Saw Palmetto induces growth arrest and apoptosis of androgen-dependent prostate cancer LNCaP cells via inactivation of STAT 3 and androgen receptor signaling

YANG YANG1, TAKAYUKI IKEZOE2, ZHIXING ZHENG1, HIROKUNI TAGUCHI2, H. PHILLIP KOEFFLER3 and WEI-GUO ZHU1

1Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100083, P.R. China; 2Department of Hematology and Respiratory Medicine, Kochi Medical School, Kochi University, Okokcho, Nankoku, Kochi 783-8505, Japan; 3Division of Hematology and Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA

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Abstract. PC-SPES is an eight-herb mixture that has an activity against prostate cancer. Recently, we purified Saw Palmetto (Serenoa repens) from PC-SPES and found that Saw Palmetto induced growth arrest of prostate cancer LNCaP, DU145, and PC3 cells with ED50s of approximately 2.0, 2.6, and 3.3 μl/ml, respectively, as measured by mitochondrial-dependent conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Saw Palmetto induced apoptosis of LNCaP cells in a time- and dose-dependent manner as measured by TUNEL assays. Also, Saw Palmetto increased the expression of p21waf1 and p53 protein in LNCaP cells. In addition, we found that Saw Palmetto down-regulated DHT- or IL-6-induced expression of prostate specific antigen in conjunction with down-regulation of the level of androgen receptor in the nucleus as measured by Western blot analysis. Moreover, Saw Palmetto down-regulated the IL-6-induced level of the phosphorylated form of STAT 3 in LNCaP cells. Furthermore, Saw Palmetto inhibited the growth of LNCaP cells present as tumor xenografts in BALB/c nude mice without adverse effect. These results indicate that Saw Palmetto might be useful for the treatment of individuals with prostate cancer.

Introduction

Androgens play an important role in both development and maintenance of the normal prostate as well as the initiation and progression of prostate cancer (1,2). Androgen deprivation therapy remains the mainstay of prostate cancer treatment (2). Androgen receptor (AR) belongs to the steroid hormone subfamily of nuclear receptors and mediates the signal of androgens. AR is complexed in the cytoplasm to chaperone proteins that keep the receptor in a transcriptional inactive form. Upon binding to the ligands, AR dissociates from the chaperone and translocates to the nucleus where it binds to androgen response elements (AREs) and activates target genes such as prostate specific antigen (PSA) (1,2).

PSA belongs to the kallikrein-like serine protease family; it is produced almost exclusively by prostate epithelial cells, and is used as a serum marker for diagnosis and progression of prostate cancer (3). The 5'-upstream promoter and enhancer region of the PSA gene contains several AREs to which ligand activated AR binds and induces expression of PSA (4-6).

Signal transducers and activators of transcription (STAT) are part of the signal transduction pathway of many growth factors and cytokines and are activated by phosphorylation of tyrosine and serine residues by upstream kinases (7). For example, signaling by IL-6 generally induces phosphorylation of STAT 3 (7). This signaling has been demonstrated to play a major role in tumor formation (8). Aberrant signaling by STAT3 is found in many types of malignancies, including multiple myeloma, head and neck cancer, breast cancer, and prostate cancer (9-13).

Herbs have been used for many centuries for treatment of cancer (14,15). Use of these therapies has been dramatically rising in recent years in the United States (16). One of the herbal mixtures frequently used by individuals with prostate cancer is the Chinese herbal mixture denoted PC-SPES (17-24). It contains a partially extracted mixture of eight herbs: Dendrantherma morifolium, Tzvel; Ganoderma Lucidum, Karst; Glycyrrhiza glabra L; Isatis indigotica, Fort; Panax pseudo-ginseng, Wall; Rabdosia rubescens;
Scutellaria baicalensis, Georgi and Serenoa repens (17-24). Previous studies showed that PC-SPES mediated an anti-proliferative effect on prostate cancer cells in vivo and in vitro (16-25). In addition, recent clinical studies showed that PC-SPES reduced prostate specific antigen (PSA) levels in more than 80% of individuals with prostate cancer (26,27). Saw Palmetto (Serenoa repens) is one of the components of PC-SPES. It has been studied exclusively in P.R. China and has been shown to possess anti-tumor activity. However, the mechanism by which Saw Palmetto inhibits growth of cancer cells remains to be fully elucidated. In this study, we found that Saw Palmetto inhibited the proliferation of prostate cancer cells. We also found that Saw Palmetto inhibited levels of PSA in conjunction with disruption of the AR signal pathway and inactivated STAT 3 signaling in LNCaP cells. Saw Palmetto might be useful for treatment of individuals with prostate cancer.

Materials and methods

Cell line. Prostate cancer LNCaP, PC-3, and DU145 cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained in RPMI-1640 with 10% fetal bovine serum (FBS).

Saw Palmetto. Saw Palmetto (Serenoa repens) was purified from PC-SPES. One capsule contains 400 mg of powered herbal extracts. Stock solutions of Saw Palmetto were prepared by exposing these herbal extracts to 70% ethanol (1 capsule/1 ml of 70% ethanol). For in vitro use, Saw Palmetto was diluted in RPMI-1640. An aliquot was used only once.

MTT assays. Cells (10⁴/ml) were incubated with a variety of concentrations of Saw Palmetto (0-4 μl/ml) for 3 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product, as previously described (28).

Assessment of apoptosis. Apoptotic cell death was examined by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) method and 7-amino actinomycin-D (7-AAD) staining. TUNEL assay was performed using the
The difference between 2 groups under multiple conditions was assessed by one-way analysis of variance (ANOVA) using PRISM statistical analysis software (GraphPad Software, San Diego, CA). The non-parametric Mann-Whitney U test was performed to assess the difference between 2 groups in the in vivo studies.

Results

Effect of Saw Palmetto on proliferation of prostate cell lines. To explore the anti-proliferative effects of Saw Palmetto, prostate cancer cell lines (LNCaP, DU145, PC3) were cultured in the presence of various concentrations of Saw Palmetto (0-4 μl/ml, 48 h). Saw Palmetto effectively inhibited the growth of LNCaP, DU145, and PC3 cells with the effective dose that inhibited 50% growth (ED50s) of approximately 2.0, 2.6, and 3.3 μl/ml, respectively as measured by MTT assay on the third day of culture (Fig. 1A).

Saw Palmetto induced apoptosis of LNCaP cells. TUNEL assay was utilized to examine the pro-apoptotic effect of Saw Palmetto. Saw Palmetto induced apoptosis of these cells in a dose- and time-dependent manner (Fig. 1B). Saw Palmetto (0.5 μl/ml or 1.0 μl/ml) induced a mean 8.7±2.0% (± SD) and 14.3±2.2% of the LNCaP cells to become apoptotic at 24 h, which increased to a mean 9.9±1.6% and 35.1±4.4% at 48 h, respectively (p<0.005) (Fig. 1B). In addition, the proportion of cells with fractional DNA content, a feature characteristic of apoptosis, was measured by 7-AAD staining using the LNCaP cells (Fig. 1C). Saw Palmetto (0.5 μl/ml or 1.0 μl/ml, 24 h) induced a mean 4.9±1.7% or 9.9±1.5% of cells to become apoptotic (p<0.05) (Fig. 1C).

Effect of Saw Palmetto on expression of p21^{waf1}, p27^{kip1}, and p53 in LNCaP cells. The modulation of expression of the cell cycle checkpoint proteins, p21^{waf1}, p27^{kip1}, and p53 were examined by Western blot analysis (Fig. 1D). LNCaP cells constitutively expressed wild-type p53 protein. Exposure of these cells to Saw Palmetto (1 μl/ml, 24 h) increased their levels of p53 by 2-fold compared with control cells. Whereas, Saw Palmetto (1 μl/ml, 24 h) did not modulate the level of p21^{waf1} in LNCaP cells (Fig. 1D). The level of p21^{waf1} protein was negligible in the untreated LNCaP cells; however, exposure of these cells to Saw Palmetto (1 μl/ml, 24 h) resulted in a dramatic increased level of p21^{waf1} protein (Fig. 1D).

Effect of Saw Palmetto on level of PSA and AR protein. LNCaP cells cultured in the presence of 10% FCS constitutively expressed PSA and AR proteins in whole cell protein, and Saw Palmetto (0.5 μl/ml and 1 μl/ml, 24 h) down-regulated the level of PSA or AR by 90% or 80%, respectively (Fig. 2A). Next, we explored the expression of PSA and AR proteins in cytoplasmic and nuclear proteins. As shown in Fig. 2B, Saw Palmetto (1 μl/ml, 24 h) down-regulated the level of PSA by 90% in cytoplasmic protein. Saw Palmetto (1 μl/ml, 24 h) did not modulate the level of AR expression in the cytoplasmic protein of LNCaP cells. Whereas, the expression level of AR protein was reduced by 0.4-fold at the same dose in intranuclear levels (Fig. 2B).
Figure 2. Effect of Saw Palmetto on the level of PSA and AR proteins. Western blot analysis. (A) LNCaP cells were cultured with either Saw Palmetto (0.5 μl/ml and 1 μl/ml) or control diluent. After 24 h, cells were harvested and subjected to Western blot analysis. The membrane was sequentially probed with anti-PSA, -AR, and -β-actin antibodies, and band intensities were measured by densitometry. (B) LNCaP cells were cultured with either Saw Palmetto (1 μl/ml) or control diluent. After 24 h, cytoplasmic proteins were extracted, subjected to Western blot analysis, and the membrane was sequentially probed with anti-PSA, -AR, and -β-actin antibodies. Band intensities were measured by densitometry. (C) LNCaP cells were cultured with either Saw Palmetto (1 μl/ml) or control diluent. After 24 h, cells were harvested, and nuclear proteins were prepared and subjected to Western blot analysis, and the membrane was sequentially probed with anti-AR, and -β-actin antibodies. Band intensities were measured by densitometry. SP, Saw Palmetto.

Figure 3. Effect of Saw Palmetto on levels of DHT- or IL-6-induced PSA and AR protein. Western blot analysis. (A) Effect of Saw Palmetto on levels of DHT-induced PSA and AR protein. LNCaP cells were cultured with either Saw Palmetto (1 μl/ml) or, either alone or in combination, DHT (10 nM) or control diluent. After 24 h, cells were harvested and cytoplasmic and nuclear proteins were extracted, subjected to Western blot analysis, and the membrane was sequentially probed with anti-PSA, -AR, and -β-actin antibodies. Band intensities were measured by densitometry. (B) Effect of Saw Palmetto on levels of IL-6-induced PSA and AR protein. LNCaP cells were cultured with either Saw Palmetto (1 μl/ml) or control diluent. After 24 h, cells were exposed to IL-6 (20 ng/ml) for 30 min, harvested, and cytoplasmic and nuclear proteins were extracted, subjected to Western blot analysis, and membrane was sequentially probed with anti-PSA, -AR, and -β-actin antibodies. Band intensities were measured by densitometry. SP, Saw Palmetto.
Figure 4. Effect of Saw Palmetto on STAT 3 activity in LNCaP cells. (A) Western blot analysis. LNCaP cells were cultured with either Saw Palmetto (1 μl/ml) or control diluent. After 3 h, cells were exposed to IL-6 (20 ng/ml) for 30 min, harvested, subjected to Western blot analysis, and membrane was sequentially probed with anti-p-STAT 3, -STAT 3 antibodies. Band intensities were measured by densitometry. (B) MTT assay. LNCaP cells (10^3/ml) were plated in 96-well plates and cultured either with or without AG490 (10 nM). After 3 h, cells were exposed to Saw Palmetto (1 μl/ml) for 48 h, and their cell viability was measured by MTT assay. Results represent the mean ± SD of 3 experiments performed in triplicate. SP, Saw Palmetto.

Effect of Saw Palmetto on IL-6-stimulated p-STAT 3 in LNCaP cells. We next investigated the effect of Saw Palmetto on STAT 3 signaling which is activated by IL-6. The level of the phosphorylated form of STAT 3 in the control LNCaP cells was negligible (Fig. 4A). LNCaP cells were serum-starved for 24 h and exposure of these cells to IL-6 (20 ng/ml, 30 min) dramatically increased the level of the phosphorylated form of STAT 3. Pretreatment (3 h) of these cells with Saw Palmetto (1 μl/ml, 24 h) blocked the IL-6-induced phosphorylation of STAT 3 by 60% (Fig. 4A). Saw Palmetto did not modulate basal and IL-6 stimulated levels of total STAT 3 (Fig. 4A).

Combination effect of Janus family of tyrosin kinase (JAK) inhibitor AG490 and Saw Palmetto on growth of LNCaP cells. We next studied the interaction of Janus family of tyrosin kinase (JAK) inhibitor AG490 and Saw Palmetto on growth of LNCaP cells. LNCaP cells were pre-incubated with AG490 (10 nM) for 3 h and then exposed to Saw Palmetto (1 μl/ml) and cultured for 48 h. AG490 or Saw Palmetto alone inhibited the growth of LNCaP cells by 12.6±2.5% and 54.6±1.2% respectively. The combination of AG490 and Saw Palmetto inhibited the growth of LNCaP cells by 72.6±7.3% compared with control cells (p<0.005) (Fig. 4B).

Effect of Saw Palmetto on LNCaP xenografts in vivo. We evaluated the ability of Saw Palmetto to inhibit the growth of LNCaP cells growing as xenografts in BALB/c nude mice. Tumor volume was measured every week (Fig. 5A), and tumor weight was determined at autopsy (Fig. 5B). Saw Palmetto markedly suppressed the growth and weight of LNCaP tumors. As shown in Fig. 5A, the volume of LNCaP tumors in the mice who received Saw Palmetto was significantly decreased compared with control mice (p<0.05). In addition, the difference of mean tumor weights between these two groups was significant (p<0.02). The tumors from control mice weighed 98.6±36.0 mg. On the other hand, tumors from mice who received Saw Palmetto weighed only 32.8±22.7 mg (Fig. 5B).

We next explored the ability of Saw Palmetto to decrease serum levels of PSA of LNCaP cells in vivo. After 4 weeks of treatment, blood was withdrawn from each mouse and serum levels of PSA were measured. As shown in Fig. 5C, the concentration of PSA in control cells was 5.3±2.2 ng/ml and in Saw Palmetto-treated cells was 1.8±2.0 ng/ml. Serum levels of PSA were reduced by approximately 66% (p<0.05) (Fig. 5C). Serum levels of PSA in normal mice at the same age were <0.03 ng/ml (data not shown).

The tumors and organs of the BALB/c nude mice were fixed, stained, and viewed by light microscopy. The tumors from control mice showed a typical histological appearance of infiltrating, poorly differentiated adenocarcinomas of the prostate (Fig. 5D). Tumors from mice receiving Saw Palmetto had marked fibrosis and inflammatory cells such as giant cells (Fig. 5E). Organs from mice treated with Saw Palmetto did not show any changes compared with controls, including in their livers, kidneys, and spleens. During the study, all the mice were weighed each week. The mean weights of the experimental groups were statistically the same as those of the control mice (data not shown).
Discussion

Herbs have been used for many centuries for treatment of cancer. One of the herbal mixtures frequently used by individuals with prostate cancer is the Chinese herbal mixture denoted PC-SPES. Our previous study had shown that PC-SPES inhibited the growth of prostate cancer cells and downregulated the basal and DHT-induced PSA expression in LNCaP cells (29). PC-SPES contains a partially extracted mixture of eight herbs, and which herb has the main effect in

Figure 5. Effect of Saw Palmetto on growth of LNCaP tumors in nude mice. (A) Tumor volumes. LNCaP cells were injected bilaterally s.c. into BALB/c nude mice, forming two tumors/mouse. Saw Palmetto (10 mg/kg/mouse) was administered orally five times a week. Control mice received diluent only. Tumors were measured every week. Each point represents the mean ± SE of 10 tumors. (B) Tumor weights at autopsy. After 4 weeks of treatment, tumors were removed and weighed. Results represent mean ± SE of tumor weights. Statistical significance of the differences was analyzed by Mann-Whitney U test. Bars, SE. (C) Serum levels of PSA. After 4 weeks of treatment, blood was withdrawn from each mouse and serum levels of PSA were measured. (D and E) Histology of LNCaP human prostate tumors from mice treated with or without Saw Palmetto in vivo. After 4 weeks of growth in nude mice treated either with or without Saw Palmetto, LNCaP tumors were removed, fixed in formalin, and stained with H&E. (D) Control tumors from mice that received diluent displayed poorly differentiated adenocarcinoma (x200). (E) Tumors from mice treated with Saw Palmetto (10 mg/kg) five times a week for 4 weeks showed marked fibrosis and appearance of inflammatory cells such as giant cells (x200).
the treatment of prostate cancer is unclear. Recently, several investigators focused on the study of potent components of PC-SPES. *Rabdosia rubescens* was reported to induce apoptosis of lung cancer cells and inhibit the growth of breast cancer cells (30,31). Oridonin purified from *Rabdosia rubescens* induced growth inhibition and apoptosis of a variety of human cancer cells (32). Baicalin, a major component of PC-SPES, inhibited the proliferation of human cancer cells such as prostate cancer cell lines, breast cancer cell lines, myeloblastic leukemia cell lines and promyelocytic leukemia cell lines (33). In this study, we explored the effect of Saw Palmetto, one component of PC-SPES, on prostate cancer cells. We found that Saw Palmetto can effectively inhibit the growth of LNCaP, DU145, and PC3 cells and induced apoptosis of LNCaP cells in a dose- and time-dependent manner (Fig. 1B and C). Saw Palmetto down-regulated the level of PSA or AR in LNCaP cells (Fig. 2A). Also, Saw Palmetto down-regulated basal and DHT- or IL-6-induced PSA expression in cytoplasmic protein and AR expression in nuclear proteins in LNCaP cells (Figs. 2B and 3). These results suggested that Saw Palmetto is one of the representative components of PC-SPES on the treatment of prostate cancer. Saw Palmetto might inhibit LNCaP cell growth through blocking AR signaling.

Previous studies have demonstrated that STAT 3 signaling has a critical role in the tumor formation of prostate cancer (34,35) and IL-6 treatment results in the activation of STAT 3 in prostate cancer cells (36). In this study, we found for the first time that Saw Palmetto down-regulated the IL-6-induced level of the phosphorylated form of STAT 3 in LNCaP cells (Fig. 4A). To block this pathway, a Janus family of tyrosine kinase (JAK) inhibitor AG490 was used and the proliferation of LNCaP was effectively inhibited in a dose-dependent manner (data not shown). The combination of AG490 and Saw Palmetto significantly inhibited the growth of LNCaP cells (p<0.005) (Fig. 4B). These results suggest that STAT 3 signaling also plays an important role in the survival of LNCaP cells and Saw Palmetto inhibits their cell growth via inhibition of STAT 3 signaling.

In summary, we found that Saw Palmetto is an important component of PC-SPES and active against prostate cancer cells in vitro as well as in vivo through inhibition of AR and STAT 3 signaling. Saw Palmetto may be useful as an adjunctive therapeutic agent for treatment of individuals with prostate cancer and other types of cancer in which AR or STAT 3 signaling is activated.

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