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α-Tocopheryl succinate induces cytostasis and apoptosis in osteosarcoma cells: the role of E2F1

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Abstract

α-Tocopheryl succinate (α-TOS), a redox-inactive analog of vitamin E, induces cell cycle arrest, differentiation, and triggers apoptosis. We examined the ability of α-TOS to induce cytostasis and/or apoptosis in two human osteosarcoma cell lines, which carry wild-type pRb but differ in the p53 status. In the wt-p53 cells, α-TOS induced apoptosis, which was associated with p53 activation and enhanced E2F1 expression. Mutant p53 cells failed to undergo apoptosis when challenged with α-TOS. The cell growth arrest after α-TOS treatment was associated with a reduced expression of E2F1. Knocking down E2F1 rendered the α-TOS-sensitive cells rather resistant to the apoptotic stimulus inducing a marked and prolonged cell growth arrest. We conclude that α-TOS induces cell growth arrest or apoptosis involving E2F1.

Keywords: Osteosarcoma; Proliferation; Apoptosis; α-Tocopheryl succinate; E2F1; p53

α-Tocopheryl succinate (α-TOS), a redox-silent analog of vitamin E, is a highly selective cytotoxic agent, both reducing proliferation and inducing apoptosis in many types of cancer cells [1–3]. Proliferation and apoptosis are intimately coupled, and some cell cycle regulators can influence both cell division and programmed cell death [4,5]. The role of the Rb/E2F pathway in the regulation of cell cycle progression is well established [6]. Among members of the E2F family, E2F1 appears to play a dual role in the control of cell proliferation and apoptosis [7]. Over-expression of E2F1 can drive quiescent cells into S phase [8], and cells with multiple E2F protein deletions exhibit enhanced proliferation [9]. In addition, E2F1 seems to be able to induce apoptosis when expressed in the absence of proliferative signals [10,11]. The induction of the apoptotic pathway is associated with the capacity of E2F1 to induce accumulation of p53 as well as other genes [10]. p53 is a key component of a cellular emergency-response mechanism [12,13]. A variety of stress signals induce cell growth arrest or apoptosis, thereby eliminating damaged and potentially harmful cells [14]. The p53 gene is lost or mutated in ~50% of human cancers [15,16]. Lack of functional p53 is accompanied by high rates of genomic instability, rapid tumor progression, and resistance to therapy [17,18]. To reconcile with this important pathway, several therapeutic strategies have been designed to restore the function of deleted or mutated genes to overcome resistance of cancer cells to apoptosis.

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Here, we examined the ability of α-TOS to induce apoptosis and inhibit proliferation in cancer cells that differ in their p53 status. Our results show that α-TOS induces an apoptotic response in cells carrying wt-p53. α-TOS-induced apoptosis was associated with p53 activation followed by a marked induction of E2F1 expression and down-regulation of the Bcl-2 protein. In contrast, α-TOS treatment resulted in cell growth arrest of cells carrying mutated p53 (mut-p53), associated with E2F1 down-regulation. Although p53 is important for apoptosis induction, the vitamin E analog induces cell death in a p53-independent manner. Down-regulation of E2F1 rendered the α-TOS-sensitive cells rather resistant to the apoptotic stimulus inducing a marked and prolonged cell growth arrest. These results contribute to our understanding of the molecular mechanism(s) that regulate the cellular switch between apoptosis and growth arrest in response to α-TOS treatment.

Materials and methods

Reagents. *RRR*-α-TOS, annexin V-FITC, 3-,4-,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and G-418 were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Roche Diagnostic (Basel, Switzerland). Anti-CDK2, anti-p53, anti-phospho-p53 (Ser-20), anti-cyclin A, and anti-cyclin E were obtained from Oncogene Research (Cambridge, MA). Anti-E2F1 and anti-p21$^{Waf1/Cip1}$ monoclonal IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-Bcl-2 IgG was from Upstate Biotechnology (Lake Placid, NY).

Cell culture and treatment. The wt-p53 U2OS and the mut-p53 MG63 human osteosarcoma (OS) cell lines, obtained from the American Type Culture Collection (Rockville, MD), were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with α-TOS up to 100 μM. α-TOS was dissolved in ethanol, diluted in complete DMEM, and added to the final concentration at 0.01% (v/v) of ethanol.

Annexin V-FITC assay. Apoptosis was quantified by the annexin V-FITC method, which detects phosphatidylserine (PS) externalized in the early phases of apoptosis [19]. Briefly, cells were plated at 0.1×10^6 per well in 24-well cell culture plates. After an overnight recuperation, cells were treated with α-TOS. Floating and attached cells were collected, washed twice with PBS, re-suspended in 0.1 ml binding buffer (10 mM Hepes, 140 mM NaCl, and 5 mM $CaCl_2$, pH 7.4), incubated for 20 min at room temperature with 2 μl annexin V-FITC supplemented with 10 μl PI (10 μg/ml), and analyzed by flow cytometry (Becton–Dickinson FACScalibur) using channel 1 for annexin V-FITC binding and channel 2 for PI.

Cytotoxicity and cell proliferation assay. Cells were plated in 96-well flat-bottomed tissue culture plates at 2.5×10^3 per well, allowed to attach overnight, incubated for up to 3 days with α-TOS, and cell viability and proliferation was assessed as follows. Cell viability was determined using the MTT assay [20]. Briefly, treated cells were incubated with 10 μl of 3-4-,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), 5 mg/ml in PBS for 4 h at 37 °C. The medium was then removed and combined with 200 μl of 1% SDS. Absorbance was read at 550 nm using an ELISA plate reader. A cell proliferation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to determine the amount of 5-bromo-2′-deoxyuridine (BrdU) incorporated in DNA according to the manufacturer's instructions. Cells (10^3) were incubated with 100 μM BrdU for 2 h at 37 °C. The cells were fixed and anti-BrdU-POD (monoclonal, peroxidase-conjugated IgG), which binds to the incorporated BrdU in the newly synthesized DNA, was added. The immune complex was detected by the subsequent substrate reaction (tetramethylbenzidine), and the absorbance of the blue-green product was assessed at 370 nm in a microtiter plate reader.

Western blot analysis. Cells were treated as indicated and lysed in a buffer containing 250 mM NaCl, 25 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8), 0.1% Nonidet P-40, and a cocktail of protease inhibitors (2 mM sodium orthovanadate, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride). Protein cell extracts were quantified using the Bio-Rad Protein assay (Richmond, CA). Protein (50 μg) from total cell lysates was resolved using 12% SDS–PAGE and transferred to a nitrocellulose membrane. After blocking (PBS containing 0.1% Tween 20 and 5% skimmed milk) for 1 h, the membrane was incubated overnight with anti-phospho-p53 (ser-20), anti-p21$^{Waf1/Cip1}$, or anti-Bcl-2 IgG, and protein loading was corrected for β-actin. The E2F1 expression and CDK2/cyclin A complex were evaluated by immunoprecipitation according to manufacturer's protocol (Oncogene Research). Briefly, 100 μg of whole cell extracts was treated with 5 μl of the appropriate primary antibody in a volume of 100 μl at 4 °C for 1 h. Three milligrams of protein A–Sepharose in 100 μl volume was then added and incubated for additional 1 h. The beads were washed six times, boiled in SDS sample buffer, and the proteins were separated on 12% polyacrylamide gels. The signal was visualized by the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) and quantified by the GS-670 imaging densitometer (Bio-Rad, Hercules, CA).

Cyclin protein levels. Expression of cyclin A and cyclin E was evaluated by flow cytometry before and after treatment with α-TOS. U2OS and MG63 cells were seeded in six-well plates at 3×10^5 per well, treated, floating and attached cells were collected, permeabilized in cold (−20 °C) 70% ethanol for 1 h, and incubated at 4 °C with antibodies against cyclin A and cyclin E, followed by a secondary FITC-conjugated IgG. The level of protein expression was then evaluated by flow cytometry (Becton–Dickinson FACScalibur).

Immunocytochemistry. For the analysis of the localization of E2F1, the cells were placed overnight in 35-mm dishes on poly-lysine-coated glass cover-slip. After 4 h of incubation with α-TOS (40 μM), the cells were washed two times with PBS, fixed with freshly prepared 3% formaldehyde in PBS, and permeabilized with a saponin solution (0.05% saponin in PBS containing 2% FBS). Cells were then incubated with anti-E2F1 IgG in saponin solution for 1 h at room temperature. FITC-conjugated, secondary IgG was then added. The cover-slips were mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA) plus DAPI (4′,6-diamino-2-phenylindole) and viewed in a confocal microscope (MRC 1024, Bio-Rad, Hercules, CA), and the E2F1 positive nuclei were counted.

Cell transfection. Stable transfections were performed in wt-p53 U2OS cells using GenePorter II transfection reagent (GTS, Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocol. Briefly, U2OS cells at approximately 70% of confluency were incubated with 1 ml of serum-free medium containing the empty plasmid (mock transfection) or the DN-E2F1 plasmid pre-incubated with the transfection reagent complex. In brief, 3 μg of plasmid DNA was mixed with 500 μl serum-free medium, and this was combined with 20 μl of transfection reagent mixed with 500 μl serum-free medium. The mixture was then incubated for 45 min at room temperature and added to the cells. After 4–5 h, the transfected cells were washed and incubated with the complete culture medium. After 48 h, the transfected cells were selected by culturing in the complete medium supplemented with gentamicin (G-418; Sigma, St. Louis, MO) at the concentration of 0.4 mg/ml. After 4–5 generations, when cell death was minimal, the cells were considered stably transfected.