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. Eicosanoid products of both the cyclooxygenase (COX) and lipooxygenase (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 A2 (PLA2) enzymes regulate the provision of arachidonic acid to both COX- and LOX-derived eicosanoids, and a secreted form of the enzyme (sPLA2-IIA) is elevated in prostate cancer tissues. Here, we show by immunohistochernistry, in patients receiving androgen ablation therapy, that sPLA2-IIA remains elevated in remaining cancer cells relative to benign glands after treatment. Furthermore, sPLA2-IIA expression seen in benign glands is substantially decreased after androgen depletion, whereas cytosolic PLA2-α (cPLA2-α) levels are unchanged. sPLA2-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 sPLA2-IIA (1–100 nmol/L), but not an inactive sPLA2-IIA mutant (H48Q), results in a dose-dependent increase in cell numbers or the fraction of cells in G2-M phase, which is inhibited by sPLA2-IIA-selective inhibitors. The effect of exogenous sPLA2-IIA can also be blocked by inhibition of cPLA2-α, suggesting a role for cPLA2-α in mediating sPLA2-IIA action. sPLA2-IIA inhibitors suppressed basal proliferation in LNCaP cells and in the androgen-independent, sPLA2-IIA-positive cell line PC3 but not in the sPLA2-IIA-negative androgen-independent cell line DU145. Established PC3 xenograft tumors grew more slowly in mice treated with sPLA2-IIA inhibitors than those treated with saline only. The PLA2 enzymes, and sPLA2-IIA in particular, thus represent important targets for the treatment of sPLA2-IIA-positive androgen-independent prostate cancer.

INTRODUCTION

Previous studies have demonstrated that the eicosanoid pathway is activated in prostate cancer, and cyclooxygenase (COX) and lipooxygenase (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 A2 (PLA2), 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 prostanoids by COX or hydroxyeicosatetraenoic acids by LOX. PLA2 is a growing family of proteins, and in human, there are 10 secreted PLA2 forms (sPLA2), at least three isoforms of cytosolic PLA2 (cPLA2), and multiple isoforms of calcium-independent intracellular PLA2 (5–9).

The aim of this study was, first, to determine the expression levels of sPLA2 and cPLA2 in prostate cancer patients treated with androgen ablation therapy; second, to determine oncogenic potential of sPLA2 and cPLA2; and last, to determine the effect of specific sPLA2-IIA inhibitors on prostate cancer cell growth. We have now obtained evidence that sPLA2-IIA is constitutively expressed in remaining prostate cancer cells after 3 months of androgen ablation therapy. The increased sPLA2-IIA has an oncogenic action that requires the activity of sPLA2-IIA and of cPLA2-α. Our newly designed sPLA2-IIA inhibitors can block basal and sPLA2-IIA–induced prostate cancer cell proliferation in vitro and in vivo.

MATERIALS AND METHODS

Reagents. sPLA2-IIA was purified from conditioned media produced by a Chinese hamster ovary cell line (5A2) stably transfected with the human sPLA2-IIA cDNA, as described previously (10), and quantified by enzyme-linked immunosorbent assay (11). sPLA2-IIA contained <0.1 ng of endotoxin per milligram of protein (Limulus ameboocyte 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 sPLA2-IIA mutant enzyme H48Q will be described elsewhere. H48Q 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 sPLA2-IIA inhibitors were synthesized using N-(9-fluorenylmethoxycarbonyl) solid phase chemistry (Auspep, Melbourne, Australia) as described previously (10). c(2Nap)LS(2Nap)R is at least 10-fold more potent in binding sPLA2-IIA and inhibiting sPLA2-IIA enzyme activity than cFLSYR. Peptides for in vivo use were exchanged into acetate buffer before lyophilization. The cPLA2-α inhibitor pyrrolidine-1 was synthesized as described previously (13). Polyclonal anti-sPLA2-IIA (160502; Cayman Chemicals, Sydney, Australia) and anti-cPLA2-α (SC-438; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased. The monoclonal anti-sPLA2-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 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 percent-age of cells with the highest staining intensity was stratified into three scores:

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Conventional end-time PCR was performed at an annealing temperature of 55°C. Hypoxanthine phosphoribosyltransferase was used as the house-keeping gene, (HPRT) (17). For endogenous sPLA2-IIA mRNA level, total RNA from LNCaP cells was isolated using Trizol reagent. The first-strand cDNA was synthesized from 2 micrograms total RNA using Moloney murine leukemia virus reverse transcriptase and oliogot(dT) as described previously (16). Primers were designed based on the human sPLA2-IIA mRNA (NM_003000). Forward, 5′-TTTGTG-CACCCAAGAAGCTCTAC-3′. Reverse, 5′-GGGAGGGAGGGTATGAGA-3′. Pyrohexine phospholipidtransferase 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 mMol/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 7000 sequence detection system: 50°C for 2 minutes, 95°C for 15 seconds, 55°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 phospholipidtransferase, the ΔΔ method was used to calculate relative changes in mRNA levels of sPLA2-IIA corrected for hypoxanthine phospholipidtransferase (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 1×10⁴ 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 2 hours. 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 (1×10⁶) 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.

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,9 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 family showed no or reverse expression. Analysis of sPLA2-IIA mRNA level 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 cytoplasmic staining in benign glands (Fig. 9 Internet address: www.ncbi.nlm.nih.gov/SAGE.)
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.
find an androgen response element within 3 kb of the 5-flanking region of the sPLA2-IIA gene using MatInspector Release 5.3 (Genomatix, München, Germany) and found an androgen response element GAGGTTAAAAGTATTTC 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...
Currently, two models are proposed to explain the action of sPLA2 on role of cPLA2 - dose required to block arachidonate release in a number of cPLA2 - molar to low micromolar range of pyrrolidine-1 is consistent with the B growth-promoting sPLA2 -IIA is constitutively expressed in prostate from the G1 phase.

...70 to 74 of the native sPLA2-IIA protein (70 FLSYK74; ref. 26). To determine whether sPLA2-IIA–induced prostate cancer cell proliferation depends on its enzyme activity, H48Q purified by immunoaffinity chromatography was used to treat LNCaP cells. Over the same dose range as the wild-type sPLA2-IIA, H48Q, 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-α-induced cell growth completely, demonstrating that cPLA2-α activity is necessary for sPLA2-α–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 (24) 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.

Cyclic Peptide Inhibitors of Secreted Phospholipase A2-α 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 PL2α is, in most cases, the rate-limiting step in eicosanoid synthesis, we have considered the potential of sPLA2-α as a target for treatment of prostate cancer. We reason that a better outcome can be achieved with the PL2α inhibitor than with a COX inhibitor alone because the latter suppresses the production of prostaglandins only. The first sPLA2-III inhibitor, LY311727, was rationally designed by Eli Lilly. Studies examining structure-activity relationships show that the Lilly inhibitor is relatively nonselective (25). We have previously shown that human sPLA2-α is dose-dependently inhibited by a pentapeptide sequence comprising residues 70 to 74 of the native sPLA2-III protein (26). Because of the inherent flexibility of the linear peptide sequence, inhibition was weak in in vitro assay. 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-III inhibition assays and do not bind to a structurally related enzyme sPLA2-IIB.

To test the effect of blocking endogenous sPLA2-III on cell growth, we first determined the basal mRNA levels of sPLA2-III in three human prostate cancer cell lines. mRNA encoding sPLA2-III 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-III.

It is interesting to note the difference in response of LNCaP cells to the sPLA2 inhibitors between Figs. 4 and 6. In the absence of exogenous sPLA2-III 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 resulted in ~40% fewer cells than in control. Recent studies have shown that the effects of exogenous sPLA2-III are mechanistically separable from those of endogenous intracellular sPLA2-III in model cell systems (27). It is thus possible that sPLA2 and cPLA2 may modulate cell growth by separate mechanisms depending on their 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-III.

In vivo, cFLSYR, at either 1 mg/kg or 10 mg/kg, and
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 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 PLA2 inhibitors in the treatment of prostate cancer, particularly in the hormone refractory form.

REFERENCE


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 as a positive 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 (●), A0 untreated cells 0.50 ± 0.03; DU145 (■), A0 untreated cells 0.73 ± 0.05; PC3 (■), A0 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 × 107 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, 68.6 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, 152.6 mm3; range, 0–199.81 mm3; cFLSYR (10 mg/kg): median, 865.9 mm3; range, 0–1991 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.

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