Oncogenic Action of Secreted Phospholipase A2 in Prostate Cancer

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ABSTRACT

Mortality from prostate cancer is associated with progression of tumors to androgen-independent growth and metastasis. Eicosanoids are important mediators of the proliferation of prostate cancer cells in culture and are thought to be involved in mediating tumor metastasis. The in vitro localization of eicosanoids in prostate cancer cell proliferation

INTRODUCTION

Previous studies have demonstrated that the eicosanoid pathway is activated in prostate cancer cell lines, and cyclooxygenase (COX) and lipoxygenase (LOX) products are important mediators of the progression of prostate cancer. The cPLA2 enzyme regulates the production of arachidonic acid from glycerophospholipids, leading to production of free fatty acids and phospholipidmediated eicosanoids. The sPLA2-IA inhibitor pyrrolidine-1 was synthesized as described previously (13). Polyclonal anti-sPLA2-IA (160502; Cayman Chemicals, Sydney, Australia) and anti-cPLA2-IP (53/438; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased. The monoclonal anti-sPLA2-IA 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 exceeded that observed with the preimmune 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 intensity staining was stratified into three scores:


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Conventional end-time PCR was performed at an annealing temperature of LNCaP, DU145, and PC3 cells was isolated and reverse transcribed as above. The first-strand cDNA was synthesized from 2 ug of total RNA using Trizol reagent. The concentration of testosterone, estradiol, and progesterone in charcoal-stripped secreted PLA2-IIA mRNA (NM_000300). Forward, 5'-TTTGTG-CACCCAAAGACTCTTAC-3'. Reverse, 5'-GGGAGGAGGAGGTTAGAGA3'. Hypoxanthine phosphoribosyltransferase was used as the house-keeping gene, and its primer sequences were published previously (14). The PCR reaction (15 uL) contained 5.5 uL of water, 7.5 uL of 2x platinum quantitative PCR superMix-UDG (Invitrogen, Melbourne, Australia), 0.25 uL each of 0.01 mmol/L primers, 0.5 uL of 25 uM SYBR Green 1 dye (Molecular Probe, Sydney, Australia), and 1 uL of cDNA. The following protocol was used on ABI PRISM 7700 Sequence Detection System. 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 ∆∆ 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. Treatment performed on cells at 70 to 80% confluence, in medium containing 5% FBS for 72 hours. After treatments, the number of viable cells was determined by 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-ml 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 1×10^5 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/6(d1.d2)^2/3; 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 (c2NapA)L5S(2NapA)R; 1 mg/kg), and saline. cFLSYR was dissolved directly in sterile saline, whereas c2NapA)L5S(2NapA)R was diluted into sterile saline from a stock solution (40 mg/mL in dimethyformamide) immediately before use. The saline group received sterile saline containing 0.75% dimethyformamide. 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 chi² 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-(-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 (21). 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 absence of androgens (n = 50), there was patchy cytoplasmic staining in benign glands (Fig. 9)}
Fig. 1. Immunohistochemical analysis of sPLA₂-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-sPLA₂-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.
IA) 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).

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...
(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. Lyso- phospholipids 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.

To determine whether sPLA2-IIA–induced prostate cancer cell pro- liferation depends on its enzyme activity, H4qQ purified by immuno- affinity chromatography was used to treat LNCaP cells. Over the same dose range as the wild-type sPLA2-IIA, H4qQ, 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 and 2 (14) in prostate cancer and the known inhibitory effect of the two annexins on cPLA2-α activity, the activity of cPLA2-α could be increased significantly in prostate cancer cells.

**Cyclophilin Peptide Inhibitors of Secreted Phospholipase A2-IIA Suppress Endogenous Secreted Phospholipase A2-IIA–Dependent Proliferation In vitro and In vivo.** Based on our finding that the growth-promoting sPLA2-IIA is constitutively expressed in prostate cancer cells and the knowledge that production of arachidonic acid and lysophospholipids by PLA2 is, in most cases, the rate-limiting step in eicosanoid synthesis, we have considered the potential of sPLA2-IIA as a target for treatment of prostate cancer. We reason that a better outcome can be achieved with the PLA2 inhibitor than with a COX inhibitor alone because the latter suppresses the production of prostaglandins only. The first sPLA2-IIA inhibitor, LY311727, was rationally designed by Eli Lilly. Studies examining structure-activity relationships show that the Lilly inhibitor is relatively nonspecific (25). We have previously shown that human sPLA2-IIA is dose-dependently inhibited by a pentapeptide sequence comprising residues 70 to 74 of the native sPLA2-IIA protein (16). Because of the inherent flexibility of the linear peptide sequence, 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(2NapA)LS(2NapA)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 was undetectable in DU145 compared with LNCaP and PC3 cells (Fig. 6A). We then tested the effect of individual inhibitors cFLSYR and c(2NapA)LS(2NapA)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(2NapA)LS(2NapA)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...
c(2NapA)LS(2NapA)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 has reached the size of 5 × 5 mm. We found that inhibitor c(2NapA)LS(2NapA)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 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 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 needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed. It is nonetheless remarkable that blockade of xenograft-derived sPLA2-IIA alone, as host-derived sPLA2-IIA expression is needed.
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