA₂ inhibition.¹⁷ The combination of decreased invasion and cancer cell viability from sPLA₂ inhibition favors its potential as a therapeutic agent. As specific group IIa sPLA₂ inhibitors have already been studied in clinical trials for rheumatoid arthritis and sepsis, their potential application in malignancy is particularly appealing.^{24,25}

The mechanism of sPLA₂ inhibition on ICAM-1 expression is likely dependent on the transcription factor nuclear factor- κ B.^{9,10,16} Elucidation of intracellular pathways affected by group IIa sPLA₂ inhibition in lung cancer cells is the topic of ongoing research.

In summary, these data suggest that sPLA₂ plays a significant role in mediating the inflammatory signals that induce ICAM-1 expression in lung cancer cells. In addition, this study demonstrates that pharmacologic inhibition of the enzyme can significantly decrease ICAM-1 expression and subsequent cancer cell invasion. This lays the groundwork for further investigation into the cellular mechanisms of sPLA₂ and the effect of sPLA₂ on lung cancer proliferation in vitro and in vivo.

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Discussion

Dr Michael Mulligan (Seattle, Wash). Congratulations on your paper and a very nice presentation. I always appreciate seeing a lot of effort put into work, and clearly you did that. I noticed the article that your group put out via e-publication last month evaluating ICAM regulation with sPLA₂ in esophageal cancer cells, and this is a nice extension of that work. It is always difficult to know what the next great research question is and you seem to be onto something here.

I have 3 questions, which I will ask one at the time.

The matrigel assay is used to screen cell lines and measure invasiveness, but ultimately it is a dead matrix and is devoid of either known or candidate ligands for ICAM-1. If you do a quick search, you can find that there is now a body of researchers who are looking at ICAM-1 not so much as an adhesion receptor, particularly in the invasion paradigm, but as a signaling molecule. That is a little

bit different. Indeed, your findings suggest that your greatest effects on invasiveness were with direct inhibition of ICAM-1 and not with sPLA₂ inhibition indirectly, thereby affecting expression of ICAM-1. Can you postulate how ICAM-1 is regulating invasiveness? Is it merely adjusting the phenotype or something more invasive, or how does blockade of ICAM-1 allow you to digest through a collagen matrix differently than without?

Dr Yu. ICAM-1 can be considered a phenotype of invasive cells. How it helps the tumor cells invade through the matrix or, more important, in an in vivo model promote metastatic disease, is not well defined. There are other mediators of invasion that we did not necessarily address here, such as metalloproteinases that help digest the matrix. ICAM-1 is a reasonable marker of invasion given all of the evidence in serum and in tumor tissue. There are always limitations to in vitro studies, and I think the next move is to try sPLA₂ inhibition in an in vivo model.

Dr Mulligan. It is exciting because with direct blockade you definitely showed an effect; yet there is no ICAM ligand in the assay, so it has to be signaling somehow. There is a lot of potential there for another award.

The second question relates to specificity and loss of specificity with S3319, also known as KHO64. If you look at the dose ranges or the therapeutic concentrations for most of the small molecule targeted interventions that we use, like Tarceva (Hoffmann-La Roche, Basel, Switzerland) or any other “nibs,” they are always in the low nanomolar range, yet you were using up to 40 $\mu\text{mol/L}$. What data do you have to assess for loss of specificity or potential off-target effects?

Dr Yu. It is difficult to correlate concentrations that are used in vitro with an eventual in vivo concentration. One thing we did do was look at the cytotoxicity of the small molecule inhibitor in the lung cancer cells using a lactic dehydrogenase cytotoxicity assay. All the concentrations and time points we used were viable for the cells.

Dr Mulligan. However, you are seeing something weird, too, in that you are seeing an enzyme inhibitor having a sort of an auto-crine reverse effect on transcription of the enzyme itself. It could be that you have disrupted all sorts of transcriptional mechanisms that you just have not tracked yet, so it would be good to go back and look at that.

The last question is a more global one. Activation of sPLA₂ is a fairly upstream activation event, particularly if you try to relate it to ICAM-1 activation. There are a lot of intervening steps, including activation of cyclooxygenase. For the past 20 years there has been a lot of work looking at inhibition of those intermediate steps that should theoretically work reasonably well, but they do not work. I found about 100 publications or more this morning that demonstrated efficacy in cell systems and more than 50 clinical trials that showed that nothing worked when they tried to take it forward. What makes this different? Why should this work and is it safe? Because it is such an omnipresent enzyme that has so many functions, if we knock it out, what are we going to do to the wild type or to the patient?

Dr Yu. sPLA₂ is upstream. One of the interesting things about this particular enzyme is that it generates arachidonic acid, but it also has some other effects. For example, sPLA₂ can function as a ligand and not just as an intracellular enzyme. These other functions may be targeted with inhibition of sPLA₂. In terms of sPLA₂ being used in humans and whether or not it is toxic, sPLA₂ inhibitors have been used in trials for rheumatoid arthritis and sepsis. There were no noticeable toxicities. Finally, regarding the idea that there could be a single shot approach to addressing anything involving cancer or invasiveness in particular, sPLA₂ inhibition is useful as a research tool and may eventually be part of multimodality therapy targeting different pathways that promote malignancy.

Dr Mulligan. Perhaps this is one pathway but maybe not *the* pathway.