Available online at www.sciencedirect.com
SCIENCE DIRECT
Experimental and Molecular Pathology 81 (2006) 55–61
Experimental and Molecular Pathology
www.elsevier.com/locate/yexmp

Induction of cytokines by radioprotective tocopherol analogs

Vijay K. Singh [a,b,*], Randi L. Shafran [a], William E. Jackson III [a], Thomas M. Seed [c], K. Sree Kumar [a]

[a] Armed Forces Radiobiology Research Institute, Bethesda, MD 20889, USA
[b] Department of Radiation Biology, USUHS, Bethesda, MD 20814, USA
[c] Radiation Biophysics, Catholic University of America, Washington, DC 20064, USA

Received 17 October 2005, and in revised form 14 November 2005
Available online 19 January 2006

Abstract

Tocols are a family of eight isomers consisting of four tocopherols and four tocotrienols that exist in four isomeric forms: alpha (α), beta (β), gamma (γ), and delta (δ). Recently, tocols were found to have important and unique biological effects on nutrition and health other than antioxidant properties and are, therefore, now receiving increased attention. We have demonstrated the radioprotective efficacy of various tocol analogs and some of their esters. Three forms of tocols – α-tocopherol, α-tocopherol succinate, and γ-tocotrienol – significantly protected mice against lethal gamma irradiation when administered subcutaneously 24 h before irradiation. The radioprotective effects of tocols on survival were associated with peripheral blood cell recovery after radiation induced cytopenia. Hematopoietic cytokines are known to promote the proliferation and differentiation of blood cell progenitors. Therefore, we hypothesized that peripheral blood cell recovery is preceded by hematopoietic cytokine induction. To test this hypothesis and compare the various radioprotective and non-radioprotective analogs, we measured serum cytokines using a sandwich ELISA, Luminex, and cytokine array in mice treated with various tocols (α-tocopherol succinate, α-tocopherol, δ-tocopherol, γ-tocopherol, γ-tocotrienol, and tocopherol acetate). Among the serum cytokines measured, ELISA and Luminex studies indicated that α-tocopherol, α-tocopherol succinate, and γ-tocotrienol increased G-CSF levels in mice. Alpha-tocopherol succinate was most effective in stimulating G-CSF. IL-6 was detected by Luminex in sera samples from mice treated with the above three analogs. The results of the cytokine array suggest that other cytokines and chemokines in addition to G-CSF and IL-6 are induced. Since G-CSF, IL-6, and certain chemokines are important hematopoietic factors, these results support our hypothesis that the protection of mice from radiation-induced hematopoietic death is mediated by cytokines and chemokines. These studies may indicate that α-tocopherol succinate can be used as an adjunct in cancer chemotherapy, where neutropenia is a serious problem with threatening infectious complications.

Keywords: Cytokines; Cytokine array; ELISA; G-CSF; Luminex; Mice; Radiation; Tocols

Introduction

Vitamin E is a generic term used for four naturally occurring tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ) as well as derivatives (Packer, 1991). The base chemical structure of natural source forms of vitamin E consists of a hydroxyl-bearing aromatic ring system (1 phenolic and 1 heterocyclic, known as the chroman head) and either a saturated phytyl (tocopherols) or polyunsaturated (tocotrienols) side chain or tail (Kamal-Eldin and Appelqvist, 1996; Packer, 1991). A major antioxidant function of α-tocopherol in humans is the inhibition of lipid peroxidation (Noguchi and Niki, 1999). Unlike other fat-soluble vitamins, toxicity of α-tocopherol is very low, probably because it is not stored in the liver (Papas, 1999). α-Tocopherol has been the focus of research because it is the predominant form in human and animal tissues. In addition, it is by far the most bioactive form based on the rat fetal resorption test, which is the classical assay for vitamin E activity (Desai, 1980). The other tocopherols and tocotrienols, however, have important and unique biological effects other than antioxidant function in nutrition and health and are now receiving increased attention (Christen et al., 1997; Cooney et al., 1993; Stone and Papas, 1997).

* Corresponding author. Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603, USA. Fax: +1 301 295 0292.
E-mail address: singh@afrri.usuhs.mil (V.K. Singh).

0014-4800/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2005.11.002

Radiation hazards can vary from late life pathologies to acute mortality (Pellmar and Rockwell, 2005; Singh and Seed, 2003). During the last five decades, a number of compounds of diverse structure have been considered as countermeasures for radiation (MacVittie and Farese, 2002a; Pellmar and Rockwell, 2005; Singh and Seed, 2003). Despite promising observations with various agents to date, none have been approved as a countermeasure for radiation for either military personnel or first responders. Recently, we have investigated the radioprotective efficacy of α-tocopherol (Kumar et al., 2002) which indicated that α-tocopherol is effective when given 24 h before irradiation at a dose of 400 mg/kg body weight. Among selected analogs and esters of α-tocopherol tested for radioprotection, α-hemisuccinate ester (α-tocopherol succinate) and an unsaturated analog (γ-tocotrienol) when administered subcutaneously (sc) 24 h before irradiation significantly protected mice against lethal cobalt-60 gamma irradiation (Singh et al., 2005a).

The use of cytokines and growth factors in radiation injury has focused on enhancing recovery from the acute radiation syndrome. A number of cytokines and growth factors have shown significant in vivo and in vitro preclinical efficacy against radiation injury to normal cells (Singh and Yadav, 2005). The FDA has approved some of them for the profound neutropenia and thrombocytopenia that can occur with cancer chemotherapy, some are in different stages of clinical trials for radiation injury, and few are moving through preclinical evaluation (MacVittie and Farese, 2002b; Pellmar and Rockwell, 2005; Singh and Seed, 2003). These developments suggest important roles for cytokines in recovery from acute radiation syndrome.

To test whether protection by these tocols is due to the induction of hematopoietic cytokines, and to compare radioprotective analogs with non-radioprotective analogs with respect to induction of cytokines, we studied the cytokine profiles induced by α-tocopherol, δ-tocopherol, γ-tocopherol, γ-tocotrienol, α-tocopherol succinate, and tocopherol acetate) in mice. We have measured various cytokines in serum by sandwich ELISA and multiplex Luminex. We also carried out a cytokine array for evaluating a large number of cytokines. Our results demonstrate that α-tocopherol, γ-tocotrienol, and α-tocopherol succinate stimulated production of circulating G-CSF and IL-6 in irradiated and unirradiated animals. Furthermore, there are indications from the cytokine array that these compounds modulate production of additional cytokines and chemokines.

Materials and methods

Mice

Six- to eight-week-old male CD2F1 mice were purchased from Charles River, Wilmington, MA, and were housed (eight per cage) in AAALAC accredited facility (Association for Assessment and Accreditation of Laboratory Animal Care International). The mouse holding room was maintained at 21 ± 2°C with 10–15 hourly cycles of fresh air and a relative humidity of 50% ± 10%. The mice, upon arrival from the vendor, were held in quarantine for 2 weeks. Microbiology, serology, and histopathology examination of representative samples ensured the absence of *Pseudomonas aeruginosa* and common murine diseases. Mice were provided certified rodent rations (Harlan Teklad Rodent Diet, Harlan Teklad, WI) and acidified water (with HCl, pH 2.5–2.8) ad libitum. All mice were kept in rooms with a 12-h light/dark cycle.

All animal procedures were performed based on a protocol approved by the Armed Forces Radiobiology Research Institute (AFRRI), Institutional Animal Care and Use Committee (IACUC). Research was conducted according to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council, US National Academy of Sciences.

Treatment of mice

In this study α-tocopherol succinate, α-tocopherol, δ-tocopherol, γ-tocopherol, γ-tocotrienol, and tocopherol acetate were used at a dose of 400 mg/kg bodyweight. Tocols were dispersed in a mixture of polyethylene glycol (PEG-400) and a proprietary emulsifying agent provided by Stuart Products, TX. Tocols in this vehicle were administered as a single dose by the sc route to mice 22–24 h before exposure to whole-body gamma radiation. The control group received only vehicle supplemented with olive oil to replace drug.

Radiation

Mice were bilaterally irradiated in well-ventilated lucite boxes (eight mice in each box) at a dose rate of 60 cGy per min in the cobalt-60 gamma radiation facility of the Armed Forces Radiobiology Research Institute (Singh et al., 2005b). Following irradiation, mice were returned to their cages and monitored. Total radiation doses of 3 or 7 Gy were used in this study.

Bleeding of mice and serum collection

Mice were terminally anesthetized with Isoflurane (Abbott Laboratories, Chicago, IL) before blood was collected from the abdominal vein with a 23-gauge needle. After collection, blood was allowed to clot in a Petri dish. Serum was transferred to microcentrifuge tubes and centrifuged at 1000 rpm for 10 min. The serum was then collected and stored at −70°C until use.

ELISA for estimation of cytokines in serum samples

Cytokine measurements were carried out as described earlier (Mehrotra et al., 2003). Serum samples were collected and stored at −70°C until tested. Briefly, ELISA for various cytokines was carried out as solid phase enzyme immunoassays with DuoSets (R&D systems, Inc., Minneapolis, MN) based on the multiple antibody sandwich principle. Absorbance was measured in an ELISA reader (Multiscan Accent, Huntsville, AL) at 450 nm and was proportional to the concentration of cytokine present in samples. Standard curves were obtained by plotting known concentrations of respective cytokines versus absorbance. Results are presented as concentrations in pg/ml in six-fold diluted serum.

Estimation of cytokines by Luminex

The general multiplex assay protocol is a sandwich immunoassay system employing microspheres. This novel method allowed the simultaneous detection of different cytokines in the Luminex-100 (Luminex Corp, Austin, TX), a dual-laser flow analyzer. Mouse serum samples were analyzed for various cytokines by multiplexing (Arroyo et al., 2004). In brief, the microspheres (Luminex Corp, Austin, TX) were activated by *N*-hydroxysuccinamide (Pierce Biotechnology, Inc., Rockford, IL) followed by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (Pierce Biotechnology, Inc., Rockford, IL). Recombinant cytokine antibodies (R&D systems, Inc., Minneapolis, MN) were then coupled to the activated microspheres. Cytokine antibody coupled microspheres were verified with secondary antibody conjugated with phycoerythrin (Molecular Probes, Inc., Eugene, OR) for coupling before proceeding to the actual assay. Five-fold diluted sera samples were pipetted into the wells of a filter-bottom microplate (MultiScreen-BV, 1.2 μm hydrophilic, low protein binding Durapore membrane), placed in filter plates (Millipore Corp., Billerica, MA), and cytokine antibody conjugated microspheres were added to each well, and washed and diluted biotinylated antibody (R&D systems, Inc., Minneapolis, MN) was added. After removal of excess biotinylated antibody, streptavidin–phycoerythrin (Molecular Probes, Inc., Eugene, OR) was added. Further, after