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Original Contribution

γ-Tocotrienol induces growth arrest through a novel pathway with TGFβ2 in prostate cancer

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ABSTRACT

Regions along the Mediterranean and in southern Asia have lower prostate cancer incidence compared to the rest of the world. It has been hypothesized that one of the potential contributing factors for this low incidence includes a higher intake of tocotrienols. Here we examine the potential of γ-tocotrienol (GT3) to reduce prostate cancer proliferation and focus on elucidating pathways by which GT3 could exert a growth-inhibitory effect on prostate cancer cells. We find that the γ and δ isoforms of tocotrienol are more effective at inhibiting the growth of prostate cancer cell lines (PC-3 and LNCaP) compared with the γ and δ forms of tocopherol. Knockout of PPAR-γ and GT3 treatment show inhibition of prostate cancer cell growth, through a partially PPAR-γ-dependent mechanism. GT3 treatment increases the levels of the 15-lipoxygenase-2 enzyme, which is responsible for the conversion of arachidonic acid to the PPAR-γ-activating ligand 15-*S*-hydroxyeicosatrienoic acid. In addition, the latent precursor and the mature forms of TGFβ2 are down-regulated after treatment with GT3, with concomitant disruptions in TGFβ receptor I, SMAD-2, p38, and NF-κB signaling.

Prostate cancer has the greatest country-to-country variation in incidence of any reportable cancer, suggesting the importance of nutritional factors [1]. Micronutrient antioxidants such as vitamin E scavenge free radicals and may play a role in preventing oxidative damage to the prostate epithelium. The term vitamin E refers to four tocopherol isoforms, α-tocopherol (AT), γ-tocopherol (GT), δ-tocopherol (DT), and β-tocopherol (BT) (containing a phytyl tail), and four tocotrienol isoforms, α-tocotrienol (AT3), γ-tocotrienol (GT3), δ-tocotrienol (DT3), and β-tocotrienol (containing an isoprenoid tail). These isoforms can be found in vegetable oils, nuts, and whole grains. Much of the previous research connecting vitamin E to cancer has been focused on AT, whereas other isoforms of vitamin E have been largely ignored [2]. The ability of vitamin E to induce apoptosis in cancer cells varies among the isoforms and is not necessarily correlated with their antioxidant potencies [3–7]. This suggests that vitamin E isoforms may influence cancer growth by modulating gene-regulatory functions through mechanisms unrelated to their antioxidant properties [3–5]. For example, GT, but not AT, down-regulates cyclin D_1 and cyclin E levels in several cancer cell lines [6]. AT and BT differentially inhibit integrins [8]. Tocotrienols, with the isoprenoid tail, inhibit the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG CoA) reductase pathway, leading to mevalonate depletion and inhibition of prenylation of critical signaling proteins through an Insig-dependent ubiquitination and degradation of the HMG CoA reductase enzyme [9]. By regulating HMG CoA reductase, tocotrienols also regulate

Abbreviations: GT3, *R*-γ-tocotrienol; 15-LOX-2, 15-lipoxygenase-2; 15-S-HETE, 15-*S*-hydroxyeicosatetraenoic acid; NF-κB, nuclear factor κB; TGFβ, transforming growth factor β; TAK-1, TGFβ-activating kinase-1; TAB-1, TGFβ-activating kinase-1 subunit; AT, *RRR*-α-tocopherol; GT, *RRR*-γ-tocopherol; PPAR, peroxisome proliferator-activated receptor; DT, *RRR*-δ-tocopherol; DT3, *R*-δ-tocotrienol; QPCR, quantitative polymerase chain reaction; DN, dominant negative; LBD, ligand-binding domain.

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cholesterol production in mammalian cells [10]. Targeting the mevalonate pathway for cancer therapy and prevention is currently under investigation [11–13]. Our laboratories have demonstrated that AT and GT have the ability to up-regulate the expression of peroxisome proliferator activated receptor (PPAR)-γ protein and mRNA in colon cancer cell lines, with GT being more effective than AT [14], and that GT-mediated growth arrest in prostate cancer cells is PPAR-γ-dependent through the up-regulation of 15-LOX-2, resulting in 15-S-HETE expression [15]. These studies support the view that different isoforms of vitamin E could exert anticancer effects by mechanisms unrelated to their antioxidant activity. The purpose of this study was to compare the prostate cancer growth inhibition of tocopherols to that of tocotrienols and determine which of these isoforms is more potent at growth inhibition in both LNCaP (androgen-dependent) and PC-3 (androgen-independent) prostate cancer cells, as well as to determine if the mechanism of action of GT3 is similar to that previously determined for GT [15].

Experimental procedures

Materials

The GT (99% pure), DT (99% pure), and AT (99% pure) were generously donated by the Cognis Corp. (LaGrange, IL, USA). The GT3 and DT3, with a purity of 98 and 75%, respectively, were generously donated by Carotech Corp. (Kuala Lumpur, Malaysia). 15-Deoxy-Δ-12,14-prostaglandin J_2 was purchased from BioMol Research Laboratories (Plymouth Meeting, PA, USA). The PPAR-γ dominant-negative (DN) vector was a generous gift from Dr. V. Krishna K. Chatterjee (Department of Medicine, University of Cambridge, Cambridge, UK).

Cell culture

The PC-3 prostate cancer cell line, CRL-1435 (derived from a bone metastasis of a grade IV human prostatic adenocarcinoma displaying epithelial morphology), and the LNCaP prostate cancer cells, CRL-1740 (derived from a left supraclavicular lymph node of human prostate carcinoma), were purchased from the American Type Culture Collection (Manassas, VA, USA). Prostate cancer cell lines were maintained as monolayer cultures in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C and were subcultured at 50–75% confluence (75% confluence for PC-3 cells and 50% confluence for LNCaP cells). PrEC primary prostate epithelial cells were purchased from Lonza Bioscience and maintained by strict adherence to the manufacturer's instructions in an atmosphere of 5% CO_2 at 37 °C.

Enrichment of vitamin E media

Concentrations of vitamin E (tocopherols or tocotrienols) were determined in ethanol using an HP-8542A diode array spectrophotometer with the following maximum wavelengths (λ_{max}) and molar extinction coefficients (ε): AT λ_{max} 292 nm, ε = 3270; GT λ_{max} 298 nm, ε = 3810; DT λ_{max} 298 nm, ε = 3520; AT3 λ_{max} 292 nm, ε = 3870; GT3 λ_{max} 298 nm, ε = 4230; DT3 λ_{max} 292 nm, ε = 3300. Before treatment, the cell culture medium was enriched with tocopherol or tocotrienol by adding the appropriate amount of tocopherol in ethanol (ethanol concentrations never exceeded 50 μl ethanol/ml of medium). In the vehicle-treated cells, ethanol was added to the complete culture medium at the same concentration that was added to the treatments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

PC-3 or PrEC cells were plated at a concentration of 1×10^5 cells/well in a 96-well flat-bottom plate and allowed to adhere for 24 h before treatment. The vitamin E isoforms were added at various concentrations for the indicated times. After treatment, the MTT reagents were applied according to the manufacturer's (Molecular Probes, Carlsbad, CA, USA) instructions. The absorbance at 570 nm was monitored using a Spectramax Plus 190 UV–Vis spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Caspase activity assay

Cells were plated at 2×10^6 cells/well in six-well plates 24 h before treatment. Cells were treated with tocopherol- or tocotrienol-enriched medium for varying times (0–24 h). After treatment, the cells were collected and washed twice with PBS. For optimal lysis, the cells were incubated in lysis buffer (10 mM Tris–HCl, 0.01% Triton X-100, 1 mM EDTA, 100 mM NaCl, pH 7.5) and subjected to two freeze/thaw cycles at −80 °C. Cell lysates, in 40-μl aliquots, were added to a microtiter plate well followed by addition of working solution, which included reaction buffer (10 mM Pipes, 2 mM EDTA, 0.1% Chaps, pH 7.4) and 50 mM substrate. Cellular caspase-3 activity was measured using the caspase-3 substrate, Ac-DEVD-AFC (Sigma, St. Louis, MO, USA), according to the manufacturer's protocol and measuring fluorescence with a BioTek FLX-800 microplate fluorimeter (λ_{ex} 400 nm, λ_{em} 505 nm).

Analysis of cell viability, apoptosis, and mitochondrial distribution by fluorescence microscopy assay

The cells were plated in Costar black plastic 96-well plates with clear bottoms and treated for 6, 24, and 72 h with 0, 0.1, 1, 5, 10, 20, and 40 μM γ-tocotrienol. After treatment, the cells were triple-stained with calcein AM (2 μM), DAPI (1 ng/ml), and MitoTracker Red CM-H2XRos (1 μM) in phenol-red-free and serum-free Dulbecco's minimal essential medium (Invitrogen, Carlsbad, CA, USA). Apoptosis and nuclear morphology, DNA dye uptake, and cellular staining were assessed by fluorescence microscopy using an Olympus IX-70 inverted microscope. Image acquisition was achieved using a Quantix charged-coupled device camera and IP Labs software (Scanalytics, Inc., Fairfax, VA, USA) on a Macintosh computer (Apple Computer Corp., Cupertino, CA, USA).

Treatment of cells for RNA/protein extraction

PC-3 human prostate cancer cells were seeded at 5×10^6 cells/plate in 100×20-mm plates 24 h before treatment. Then the cells were treated with vitamin E isoforms or vehicle (+ ethanol) for the indicated times.

Western blot analysis

The protein concentrations of the cell lysates were determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Cell lysates were separated by electrophoresis on a 10% SDS–polyacrylamide gel and electrotransferred onto Hybond-ECL nitrocellulose membrane as previously described [14]. Blotted membranes were blocked overnight with 5% bovine serum albumin, fraction V, or 5% skimmed milk and incubated with the primary antibodies. Antibodies from Santa Cruz Biotechnology (San Cruz, CA, USA) included PPAR-γ E-8, 15-LOX-2 E-13, phosphorylated NF-κB (sc-33020), and phosphorylated p38 (sc-17852). Antibodies from Cell Signaling (Danvers, MA, USA) included TGFβ receptor I (3712), phosphorylated SMAD-2 (3104), SMAD-2 (3102), TAK-1 (4505), p38 (9212), phosphorylated p38 (9215), TAB-1 (3226), MKK3/6 (9231), phosphorylated NF-κB (3031), NF-κB p65 (3034), PARP (9542), caspase-9 (9501), and caspase-3 (9665). Antibodies purchased from R&D Systems (Minneapolis, MN, USA) included TGFβ2 (AB-12-NA) and XIAP (AF-8221). β-Actin was obtained from Sigma (F3022). After primary antibody incubation the blots were probed with the appropriate secondary antibody conjugated with horseradish peroxidase. The signal was measured using Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology). To