Modulation of growth in human esophageal adenocarcinoma cells by group IIa secretory phospholipase A_2

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Objective: Esophageal adenocarcinoma is thought to arise from lesions produced by chronic esophageal inflammation. Secretory phospholipase A_2 is an important mediator of mucosal response to gastroesophageal reflux, but its role in the function of mature cancer cells is unclear. We sought to determine the influence of group IIa secretory phospholipase A_2 on proliferation of human esophageal adenocarcinoma cells.

Methods: FLO-1 and OE33 cells derived from human esophageal adenocarcinoma were cultured with standard techniques. Cells were treated with 1-, 5-, 10-, and 20-μmol/L doses of 5-(4-benzyloxyphenyl)-4S-(7-phenylheptanoylamino)pentanoic acid, a specific inhibitor of group IIa secretory phospholipase A_2, for 72 hours. Gene for group IIa secretory phospholipase A_2 *(PLA2G2A)* was overexpressed and silenced with lentiviral infection techniques. Cell proliferation and viability were measured with standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and bromodeoxyuridine incorporation assays. All assays were performed in triplicate. *PLA2G2A* expression was measured with quantitative reverse transcriptase polymerase chain reaction; protein levels were detected with immunofluorescence microscopy. Statistical analysis was by analysis of variance with Fisher post hoc analysis.

Results: Secretory phospholipase A_2 protein was found in both malignant esophageal adenocarcinoma cell lines. Treatment with specific group IIa secretory phospholipase A_2 inhibitor resulted in dose-dependent reductions in growth and cell number in both cell lines. Overexpression of *PLA2G2A* resulted in enhanced cancer cell growth, whereas gene knockdown attenuated growth.

Conclusions: Group IIa secretory phospholipase A_2 appears significant in growth and proliferation of human esophageal adenocarcinoma cells. Secretory phospholipase A_2 inhibition should be studied further regarding potential chemopreventive and therapeutic properties in esophageal adenocarcinoma. (J Thorac Cardiovasc Surg 2010;139:591-9)

Esophageal adenocarcinoma of the gastroesophageal junction is currently the cancer with the fastest increasing incidence in the United States.[1] Since 1970, its incidence in some populations has increased more than 800%.[1] The development of esophageal cancer is thought to be a multistep process involving the accumulation of genetic events that result in abnormalities of cell cycle regulation. This results in formation of preneoplastic and eventually malignant lesions in the esophageal mucosa.[2] The central factor in the development of these genetic events and malignant lesions is inflammation related to gastroesophageal reflux disease.[3] Reflux disease is the most significant risk factor for the development of esophageal adenocarcinoma, and people with long-standing reflux disease are 43 times more likely to have adenocarcinoma of the esophagus develop.[2]

Several molecular factors are thought to mediate the generation of mucosal inflammation in reflux disease and aid in the development of malignancy. Such factors include cyclooxygenase-2 (COX-2), tumor necrosis factor α, and inducible nitric oxide synthase.[4,5] Recently, we confirmed the enzyme secretory phospholipase A_2 ($sPLA_2$) as another important mediator of esophageal mucosal inflammation.[6] The enzymatic activity of phospholipase enzymes results in the generation of bioactive molecules such as lysophospholipids, prostaglandins, leukotrienes, and free fatty acids. In particular, $sPLA_2$ has been implicated as a pathogenic factor in a variety of inflammatory diseases, including acute pancreatitis, septic shock, adult respiratory distress syndrome, Crohn disease, ulcerative colitis, bronchial asthma, and allergic rhinitis.[7]

The role of $sPLA_2$ in the function of the cancer cell is not clear. Upregulation of $sPLA_2$ has been described in several human cancers, including prostate, ovarian, and intestinal tumors, and apparently its presence has integral effects on both growth regulation and level of virulence of these cancers.[7] The findings by others demonstrating that $sPLA_2$ levels are increased in the mucosa of patients with both

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Disclosures: None.

Supported by University of Colorado Academic Enrichment Funds.

Received for publication June 25, 2009; revisions received Sept 4, 2009; accepted for publication Oct 3, 2009.

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0022-5223/$36.00

doi:10.1016/j.jtcvs.2009.10.061

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Abbreviations and Acronyms

5-LOX = 5-lipoxygenase
BrdU = bromodeoxyuridine
COX-2 = cyclooxygenase-2
FBS, fetal bovine serum
GFP = transgene for green fluorescent protein
LDH = lactate dehydrogenase
LVUT = lentiviral expression vector pLVUT-tTR-KRAB
MTT = 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
PBS = phosphate-buffered saline solution
PCR = polymerase chain reaction
PGE_2 = prostaglandin E_2
PLA2G2A = gene for group IIa secretory phospholipase A_2
RT-PCR = reverse transcriptase polymerase chain reaction
shRNA = short hairpin RNA
$sPLA_2$ = secretory phospholipase A_2

Barrett esophagus and esophageal carcinoma suggest that it may play a role in more advanced metaplastic states as well as in the early inflammatory process in esophageal disease.[8] Because of our findings demonstrating the role of $sPLA_2$ in esophageal mucosal inflammation, we hypothesized that $sPLA_2$ may influence the growth and proliferation of esophageal cancer cells.

MATERIALS AND METHODS

Cell Lines and Treatment

The esophageal adenocarcinoma cell line OE33 was purchased from Sigma (Sigma Chemical Company, St Louis, Mo), and the cell line FLO-1 was a generous gift from Dr David Beer (Department of Surgery, University of Michigan, Ann Arbor, Mich). OE33 cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. FLO-1 cells were grown in Dulbecco modified Eagle minimal essential medium supplemented with 10% FBS and antibiotics. HEK293 T cells were obtained from the American Type Culture Collection (Manassas, Va) and grown in Dulbecco modified Eagle minimal essential medium supplemented with 10% FBS and antibiotics. All cells were grown incubated at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. The $sPLA_2$ inhibitor 5-(4-benzyloxyphenyl)-4S-(7-phenylheptanoylamino)pentanoic acid was purchased from Sigma and dissolved in dimethyl sulfoxide. Stock solutions of the drug were diluted in medium before each experiment such that the concentration of dimethyl sulfoxide never exceeded 0.1%.

Reverse Transcriptase Polymerase Chain Reaction

OE33 and FLO-1 cells were grown in 6-well plates until confluent. Cells were then rinsed twice with cold phosphate-buffered saline solution (PBS) and lysed with Buffer RLT from RNeasy kit (Qiagen, Valencia, Calif). RNA was extracted according to the protocol provided with the kit. Reverse transcription was performed in the usual fashion. Primers used to amplify the genes for group IIa $sPLA_2$ *(PLA2G2A)* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* are shown in Table 1. Polymerase chain reaction (PCR) product bands were quantified from ethidium-stained gels with National Institutes of Health ImageJ software (http://rsbweb.nih.gov/ij/).

Quantitative PCR was carried out with the Rotor-Gene 6000 Real-Time DNA Detection System (Qiagen) with SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, Calif). All reactions were performed in a total volume of 20 μL with 20 ng complementary DNA (based on the original RNA concentration) and 0.5 μg concentrations of the forward and reverse primers. Negative (no added template) controls were run for each set of experiments. PCR reactions were set at 95°C for 5 minutes, followed by 40 cycles including a denaturation step at 95°C for 5 seconds, an annealing step at 63°C for 10 seconds, and an extension step at 72°C for 15 seconds. The last step included a slow increase in temperature from 72°C to 95°C, or melt curve. Verification of the appropriate product was conducted by melt curve analysis as well as agarose gel electrophoresis. Data were calculated as relative abundance of heat shock protein 25 gene *(HSPB1)* relative to that of the glyceraldehyde-3-phosphate dehydrogenase gene *(GAPDH)* with the $\Delta\Delta$Ct method.

Immunofluorescence Microscopy

OE33 and FLO-1 cells were plated on 8-well glass chamber slides at a density of 5×10^3 cells/chamber. Cells were incubated in growth medium for 72 hours before fixation with a mixture of 70% acetone and 30% methanol for 5 minutes, followed by fixation with 4% paraformaldehyde for 10 minutes. Sections were washed with PBS, blocked with 10% normal serum for 30 minutes, and incubated overnight with 5-μg/mL polyclonal rabbit anti–human group IIa $sPLA_2$ in PBS containing 1% bovine serum albumin antibody (Biovendor, Candler, NC). After washing with PBS, sections were incubated with Cy3-conjugated matched IgG at 1:150 dilution with PBS containing 1% bovine serum albumin (Jackson Immunoresearch, West Grove, Pa). To assess specificity, cells in adjacent chambers were incubated with non–immune-matched IgG (5 μg/mL in PBS containing 1% bovine serum albumin) and otherwise processed identically (negative controls). Primary incubation was performed at 4°C, and all other incubations were performed at room temperature. Bis-benzimide was used to stain nuclei (4′,6-diamidino-2-phenylindole, imaged on the blue channel) and wheat germ agglutinin to stain cell membranes (labeled with Alexa 488 and imaged on the green channel). Group IIa $sPLA_2$ was imaged on the red (Cy3) channel. Microscopic observation and photography were performed with a Leica DMRXA confocal microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Quantitation of Viable Cell Number and Cellular Proliferation After $sPLA_2$ Inhibition

OE33 and FLO-1 cells were subcultured in 96-well plates at a density of 1×10^4 cells/well in 10% FBS-containing medium for 48 hours. Cells were then incubated in reduced serum medium (0.5% FBS) for 24 hours to induce cell cycle arrest. The medium was then replaced with reduced-serum medium containing dimethyl sulfoxide (0.1%) or the $sPLA_2$ inhibitor at 1, 5, 10, and 20 μmol/L and incubated for 72 hours. At the end of the treatment, viable cell numbers were determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche Diagnostics GmbH, Mannheim, Germany). To verify that any changes in viable cell number in the treatment groups were not due to cytotoxic effects of the inhibitory drug, the lactate dehydrogenase (LDH) activities of the cell supernatants were measured after the 72 hour treatment. LDH is contained in the cytoplasm of cells and is released into the media of cells undergoing necrosis.

To confirm that the effects on relative viable cell number were due to effects on proliferation, cells were treated as described here, and a bromodeoxyuridine (BrdU) incorporation assay (Roche) was performed according to manufacturer instructions. This assay quantifies de novo DNA synthesis as a marker of proliferation.

TABLE 1. Gene sequences used

	Forward	Reverse
PLA2G2A RT-PCR primers	5′-CCACGGATCGCTGCTGTGTCA	5′-TCCTGTTTTGCACAGGTGATT
GAPDH RT-PCR primers	5′-GAGTCAACGGATTTGGTGGT	5′-GACAAGCTTCCCGTTCTCAG
PLA2G2A full-length transgene primers	5′-GTCGAACCGGTATGAAGACCCTCCTACTGTTG	5′-GTCGAGGGCCCTCAGCAACGAGGGGTGCTCCC
PLA2G2A short hairpin RNA sequences	5′-GGCCCATGGGAATTTGGTGAATT	
	5′-GGTGCTAGAAACAAGACGACCTA	
	5′-GGGCTGTGTCACTCATGACTGTT	
	5′-GGGATCAAGTTGACGACAGGAAA	

PLA2G2A, Gene for secretory phospholipase A_2; *RT-PCR,* reverse transcriptase polymerase chain reaction; GAPDH, gene for glyceraldehyde-3-phosphate dehydrogenase.

Construction of Expression Vector for Group IIa $sPLA_2$ Gene

The lentiviral expression vector pLVUT-tTR-KRAB (LVUT) was used for overexpression of the gene for group IIa $sPLA_2$ *(PLA2G2A).* This vector contains a transgene coding for green fluorescent protein *(GFP)* under the control of the human ubiquitin C RNA polymerase II promoter. The *GFP* transgene was removed by restriction digestion with Apa1 and Age1 restriction enzymes (New England Biolabs, Ipswich, Mass) and was replaced with a transgene coding for *PLA2G2A* ($LVUT^{PLA2G2A}$). The *PLA2G2A* transgene was amplified from complementary DNA of FLO-1 cells by reverse transcriptase PCR (RT-PCR) with specifically designed primers containing the Age1 restriction sequence on the 5′ primer and the Apa1 sequence on the 3′ primer (Table 1). Insertion of the *PLA2G2A* transgene was performed with the aid of a Quick Ligation Kit (New England Biolabs).

Production of Infectious Lentiviral Particles

Infectious lentiviral particles containing $LVUT^{PLA2G2A}$ were produced as previously described.[9] Briefly, HEK293 T cells were plated in 60-mm

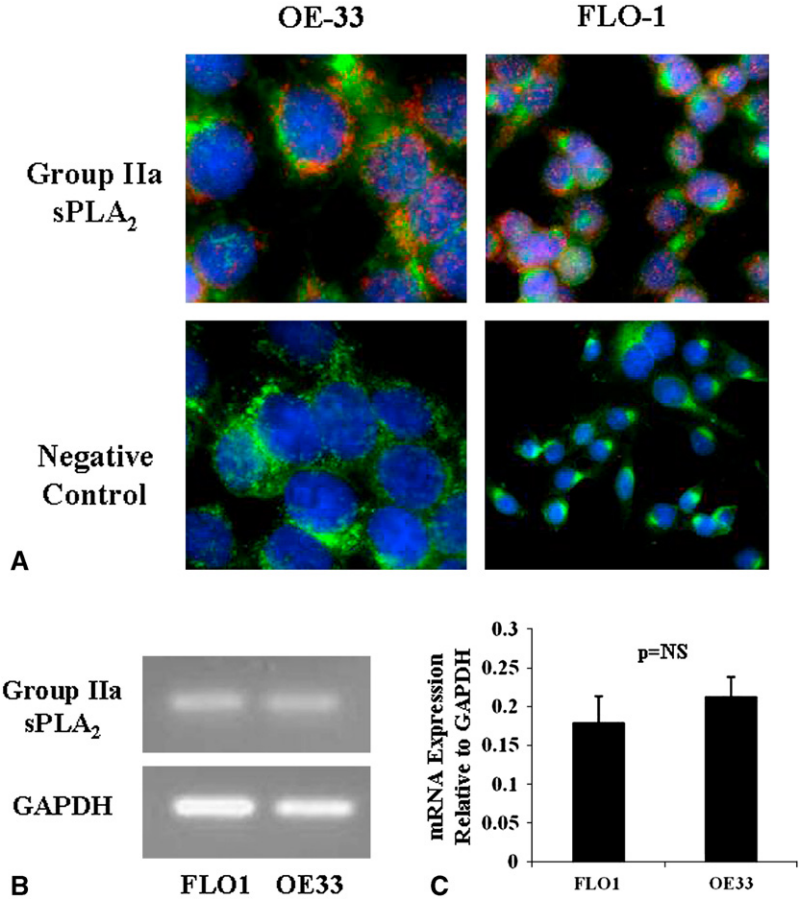

FIGURE 1. Expression of group IIa secretory phospholipase A_2 *(sPLA2)* protein in esophageal adenocarcinoma cell lines. A, Immunofluorescence microscopy shows expression of group IIa secretory phospholipase A_2 *(red)* in esophageal adenocarcinoma cell lines FLO-1 and OE33. Nuclei are stained *blue*, and cell membranes are stained *green*. B, Reverse transcriptase polymerase chain reaction shows near equal expression of group IIa secretory phospholipase A_2 in esophageal cancer cells. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase; *mRNA,* messenger RNA; *NS,* not statistically significant.

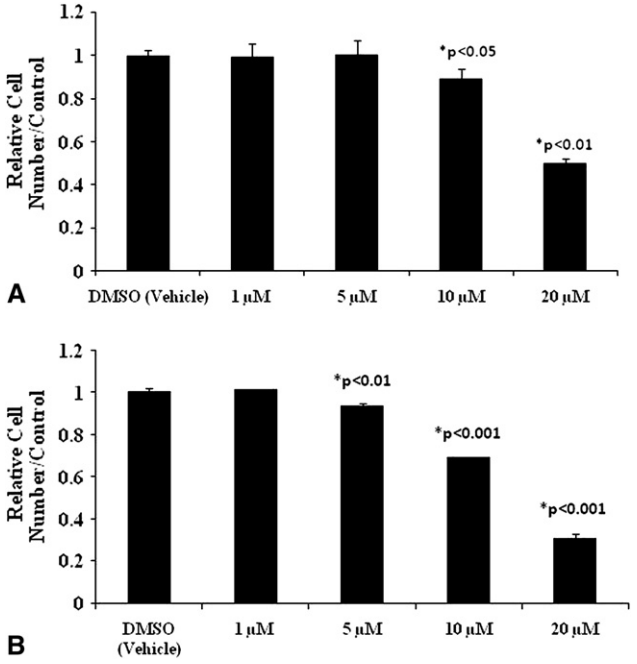

FIGURE 2. Effect of group IIa secretory phospholipase A_2 inhibition on esophageal adenocarcinoma cell number. Relative cell numbers after 72 hours of treatment of FLO-1 cells (A) and OE33 cells (B) relative to controls, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Statistical significance relative to untreated controls and vehicle (dimethyl sulfoxide, *DMSO*) controls was reached with 10- and 20-μmol/L doses for FLO-1 cells and with 5-, 10-, and 20-μmol/L doses for OE33 cells.

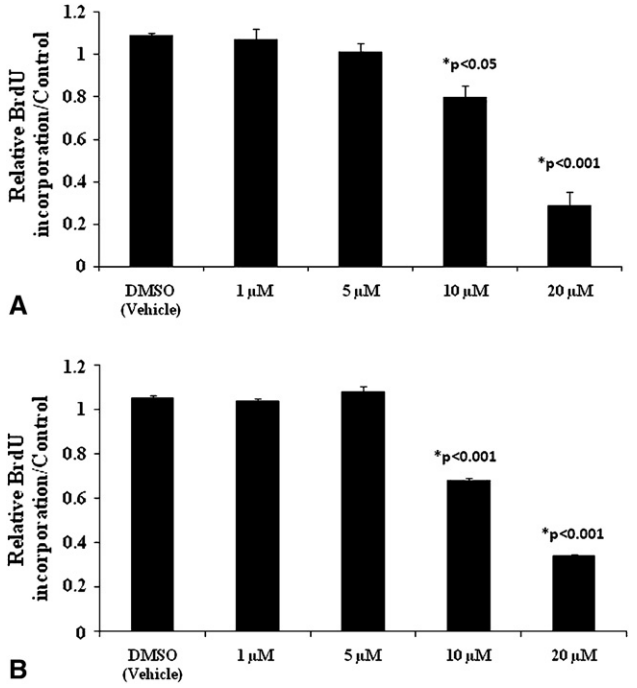

FIGURE 3. Effect of group IIa secretory phospholipase A_2 inhibition on esophageal adenocarcinoma cellular proliferation. Relative bromodeoxyuridine *(BrdU)* incorporation after 72 hours of treatment in FLO-1 cells (A) and OE33 cells (B) relative to controls. Statistical significance versus both untreated and vehicle (dimethyl sulfoxide, *DMSO*) controls was reached with 10- and 20-μmol/L doses in both cell types.

dishes at a density of 7×10^5 per plate, cultured for 24 hours, and then co-transfected with the LVUT^{PLA2G2A} construct, a packaging vector (pPAX2; Addgene, Cambridge, Mass), and an envelope vector (pMD2; Addgene) with Effectene transfection reagent (Qiagen). At approximately 24 hours after transfection, the cell culture medium was replaced with viral collection medium and incubated for a further 30 hours. This medium was then collected and filtered through a 0.45-μm filter to remove any cellular debris. Viral stocks were stored at –80°C until further use.

Overexpression of *PLA2G2A*

FLO-1 and OE33 cells were cultured in 6-well plates at a density of 3×10^5 cells per well. After 48 hours, growth medium was removed and replaced with fresh growth medium containing 0.25 mL filtered LVUT^{PLA2G2A} infectious viral particles. This volume was based on *GFP* expression in FLO-1 and OE33 cells from titration experiments with LVUT viral stocks. Approximately 24 hours after infection, medium containing viral particles was removed and replaced with fresh growth medium. Overexpression of group IIa sPLA$_2$ protein was assessed with immunofluorescence microscopy as described previously.

Lentiviral Knockdown of *PLA2G2A*

Four short hairpin RNA (shRNA) constructs for *PLA2G2A* as well as a nontargeting shRNA construct in a pLKO.1 puro lentiviral vector were purchased from Sigma. The sense sequences of these constructs are shown in Table 1. Infectious lentiviral stocks were produced with these constructs as described previously. Titration experiments were performed with infectious stocks containing a *GFP*-containing pLKO.1 puro lentivirus. FLO-1 and OE33 cells were cultured in 6-well plates at a density of 3×10^5 cells/well and infected with growth medium containing 0.5 mL filtered viral stock of each *PLA2G2A* construct. After 24 hours of infection, medium containing viral particles was removed and replaced with fresh growth medium. Knockdown of *PLA2G2A* expression was determined with quantitative RT-PCR, and knockdown of protein expression was assessed with immunofluorescence microscopy.

Quantitation of Viable Cell Number and Cellular Proliferation of Cells Infected With Lentivirus

Cells infected with lentiviral vectors were subcultured in 96-well plates at a density of 1×10^4 cells/well in growth medium for 48 hours, at which point the medium was replaced with serum-reduced medium. Cells were grown in serum-reduced medium for 72 hours before assessment of viable cell number and cellular proliferation with MTT or BrdU assays, respectively. All experiments with infected cells included 3 wells of untransfected control cells. Cells infected with LVUT^{PLA2G2A} were also compared with cells transfected with LVUT^{GFP} as a transfection control. Cells infected with *PLA2G2A* shRNA lentiviral constructs were compared with cells infected with a nontargeting shRNA construct as a transfection control.

Statistical Analysis

Cell viability and proliferation studies were performed in triplicate wells. The mean of all observations from a single experiment was compared with that of control cells on the same tissue culture plate and regarded as $n = 1$. Each experiment was repeated 3 times. LDH cytotoxicity studies were performed in duplicate wells, and the treatment groups were compared with both the control group and blank. Statistics were performed with analysis of variance with a Fisher post hoc analysis.

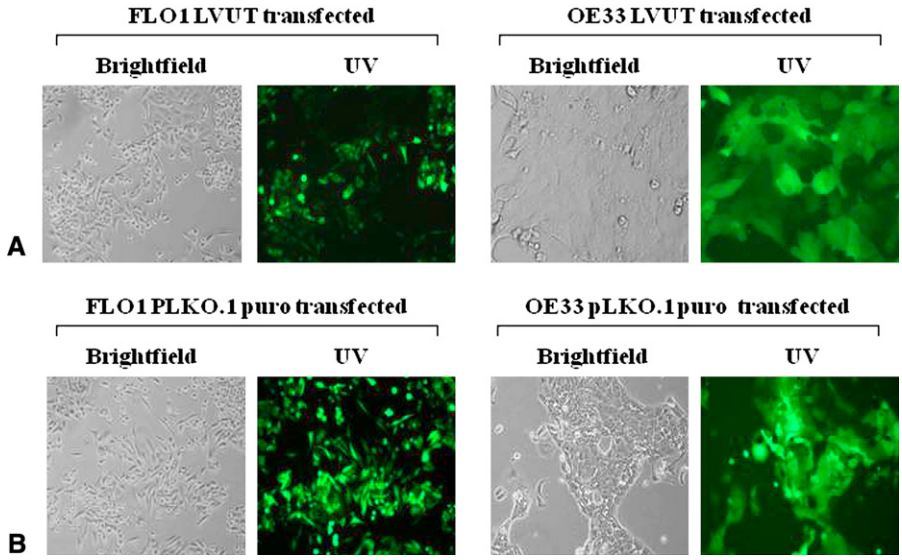

FIGURE 4. Infection of esophageal adenocarcinoma cells with lentiviral particles. Both FLO-1 and OE33 cells are efficiently infected with lentiviral particles derived from lentiviral expression vector pLVUT-tTR-KRAB *(LVUT)* and pLKO.1 puro constructs, as shown by green fluorescent protein expression under ultraviolet *(UV)* light *(green)*. Infection with lentiviral particles did not affect cell shape or size.

RESULTS

Expression of Group IIa $sPLA_2$ in Esophageal Cancer Cells

Immunofluorescence microscopy demonstrated that both OE33 and FLO-1 cells showed expression of $sPLA_2$ protein within the cytoplasm (Figure 1, *A*). Further, we confirmed that the cellular levels of $sPLA_2$ messenger RNA expression were similar between the 2 cancer cell lines according to RT-PCR. Representative PCR product bands from ethidium-stained gels as well as band densitometry are shown in Figure 1 (*B* and *C*). The $sPLA_2$ primers were verified with a plasmid containing the coding sequence for the *PLA2G2A* gene.

Inhibition of $sPLA_2$ Reduces Viable Cell Number and Cell Proliferation in OE33 and FLO-1 Cells

Treatment of both OE33 and FLO-1 esophageal adenocarcinoma cells with the $sPLA_2$ inhibitor produced a dose-dependent decrease in viable cell number across the dose range of 1 to 20 μmol/L. The 10- and 20-μmol/L doses produced 11% and 50% reductions in viable cell number, respectively, in FLO-1 cells (Figure 2, *A*). OE33 cells were more sensitive to $sPLA_2$ inhibition, showing 7%, 31%, and 69% reductions in viable cell number with 5-, 10-, and 20-μmol/L doses, respectively (Figure 2, *B*).

To examine whether the reduced viable cell number measured by the MTT assay reflected decreased proliferation, we quantified cellular proliferation with a BrdU incorporation assay. FLO-1 cells showed 20% and 70% decreases in proliferation relative to control with 10-μmol/L and 20-μmol/L doses of $sPLA_2$ inhibitor, respectively (Figure 3, *A*). Similarly, OE33 cells demonstrated 30% and 65% reductions in proliferation with 10- and 20-μmol/L doses, respectively (Figure 3, *B*). The $sPLA_2$ inhibitor did not cause any cell necrosis at any of the doses used in this study, as determined by LDH activity assay (data not shown).

Overexpression of Group IIa $sPLA_2$ Results in Augmented Growth of Esophageal Adenocarcinoma Cells

Both FLO-1 and OE33 cells were efficiently infected with LVUT lentiviral particles without effect on the health of the cells (Figure 4, *A*). FLO-1 and OE33 cells infected with the $LVUT^{PLA2G2A}$ particles showed 20-fold and 16-fold increases in expression of the enzyme, as shown by quantitative RT-PCR (Figure 5, *A*). Immunofluorescent staining showed increased group IIa $sPLA_2$ expression in cells infected with $LVUT^{PLA2G2A}$ particles (Figure 5, *B*). Overexpression of group IIa $sPLA_2$ in FLO-1 cells led to an 18% increase in number of viable cells and a 25% increase in cell proliferation. This effect was similar in OE33 cells, with a 16% increase in viable cell number and a 17% increase in cell proliferation (Figure 5, *C* and *D*).

Gene Knockdown of *PLA2G2A* Results in Attenuated Growth of Esophageal Adenocarcinoma Cells

Both FLO-1 and OE33 cells were efficiently infected with pLKO.1 puro lentiviral particles without affecting cell health (Figure 4, *B*). Infection with *PLA2G2A* shRNA viral particles led to 80% and 75% reductions in enzyme expression

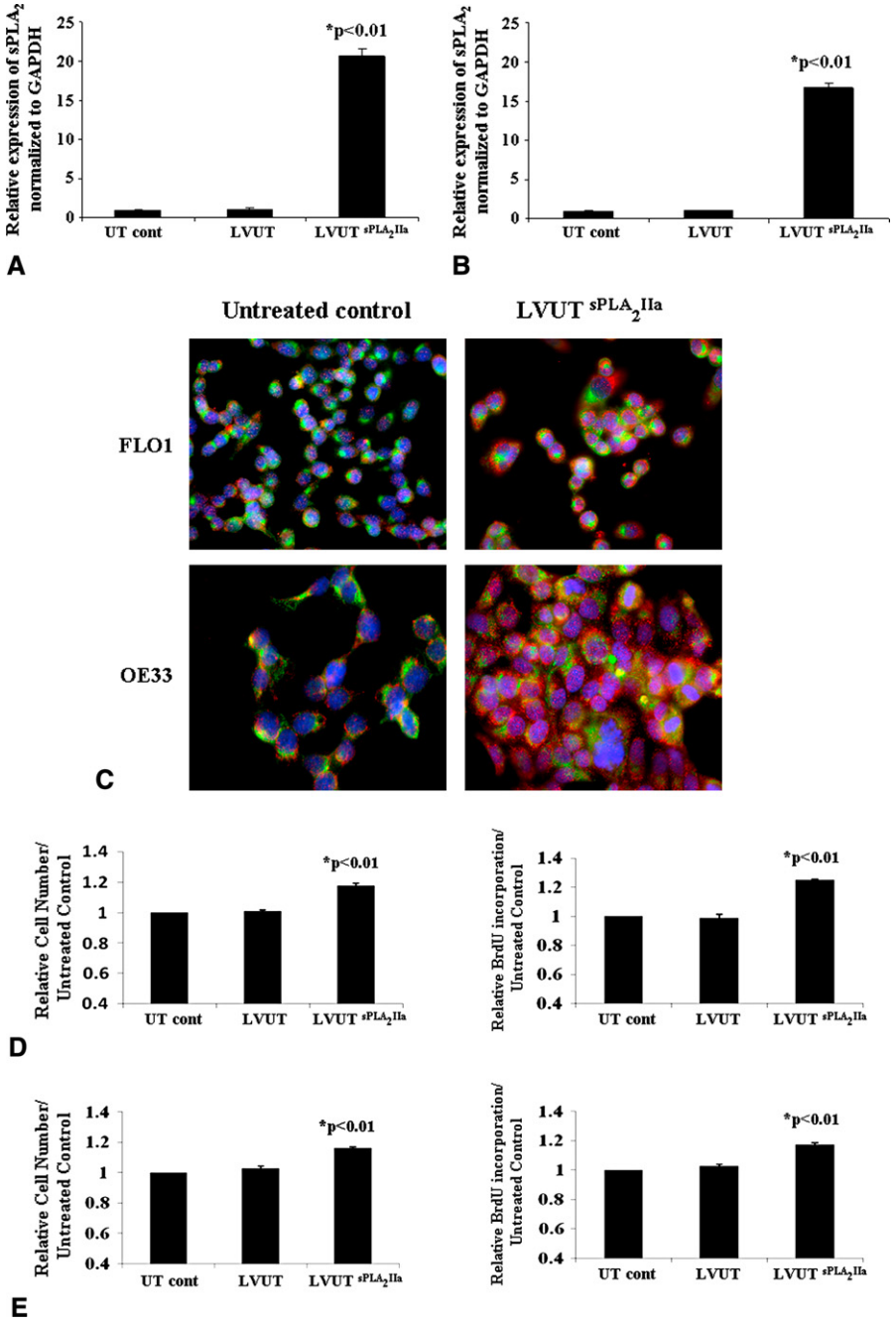

FIGURE 5. Overexpression of group IIa secretory phospholipase A_2 *(sPLA$_2$)* in esophageal adenocarcinoma cells augments cell growth. A, In quantitative reverse transcriptase polymerase chain reaction analysis of FLO-1 and OE33 cells infected with lentiviral expression vector pLVUT-tTR-KRAB *(LVUT)* with gene for secretory phospholipase A_2, viral particles show significant overexpression of group IIa secretory phospholipase A_2 relative to controls. B, Immunofluorescent microscopy of both FLO-1 and OE33 cells shows increased protein levels of group IIa secretory phospholipase A_2 *(red)* in cytoplasm of cells infected with viral particles derived from pLVUT-tTR-KRAB–secretory phospholipase A_2 gene construct. C, Viable cell number is increased in both FLO-1 and OE33 cells that overexpress group IIa secretory phospholipase A_2 relative to uninfected and pLVUT-tTR-KRAB–infected controls. D, FLO-1 and OE33 cells that overexpress group IIa secretory phospholipase A_2 demonstrate greater cell proliferation than controls. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *UT,* untreated; *BrdU,* bromodeoxyuridine.

in FLO-1 and OE33 cells, respectively, as shown by quantitative RT-PCR (Figure 6, *A*). Immunofluorescent staining showed decreased group IIa $sPLA_2$ protein levels in cells infected with pLKO.1 puro PLA2G2A shRNA particles (Figure 6, *B*), indicating effective knockdown of the gene. Knockdown of *PLA2G2A* expression in FLO-1 cells

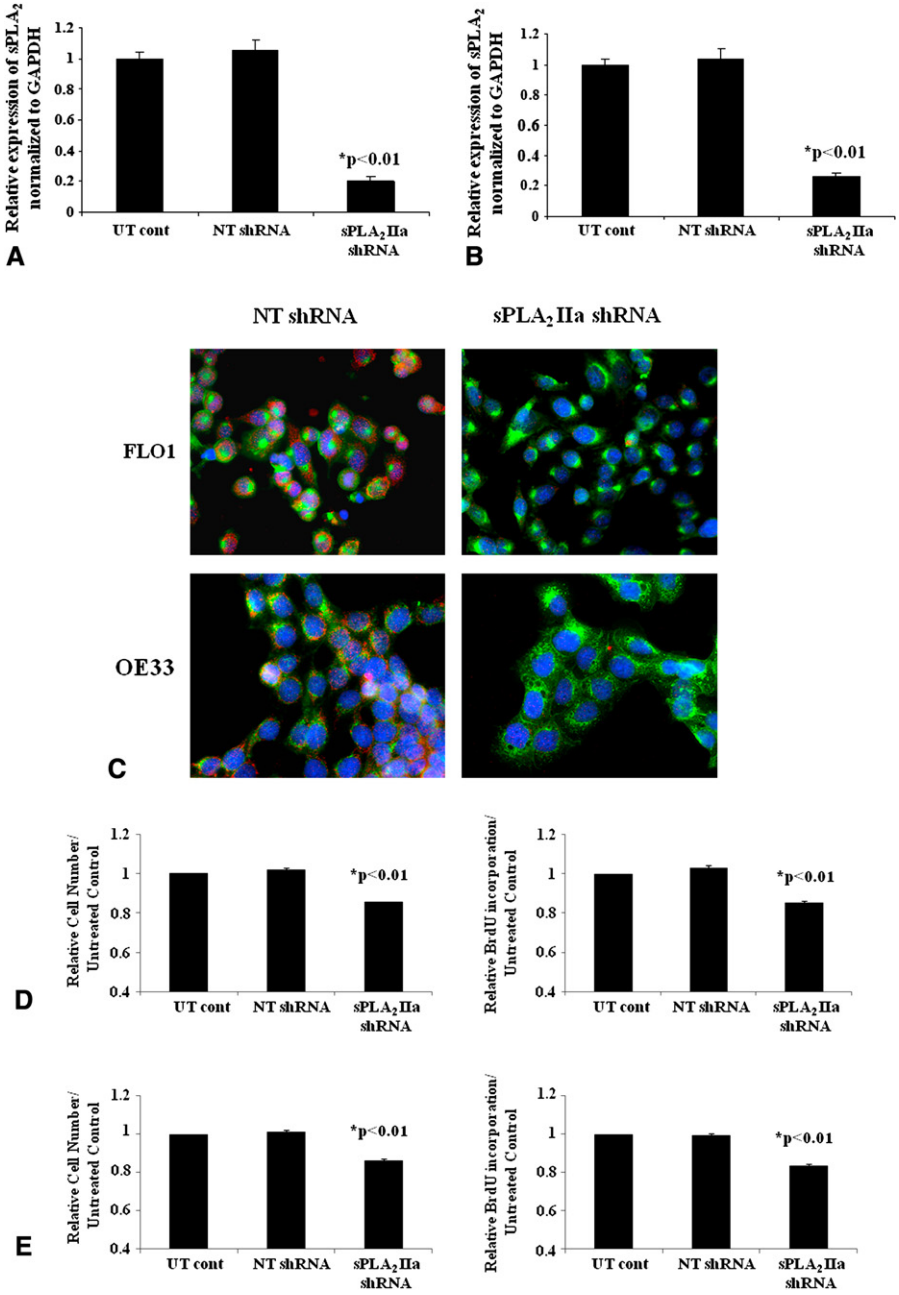

FIGURE 6. Genetic knockdown of group IIa secretory phospholipase A_2 *(sPLA$_2$)* attenuates growth of esophageal adenocarcinoma cells. A, Quantitative reverse transcriptase polymerase chain reaction analysis of FLO-1 and OE33 cells infected with group IIa secretory phospholipase A_2 short hairpin RNA *(shRNA)* viral particles shows significant reduction in expression relative to nontargeting *(NT)* and untreated *(UT)* controls. B, Immunofluorescent microscopy of both FLO-1 and OE33 cells shows decreased protein levels of group IIa secretory phospholipase A_2 *(red)* in cells infected with secretory phospholipase A_2 short hairpin RNA viral particles relative to nontargeting short hairpin RNA control. C, Viable cell number is diminished in both FLO-1 and OE33 cells that have genetic knockdown of group IIa secretory phospholipase A_2 transcript relative to uninfected and nontargeting controls. D, Knockdown of group IIa secretory phospholipase A_2 expression results in attenuated cell proliferation relative to uninfected and nontargeting short hairpin RNA controls. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *BrdU,* bromodeoxyuridine.

resulted in 15% reduction in viable cell number and cell proliferation. OE33 cells showed similar reductions in both cell number and proliferation after knockdown of *PLA2G2A* expression (Figure 6, *C* and *D*).

DISCUSSION

Overexpression of $sPLA_2$ protein has been found in several types of malignancy, including breast, ovarian, prostate, and pancreatic cancers[10]; however, the role of this enzyme in

the cancer cell is not clear. This study shows that group IIa $sPLA_2$ protein is expressed in the esophageal adenocarcinoma cell lines OE33 and FLO-1. With lentiviral infection techniques, we were able to create separate cell lines of both OE33 and FLO-1 cells that either stably overexpressed or minimally expressed the group IIa $sPLA_2$ enzyme. For the first time, we have illustrated the important role of this protein in growth regulation of esophageal adenocarcinoma cells by demonstrating that pharmacologic inhibition or genetic knockdown of this enzyme results in decreases in both viable cell number and cellular proliferation in esophageal adenocarcinoma cells. Furthermore, overexpression of this protein leads to augmented cell growth, as indicated by viable cell number and proliferation.

Our results accord with those of an earlier study that looked at the effect of overexpression of $sPLA_2$ on colon tumor cells.[11] In that study, the authors transfected colon tumor cells with *PLA2G2A* and found that cells overexpressing the enzyme developed into larger, more invasive tumors than vector controls when injected into nude mice.[11] In contradistinction, the homologous murine *Pla2g2a* gene has been shown to confer resistance to intestinal tumorigenesis.[12,13] Additionally, elevated expression of the enzyme inhibits gastric cancer invasion and metastasis.[14] The discrepancy between these studies likely has to do with differences in tumor histology and level of enzyme expression. Group IIa $sPLA_2$ has both inflammatory and anti-inflammatory properties, which could also contribute to these differences.[7]

Overexpression of group IIa $sPLA_2$ is often accompanied by an increase in production of COX-2, an enzyme that catalyzes the synthesis of bioactive prostaglandins from arachidonic acid.[15,16] COX-2 has been implicated in the development and progression of cancers of the gastrointestinal tract, including colorectal, gastric, and esophageal adenocarcinomas.[17] Prostaglandin E_2 (PGE_2), a downstream product of COX-2, is the most important COX-2–derived prostaglandin involved in oncogenesis. It has been shown to play roles in tumor cell proliferation, invasion, angiogenesis, and immunosuppression.[18] Several cell culture studies have illustrated the roles of COX-2 and PGE_2 in cellular proliferation in esophageal cancer.[19-21] One study showed that cultured esophageal squamous cell cancer cells expressed high levels of the PGE_2 receptor EP_2. Those authors were also able to show significant reduction in cellular proliferation with pharmacologic blockade or RNA knockdown of the EP_2 receptor.[20] Other authors have used OE33 cells to show that treatment with an agonist of the EP_2 receptor is able to prevent apoptosis and enhance cellular migration.[19] It would be reasonable to speculate that the finding of decreased cellular proliferation after treatment with an inhibitor of group IIa $sPLA_2$ could be directly related to decreased production of PGE_2.

Arachidonic acid liberated by group IIa $sPLA_2$ can also be converted to mitogenic leukotrienes by 5-lipoxygenase (5-LOX). 5-LOX has been shown to be important in the development of esophageal adenocarcinoma in vivo. The expression of 5-LOX was increased in esophageal tumors in both human samples and samples from rats that had been exposed to surgically induced mixed reflux. Furthermore, incidence of tumor development was decreased by 36% in animals with reflux treated with zileuton, a specific inhibitor of 5-LOX.[22] Another group showed that several esophageal cancer cell lines express 5-LOX and that inhibition of this enzyme leads to decreased production of leukotriene B_4 and increased rates of apoptosis.[23] It is possible that some of the decrease in viable cell number seen with inhibition of group IIa $sPLA_2$ in our study is related to decreased leukotriene B_4 production and increased apoptosis.

Group IIa $sPLA_2$ has also been shown to modulate cellular proliferation in arachidonic acid–independent mechanisms. Xie and colleagues[24] found that inhibition of hepatocellular carcinoma cells with the $sPLA_2$ inhibitor 12-epi-scalaradial blocked epidermal growth factor receptor–regulated activation of *Akt,* the phosphorylation of which can lead to increased cellular proliferation or decreased apoptosis.[24] This was found to act in an arachidonic acid–independent fashion. The authors of that paper also found scalaradial to inhibit phosphorylation of the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2, which has been shown to regulate cellular proliferation in several cell types.[25]

The observations in this study suggest that group IIa $sPLA_2$ is an important contributor to cellular proliferation in esophageal adenocarcinoma cells. To our knowledge, this is the first report of pharmacologic inhibition and genetic knockdown of group IIa $sPLA_2$ attenuating proliferation in esophageal adenocarcinoma cells. Further animal studies are needed to assess the importance of this enzyme in vivo; however, group IIa $sPLA_2$ remains a potential target for treatment of malignancy.

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Discussion

Dr Ross M. Bremner *(Phoenix, Ariz).* I thank Dr Mauchley and colleagues for providing me with a manuscript well in advance of the meeting, but even more for providing me with a new and improved manuscript just this morning. This manuscript contains all their latest data, and it is a tribute to the Society that the opportunity of presenting this work today has stimulated Dr Mauchley and colleagues to produce another set of fairly intricate experiments to greatly add to this article. In fact, the manuscript is quite impressive.

The group has previously, as we were reminded this morning, taught us that this enzyme, $sPLA_2$, is important in reflux disease. This morning they have answered a question regarding whether this enzyme is also important in mature adenocarcinoma cells.

The study was clearly planned and expertly performed, but I do have a few questions. The first question regards the specificity of the inhibitor. Dr Mauchley, how have you convinced yourselves that the inhibitor is working at the doses you used by looking at downstream indicators such as LOX or COX activity or leukotriene or prostaglandin production?

Dr Mauchley. First, I thank you for your comments. To answer your first question, this inhibitor was developed by a group in Australia. According to 2 of their initial publications, they determined that it was a group IIA–specific inhibitor by testing it against human recombinant group IIA $sPLA_2$ as well as a few of the other isoforms.

With respect to looking at downstream players in the whole $sPLA_2$ signal transduction pathway, we have not done that yet, although we plan to do so. In particular, I think that it is important to look at PGE_2 and leukotriene B_4 production. We have collaborated with a lipid group at the university that does mass spectrometry to help us look into this.

Dr Bremner. I think that will be important. The studies showed that the inhibition of $sPLA_2$ decreased the number of viable cells in your cultures. Do you think that this is really a proapoptotic effect, or do you think that this is cell cycle arrest?

Dr Mauchley. That is a good question. So far, most of our work in this area has been to assess proliferation; however, we do have some early preliminary results with the FLO-1 cells that indicate that this is more a proliferative process and less of an apoptotic process. We used flow cytometry to try to stain for apoptotic cells treated in a similar fashion and found very little difference.

Dr Bremner. This latest set of experiments that you did transfecting cells with $sPLA_2$ and then having a group of experiments with knockdown of $sPLA_2$ was very interesting. In your knockdown group, though, I was surprised that you showed only a modest reduction in cell proliferation, about 15%. Did you consider this disappointing? Because it seems that blocking $sPLA_2$ is not really essential for cell proliferation, have you considered adding a sequential downstream inhibitor, such as a LOX or COX-2 inhibitor, to see what the effect would be?

Dr Mauchley. First, I was not discouraged at all by the findings. Although we showed efficient transfection with the *GFP*-containing construct, it was impossible to know whether transfection was as efficient with the *PLA2G2A* construct. This could explain the modesty of the reduction.

With respect to using other inhibitors, we have talked about that in the past. We have experiments planned but have not conducted any at this point.

Dr Bremner. Finally, your studies really sidestep the question of how this inhibitor would work in an animal system. Is this inhibitor toxic at all, and do you plan animal experiments to see how it works on a tumor model in a mouse, for example?

Dr Mauchley. Yes, we are in the process of getting a protocol amendment approved so that we can inject these cells into nude mice subcutaneously and assess their growth. The inhibitor that we use is actually orally bioavailable, and in some of our previous work we were able to get significant inhibition in vivo by gavage-feeding mice with the inhibitor. We are planning on feeding the inhibitor to mice to see whether we can attenuate growth in vivo as well as to use the transfected cell lines to see whether we can get increased growth with overexpression.

Dr Bremner. Fascinating work. Thank you very much.