Oncogenic Action of Secreted Phospholipase A<sub>2</sub> in Prostate Cancer

Paul Sved, Kieran F. Scott, Duncan McLeod, Nicholas J. C. King, Jas Singh, Tania Tsatsralis, Blagoy Nikolov, John Boulos, Laxman Nallan, Michael H. Gelb, Mila Sajinovic, Garry G. Graham, Pamela J. Russell, and Qihan Dong

1Department of Medicine and Sydney Cancer Centre, 2Institute of Biomedical Research, School of Biomedical Sciences and Pathology, The University of Sydney, Australia; 3St Vincent’s Hospital Clinical School, School of Medical Sciences and Department of Clinical Medicine, The University of New South Wales, Australia; 4Department of Anatomical Pathology, Royal Prince Alfred Hospital, Sydney, Australia; 5Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington; 6Oncology Research Centre, Prince of Wales Hospital, Sydney, Australia; and 7Therapeutics Centre, St. Vincent’s Hospital, Sydney, Australia

INTRODUCTION

Previous studies have demonstrated that the eicosanoid pathway is activated in prostate cancer, and cyclooxygenase (COX) and lipoxygenase (LOX) products contribute to the progression of the disease via promoting cell proliferation, motility, invasion, and angiogenesis (1–4). The underlying mechanism leading to eicosanoid pathway activation remains to be elucidated.

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) catalyzes the hydrolysis of membrane glycerophospholipids, leading to production of free fatty acids and lysophospholipids. If the esterified fatty acid is arachidonic acid, this is converted in the prostate to prostaglandins by COX or hydroxylated.

Received 9/24/03; revised 6/7/04; accepted 7/19/04.

Grant support: Department of Veteran Affairs grant 302082 (Q. Dong, G. Graham, P. Russell), New South Wales Cancer Council grant RG4602 (Q. Dong, J. Singh), National Health and Medical Research Council grant 222870 (K. Scott), and NH and NH grants HLS0040 and HLS025 (M. Gelb).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: P. Sved and K. Scott contributed equally to this work.

Requests for reprints: Qihan Dong, Department of Medicine, D06, University of Sydney, NSW 2006, Australia. Phone: 612-95155186; Fax: 612-95161273; E-mail: qhd@med.usyd.edu.au.

©2004 American Association for Cancer Research.

18 U.S.C. Section 1734 solely to indicate this fact.

ABSTRACT

Mortality from prostate cancer is associated with progression of tumors to androgen-independent growth and metastasis. Eicosanoid products of both the cyclooxygenase (COX) and lipoxygenase (LOX) pathways are important mediators of the proliferation of prostate cancer cells in culture and regulate tumor vascularization and metastasis in animal models. Pharmacologic agents that block either COX or LOX products effectively reduce the size of prostate cancer xenografts. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes regulate the provision of arachidonic acid to both COX- and LOX-derived eicosanoids, and a secreted form of the enzyme (sPLA<sub>2</sub>-IIA) is elevated in prostate cancer tissues. Here, we show by immunohistochemistry, in patients receiving androgen ablation therapy, that sPLA<sub>2</sub>-IIA remains elevated in remaining cancer cells relative to benign glands after treatment. Furthermore, sPLA<sub>2</sub>-IIA expression seen in benign glands is substantially decreased after androgen depletion, whereas cytosolic PLA<sub>2</sub>-α (cPLA<sub>2</sub>-α) levels are unchanged. sPLA<sub>2</sub>-IIA mRNA expression is detectable and inducible by androgen (0.01–10 nmol/L) in the androgen-sensitive cell line LNCaP, and exogenous addition of sPLA<sub>2</sub>-IIA (1–100 nmol/L), but not an inactive sPLA<sub>2</sub>-IIA mutant (H<sub>48</sub>Q), results in a dose-dependent increase in cell number or the fraction of cells in G<sub>2</sub>-M phase, which is inhibited by sPLA<sub>2</sub>-IIA-selective inhibitors. The effect of exogenous sPLA<sub>2</sub>-IIA can also be blocked by inhibition of cPLA<sub>2</sub>-α, suggesting a role for cPLA<sub>2</sub>-α in mediating sPLA<sub>2</sub>-IIA action. sPLA<sub>2</sub>-IIA inhibitors suppressed basal proliferation in LNCaP cells and in the androgen-independent, sPLA<sub>2</sub>-IIA-positive cell line PC3 but not in the sPLA<sub>2</sub>-IIA-negative androgen-independent prostate cell line DU145. Established PC3 xenograft tumors grew more slowly in mice treated with sPLA<sub>2</sub>-IIA inhibitors than those treated with saline only. The PLA<sub>2</sub> enzymes, and sPLA<sub>2</sub>-IIA in particular, thus represent important targets for the treatment of sPLA<sub>2</sub>-IIA-positive androgen-independent prostate cancer.

MATERIALS AND METHODS

Reagents. sPLA<sub>2</sub>-IIA was purified from conditioned media produced by a Chinese hamster ovary cell line (SA2) stably transfected with the human sPLA<sub>2</sub>-IIA cDNA, as described previously (10), and quantified by enzyme-linked immunosorbent assay (11). sPLA<sub>2</sub>-IIA contained <0.1 ng of endotoxin per milligram of protein (Limulus amebocyte lysate pyrochrome assay; Associates of Cape Cod, Falmouth, MA) and was enzymatically active in a [3H]arachidonate-labeled Escherichia coli membrane assay (10). Construction by site-directed mutagenesis, expression, purification, and characterization of the sPLA<sub>2</sub>-IIA mutant enzyme H<sub>48</sub>Q will be described elsewhere. H<sub>48</sub>Q was quantified by enzyme-linked immunosorbent assay (11). The mutant protein had no detectable enzyme activity in our E. coli membrane assay, but an activity of 2 to 4% of wild-type was reported in other studies (12). Two cyclic peptide sPLA<sub>2</sub>-IIA inhibitors were synthesized using N-9-(fluorenylimino)octoxycarbonyl solid phase chemistry (Auspep, Melbourne, Australia) as described previously (10). c(2Nap)LS(2Nap)R is at least 10-fold more potent in binding sPLA<sub>2</sub>-IIA and inhibiting sPLA<sub>2</sub> enzyme activity than cFLSRY. Peptides for in vivo use were exchanged into acetate buffer before lyophilization. The cPLA<sub>2</sub>-α inhibitor pyrrolidine-1 was synthesized as described previously (13). Polyclonal anti-sPLA<sub>2</sub>-IIA (160502; Cayman Chemicals, Sydney, Australia) and anti-cPLA<sub>2</sub>-α (SC-438; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased. The monoclonal anti-sPLA<sub>2</sub>-IIA antibody 4A1 was raised and purified (Bioquest, Ltd., Sydney, Australia) as described previously (11).

Immunohistochemistry. Prostate tissues were all fixed in buffered 10% formaldehyde solution for approximately 24 hours and paraffin-embedded. Immunohistochemistry was performed on tissue sections (5 µm) using an avidin-biotin complex method with diaminobenzidine as a chromogen as described previously (14). The sections were subjected to microwave epitope retrieval before staining. Immunostaining was considered positive and specific when the intensity of staining with the post-immune IgG (isotype control) and with no IgG (method control). Specific staining was graded as described previously (15). In brief, the staining intensity was graded as low, intermediate, and high. The percentage of cells with the highest staining intensity was stratified into three scores:

Conventional end-time PCR was performed at an annealing temperature of 62 °C. LNCaP, DU145, and PC3 cells was isolated and reverse transcribed as above. Hypoxanthine phosphoribosyltransferase was used as the house-keeping gene.

Charcoal-Stripped Fetal Bovine Serum. Activated charcoal (40 g; 250–350 mesh; Sigma) was mixed with distilled water (1 L) and soaked for 1 hour at room temperature, and floating charcoal was removed. The soaking process was repeated until all floating charcoal was removed. Charcoal was then soaked in a minimal volume of acetone and allowed to dry by evaporation overnight in a fume hood. Dried charcoal was suspended in FBS to 0.4 mg/mL, incubated at 55°C for 10 minutes, and centrifuged for 5 minutes at 2000 × g; and the supernatant was filtered through a 0.2-μm filter. Because FBS has a low concentration of sex steroids, which makes it difficult to determine the efficiency of charcoal stripping, pooled human serum was used for validation of our protocol for charcoal stripping. Relative to unstripped serum, the concentration of testosterone, estradiol, and progesterone in charcoal-stripped serum was under the detection limit by competitive immunoassay (IMMULITE 2000; DPC, Los Angeles, CA), whereas the concentration of luteinizing hormone and follicular stimulating hormone, representatives of larger Mr. proteins, were unchanged by microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL).

Reverse Transcription-Polymerase Chain Reaction. The effect of added androgen on the steady-state level of sPLA2-IIA mRNA in LNCaP cells was measured by quantitative real-time reverse transcription-PCR. After cell treatments, total RNA was isolated using Trizol reagent. The first-strand cDNA was synthesized from 2 μg of total RNA using a combination of random hexamers and oligo(dT) as described previously (16). Primers were designed based on the human sPLA2-IIA mRNA (NM_000300). Forward, 5’-TTTGGT-CACCCAGAAGACTCTTAC-3’; Reverse, 5’-GGGAGGGAGGGTATGAGA-3’. Hypoxanthine phosphoribosyltransferase was used as the house-keeping gene, and its primer sequences were published previously (14). The PCR reaction (15 μL) contained 5.5 μL of water, 7.5 μL of 2× platinum quantitative PCR superMix-UDG (Invitrogen, Melbourne, Australia), 0.25 μL each of 0.01 μm/L primers, 0.5 μL of 25× SYBR Green 1 dye (Molecular Probe, Sydney, Australia), and 1 μL of cDNA. The following protocol was used on ABI PRISM 7700: 50°C for 2 minutes; 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. After verification of parallelism in amplification efficiency (<10% variation in slopes on plots of crossing point cycles versus cDNA concentration) between sPLA2-IIA and hypoxanthine phosphoribosyltransferase, the ΔΔ method was used to calculate relative changes in mRNA levels of sPLA2-IIA corrected for hypoxanthine phosphoribosyltransferase (HPRT) (17). For endogenous sPLA2-IIA mRNA level, total RNA from LNCaP, DU145, and PC3 cells was isolated and reverse transcribed as above. Conventional end-time PCR was performed at an annealing temperature of 55°C.

Cell Proliferation Assay. Cells were plated at 1 × 10^4 per well in 96-well plates with 0.1 mL of media. Treatments were performed on cells at 70 to 80% confluence, in medium containing 5% FBS for 72 hours. After treatment, the number of viable cells was determined with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Sydney, Australia). In brief, 20 μL of solution was added to each well and incubated for 1 hour. The absorbance at 490 nm was measured with a microplate reader (Multiscan EX; Labsystems, Helsinki, Finland). Each experiment was performed in quadruplicate and repeated at least three times.

Flow Cytometric Analysis. LNCaP cells were seeded in 50-μL flasks and grown to 70 to 80% confluence before treatment for 72 hours. After trypsinization and cell counting, the treated or untreated cells (1 × 10^6) were suspended in PBS (1 mL) and incubated after the addition of 200 μL of Triton X-100 (0.4% stock) for 5 minutes at room temperature in the presence of 50 μL of propidium iodide solution (1 mg/mL stock) and 20 μL of RNase A Type I-A (50 mg/mL stock; Sigma-Aldrich). DNA content per cell was measured by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Analysis was performed on 10,000 events per sample.

PC3 Xenograft Study in Nude Mice. Animal studies were performed according to the guidelines for the ethical use of animals published by the National Health and Medical Research Council of Australia and with the approval of the University of New South Wales Animal Ethics Committee. Four- to 6-week-old athymic male nude mice (BALB/c) were inoculated subcutaneously with 1 × 10^6 PC3 cells. The cells were suspended in 0.1 mL of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum before injection and injected via a 26-gauge needle. When the size of the xenograft reached 5 × 5 mm (tumor volume, 65.5 mm^3 calculated as V = π/6d1d2^3/2; ref. 18), mice were randomly assigned into four groups (n = 15 per group), i.e., cFLSYR (1 mg/kg), cFLSYR (10 mg/kg), 2-naphthylalanine (c[2NapA]LS[2NapA]R; 1 mg/kg), and saline. cFLSYR was dissolved directly in sterile saline, whereas c[2NapA]LS[2NapA]R was diluted into sterile saline from a stock solution (40 mg/mL in dimethylformamide) immediately before use. The saline group received sterile saline containing 0.75% dimethylformamide. Subcutaneous injections were given three times weekly on the opposite flank to the xenograft site, and treatments were blinded to experimentalists throughout the study. Tumor growth was normalized for differing starting sizes by calculating relative tumor volume, i.e., the ratio of each tumor volume on a given day to its volume on day 1 of treatment. The mice were treated for a period of 8 weeks unless culled due to tumor volume reaching 15 × 15 mm (1768 mm^3).

Statistical Analysis. The Number Cruncher Statistical System (NCSS, Kaysville, UT) was used for statistical analysis. Data were analyzed by χ^2 or analysis of variance (ANOVA) as appropriate. A two-tailed P value <0.05 was considered significant.

RESULTS AND DISCUSSION

Secreted Phospholipase A2-IIA Expression Is Constitutively Activated in the Prostate Cancer Cells Remaining after Androgen Ablation Therapy. We searched the database of Serial Analysis of Gene Expression (SAGE) to determine the number of transcripts coding for individual PLA2 enzymes in prostate cancer. The cDNA libraries used for SAGE were PR317 normal prostate and PR317 prostate cancer, respectively, because both are derived from microdissected prostate tissues. We found that sPLA2-IIA mRNA was 22 times higher in prostate cancer than normal prostate, whereas other members of the sPLA2. (–, III, –II,F, –IIF, –IIIF, –III, -V, -X, -XII, and -XIII, also known as XIIB), cPLA2 (–α, –β, and –γ), intracellular PLA2 (–A), and platelet-activating factor acetylhydrolase families were either unchanged or not present. There are three published reports describing an increased sPLA2-IIA mRNA (19) and protein levels (20, 21) in prostate cancer tissue and the enhanced sPLA2-IIA expression was inversely related to 5-year patient survival (22). The chromosomal location of sPLA2-IIA (1p35-ter) was found to overlap with a prostate cancer susceptibility locus CAPB (22). To verify the SAGE results and to extend them to cancer cells in the absence of androgens, we examined, immunohistochemically, sPLA2-IIA expression in prostate cancer tissues from patients treated with androgen-ablation therapy for 3 months (100 mg of cyproterone acetate, twice daily) before radical prostatectomy. An undetectable serum prostate-specific antigen was confirmed in each patient before radical prostatectomy. Although the remaining cancer cells in these specimens are regarded qualitatively as being androgen independent, it is likely that the degree of androgen independence varies among these cells. Two antibodies were used for immunohistochemistry, and both showed the same staining pattern on consecutive sections. In the presence of androgens (n = 50), there was patchy cytoplasmatic staining in benign glands (Fig. 8).

Internet address: www.ncbi.nlm.nih.gov/SAGE.

6935
Fig. 1. Immunohistochemical analysis of sPLA$_2$-IIA in prostate tissues. In each panel, the left section is stained with hematoxylin and eosin, and the right section is stained with polyclonal anti-sPLA$_2$-IIA antibody (Cayman Chemicals). A, tissue sections from a patient without androgen-ablation therapy showing cytoplasmic brown staining in normal glands. B, sections from a patient without androgen-ablation therapy showing cytoplasmic brown staining in cancer cells. C, sections from a patient after androgen ablation therapy showing both normal (no staining) and neoplastic (brown staining) tissue. Magnification, ×40.
1A) adjacent to cancer cells and extensive cytoplasmic staining in cancer cells (Fig. 1B). With androgen ablation therapy (n = 26), benign glands showed substantially decreased staining, whereas cancer cells maintained sPLA2-IIA expression (Fig. 1C). The percentage of stained cancer and of adjacent benign glands was then stratified into three scores (Fig. 2). No difference was found in immunohistochemical staining of cPLA2-α between normal and cancer cells irrespective of androgen status (data not shown).

The lack of sPLA2-IIA expression in benign glands after androgen deprivation (Fig. 2) suggests that expression of sPLA2-IIA gene requires androgens. To verify that, we first searched the 5'-flanking region of the sPLA2-IIA gene using MatInspector Release 5.3 (Genomatix, München, Germany) and found an androgen response element GAGGTAAATGGTATTCTC from −546 to −527. Second, we treated the androgen-responsive cell line, LNCaP, with metabolically stable synthetic androgen (methyltrienolone; R1881; 0.01–10 nmol/L) for 4, 8, and 24 hours and measured sPLA2-IIA mRNA by real-time PCR. Indeed, there was a significant increase in the steady-state mRNA level of sPLA2-IIA at 8 hours (Fig. 3). However, we could not find an androgen response element within 3 kb of the 5'-flanking region of the cPLA2 genomic DNA. Androgen treatment had no effect on cPLA2 mRNA by real-time PCR (data not shown). Together, these findings suggest that expression of sPLA2-IIA but not cPLA2 is likely to be dependent on androgens in normal prostate. In cancer cells remaining after androgen ablation therapy, sPLA2-IIA expression is constitutively active via an as yet unknown mechanism.

**Oncogenic Action of Secreted Phospholipase A2-IIA in Prostate Cancer Cells.** To examine the biological relevance of constitutively expressed sPLA2-IIA to cell growth, we treated LNCaP cells with various doses of human recombinant sPLA2-IIA purified by immunoaffinity chromatography and monitored cell growth. We observed a consistent and dose-dependent stimulatory effect of sPLA2-IIA on LNCaP cell growth as measured by the cell proliferation assay at doses as low as 1 nmol/L (Fig. 4A). This potent effect was blocked by the sPLA2-IIA inhibitor, cFLSYR, at a 1:1 molar ratio of inhibitor to added enzyme (Fig. 4B).

![Image](image-url)

**Fig. 2.** Summary of immunohistochemical analysis. The extent of sPLA2-IIA staining in benign glands (I) or cancer cells (II) from patients with (+, n = 26) or without (−, n = 50) androgen ablation therapy was graded on a three-point score. (1, 0–33% cells positive; 2, 33–66% cells positive; and 3, >66% cells positive). Data are presented as the percentage of cases at each score. *P < 0.05 versus benign glands without androgen ablation therapy by χ² analysis.

**Fig. 3.** Treatment of LNCaP cells with androgen-induced steady-state mRNA level of sPLA2-IIA. LNCaP cells were grown in phenol red-free RPMI with 5% charcoal-stripped FBS and treated for 72 hours in media containing zero or increasing concentrations of R1881. mRNA levels of sPLA2-IIA in treated cells relative to untreated control cells were determined by real-time reverse transcription-PCR. The data are shown as mean ± SD of triplicate determinations normalized to 100% for untreated cells and are representative of two independent experiments. *P < 0.001 versus untreated cells by one-way ANOVA.

**Fig. 4.** Effect of addition of sPLA2-IIA on prostate cell growth. LNCaP cells were grown in RPMI with 5% FBS and treated for 72 hours in media containing increasing concentrations of sPLA2-IIA alone (A: ■) or a fixed concentration of sPLA2-IIA and increasing concentrations of the sPLA2-IIA inhibitor cFLSYR (B). Cell number relative to untreated control cells was determined by cell proliferation assay. Data are mean ± SD of quadruplicate determinations normalized to 100% for untreated cells. A490 of unstimulated cells was 0.29 ± 0.06 in A and 0.24 ± 0.03 for B. *P < 0.05 versus untreated control by one-way ANOVA. Data are representative of three separate experiments. C, flow cytometric analysis of DNA content. LNCaP cells were grown as above and stimulated for 72 hours in the absence (dotted lines) and presence (solid lines) of the sPLA2-IIA (100 nmol/L). D, same as C except that the solid lines represent the treatment of both sPLA2-IIA (100 nmol/L) and sPLA2-IIA inhibitor cFLSYR (100 nmol/L).

![Image](image-url)

We next used flow cytometric analysis to determine how sPLA2-IIA affects the distribution of LNCaP cells in different phases of the cell cycle. In sPLA2-IIA–containing medium, the proportion of LNCaP cells in the G1 phase decreased from 74% to 62% with a corresponding increase of cells in G2-M phase in comparison with untreated cells (Fig. 4C). In the presence of both sPLA2 and its inhibitor...
Currently, two models are proposed to explain the action of sPLA2 on dose required to block arachidonate release in a number of cPLA2 -molar to low micromolar range of pyrrolidine-1 is consistent with the proliferation (Fig. 5). Growth-promoting sPLA2-IIA is constitutively expressed in prostate from the G1 phase. To determine whether sPLA2-IIA-induced prostate cancer cell proliferation depends on its enzyme activity, H4Q purified by immuno-affinity chromatography was used to treat LNCaP cells. Over the same dose range as the wild-type sPLA2-IIA, H4Q, which has no more than 2 to 4% of wild-type activity (12), had no growth-promoting effect (Fig. 5A), demonstrating that sPLA2-IIA enzyme activity and thus its products are required for the proliferative effect of sPLA2-IIA. To evaluate whether cPLA2-α mediates exogenously added sPLA2-IIA action, we treated LNCaP cells with various doses of the selective cPLA2-α inhibitor pyrrolidine-1 (13) with or without a constant effective dose of sPLA2-IIA (1 nmol/L). Blockade of cPLA2 abolishes sPLA2-IIA-induced cell growth completely, demonstrating that cPLA2-α activity is necessary for sPLA2-IIA-dependent prostate cancer cell proliferation (Fig. 5B). Furthermore, the dose response at sub-micromolar to low micromolar range of pyrrolidine-1 is consistent with the dose required to block arachidonate release in a number of cPLA2-α-expressing cells (13). Considering the common loss of annexins 1 also contribute to the observation. Additional work will examine the inhibition was weak in in vitro activity assays. We have recently designed two novel cyclic peptides (10), cFLSYR and a cyclic peptide in which F and Y are substituted with c(2Nap)LS(2Nap)R. Both have shown significant improvement in potency over linear peptides in sPLA2-IIA inhibition assays and do not bind to a structurally related enzyme sPLA2-IB. To test the effect of blocking endogenous sPLA2-IIA on cell growth, we first determined the basal mRNA levels of sPLA2-IIA in three human prostate cancer cell lines. mRNA encoding sPLA2-IIA is found in DU145 and PC3 cells (Fig. 6A). We then tested the effect of individual inhibitors cFLSYR and c(2Nap)LS(2Nap)R on cell growth over a range of doses (1–100 nmol/L). LNCaP and PC-3 cell numbers were significantly decreased in the presence of inhibitor, and the smallest effective dose was 1 nmol/L (Fig. 6B). In contrast, neither of the inhibitors had an effect on DU145, presumably due to the lack of endogenous sPLA2-IIA. It is interesting to note the difference in response of LNCaP cells to the sPLA2 inhibitors between Figs. 4 and 6. In the presence of exogenous sPLA2-IIA in Fig. 4, cFLSYR did not reduce cell number below that of untreated cells at concentrations up to 100 nmol/L. However, in Fig. 6, c(2Nap)LS(2Nap)R at 1 nmol/L results in ~40% fewer cells than in control. Recent studies have shown that the effects of exogenous sPLA2-IIA are mechanistically separable from those of endogenous intracellular sPLA2-IIA in model cell systems (27). It is thus possible that sPLA2-IIA may modulate cell growth by separate mechanisms depending on its cellular location. Also, the difference in potency between the two inhibitors (see Materials and Methods) may also contribute to the observation. Additional work will examine the detailed mechanism underlying the effect of both inhibitors in the presence or absence of exogenous sPLA2-IIA.

In vivo, cFLSYR, at either 1 mg/kg or 10 mg/kg, and

(cFLSYR), the proportion of cells in G1 and G2 phase returned to basal levels (Fig. 4D). These results suggest the biological importance of the highly expressed sPLA2-IIA in prostate cancer and demonstrate that the sPLA2-IIA–induced cell proliferation can be attributed at least partly to an increased proportion of cells entering the G2-M phase from the G1 phase.

Oncogenic Action of Exogenous Secreted Phospholipase A2-IIA Requires the Activity of Both Secreted Phospholipase A2-IIA and Cytosolic Phospholipase A2-α. In contrast to the essential in vivo role of cPLA2-α in eicosanoid production under most physiologic conditions and in response to inflammatory stimuli, whether and if so, how sPLA2-IIA increases eicosanoid production in vivo is unclear. Currently, two models are proposed to explain the action of sPLA2 on eicosanoid production in cellular systems (23). In the first model, sPLA2 binds directly to membrane phospholipids, and the released arachidonic acid serves as a substrate to produce eicosanoids. Lysophospholipids mediate indirect activation of endogenous cPLA2-α via mobilization of calcium. The enhanced cPLA2-α activity can in turn cause an increased production of eicosanoids. The second model is indirect modulation of intracellular eicosanoid pathways via cell surface glycosyl phosphatidyl inositol (GPI)-linked heparan sulfate proteoglycan receptors. The internalized sPLA2 supplies arachidonic acid to downstream enzymes either directly or indirectly via activation of cPLA2-α through mitogen-activated protein kinase–mediated phosphorylation. Enzyme activity of sPLA2 is not obligatory for activation of the mitogen-activated protein kinase pathway.
c2(NapA)LS2(NapA)R at 1 mg/kg were injected three times a week subcutaneously to the flank opposite to the site of the PC3 xenograft. The treatment commenced only when the xenograft had reached the size of 5 × 5 mm. We found that inhibitor c2(NapA)LS2(NapA)R at 1 mg/kg and inhibitor cFLSYR at 10 mg/kg slowed the rate of growth of tumors from 5 weeks post-treatment commencement resulting in significant suppression of PC3 xenograft volume by 8 weeks of treatment compared with saline-treated mice (Fig. 7). cFLSYR had no effect at 1 mg/kg (data not shown). This in vivo order of potency of the two peptides correlates well with their relative potencies in in vitro enzyme activity assays (10).

There were no visible side effects of either treatment including eating, drinking, and general behavior and no evidence of toxicity at the injection site. Although the inhibitory effect of both peptides is clear at 8 weeks after treatment-commencement, it is worth noting that, because pharmacokinetic data on these peptide inhibitors are not available at present, additional efficacy and toxicity studies with optimized delivery route, dose, and dosing regimen are needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is largely confined to secretory cells in the gut in BALB/c mice and does not respond to agonist stimulation (28), seems to be sufficient to slow xenograft growth. In addition, evaluation of this effect relative to a COX-2-selective inhibitor would be valuable to corroborate the hypothesis that suppression of phospholipase A2 is more beneficial than suppression of COX alone.

In summary, the normally androgen-responsive sPLA2-IIA gene seems constitutively expressed in prostate cancer cells remaining after androgen ablation therapy. Exogenously added sPLA2-IIA promotes prostate cancer cell proliferation by a mechanism that is dependent on the activity of both sPLA2-IIA and cPLA2-α. The loss of Annexin 1 and 2 in prostate cancer could enhance sPLA2-IIA action by increasing the activity of cPLA2-α. A better therapeutic outcome might be achieved through the use of PL2 inhibitors in the treatment of prostate cancer, particularly in the hormone refractory form.

Fig. 6. Effect of sPLA2-IIA inhibition on unstimulated prostate cancer cells. A, The endogenous expression of sPLA2-IIA mRNA was evaluated by reverse transcription-PCR in three unstimulated prostate cancer cell lines (LNCaP, DU145, and PC3) grown in RPMI. Hypoxanthine phosphoribosyltransferase (HPRT) was used to control for RNA integrity and loading. B, Cells were then treated for 72 hours in the presence and absence of the sPLA2-IIA inhibitor c2(NapA)LS2(NapA)R, and cell number was determined by cell proliferation assay. Data are mean ±SD of quadruplicate determinations expressed as percentages relative to untreated control cells (100%). LNCaP ( ), A549 untreated cells, 0.50 ± 0.03; DU145 ( ), A549 untreated cells, 0.73 ± 0.05; PC3 ( ), A549 untreated cells, 0.66 ± 0.01. *p < 0.05 versus untreated control by one-way ANOVA.

Fig. 7. Effect of sPLA2-IIA inhibition on PC3 xenograft volume in nude male mice. Four- to 6-week-old athymic male nude mice were inoculated subcutaneously with 1 × 106 PC3 cells. Mice (n = 15 per group) that received injections of either of the two inhibitors showed a slower rate of growth from 5 weeks post-treatment commencement and had significantly smaller median relative tumor volume after 8 weeks of treatment compared with animals treated with saline. Median tumor volume at T = 0 for each group was as follows: Saline: median, 66.8 mm3; range, 58.5–87.4 mm3; cFLSYR (10 mg/kg): median, 69.8 mm3; range, 57.9–105.1 mm3; c2(NapA)LS2(NapA)R (1 mg/kg): median, 69.3 mm3; range, 59.3–123.8 mm3. Median tumor volumes for each group at 8 weeks were as follows: Saline: median, 1352.6 mm3; range, 0–1991.8 mm3; cFLSYR (10 mg/kg): median, 865.9 mm3; range, 0–1991.8 mm3; c2(NapA)LS2(NapA)R (1 mg/kg): median, 742.5 mm3; range, 112.9–1945.6 mm3. *p < 0.05 for either treatment versus saline by repeated measures of two-way ANOVA (time × treatment) followed by multiple comparison test.

ACKNOWLEDGMENTS

We thank E. Kingsley for her assistance in cell culture and testing inhibitors and the Diagnostic Laboratory, Department of Endocrinology, Royal Prince Alfred Hospital for measuring steroids and pituitary hormones.

REFERENCES

7. Mizenina O, Musakina E, Yanushevich Y, et al. A novel group IIA phospholipase A2 inhibitor c2(Nap)LS2(Nap)R at 1 mg/kg: median, 1991 mm3. cFLSYR (10 mg/kg): median, 865.9 mm3; range, 0–1991.8 mm3; c2(Nap)LS2(Nap)R (1 mg/kg): median, 742.5 mm3; range, 112.9–1945.6 mm3. *p < 0.05 for either treatment versus saline by repeated measures of two-way ANOVA (time × treatment) followed by multiple comparison test.

Fig. 7. Effect of sPLA2-IIA inhibition on PC3 xenograft volume in nude male mice. Four- to 6-week-old athymic male nude mice were inoculated subcutaneously with 1 × 106 PC3 cells. Mice (n = 15 per group) that received injections of either of the two inhibitors showed a slower rate of growth from 5 weeks post-treatment commencement and had significantly smaller median relative tumor volume after 8 weeks of treatment compared with animals treated with saline. Median tumor volume at T = 0 for each group was as follows: Saline: median, 66.8 mm3; range, 58.5–87.4 mm3; cFLSYR (10 mg/kg): median, 69.8 mm3; range, 57.9–105.1 mm3; c2(Nap)LS2(Nap)R (1 mg/kg): median, 69.3 mm3; range, 59.3–123.8 mm3. Median tumor volumes for each group at 8 weeks were as follows: Saline: median, 1352.6 mm3; range, 0–1991.8 mm3; cFLSYR (10 mg/kg): median, 865.9 mm3; range, 0–1991.8 mm3; c2(Nap)LS2(Nap)R (1 mg/kg): median, 742.5 mm3; range, 112.9–1945.6 mm3. *p < 0.05 for either treatment versus saline by repeated measures of two-way ANOVA (time × treatment) followed by multiple comparison test.

ACKNOWLEDGMENTS

We thank E. Kingsley for her assistance in cell culture and testing inhibitors and the Diagnostic Laboratory, Department of Endocrinology, Royal Prince Alfred Hospital for measuring steroids and pituitary hormones.

REFERENCES

12. Edwards SH, Thompson D, Baker SF, Wood SP, Wilton DC. The crystal structure of the H48Q active site mutant of human group IIA secreted phospholipase A2 at 1.5 A resolution provides an insight into the catalytic mechanism. Biochemistry 2002;41:15468–76.


Oncogenic Action of Secreted Phospholipase A₂ in Prostate Cancer

Paul Sved, Kieran F. Scott, Duncan McLeod, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/19/6934

Cited articles
This article cites 28 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/19/6934.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/64/19/6934.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/64/19/6934.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.