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Original Contribution

Role of immunoregulatory transcription factors in differential immunomodulatory effects of tocotrienols

Chandan Wilankar [a], Deepak Sharma [a], Rahul Checker [a], Nazir M. Khan [a], Raghavendra Patwardhan [a], Anand Patil [b], Santosh Kumar Sandur [a], T.P.A. Devasagayam [a,*]

[a] Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400085, India
[b] Clinical Pharmacology, ACTREC, Tata Memorial Centre, Kharghar, Navi Mumbai 410210, India

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ABSTRACT

Tocotrienols have been shown to possess antioxidant, antitumor, cardioprotective, and antiproliferative effects. This report describes novel immunomodulatory effects of tocotrienols in murine lymphocytes. γ-Tocotrienol (GT) was more effective in suppressing concanavalin A (Con A)-induced T cell proliferation and cytokine production compared to α-tocotrienol (AT) when present continuously in the culture. GT inhibited T cell activation markers and costimulatory molecule. GT modulated intracellular glutathione in lymphocytes, and the suppressive effects of GT could not be abrogated by thiol or nonthiol antioxidants, indicating a poor link between anti-inflammatory properties of tocotrienols and cellular redox status. It was also observed that GT suppressed Con A-induced activation of NF-κB, AP-1, and NF-κB-dependent gene expression. Cellular uptake studies with tocotrienols showed higher accumulation of GT compared to AT. Similar immunosuppressive effects of GT were also observed when administered to mice. In contrast, transient exposure of lymphocytes to GT (4 h) resulted in higher survival and proliferation of lymphocytes in vitro and in vivo in syngeneic and allogeneic hosts. This was attributed to the ability of GT to induce NF-κB, AP-1, and mTOR activation in lymphocytes upon transient exposure. Our results demonstrated that antioxidants such as tocotrienols may exhibit pleiotropic effects by activating multiple mechanisms in cells.

Natural vitamin E is a mixture of two classes of compounds, tocopherols and tocotrienols, each consisting of four different forms: α-, β-, γ-, and δ. Whereas tocopherols have a saturated phytol-derived tail, tocotrienols have an unsaturated phytol-derived tail. Tocotrienols are isoprenoids, whereas tocopherols are not. Barley, palm oil, rice, and oat bran have been reported to be rich in tocotrienols [1,2]. The unsaturated side chain present in tocotrienols facilitates their entry through the membrane bilayer to a greater extent than the saturated chain of tocopherols [3–6]. Thus, uptake and accumulation of tocotrienols in the cells are much greater than those of tocopherols [7]. Some reports suggest that tocotrienols are better antioxidants than tocopherols [2,8–10]. For these reasons, tocotrienols exhibit higher biological effects in vitro than tocopherols. However, there are also reports that show a lack of better antioxidant activity of tocotrienols [11].

There are several reports on the antiproliferative and antitumor effects of tocotrienols on human prostate cancer cells [12], neoplastic mammary epithelial cells [13], human gastric adenocarcinoma cells [14], human breast cancer cell lines [15,16], and human hepatoma cells [17]. The in vivo antitumor activity of tocotrienols was also demonstrated in a murine model of liver carcinogenesis [18]. α-Tocotrienol showed protection against homocysteic acid-induced neuronal cell death via antioxidant-dependent and -independent mechanisms [19]. Another study by Osakada et al. [20] demonstrated that α-tocotrienol was more effective at inhibiting H_2O_2-induced neuronal cell death than other isoforms. However, the antiproliferative effects of γ-tocotrienol were better than those of α-tocotrienol [15]. Among the isomers, α- and γ-tocotrienols were found to be better antioxidants [9].

The antitumor effects of γ-tocotrienol have recently been shown to be mediated via inhibition of nuclear factor-κB (NF-κB) and its dependent antiapoptotic gene products [21]. NF-κB is a transcription factor that participates in the induction of numerous immunoregulatory genes whose products include proinflammatory cytokines, growth factors, chemokines, adhesion molecules, and enzymes that produce secondary inflammatory mediators [22]. NF-κB is implicated as a key mediator of acute and chronic inflammatory disorders such as septic shock and allergic asthma. Activation of NF-κB is an essential component of T cell activation in response to a variety of stimuli.

T cells play pivotal roles in immune responses. Activation of T cells upon interaction with an antigen-presenting cell leads to proliferation and the induction of cytokines. Proliferation of antigen-specific clones of lymphocytes helps in the control or killing of pathogens by various effector mechanisms [23]. At the same time, proliferation of self-

* Corresponding author. Fax: +91 22 25505151.
E-mail address: tpad@barc.gov.in (T.P.A. Devasagayam).

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reactive T cell clones leads to the pathogenesis of a variety of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [24,25]. Immunosuppressive drugs such as rapamycin, cyclosporin, and FK506 have several side effects, including nephrotoxicity [26,27]. There is a need to identify novel immunosuppressors from natural sources, which have fewer side effects, that can be used in combination with classical immunosuppressive drugs.

Based on the previous report by Ahn et al. [21] showing the inhibition of NF-κB in tumor cells, we hypothesized that tocotrienols may exhibit immunomodulatory activity. In this study, the novel effects of α- and γ-tocotrienols on T cell proliferation and cytokine production and their possible mechanisms are described.

Materials and methods

Reagents and chemicals

α-Tocotrienol (AT; 99.3% pure) and γ-tocotrienol (GT; 98.2% pure) were kindly gifted by Professor Naito and Professor Yoshikawa, Kyoto Prefectural University (Kyoto, Japan). The following chemicals were obtained from Sigma Chemical Co. (USA): ethylenediamine tetraacetic acid (EDTA), 2-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), Igepal (NP-40), Trizma base, propidium iodide (PI), sodium azide, Triton X-100, Tween 20, Hepes, ethylene glycol bis(2-aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA), sodium chloride, sodium hydrogen phosphate, disodium hydrophosphate, RPMI 1640 medium, sodium bicarbonate, penicillin and streptomycin, glutathione, *N*-acetylcysteine, dithiothreitol (DTT), and Trolox. Fetal calf serum (FCS) was obtained from GIBCO BRL. Concanavalin A (Con A) was purchased from Calbiochem (USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes (The Netherlands). Monochlorobimane was obtained from Invitrogen. The following primary antibodies for Western blotting were purchased from Cell Signaling Technologies (USA): antibodies to mouse phospho-ERK, mTOR, Bcl-2, Bcl-xL, PCNA, cyclin D1, phospho-p38, phospho-p65, and β-actin. Horseradish peroxidase (HRP)-conjugated secondary antibody and Western blotting kits were obtained from Roche (Germany). The phycoerythrin (PE)-conjugated anti-mouse CD69, CD25, CD28, and CD134 and ELISA sets for detection of various cytokines were obtained from BD Bioscience (USA). Magnetic nanoparticle-conjugated antibodies for enrichment and purification of $CD4^+$ T cells were from Stem Cell Technology (USA). All other chemicals used in our studies were obtained from reputed manufacturers and were of analytical grade.

Animals

Eight- to ten-week-old inbred mice of the BALB/c, C57BL/6, and Swiss albino strains weighing approximately 20–25 g were used. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and euthanasia of small animals were strictly followed.

Treatment with AT and GT

Solutions of 100 mM AT and GT were prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. In all in vitro experiments, cells were treated with AT or GT in RPMI 1640 medium and were further stimulated with Con A (5 μg/ml) without being washed. DMSO was used as vehicle control. Transient exposure with GT refers to washing of lymphocytes 4 h after incubation with GT, and continuous exposure refers to the presence of GT in the culture for at least 12 h. In all in vivo experiments, mice were injected ip with GT in 5% Tween 80 (200 mg/kg body wt) in 0.1-ml volume. The mice in the control group were treated with an equal volume of vehicle (5% Tween 80 in phosphate-buffered saline).

Proliferation assay

Spleen cells were obtained by squeezing the spleen through a nylon mesh into a petri plate containing RPMI 1640 medium. The red blood cells were lysed by brief hypotonic shock. Lymphocytes were stained with CFSE (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI 1640 medium containing 10% FCS (complete medium). Two million lymphocytes were preincubated with α- or γ-tocotrienol or α-tocopherol (Toco) or α-tocopherol acetate (TA) and stimulated with Con A (5 μg/ml) or lipopolysaccharide (LPS; 50 μg/ml) or anti-CD3/CD28 (1 μg/ml). Cells were cultured for 72 h at 37 °C in a 95% air/5% CO_2 atmosphere. Vehicle-treated cells served as controls. In another experiment, 2×10^6 lymphocytes were treated with various antioxidants for 2 h followed by GT (50 μM) and were stimulated with Con A (5 μg/ml) for 72 h at 37 °C in RPMI 1640 with 10% FCS in a 95% air/5% CO_2 atmosphere. The cell proliferation was measured by CFSE dye dilution in a Partec Cyflow flow cytometer [28].

Mice were injected ip with GT in 5% Tween 80 (200 mg/kg body wt). The mice in the control group were treated with an equal volume of vehicle (5% Tween 80). Splenic lymphocytes were isolated 24 h after injection, stained with CFSE, and stimulated with anti-CD3/CD28 (1 μg/ml) at 37 °C in RPMI 1640 for 24 h, for cytokine measurement, and 72 h, for estimating proliferation at 37 °C in a 95% air/5% CO_2 atmosphere. Untreated cells served as controls. The cell proliferation was measured by CFSE dye dilution in a Partec Cyflow flow cytometer.

Estimation of cell cycle

The percentage of cells in different phases of the cell cycle (G1, S + G2/M) and percentage of apoptotic cells were estimated by flow cytometry. For cell cycle analysis splenocytes were treated with GT (10, 25, and 50 μM) and stimulated with Con A (5 μg/ml) for 72 h at 37 °C in RPMI 1640 medium supplemented with 10% FCS. Vehicle-treated cells served as controls. At the end of the incubation period the cells were washed with PBS and incubated with 1 ml of staining solution (5 μg/ml propidium iodide, 10 μg/ml ribonuclease A, 0.1% sodium citrate, and 0.1% Triton X-100) overnight [29]. A total of 20,000 cells were acquired in a Partec Cyflow flow cytometer and analyzed using FloMax software. Undivided cells were in the G1 phase of the cell cycle (2*n* DNA content). The population showing more than 2*n* DNA represented cells in the S + G2/M phase of the cell cycle.

Measurement of cytokine secretion in vitro

Splenic lymphocytes were isolated from Swiss mice and treated with AT or GT in the absence or presence of Con A or anti-CD3/CD28 for 24 h in RPMI 1640 medium supplemented with 10% fetal calf serum in 95% air/5% CO_2 atmosphere at 37 °C. The concentrations of IL-2, IL-4, IFN-γ, and IL-6 were estimated in the culture supernatants using cytokine ELISA sets (BD Pharmingen, USA) [29]. DMSO was used as vehicle control. The supernatant obtained from Con A- or anti-CD3/CD28-stimulated cells was used as positive control.

Intracellular glutathione (GSH) assay

The GSH content in cells was determined using monochlorobimane (MCB). Splenic lymphocytes were treated with GT (10, 25, and 50 μM) or diethylmaleate (90 μM) for 4 h at 37 °C. These cells were further incubated with MCB for 30 min at 37 °C. Fluorescence intensity was read from a 96-well plate using a plate reader (Fluostar Optima; BMG Labtech) with excitation at 394 nm and emission at 490 nm [30].