行政院國家科學委員會專題研究計畫　成果報告

維生素 E 對樹突細胞免疫功能的影響(2/2)

計畫類別： 個別型計畫
計畫編號： NSC93-2320-B-002-037-
執行期間： 93 年 08 月 01 日至 94 年 07 月 31 日
執行單位： 國立臺灣大學生化科技學系

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報告類型： 完整報告

處理方式： 本計畫可公開查詢

中　華　民　國 94 年 10 月 31 日

Vitamin E Affects Bone Marrow-Derived Dendritic Cells Cytokine Secretion and Ability to Polarize Naïve CD4+ T Cells

ABSTRACT

Vitamin E, one of the fat soluble vitamins, has been known for its immunomodulative functions. However, little is known about the effect of vitamin E on dendritic cells (DCs), the most potent antigen-presenting cells. To investigate whether the vitamin E could modulate DC function and subsequent immune response, various concentrations of α- and γ-tocopherol (Toc) were added to bone marrow-derived DC (BM-DCs) culture simultaneously with LPS activation. Surface molecules, prostaglandin E_2 (PGE_2), nitric oxide (NO), IL-12 of the BM-DCs were measured. The results showed that α-Toc had no effect on the expression of these surface molecules but γ-Toc decreased the levels of MHC II, B7.1 and B7.2 molecules. α-Toc increased BM-DC PGE_2, and NO production but slightly decreased IL-12p70 production. γ-Toc slightly increased BM-DC PGE_2 production but reduced IL-12p70 production. To further investigate whether vitamin E-treated BM-DC would affect T cell function, BM-DCs were preincubated with vitamin E and then cocultured with naïve $CD4^+$ T cells. BM-DCs preincubated with vitamin E induced $CD4^+$ T cell proliferation and IL-2 production. Vitamin E-preincubated BM-DCs did not significantly change IL-4 and IFN-γ secretion of T cells. However, as concentration of α-Toc increased, the ratio of IL-4/ IFN-γ of T cells tended to increase. γ-Toc-treated DC significantly increased the ratio of IL-4/ IFN-γ of T cells. Our results suggested that vitamin E can modulate BM-DC immune function including the influence on BM-DC surface molecules expression, PGE_2 and cytokine levels and the ability to induce naïve $CD4^+$ T cell proliferation and Th1/Th2 polarization.

KEY WORDS: dendritic cells, vitamin E, prostaglandin E_2, interleukin-12, Th1/Th2

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) for priming naïve T cells and are uniquely capable of inducing a primary specific immune response[1-3]. DCs constitutively express high levels of class Ⅰ and class II MHC molecules as well as costimulatory molecules, such as B7.1 and B7.2 [4,5]. DC-T cell interaction is affected by MHC and costimulatory molecules and cytokines and prostaglandin (PG) produced by DCs. DCs might provide the different cytokine/molecule microenvironments to the T cells that influence the Th1/Th2 polarization of immune responses. Th1/Th2 balance is dependent on the DC production of interleukin-12 (IL-12)[6], a heterodimer formed by a p40 protein and a p35 protein. The production of IL-12 by DCs may promote a Th1 response. Prostaglandin E_2 (PGE_2) is thought to drive Th2 response through inhibition of IL-12 production. Several studies reported that PGE_2 modulates the IL-12 production ability and functions of DC[7-9].

Vitamin E, one of the fat-soluble vitamins, includes all tocol and tocotrienol derivatives that exhibit qualitatively the biological activity of α-tocopherol (α-Toc)[10]. These compounds are isoprenoid side-chain derivatives of 6-chromanol[11,12]. Vitamin E is the most important biological antioxidants[10,13-16]. Many studies showed that vitamin E deficiency impairs both

cell-mediated and humoral immune responses. Animals with vitamin E deficiency have lower antibody response[17,18], lower lymphocyte response to mitogens[19], and impaired chemotaxis in response to bacterial culture[20]. In a coculture study by Beharka *et al*., dietary vitamin E influenced the mitogenic response of T cells through regulation of macrophage PGE_2 production[21]. The addition of vitamin E decreased PGE_2 production and enhanced T cell proliferation and IL-2 production. To date, however, the effect of vitamin E on specific-Ag presenting capacity of APCs to T cell has not been well elucidated. In previous studies, α-Toc has been extensively studied both *in vitro* and *in vivo* because it is the major form of vitamin E in plasma and many tissues and the exclusive component in most vitamin E supplements. Recently, γ-Toc representing 70-80% of the vitamin E in the typical US diet[22,23] has drawn more attention than before[24]. γ-Toc has a unique antioxidant function[24,25] though α- and γ-Toc are both lipophilic antioxidants[26]. In addition to its antioxidant ability, γ-Toc plays a role in immune responses. γ-Toc was shown to reduce PGE_2 synthesis in both lipopolysaccaride (LPS)-stimulated macrophages[27] and attenuates inflammation-mediated damage[28].

Little is known about the role of vitamin E on immune activity of DCs. A report described the protective effect of vitamin E on UVA-induced immune suppression in human Langerhans cells[29]. Since DCs represent the central cells of the immune response[1,4], nutrients that can modulate surface molecules and cytokines and PGE_2 production by DCs might be key mediators of the immune response. In the present study, we investigated the hypothesis that vitamin E, one of the fat-soluble vitamins and the most important biological antioxidant, modulated the DCs immune activity.

MATERIALS AND METHODS

Mice

All mice were obtained from the Animal Center of the College of Medicine of National Taiwan University. Female Balb/c mice of 6-10 weeks of age were used as the source of BM-DCs. Female DO11.10 mice transgenic for an $OVA_{323-339}$-specific αβ TCR were used as the source of naïve $CD4^+$ T cells.

Generation of Bone marrow-derived dendritic cells

BM-DCs were produced as described previously with some modifications[30]. Briefly, female Balb/c mice of 6-10 weeks of age were killed by CO_2 suffocation. After incision of the skin of the hind legs, the muscle tissue was carefully removed and the tibia and femur were taken out. The bones were then placed in a 30-mm dish with 75% ethanol and transferred into a new dish with Hank's balanced salt solution buffer. The epiphyses of the bones were removed and both ends of the bones were punctured with scissors. Then the marrow was flushed out with warm 5% FBS RPMI 1640 medium by a syringe with a 27G needle. Clusters within the marrow suspension were disintegrated and pieces of bone and debris were removed. Bone marrow cells were collected by centrifugation at 300 g for 10 min and were then depleted of red blood cells with ammonium chloride (ACK) lysis buffer followed by washes with HBSS buffer. After washes, cell pellet was resuspended in 5% FBS RPMI 1640 medium and the viable cell concentration was determined by a trypan blue exclusion method.

Bone marrow cells of 4-6 x 10^7 were obtained per mouse. At day 0, bone marrow cells were cultured in complete medium supplemented with recombinant murine GM-CSF (500 U/mL; Pepro Tech Inc., Rocky Hill, NJ) and IL-4 (1000 U/mL; Pepro Tech Inc., Rocky Hill, NJ). Cells were incubated at 37°C in 5% CO_2 for 2 days. At day 2 of culture, floating cells were gently removed with 50% upper medium and then fresh complete medium containing GM-CSF and IL-4 was added. At day 4 and day 6 of culture, the same process as day 2 was manipulated and nonadherent cells were transferred and seeded in a new plate. At day 6 of culture, $CD11c^+$ BM-DCs were positively isolated by biotinylated-anti-CD11c-Ab (BD™, San Jose, CA, USA) and Streptavidin IMag particles-DM (PharMingen, San Jose, CA, USA) in the magnetic field of IMagnet (BD™, San Jose, CA, USA). In some experiments, BM-DCs were stimulated by LPS (1μg/mL) for 48 hr. At day 8, nonadherent cells (BM-DCs) together with medium were collected. Supernatants were collected by centrifugation at 300g for 10 min and stored at -80°C.

The effect of vitamin E on Bone marrow-derived dendritic cells

α- and γ- tocopherol were dissolved in absolute ethanol to a final concentration of 500 mM. The appropriate amount of stock tocopherol was added fetal bovine serum (FBS) (1: 9 v/v) and incubated for 1 h at 37^{O}C to allow incorporation into serum lipoprotein. Control cultures with 0.1% ethanol were run with every experiment because vitamin E was initially dissolved in ethanol. For LPS activation and cytokine analysis, BM-DCs were generated and preincubated with vitamin E at day 4 of culture. At day 6, cell density was adjusted to 5×10^5 cells/mL and then vitamin E and LPS (1 μg/mL) were added simultaneously. For coculture experiments, BM-DCs were preincubated with vitamin E from day 4 to 8 of culture.

Immunophenotypic analysis

The following commercial mAb were used: anti- $I\text{-}A^d$ (PharMingen, San Jose, CA, USA), -CD80 and -CD86 (eBioscience, San Jose, CA, USA). Stained cells were analyzed using a FACScan (Becton Dickinson, San Jose, CA, USA). Data was analyzed by CellQuest software (Becton Dickinson, San Jose, CA, USA).

Coculture of vitamin E-preincubated BM-DCs and $CD4^+$ T cells

BM-DCs preincubated with vitamin E at day 4-8 were harvested at day 8 of culture and exposed to γ-irradiation (2500 rads). Purification of DO11.10 transgenic mice splenic $CD4^+$ T cells by SpinSep™ (StemCell Technologies, Vancouver, BC, Canada) is based on density gradient centrifugation. Irradiated BM-DCs (2 x 10^4 cells/well) were washed with HBSS buffer and cultured with naïve $CD4^+$ T cells (1 x 10^5 cells/well) in the presence of 1 μg/mL $OVA_{323\text{-}339}$ peptide in AIM-V medium (Gibco, Invitrogen Corp., USA) supplemented with 1/50X TCM mouse serum replacement (Celox Laboratories, Inc., Hopkins MN, USA). At day 7 of coculture, supernatants of culture medium were collected for cytokine measurements including IL-2, IL-4 and IFN-γ analysis.

For T cell proliferative assay, vitamin E-preincubated BM-DCs (5 x 10^3 cells) were cultured with naïve $CD4^+$ T cells (2.5 x 10^4 cells) in the presence of 1 μg/mL OVA peptide in 96-well round-bottomed plates (Nunc). At day 4 of coculture, [^{3}H] thymidine was added into

each well. After 96 h of coculture, 20 μL of 1 μCi [^{3}H]thymidine was added into each well and the plate was incubated for another 18 h. Cells were harvested onto glass filters (Packard) by using Cell Harvester (Packard) and filters were counted by using a dry scintillation counter (Packard). Results were reported as counts per minute (cpm) and shown as the percentage of control (proliferation of T cells cocultured with 0.1% ethanol-preincubated BM-DC) in figures.

PGE_2 production

PGE_2 production was measured using a commercially available kit (Cayman Chemiclas, Ann Arbor, MI, USA). This assay was based on the competition between PGE_2 and a PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. The color intensity of the enzymatic reaction product is inversely proportional to the amount of free PGE_2 present in the well, which is proportional to the amount of PGE_2 tracer bound to the well during the incubation.

Nitrite assay

The release of NO was measured by colorimetric reaction. This assay relies on a diazotization reaction originally described by Griess in 1879. The amount of 50 μL of culture supernatant was transferred into 96-well plates. A reagent (1% sulfanilamide dissolved in 2.5% H_3PO_4) and B reagent (0.1% *N*-1-napthylethylene diamide dihydrochloride dissolved in 2.5% H_3PO_4) were mixed at a 1:1 (v/v) ratio to prepare Griess reagent. The amount of 100 μL of Griess reagent was added into each well at room temperature for 5 min. The product of this reaction had a distinct pink color and absorbed at 540 nm. Standard curve was generated with dilutions of $NaNO_2$ (0~100 μM).

Cytokine measurements

The cytokines, IL-2, IL-4, IL-12p70, and IFN-γ, in the supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA). IL-2, IL-4, and IFN-γ were measured by use of commercially available pairs of mAb (PharMingen, San Jose, CA, USA). IL-12p70 were measured with mouse ELISA kits (Duoset; R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The results are expressed as the mean ± SEM. Analysis of data was performed using Duncan's multiple range test; $p < 0.05$ was considered significant. Some data was analyzed using Student's *t* test; $p < 0.05$ was considered significant.

RESULTS

Phenotype of vitamin E-incubated BM-DCs

Surface molecules such as DC-specific marker CD11c, MHC II molecules, costimulatory molecules, B7.1 and B7.2 were analyzed. According to fluorescence histogram results, cells expressing MHC II or B7 molecules could be divided into two groups due to their two peaks of fluorescence histogram. One group of cells expressed high density of

MHC II or B7 molecules, and the other expressed low density of these surface molecules. α-Toc had no influence on the percentage of cells that expressed MHC II or B7 molecules on LPS-stimulated CD11c+ BM-DC (Figure 1). In contrast, γ-Toc with the concentration higher than 500 μM decreased the percentage of cells that expressed high-density MHC II molecules, B7.1 or B7.2 molecules. For the expression of low-density molecules, MHC II and B7.1 molecules increased at a dose of 1000 μM γ-Toc. The percentage of cells with low-density B7.2 molecules was not affected (Figure 1).

PGE_2 and nitric oxide production by vitamin E-incubated BM-DCs

The effect of vitamin E on BM-DCs activity was analyzed after four days generation of DCs with GM-CSF and IL-4. The supernatants from culture medium were assayed to determine the production of soluble mediators, PGE_2 and NO, and cytokines by BM-DC, with LPS activation.

α-Toc increased PGE_2 production by $CD11c^+$ BM-DCs. γ-Toc also induced PGE_2 production by LPS-stimulated $CD11c^+$ BM-DC (Figure 2). As shown in Figure 3, NO production by purified BM-DC was increased by α-Toc. γ-Toc did not have significant influence on NO production by LPS-stimulated $CD11c^+$ BM-DC.

IL-12p70 production by vitamin E-incubated BM-DCs

α-Toc tended to decrease IL-12p70 level by BM-DC (Figure 4). As shown in Figure 4, γ-Toc decreased IL-12p70 production and γ-Toc at 1000 μM caused a 91% decrease by BM-DC.

Lymphocyte proliferation induced by vitamin E-incubated BM-DCs

To evaluate the effect of vitamin E on Ag-specific presenting capacity of BM-DC, BM-DCs from Balb/c mice were cocultured with naïve $CD4^+$ T cells from DO11.10 TCR-Tg mice in the presence of $OVA_{323-339}$ peptide.

BM-DCs preincubated with α-Toc (α-Toc-treated DC) induced $CD4^+$ T cell proliferation and IL-2 secretion. γ-Toc-treated DC induced $CD4^+$ T cell proliferation but could not induce effective T cell proliferation as γ-Toc concentration increased (Figure 5).

Polarization of T lymphocytes by vitamin E-incubated BM-DCs

To evaluate the cytokine profile of Th1/Th2 polarization, IFN-γ and IL-4 secretion were measured. α-Toc-treated DC and γ-Toc-treated DC did not change IL-4 and IFN-γ secretion (Figure 6A, B). But as concentration of α-Toc increased, α-Toc-treated DC tended to increase the ratio of IL-4/ IFN-γ (Figure 6C). γ-Toc-treated DC significantly increased ($p < 0.05$) the ratio of IL-4/ IFN-γ at a dose of 1000 μM (Figure 6C). It seemed that BM-DC preincubated with vitamin E might promote Th2 response though Th1 response was also induced.

DISCUSSION

Many studies have showed that vitamin E is involved in both cell-mediated and humoral immune responses but less is known about the effect of vitamin E on dendritic cells, one kind of antigen-presenting cells with a unique ability to induce primary immune response. In the present study, we investigated whether vitamin E can exert immunomodulatory effect on murine BM-DCs. We found that vitamin E decreased the expression of MHC II, B7.1 and B7.2 molecules on BM-DCs. Vitamin E increased PGE_2 but decreased IL-12 production by BM-DC. When vitamin E-preincubated BM-DCs were cocultured with naïve $CD4^+$ T cells, they increased the ratio of IL-4/ IFN-γ secretion of T cells. It is suggested that vitamin E affects not only BM-DCs surface molecules and cytokine secretion but also $CD4^+$ T cell polarization induced by BM-DCs.

With LPS activation, the percentage of cells with high-density MHC II molecules decreased in the presence of vitamin E. It might be resulted from the induction of PGE_2 by γ-Toc. Harizi *et al.* reported that PGE_2 added to the maturing BM-DCs reduces their MHC II molecules expression[8]. There is limited information about the effect of nutrients on surface molecules of DC. Vitamin D, another fat-soluble vitamin, has been more extensively studied[31-34]. A study showed that the addition of 10^{-12} and 10^{-8} M of 1α, 25-dihydroxyvitamin D3 (1α, $25(OH)_2D_3$) to murine DC cultures resulted in a concentration-dependent reduction in levels of MHC II molecules and the costimulatory molecules B7.1 and B7.2[35]. This inhibition of DC maturation is via vitamin D receptors (VDR)–dependent pathways. It needes more studies to clarify the pathways which vitamin E affected BM-DC maturation.

PGE_2 is synthesized from arachidonic acid, which can be rapidly released from the cell membrane of phospholipids by phospholipase A_2. Arachidonic acid is catalytically transformed by cyclooxygenases (COXs), either the constitutive COX-1 or the inducible COX-2 enzymes. BM-DCs can produce PGE_2 in the absence or presence of stimuli (e.g. LPS)[7]. PGE_2 can regulate the cytokine production by DCs, thus modulating the subsequent immune responses. In our study, the PGE_2 secretion by BM-DC was significantly increased by vitamin E, both of α- and γ-form. However, γ-Toc had stronger effect than that of α-Toc. γ-Toc could increase PGE_2 secretion even without LPS activation (data not shown). α-Toc only promoted PGE_2 secretion at high concentration (> 500 μM).

IL-12p70 formed by a p35 and a p40 subunit is a biologically active form of IL-12[36]. Expression of IL-12p35 is a rate-limiting step to produce biologically active IL-12p70 while IL-12p40 is produced in large excess over the IL-12 heterodimer[37, 38]. We measured biologically active form IL-12p70 production by BM-DC treated with vitamin E. Both α- and γ- Toc affect BM-DC IL-12p70 production. Vitamin E might modulate IL-12 production via the induction of PGE_2 that has been known to be a strong inhibitor of IL-12 production through a feedback mechanism at the level of the APC[39]. PGE_2 can increase the production of endogenous IL-10, which suppresses DC functions[9]. Harizi *et al.* also reported that PGE_2 enhances IL-10, which down-regulates IL-12p70 production[9]. In our study, IL-10 concentration in culture medium was too low to be measured (data not shown). The difference might come from the *in vitro* culture method of DC generation and the period of LPS stimulation.

α-Toc increased NO generation of BM-DC in our study. Jorundsson,E. *et al.* reported that NO level increased in rats fed vitamin E though it might not directly related to immune

system[40]. However, with LPS stimulation, γ-Toc at 1000 μM reduced BM-DC NO production. Similar effect can be seen in the study that γ-Toc decreased NO production by LPS-treated macrophages[27].

In addition to DCs, neutrophils and macrophages could also grow in the presence of GM-CSF[30]. In our study, $CD11c^+$ BM-DCs were purified to exclude possible PGE_2 production by other bone marrow cells. The surface molecules, such as CD11c, the β_2 integrin family heterodimer p150/95, is expressed in high density on mouse DC and is commonly used as DC marker[3]. But, there still are $CD11c^-$ DCs that share similar phenotypes and functional activities of $CD11c^+$ DCs. Therefore, contribution of $CD11c^-$ BM-DC to PGE_2 and cytokines production in the presence of vitamin E might be absent in this study.

We reported the effect of vitamin E on Ag-specific stimulatory capacity of BM-DC to naïve $CD4^+$ T cell. BM-DCs preincubated with various concentrations of α-Toc induced T cell proliferation. BM-DCs preincubated with low concentration of γ-Toc could induce T cell proliferation but high concentration of γ-Toc decreased the proliferation response. IL-2 induces proliferation and differentiation of T cells. Different T cell proliferation profile might be resulted from different IL-2 production of T cells stimulated by vitamin E-preincubated BM-DCs. Interestingly, T cell proliferation and IL-2 production stimulated by α-Toc-DC or γ-Toc was similar to the direct effect of vitamin E on mouse splenocytes[41].

DCs are believed to direct the differentiation of $CD4^+$ T cells into either Th1 (IFN-γ producing) cells or Th2 (IL-4 producing) cells[42]. We examined IFN-γ and IL-4 production by T cells stimulated by α-Toc-treated DC or γ-Toc-treated DC to see whether vitamin E could affect the ability of BM-DC to promote Th1/Th2 response. α-Toc-treated DC and γ-Toc-treated DC did not affect IL-4 and IFN-γ secretion. But the ratio of IL-4/IFN-γ was higher in T cells stimulated by BM-DCs preincubated with high concentration of vitamin E. T cell response could be affected by different signals including MHC II and costimulatory molecules and cytokines produced by BM-DCs. Different secretion profile of DC may direct Th1/Th2 polarization[42], so vitamin E might modulate Th1/Th2 responses via the regulation of secretion profile of BM-DC. The Th2 polarization may be resulted from the increase of PGE_2 production and the decrease of IL-12 and NO secretion from vitamin E-treated BM-DCs. Above all, vitamin E influenced BM-DC maturation and secretion and could preprogram BM-DC to polarize a Th2 response though Th1 response was also induced.