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ORIGINAL CONTRIBUTION

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Alpha-tocopheryl succinate (α-TOS) modulates human prostate LNCaP xenograft growth and gene expression in BALB/c nude mice fed two levels of dietary soybean oil

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Abstract *Background* Malignancy of the prostate constitutes a leading cause of cancer-related deaths in America and Europe. Alpha-tocopheryl succinate (α-TOS) has been shown to inhibit human prostate cancer growth *in vitro*, via several mechanisms, including inhibiting prostate-specific antigen (PSA) and vascular endothelial growth factor (VEGF) expressions. The route of α-TOS administration has a profound effect on its antitumor activity, and few studies have investigated its effects on prostate cancer growth *in vivo*. *Aim of the Study* The present study tested the hypothesis that α-TOS wil reduce the growth of human prostate LNCaP tumors in mice fed low (7%) and high (20%) levels of dietary soybean oil, compared to the controls receiving vehicle, by modulating PSA and VEGF gene expressions in the tumor tissue. *Methods* BALB/c nude mice (n = 42) were subcutaneously inoculated with 1×10^6 LNCaP cells and assigned to one of four dietary groups; 7% or 20% soybean oil diet with or without α-TOS treatment. Three weeks later, mice received daily intraperitoneal injections of α-TOS (100 mg/kg body weight) in sesame seed oil (SSO) for two weeks; controls received SSO injections. Tumor volumes were recorded weekly. Sera, liver, and tumor tissues were collected at seven weeks for serum PSA, testosterone and α-tocopherol analyses, histopathological examination, and reverse transcription and polymerase chain reaction (RT-PCR) amplification of PSA and VEGF gene fragments in tumors. Relative quantification of gene expression was performed using real-time PCR. $P \leq 0.05$ was considered significant. *Results* Intraperitoneal injections of α-TOS caused decreased tumor growth in both groups (7% and 20% fat, $P < 0.05$), versus controls. α-TOS treatment significantly reduced serum PSA and testosterone levels in comparison to the SSO-treated controls ($P < 0.05$). Control tumors had a greater degree of angiogenesis than α-TOS tumors, as demonstrated by the greater number of blood-filled vessels. PSA and VEGF mRNA expressions, were also reduced with α-TOS treatment ($P < 0.05$), revealing the possible molecular mechanisms of growth inhibition of LNCaP xenografts by α-TOS. *Conclusions* Our study shows significant reduction in LNCaP xenograft growth with α-TOS treatment in nude mice fed a low (7%) and high (20%) fat soybean oil diets versus

controls. Serum PSA and testosterone, tumor angiogenesis, and PSA and VEGF mRNA expressions were markedly reduced by α-TOS administration, suggesting a possible role of α-TOS as a chemotherapeutic agent in human prostate cancer, and warrants further investigations on the dose and delivery of α-TOS in humans.

Key words alpha-tocopheryl succinate – prostate cancer – angiogenesis – prostate-specific antigen – xenograft growth

Introduction

Malignancy of the prostate constitutes the second leading cause of cancer-related deaths in American men, and is also the third most frequent cause of cancer mortality among the European countries [1, 2]. The androgen receptor (AR) is required for the development of both the normal prostate gland and prostate cancer. In the early stages of prostate cancer, almost all cancer cells are androgen-dependent and highly sensitive to antiandrogen therapy. However, prostate cancer usually recurs after a few years of androgen ablative treatment, and most cancer cells become androgen-independent, rendering antiandrogen therapy useless. As androgen-independent prostate tumors are incurable at the present time, the prevention of such aberrant AR activation is an attractive therapeutic target [3].

Studies have shown that dietary micronutrients like, carotenoids and lycopenes, retinoids and vitamin A, vitamin E [alpha (α) tocopherol and gamma (γ) tocopherol], vitamin C, selenium, and phenol-containing dietary substances have effects that extend beyond their antioxidant properties [4]. Alpha-tocopheryl succinate (α-TOS) has been reported to be a very effective chemotherapeutic agent in prostate cancer. The proposed mechanisms of α-TOS action on human prostate cancer cells by various authors include enhancing the chemotherapeutic effects of adriamycin on human prostate cancer cells [5], inducing apoptosis by causing mobilization of Fas from the cytosol to the plasma membrane in human prostate cancer cells [6], and decreasing the production of vascular endothelial growth factor (VEGF) by prostate cancer cells [7]. α-TOS has also been reported to inhibit the transcription as well as translation of prostate-specific antigen (PSA) and AR in human prostate cancer cells, exert a synergistic effect in combination with hydroxyflutamide on growth inhibition of prostate cancer [8], and also inhibit human prostate cancer cell invasiveness by inhibiting the activity of matrix metalloproteinases [9]. Zu and Ip [10] compared the antineoplastic action of α-tocopherol and its two ester derivatives, α-tocopheryl acetate and α-TOS on the growth of human prostate PC-3 cancer cells in culture. Interestingly, a concentration of 20 μM of α-TOS was sufficient to achieve a 40% growth inhibition of PC-3 cells after 72 h of treatment, whereas, neither α-tocopherol nor α-tocopheryl acetate was able to achieve the same after the same length of exposure and with a dose 10 times higher than that of α-TOS. While various *in vitro* studies report the superiority of α-TOS as a chemopreventive agent, very few studies have reported similar *in vivo* effects of α-TOS. A recent study by Venkateswaran et al. [11] showed a significant reduction in prostate cancer incidence in male *Lady* transgenic mice fed a standard-fat (25% kcal-fat) or a high-fat (40% kcal-fat) diet with antioxidant supplementation (800 IU of α-TOS, 200 μg of seleno-DL-methionine and 50 mg of lycopene) versus the controls. However, α-TOS is not an antioxidant, but becomes one once it is taken up by the intestinal epithelial cells, and hydrolyzed to α-tocopherol. While normal cells, including intestinal epithelial cells, are capable of hydrolyzing α-TOS to α-tocopherol, cancer cells lack this ability. As a result, accumulation of the intact molecule of α-TOS in cancer cells causes apoptosis [12]. Malafa and Neitzel [13] demonstrated that nude mice injected intraperitoneally (ip) with α-TOS significantly lowered final weight of human breast cancer xenografts than the control vehicle-treated group, whereas, no difference was noted between mice treated subcutaneously (sc) with α-TOS versus the control sc treated group. Thus, the route of α-TOS administration has a profound effect on its antitumor activity in vivo. This effect remains to be established in prostate cancer growth in vivo and may produce a similar or even greater inhibitory effect than reported with oral ingestion of α-TOS [11].

This study investigated the effects of α-TOS alone, administered intraperitoneally, on androgen-dependent human prostate LNCaP xenograft growth in nude mice. Our study tested the hypothesis that α-TOS will reduce the growth of human prostate LNCaP tumors in mice fed low (7%) and high (20%) levels of

dietary soybean oil, compared to the controls receiving vehicle, by modulating PSA and VEGF gene expressions in the tumor tissue.

Materials and methods

Cell culture

The LNCaP cells, serum and the medium were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in RPMI-1640 medium supplemented with 5% fetal calf serum, nonessential amino acids, and L-glutamine in an atmosphere of 5% CO_2/air at 37°C. The cells were grown in flasks and passaged when they reached subconfluence and were treated briefly with trypsin/EDTA to put them in solution for each passage. The cells were counted using a hemocytometer, tested for viability with exclusion by trypan blue dye, and were taken for injection into mice when most of the cells were in log phase growth; usually covering only 1/2 the surface of the flask. Only cells with >90% viability were used. The cells were kept on ice (4°C) until injected.

Animals and diets

Forty-two male BALB/c nude mice, aged 8 weeks, were obtained from National Cancer Institute (Frederick, MD) and housed 4–5 animals per cage, in a pathogen-free environment with microbe filter tops in filtered laminar-flow hoods. All mice were used for the tumor implantation and were randomly assigned to the dietary experimental groups. All animals were cared for in accordance with the Institutional Animal Care and Use Committee (IUCAC) guidelines of the University of Texas at Southwestern Medical Center (Dallas, TX). The water and bedding were heat sterilized (autoclaved). In order to prevent sepsis by *Pseudomonus aeruginosa* bacteria, 750 µl of Chlorox/bottle of 400 ml of drinking water were added.

The composition of the AIN-93G diets [14] was modified with added methionine and high-fat (20% soybean oil). The diets were isonitrogenous in terms of kilocalorie of the diet to ensure similar protein intake levels in both groups. The diets were pelleted and irradiated by Bio-Serv (Frenchtown, NJ).

Food consumption, tumor volume and animal weights were recorded weekly. The diets were stored at 4°C and fresh diets were given every 3–4 days. The tumor size was measured using a caliper and tumor volume was calculated using the formula (cm^3): length (L) × width (W) × height (H) × 0.5236. Tumor weight was estimated from tumor volume, as the density of tissue is 1 g/ml. Body weight of each animal was then determined by subtracting tumor weight from total body weight.

Experimental design

This was a 7-week study design, starting from inoculation of the animals with LNCaP cells. LNCaP cells in culture were resuspended in ice-cold Matrigel (Collaborative Biomedical Products, Bedford, MA) at a concentration of 1×10^6/0.2 ml. Mice were then inoculated (27 gauge needle) subcutaneously in the hindlimb with 1×10^6 cells. All animals were fed the control diet containing 7%-fat *ad libitum* for 1 week prior to inoculation. Following inoculation, the animals were randomly assigned to one of the four groups: 7% or 20% soybean oil diet, with or without α-TOS treatment. All animals remained on their respective diets until the termination of the study. Three weeks after inoculation, when measurable tumors were formed in most of the animals (at least 0.02 ± 0.005 cm^3 in 80% of the mice in each group), the experimental groups received daily intraperitoneal (ip) injections of α-TOS in sesame oil (100 mg/kg body weight) while the controls received a vehicle of sesame oil for 2 weeks and the animals remained on their respective diets for the remaining 2 weeks of the study. α-TOS [all racemic (all rac) α-tocopheryl succinate] was purchased from EMD Biosciences (San Diego, CA) and sesame oil obtained from Sigma Chemical Co. (St. Louis, MO). All animals were sacrificed 7 weeks after inoculation and blood was collected through cardiac puncture. Sera were separated and stored at −80 °C until analyses. Two hundred milligrams of the LNCaP tumor tissue were weighed and preserved in RNAlater® (Ambion Inc.) for subsequent RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). A sample tumor tissue was stored in 10% formalin in PBS for later histopathologic examination. Liver tissues were also collected and snap-frozen for α- tocopherol analyses.

Analyses

Serum PSA and testosterone: Human PSA levels in serum were determined using an enzyme immunoassay kit (ALPCO Diagnostics, Windham, NH). Total serum testosterone was determined using enzyme-linked immunosorbent assays (ALPCO Diagnostics, Windham, NH). All the above assays were performed in duplicate. The interassay variability was <5.0%.

Liver tissue α-tocopherol analysis was done according to the protocol of Devaraj and Jialal [15]. Separation was performed on a reverse-phase column (C_{18}) at 25°C and α-tocopherol was detected at

292 nm. The mobile phase used was methanol:acetonitrile:dichloroethane (60:20:20) with a flow rate of 0.8 ml/min. α-tocopherol was extracted following the lipid extraction from the liver tissues according to Hara & Radin [16].

Total RNA isolation and RT-PCR: Total cellular RNA was isolated from vehicle and α-TOS -treated LNCaP xenografts (preserved in RNAlater® Ambion Inc.) using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to protocols provided by the manufacturer. RNA purity was established by agarose gel electrophoresis, and the amount of RNA was quantified by measurement of the $A_{260/280\ nm}$ ratio. Reverse transcription (RT) of RNA into cDNA was accomplished with SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For real-time PCR, after a 1:200 dilution, two microliters of the RT reaction and the corresponding PCR primer set plus 25 μl of the SYBR® Green qPCR master mix (modified DyNAmo hot start DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP) (MJ Research, Reno, NV) was added and then adjusted to 50 μl with ultra pure (UP) H_2O. Primers (5 μl each) were added for the following: PSA forward and backward, 5′-GGAACAAAAGCGTGATCTTGC TGGG-3′ and 5′-GGGTGAACTTGCGCACACACGTC-3′, expected size 363 bp; VEGF forward and backward, 5′-ATCTTCAAGCCATCCTGTGTGC-3′ and 5′-TCACCGCCTCGGCTTGTCACAT-3′, expected size 391, 365 and 291 bp (3 isoforms); and β_2-microglobulin (housekeeping gene, internal control) forward and backward, 5′-ATGCCTGCCGTGTGAACCATG TG-3′ and 5′-AGAGCTACCTGTGGAGCAACCTG-3′, expected size 280 bp [17–19].

Reactions were assembled at room temperature, and then loaded on the DNA Engine Opticon® 2 System for continuous fluorescence detection (MJ Research, Inc. Watertown, MA). The thermal cycling protocol was 95°C for 15 min at hot start followed by 40 cycles (60°C or in accord to primer melting points less 4–6°C for 1 min; 72°C for 2 min for primer extension and 94°C for 30 s for dsDNA template denaturation). Following PCR, in order to confirm a single band dsDNA product as well as match the dsDNA size to the DNA standard, ten microliters of each PCR sample plus 2.0 μl of 30% glycerol with tracking dyes were loaded on a 2% (w/v) agarose gel and separated by size using electrophoresis at 80 V for 90 min. DNA was then visualized by fluorescence from ethidium bromide staining (0.5 μg/ml) and documented photographically.

The Opticon Monitor software, version 2.02, was used to calculate the initial quantity of template in a sample. Each total RNA sample was analyzed twice and in duplicate for amount of dsDNA from RT-PCR for each gene. The ratio of PSA or VEGF/$\beta_2$microglobulin expression were used for comparison among different treatment groups at different time points.

Tumor histology

Formalin-fixed tumor specimens were embedded in paraffin and sectioned at 5 μm thickness. Hematoxylin and eosin (H & E)-stained slides were examined by light microscopy, using a Nikon microscope. We characterized tumor morphology and cytologic features. Tissue density was determined by measuring total tumor area and quantitating the proportion (expressed as %) occupied by tumor cells, compact connective tissue matrix, blood-filled vessels, and areas of necrosis. Many blood vessels were readily identifiable and were quantified on the hematoxylin-eosin-stained sections because of the presence of eosin-stained red blood cells.

Statistical analysis

All samples were analyzed using a two-way ANOVA followed by Tukey–Kramer multiple comparison tests to investigate the effects of dietary fat levels and α-TOS treatment on the growth of human prostate LNCaP tumor tissues. A $P \leq 0.05$ was considered significant. Simple correlation and linear regression tests were performed to identify potential relationships among experimental parameters. The statistical software used was SPSS, version 11.5 for Windows.

Results

Table 1 shows average initial and final body weights, weight gain and daily intake of total feed and selected nutrients of nude mice from treatment and control groups. Mice fed *ad libitum* the 20%-fat diet consumed higher amounts of calorie and fats than those fed the 7%-fat diet. In this study, a hypercaloric, high-fat diet (20%-fat) was shown to promote prostate tumor growth in nude mice in comparison to the tumor growth in mice that had a lower fat and a higher carbohydrate intake.

The final tumor volumes at 7 weeks after inoculation (Fig. 1) were significantly lower in the α-TOS-treated groups than the controls for both levels of dietary fat. Also, the mean tumor volume was lower for the 7%-fat group (0.09 ± 0.05 cm^3) compared to those in the 20%-fat group (0.19 ± 0.1 cm^3, $P < 0.001$). The overall treatment effect ($P < 0.0001$) showed that the α-TOS-treated mice had a lower tumor volume (0.06 ± 0.02 cm^3) when compared to the vehicle-

Table 1 Average body weights, daily feed and selected nutrient intakes in BALB/c nude mice bearing LNCaP tumors from different control and experimental groups (mean ± SE)

Parameters	7% soybean oil + vehicle[a]	7% soybean oil + α-TOS[b]	20% soybean oil + vehicle	20% soybean oil + α-TOS
n	10	11	11	10
Initial body weight (g)	24.0 ± 2.39	26.0 ± 3.44	24.0 ± 2.28	24.0 ± 1.71
Final body weight (g)[c]	28.0 ± 2.76	30.0 ± 4.45	26.0 ± 2.16	26.0 ± 1.39
Weight gain (g)[d]	4.0 ± 0.5	4.0 ± 0.9	2.0 ± 0.4	2.0 ± 0.5
Feed intake (g/d)[e]	5.0 ± 0.51	6.0 ± 0.27	5.0 ± 0.27	5.0 ± 0.07
Calorie intake (kcal/d)[f]	19.0 ± 0.61	22.0 ± 0.45	24.0 ± 0.74	23.0 ± 0.54
Protein intake (g/d)	1.0 ± 0.17	1.0 ± 0.23	1.0 ± 0.28	1.0 ± 0.32
Fat intake (g/d)[g]	0.33 ± 0.1	0.39 ± 0.2	1.0 ± 0.3	1.0 ± 0.3
Carbohydrate intake (g/d)	3.0 ± 0.5	3.0 ± 0.4	2.0 ± 0.4	2.0 ± 0.5

[a] Sesame seed oil
[b] Alpha-tocopheryl succinate (100 mg/kg body weight)
[c, d, f & g] Significant overall effect of dietary fat level; 7% versus 20% fat group ($P < 0.001$, Two-way ANOVA)
[e] Significant effects of treatment; α-TOS versus sesame seed oil ($P < 0.05$) and interaction ($P < 0.0001$), Two-way ANOVA)

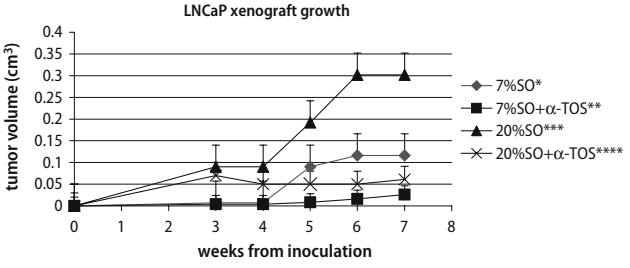


Fig. 1 LNCaP xenograft growth in BALB/c nude mice fed 7% or 20% soybean oil diet with or without α-TOS treatment. Significant effects of dietary fat and treatment (two-way ANOVA; $P < 0.05$). SO—Soybean oil diet, α-TOS—alpha-tocopheryl succinate. *7% SO & **7% SO + α-TOS differ in tumor growth ***20% SO & ****20% SO + α-TOS differ in tumor growth *7% SO & ***20% SO differ in tumor growth **7% SO + α-TOS & ****20% SO + α-TOS differ in tumor growth ($P < 0.05$)

treated mice (0.24 ± 0.12 cm^3). The 7% α-TOS-treated group had the lowest tumor volume in comparison to the other three groups. The 20%-fat fed controls had an accelerated tumor growth compared to those fed 7%-fat diet. Intraperitoneal injections of α-TOS caused tumor growth to reduce in both the dietary groups, but the mean tumor volume was higher in the 20% α-TOS-treated group (0.06 ± 0.02 cm^3) compared to those in the 7% α-TOS -treated mice (0.03 ± 0.01 cm^3).

α-TOS treatment significantly reduced serum PSA level (40.09 ± 2.9 ng/ml) in comparison to the sesame seed oil-treated controls (85.38 ± 4.1 ng/ml). The two-way ANOVA also revealed a significant effect of dietary fat level. Mice fed 20%-fat diet had a higher serum PSA level (85.45 ± 4.3 ng/ml) than those fed 7% dietary fat from soybean oil (40.02 ± 2.54 ng/ml; $P < 0.05$). Both treatment and dietary fat level were found to modulate PSA secretion from LNCaP xenografts. In case of serum total testosterone, a significant effect was observed in α-TOS-treated animals versus the controls ($P < 0.05$). Table 2 shows serum PSA and testosterone values. Hepatic α-tocopherol levels were significantly higher in the 20%-fat fed mice versus the 7%-fat group (34.4 ± 3.4 nmol/g and 25.74 ± 2.1 nmol/g, respectively, $P < 0.05$), and also in the α-TOS-treated group in comparison to the untreated controls (37.81 ± 4.4 nmol/g and 22.33 ± 4.2 nmol/g, respectively, $P < 0.05$), as shown in Table 3. α-TOS levels were not detected in the liver samples.

PSA and VEGF mRNA expressions in LNCaP xenografts were quantified using RT and real-time PCR on total RNA extracts from the tumor tissues. Amplification of the RT cDNAs from LNCaP xenograft RNAs (control or α-TOS-treated) gave three distinct VEGF isoforms of 391, 365 and 231 bp (Fig. 2) as expected from the primers used; these bands had been sequenced previously by other investigators and shown to correspond to $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$, respectively (18). Real-time PCR system, based on the binding of SYBR Green I to the double-stranded DNA grove of the gene fragment being amplified, makes quantification possible in the exponential phase of the reaction. In case of VEGF, the fluorescence signal [C (t) value] was detected three cycles later for α-TOS-treated RNA samples in comparison to the controls. When expressed relative to β_2-microglobulin [internal control, 280 bp (Fig. 3)], VEGF expression was significantly affected by dietary fat level, treatment or interaction (two-way ANOVA, $P < 0.05$; Fig. 3). This inhibition of VEGF at the transcriptional level also corresponded to a reduction in angiogenesis in α-TOS-treated xenografts when compared to controls (Fig. 4). A moderately positive correlation (Pearson r 0.473, $P < 0.05$) was observed between PSA and VEGF mRNA expressions in LNCaP xenografts (data not shown). PSA mRNA expression was significantly affected by α-TOS treatment ($P < 0.05$, Figure 3). A single PSA gene fragment (363 bp, Fig. 2), from α-TOS-treated xenografts, was detected two cycles later compared to control

Table 2 Serum prostate-specific antigen (PSA) and testosterone levels in BALB/c nude mice bearing LNCaP xenografts fed 7% or 20% soybean oil diet with or without α-TOS treatment (mean ± SE)

Parameters (ng/mL)	7% soybean oil + vehicle[a]	7% soybean oil + α-TOS[b]	20% soybean oil + vehicle	20% soybean oil + α-TOS
n	10	11	11	10
Serum PSA[c]	55.12 ± 2.7	26.28 ± 2.55	112.89 ± 3.6	55.27 ± 3.64
Serum Testosterone[d]	0.52 ± 0.08	0.33 ± 0.02	0.61 ± 0.07	0.28 ± 0.09

[a] Sesame seed oil
[b] Alpha-tocopheryl succinate (100 mg/kg body weight)
[c] Significant effect of treatment; α-TOS versus sesame seed oil ($P < 0.0001$) and dietary fat level; 7% versus 20% fat group ($P < 0.0001$, Two-way ANOVA)
[d] Significant effect of treatment; α-TOS versus sesame seed oil ($P < 0.05$, Two-way ANOVA)

Table 3 Percentage (%) angiogenesis and tumor area in LNCaP xenografts in BALB/c nude mice fed 7% or 20% soybean oil diet with or without α-TOS treatment (mean ± SE)

Parameters	7% soybean oil + vehicle[a]	7% soybean oil + α-TOS[b]	20% soybean oil + vehicle	20% soybean oil + α-TOS
n	10	11	11	10
% angiogenesis[c]	2.25 ± 0.7	0.8 ± 0.3	1.9 ± 0.2	1.45 ± 0.4
% tumor area[d]	63.75 ± 1.3	42.14 ± 1.6	68.0 ± 7.5	40.0 ± 6.6
Liver alpha-tocopherol (nmol/g)[e]	16.66 ± 1.7	34.82 ± 3.50	28.00 ± 5.6	40.80 ± 3.04

[a] Sesame seed oil
[b] Alpha-tocopheryl succinate (100 mg/kg body weight)
[c] Significant effect of treatment; α-TOS versus sesame seed oil ($P < 0.0001$) and dietary fat level; 7% versus 20% fat group ($P < 0.0001$, Two-way ANOVA)
[d] Significant effect of treatment; α-TOS versus sesame seed oil ($P < 0.05$, Two-way ANOVA)
[e] Significant effect of treatment; α-TOS versus sesame seed oil and dietary fat level; 7% versus 20% fat group ($P < 0.05$, Two-way ANOVA)

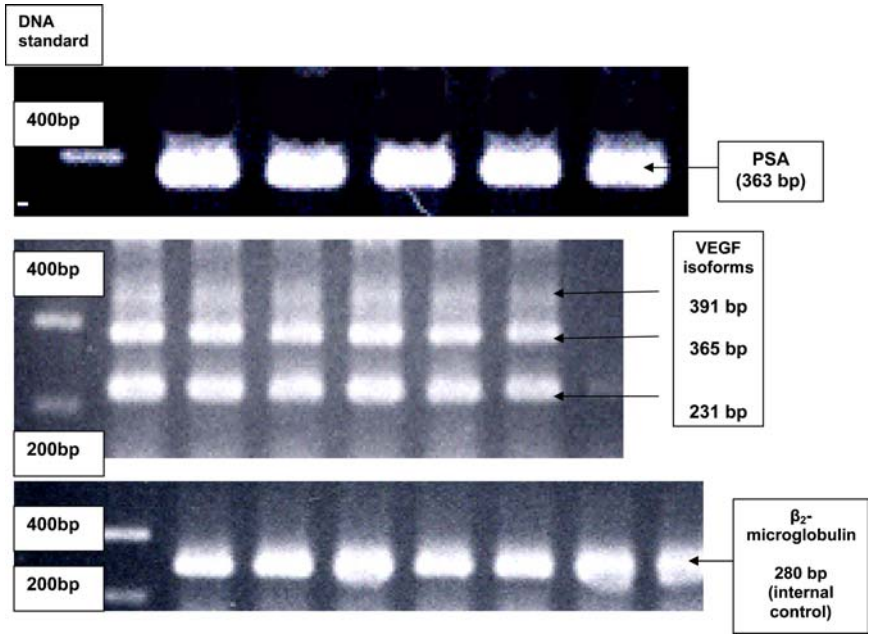
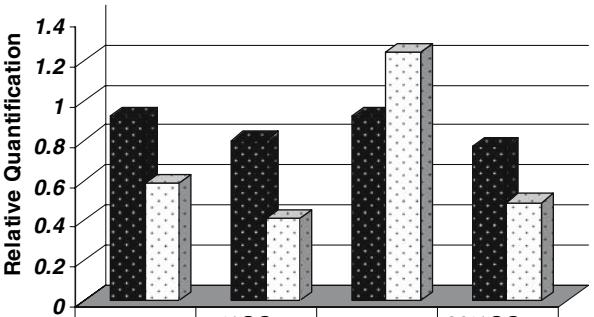


Fig. 2 RT-PCR amplification (40 cycles) of prostate-specific antigen (PSA, 363 bp), vascular endothelial growth factor (VEGF, three isoforms: 391, 365, and 231 bp), and β_2-microglobulin (internal control, 280 bp) gene fragments in LNCaP xenografts in nude mice fed 7% or 20% soybean oil diet with or without α-TOS treatment; tumors harvested at the end of 7 weeks from xenograft inoculation

xenograft RNAs. Thus, both the PSA mRNA and serum PSA levels were found to be inhibited by α-TOS ($P < 0.05$, Table 2).

There were considerable differences in tumor architecture between α-TOS and control tumors. Control LNCaP tumors had a greater degree of angiogenesis than α-TOS tumors, as demonstrated by the greater number of blood-filled vessels (Fig. 4, Table 3). There was a greater abundance of tumor cells in the sections of control tumors than α-TOS tumors, as well (Table 3). The morphology of tumor cells themselves were similar in all sections.



	7%SO	7%SO+α-TOS	20%SO	20%SO+α-TOS
PSA*	0.923	0.802	0.925	0.774
VEGF**	0.586	0.409	1.24	0.491

Fig. 3 Relative quantification of prostate-specific antigen (PSA) and vascular endothelial growth factor (VEGF) gene fragments to β_2-microglobulin using real-time PCR in LNCaP xenograft samples in nude mice fed a 7% or 20% soybean oil diet with or without α-TOS treatment; *significant effect of treatment (α-TOS versus sesame seed oil); **significant effects of dietary fat (7% versus 20% group) and treatment (α-TOS versus sesame seed oil); two-way ANOVA, $P < 0.05$. (SO—Soybean oil diet, α-TOS—alpha-tocopheryl succinate)

Discussion

This study reports the effects of α-TOS on the growth of human prostate LNCaP xenografts in nude mice, fed 7% or 20% soybean oil diet. Several cell culture studies have reported that α-TOS is an effective form of vitamin E in prostate cancer treatment [5–10]. However, *in vivo* data is scarce. Though we used sesame oil as a vehicle, its reported anticarcinogenic properties [20] did not mask the anti-tumor effects of α-TOS in our study, which produced statistically significant reductions in LNCaP growth, in comparison to controls receiving sesame oil only, in mice fed low (7%) and high (20%) levels of soybean oil diets. However, there exists the possibility of synergism between α-TOS and sesame lignans, which may partially contribute to the potency of α-TOS as a chemotherapeutic agent. Fleshner et al. [21] showed a reduction in LNCaP xenograft growth in nude mice fed a high-fat (40% kcal-fat) diet with *all rac*-α-tocopheryl acetate supplementation versus the controls but no effects between a low fat (20% kcal-fat) diet with *all-rac*-α-tocopheryl acetate supplementation versus the controls, following a 15-week supplementation period. The 7% or 20% soybean oil diet (w/w) used in our study corresponds to approximately 14%- or 40%-kcal fat and these levels are almost identical to the 20%- or 40%-kcal fat diet used in the study by Fleshner et al. In contrast to the findings of Fleshner et al. [21], our study shows that a 2-week α-TOS administration caused a significant reduction in LNCaP xenograft growth not only in high fat (40%-kcal fat) fed group, versus the controls, but also in those mice fed a low fat (14%-kcal fat) diet in comparison to the corresponding controls (Fig. 1). Thus, α-TOS appears to be a stronger chemopreventive agent than *all rac*-α-tocopheryl acetate in human prostate cancer growth. Also, the route of administration, being intraperitoneal for α-TOS in our study, and oral supplementation of *all rac*-α-tocopheryl acetate by Fleshner et al. may further explain the higher chemotherapeutic potency of the former. The effects of a high-fat diet in promoting the incidence and progression of prostate cancer and those of a low-fat diet in reversing these effects have already been reported by several studies [11, 21–23]. Our study also shows a similar effect with the 20% soybean oil (40%-kcal fat) fed control group having the highest tumor volume (Figure 1) at the end of 7 weeks, in contrast to the 7% soybean oil (14%-kcal fat) fed controls. This effect of a low fat diet in arresting the growth of human prostate LNCaP xenograft was synergistically inhibited in the 7%-fat + α-TOS treated group which showed a further significant reduction in tumor volume (Fig. 1).

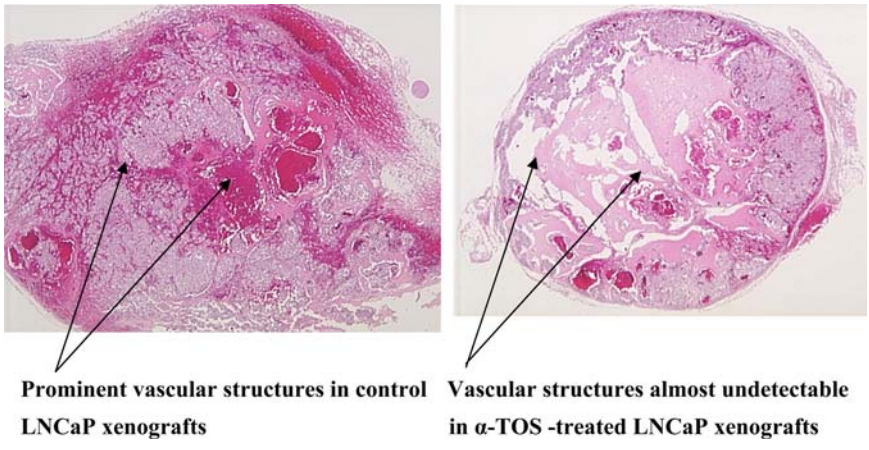


Fig. 4 Sections from control or α-TOS-treated LNCaP xenografts showing angiogenesis

Overall, feed intake was highest in the 7% α-TOS-treated group in comparison to other groups (Table 1). This finding is consistent with those of Barnett et al. who also found that mice from the α-TOS group ate more than the vehicle-treated group [24]. Our study findings also indicate no obvious toxic effects of α-TOS as well as the effects of increased tumor burden in the control group. Serum testosterone analysis revealing a significant effect of treatment (Table 2) suggests a role of α-TOS in hormone metabolism. Serum androgens have been related to the progression of prostate cancer and metastatic disease. Animal studies have shown that supplementation with high levels of α-tocopherol inhibits prostaglandin synthesis which in turn leads to lower serum androgens by interacting with the gonadal pituitary axis [25]. Such results have not been reported with α-TOS treatment. But, it is possible that in our study, chronic administration of α-TOS, everyday for 2 weeks at 100 mg/kg body weight, would enrich the circulation with α-tocopherol, upon hydrolysis by hepatic esterases, as suggested by previous researchers [12], and exert similar inhibitory effects on sex hormone concentration. A cross-sectional analysis within a subset of the ATBC clinical trial also showed that serum α-tocopherol was significantly inversely associated with serum androstenedione, testosterone, sex hormone-binding globulin, and estrone [26]. Serum testosterone levels in our study showed no significant effect of dietary fat level (Table 2). This is consistent with the data reported by Wang et al. [23] and also Aronson et al. [27], who studied the effects of high or low fat corn oil diets on the growth of LNCaP xenografts in immune deficient mice. Thus, the type and amount of dietary fat does not seem to have a significant effect on androgen metabolism in nude mice bearing human prostate xenografts. Interestingly, similar to the findings of Wang et al. [23], we also report that serum PSA levels were markedly affected by diet and also by α-TOS treatment (Table 2). This effect of α-TOS treatment on PSA levels was a unique observation in vivo, similar to the data reported earlier by Zhang et al. [8] in LNCaP cell line. PSA secretion was directly proportional to the tumor burden and so the 20%-fat fed control group with the maximum tumor volume (Fig. 1) also exhibited the maximum increase in serum PSA level (Table 2). The decrease in PSA level with low fat (7% soybean oil) diet or α-TOS treatment leads to the observation that serum PSA can be used as a biomarker for evaluation of human LNCaP prostate cancer xenograft growth in nude mice model, following dietary treatment and intraperitoneal administration of α-TOS. The decrease in PSA mRNA expression (Fig. 3) as a result of α-TOS treatment indicates reduced transcription and corresponding translation in LNCaP xenografts; a major indicator of growth reduction of PSA-secreting LNCaP xenograft in our study. Serum PSA values reflect total prostate volume, in both normal and malignant tissues, and is helpful in monitoring and evaluating therapeutic response [28].

Tissue distribution and turnover of α-TOS have been studied earlier by Weber et al. [29] who found an almost three times higher level of liver α-tocopherol (the hydrolysis product of α-TOS) in nude mice than those reported by our study (Table 3). Weber et al. also reported significant levels of α-TOS in serum, liver, kidney, and tumor tissues in these mice, which were treated with α-TOS for 10 days. This difference in liver accumulation of α-tocopherol, and our inability to detect α-TOS in liver samples, may be explained by the fact that the α-TOS treatment in our study was terminated 2 weeks prior to sacrifice, in comparison to the study by Weber et al. in which animals were sacrificed immediately after termination of α-TOS treatment. Weber et al. also reported data, which showed a superior tumor growth inhibition by α-TOS (80%), in comparison to α-tocopherol (35%) of HCT116 xenografts in nude mice [29]. We could not analyze α-tocopherol levels in plasma and tumor tissues due to insufficient samples. However, it appears that α-TOS is rapidly hydrolyzed to α-tocopherol and continuous administration may be necessary to maintain adequate serum and tissue levels, essential for its chemotherapeutic effects.

Angiogenesis, a major physiological process essential for the survival of tumor tissues was also shown to be affected by α-TOS treatment. The marked differences seen in tumor angiogenesis and % tumor area in Fig. 4 and Table 3, between α-TOS-treated or control LNCaP xenografts reinforce the observations by Mukherjee et al. [22] and Malafa and Neitzel [13]. These authors have reported the individual effects of a low-fat diet or α-TOS treatment on reduced vasculature or decreased VEGF gene expression, respectively, in human cancer xenografts in immunedeficient mice. These independent effects were shown to act synergistically in our study which led to a significant reduction in expression of VEGF mRNA in xenografts in low and high fat fed group with α-TOS treatment versus the untreated controls (Fig. 3). The positive correlation between PSA and VEGF mRNA expressions (data not shown) indicates a simultaneous increase of both with LNCaP cell multiplication and a concomitant reduction in tumor angiogenesis due to α-TOS treatment as seen in Fig. 4. It should be noted that the angiogenesis was of host origin.

Our study shows α-TOS, by itself, to be an effective chemotherapeutic agent in reducing human prostate xenograft growth in an *in vivo* model. The study design of 2-week administration of α-TOS was on the basis of previous limited studies (13, 24) who con-

ducted similar in vivo administration of α-TOS. On the basis of our novel data, future studies may involve a long-term administration of α-TOS. An important limitation of our study was the time gap between the termination of α-TOS treatment and the sacrifice of the animals, as a result of which we were unable to show the accumulation of α-TOS in liver tissues in these animals. However, the challenge remains in administering this vitamin E analog to humans, in which case daily intraperitoneal doses may not be feasible. Further studies in animals with higher doses of α-TOS, given intraperitoneally or intravenously [12] every two or three days, are warranted to detect any difference in the chemotherapeutic action of α-TOS. α-TOS has repeatedly shown to be highly selective for cancer cells, and remains non-toxic to normal cells even when accumulated at high levels in the biological system [29].

In summary, our study shows significant reduction in human prostate LNCaP xenograft growth with α-TOS treatment in nude mice fed a low (7%) and high (20%) fat soybean oil diets verus controls. Serum PSA and testosterone, tumor angiogenesis, and PSA and VEGF mRNA expressions were markedly reduced by α-TOS administration.

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