Immunomodulatory Effects of Eckol, a Pure Compound of Ecklonia Cava, on Dendritic Cells

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ABSTRACT

Background: Eckol purified from Ecklonia cava, a brown alga has been known to have cytoprotective effects on some cell lines against oxidants and ionizing radiation. However, there is no study about the effects of eckol on immune cells. **Methods:** Bone marrow (BM)-derived dendritic cells (DCs) were used to demonstrate the immunomodulatory effects of eckol on DCs, such as viability, the expression of surface markers, allogeneic stimulating capacity using MTT, flow cytometric, ^{3}H-thymidine incorporation assay. **Results:** Eckol did protect DCs against cytokine withdrawal-induced apoptosis in a concentration dependent manner based on MTT assay. And also, it increased the expression of MHC class II and CD86 (B7.2) molecules, maturation markers, on the surface of viable DCs gated in FACS analysis. Furthermore, eckol-treated DCs stimulated the proliferation of allogeneic $CD4^{+}$ T lymphocytes compared to imDCs in ^{3}H-thymidine incorporation assay. $CD4^{+}$ T lymphocytes activated with eckol-treated DCs produced the larger amount of IFN-γ and IL-4 than those cells with imDCs. **Conclusion:** Taken together, we demonstrate in this study that eckol, a pure compound of Ecklonia cava, may modulate the immune responses through the phenotypic and functional changes of DCs. **(Immune Network 2006;6(4):199-203)**

Key Words: eckol, dendritic cells, maturation, immune responses

Introduction

Eckol is a purified compound of Ecklonia cava, a brown alga that is a foodstuff in Korea. It has been demonstrated that eckol is able to function as an antioxidant (1) and inhibit mushroom-derived tyrosinase activity (2). Especially, a recent study reported that eckol protects cells against hydrogen peroxide, serum starvation, radiation-induced cell death and increases the activity of catalase, the phosphorylation of extracellular signal-regulated kinase (ERK), the activity of NF-κB transcription factor (3).

Dendritic cells (DCs) are the most potent antigen-presenting cells that play critical roles in immune systems of host, such as inducing immune tolerance, both innate and adaptive immune responses (4-7). DCs uptake antigen in periphery, migrate into immune organs including lymph nodes, and stimulate naive lymphocytes or other immune effector cells. The maturation of DCs is an essential process to enhance immune responses via the expression of MHC class I, II molecules, costimulatory molecules including B7, and the production of cytokines.

Although eckol showed a variety of functions, such as cytoprotective, anti-oxidative effects on cells, there is no study about the functional roles of eckol in immune responses yet. In this study, we tried to investigate if eckol may affect the phenotype or function of DCs and DC-involved immune responses, especially the reaction of lymphocytes. The study about the effects of eckol on DCs may provide us with valuable information for the use of eckol.

Materials and Methods

Animals and reagents. C57BL/6, BALB/c mice were purchased from ORIENT BIO, Inc. (Seongnam, Gyeonggi, Korea) and maintained in the lab animal facility. 7- to 12- week-old female mice were used for experiments. Animal experiments were performed based on the NIH guidelines (USA) for laboratory

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animal use and care. Eckol was obtained from Dr. Nam Ho Lee (Cheju National University, Jeju, Korea) and a previous report provided its structure (3). Anti-CD40 mAb (BD Biosciences, San Jose, CA, USA) was used for CD40 ligation, a physiological maturing signal for DCs. In this study, anti-CD40 mAb was used as a positive control for maturation in most experiments except for the determination of nitric oxide (NO) since it did not induce the production of NO in preliminary experiments.

Generation of DCs. DCs were generated from bone marrow cells of C57BL/6 mice using a protocol that was established in our lab (8). In brief, bone marrow cells were harvested from femur and tibia of mice by flushing with Hanks' balanced salt solution (HBSS, Invitrogen, Carlsbad, CA, USA) and erythrocytes were removed using ammonium chloride, potassium carbonate (ACK) lysis buffer. Cells were cultured at a concentration of 2×10^6 cells/ml in 6-well culture plates. RPMI-1640 medium containing 2 mM L-glutamine, 5% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin (all from Invitrogen) and 10 ng/ml recombinant mouse granulocyte-macrophage colony--stimulating factor (GM-CSF, Biosource International, Camarillo, CA, USA) was used for DC culture. At 6 to 10 day of culture, floating cells, over 85% $CD11c^+$ cells based on flow cytometric analysis, were used as DCs for experiments.

Measurement of cell viability. For the determination of cell viability, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay. After cell culture with treatment, MTT reagent was added at a concentration of 1 mg/ml, incubated for 24 hr and then 100 μl/well 10% sodium dodecyl sulfate (SDS, Sigma) solution was added to dissolve the insoluble crystal for 24 hr. The absorbance of sample was measured at 570 nm, 630 nm as reference by using a microplate reader (Molecular Devices, Sunnyvale, CA).

Flow cytometric analysis of maturation markers on the surface of DCs. For blocking Fc receptors, DCs were incubated with purified anti-mouse CD16/CD32 mAb at a concentration of 1 μg/100 μl for 15 min at 4°C. Surface staining of DCs was performed using biotin-labeled anti-mouse $I\text{-}A^b$ mAb or anti-mouse CD86 (B7.2) mAb, and sequentially fluorescein isothiocyanate (FITC)-streptavidin at a concentration of 1 μg/100 μl for 30 min at 4°C. Phycoerythrin (PE)-labeled anti-mouse CD11c mAb was used for the determination of DCs. FITC- or PE-labeled isotype-matched mAb was used as control, respectively (all from BD Biosciences). After staining, cells were analyzed with FACSCalibur™ flow cytometer and CellQuest™ software (BD Biosciences).

Splenic $CD4^+$ T lymphocyte preparation and mixed lymphocyte reactions (MLR). Spleen cells were harvested from spleen of Balb/c mice using a protocol that was previously established (9). For MLR, $CD4^+$ T lymphocytes were purified from the spleen cells using CD4 (L3T4) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. DCs of C57BL/6 and 1×10^5 $CD4^+$ T cells of Balb/c were co-cultured in 96-well flat-bottom cell culture plates (BD Biosciences) and the ratio of DC: T cell was a range of 1 : 20~1 : 540 in quadruplicate. The co-cultures were incubated for 5 days and pulsed with ^{3}H-thymidine, (PerkinElmer, Wellesley, MA, USA) at a concentration of 1 μCi/well for last 18 hr. Incorporated thymidine was measured by a scintillation counter, Wallac Microbeta® TriLux (PerkinElmer).

Determination of NO production and enzyme-linked immunosorbent assay (ELISA). The level of NO in supernatants was determined using modified Griess reagent (Sigma) according to the manufacturer's instruction. DCs were incubated for 48 hr and cell-free supernatants were harvested by centrifugation. Lipopolysaccharide (LPS, Sigma) from Escherichia coli O26 : B6 was used as a positive control to induce the production of NO from DCs. The amount of interferon (IFN)-γ interleukin (IL)-4, and IL-12 in the supernatants of cell culture was determined based on ELISA using cytokine detection kits (all from Biosource International) according to the manufacturer's instructions.

Statistical analysis. Data were expressed as mean± standard deviation (SD). Statistical analysis was performed by using one- and two-way ANOVA and Student's two-tailed t-test. Data of $p<0.05$ were recognized as statistically significant.

Results

Effects of eckol on the viability of DCs. To investigate the effects of eckol on DCs, we first measured the viability of DCs treated with eckol. In MTT assay, eckol marginally increased the viability of DCs in a concentration-dependent manner (Fig. 1). After culture of DCs in the presence of GM-CSF, DCs were used for experiments in the absence of GM-CSF. This leads to cytokine withdrawal-induced apoptosis of DCs. It is thus suggested that eckol may protect DCs from the apoptosis. To eliminate the possibility that eckol dissolved in DMSO may enhance the O.D. value by chemical reaction with MTT reagent, we subtracted the O.D. value of eckol itself from that of eckol-treated DCs. It is thus suggested that eckol may protect DCs from cytokine withdrawal-induced apoptosis.

Eckol does not affect the production of NO or IL-12 from DCs. To investigate if eckol may have a potential character of inflammatory agent, we measured the level of NO from eckol-treated DCs. Eckol did not enhance the

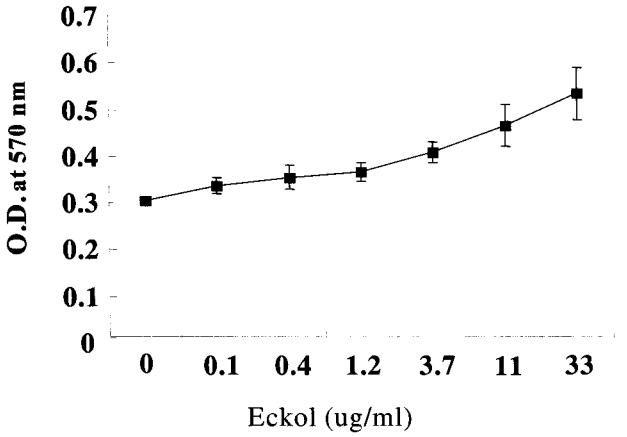

Figure 1. The effect of Eckol on the viability of DCs. For MTT assay, DCs were seeded at a concentration of 5×10^4 cells/well of 96-well culture plates and incubated in the presence of eckol at a range of concentration 0~33 ug/ml. Cells were treated with eckol for 48 hrs. Eckol was dissolved in DMSO, which not included over 0.4% (v/v) in all experiments. Data represent one of three independent similar results.

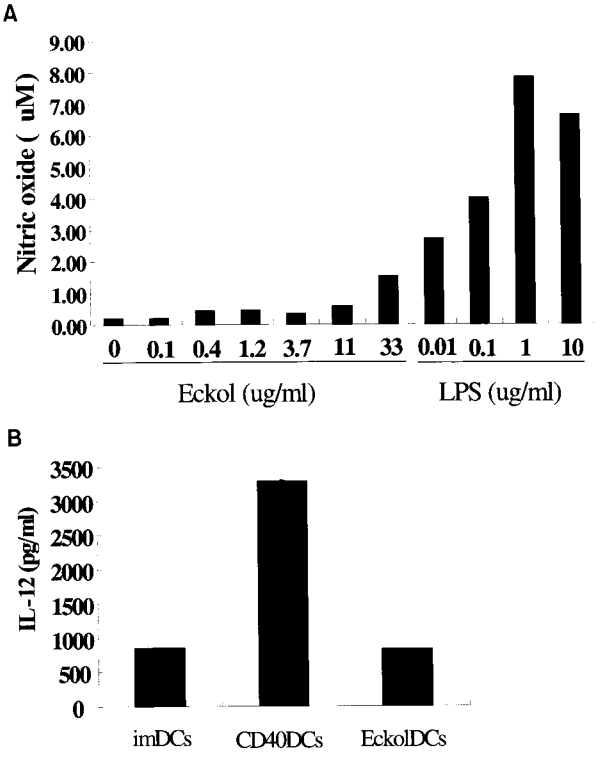

Figure 2. NO and IL-12 production of DCs were not affected by eckol. For the determination of NO, DCs were seeded at a concentration of 5×10^4 cells/well of 96-well culture plates and incubated in the absence or presence of eckol or LPS (A). For determining the amount of IL-12, DCs were seeded at a concentration of 5×10^5 cells/well of 24-well culture plates and incubated in the absence or presence of 33 ug/ml eckol or 1 ug/ml anti-CD40 mAb (B). After treatment, cells were incubated for 48 h in both assay. Data represent one of three independent similar results.

significant level of NO at a range of concentration (0~11 μg/ml) and only marginal increase at 33 μg/ml (Fig. 2A). In same experiments, LPS, as a representative pathological maturing agent for DCs, markedly increased the level of NO. In addition, we measured the level of IL-12, a major inducing cytokine for helper T cell type 1 (Th1) or cellular immunity. The level of IL-12 produced by eckol-treated DCs was very similar to that by imDCs (Fig. 2B).

The enhanced expression of maturation markers on DCs by eckol. To investigate if eckol may affect the maturation status of DCs, phenotypic analysis was performed using flow cytometric staining. MHC class II and CD86 molecules have been recognized as maturation markers for mouse dendritic cells originated from bone marrow cells. Flow cytometric analysis revealed that eckol-treated DCs expressed more MHC class II and CD86 molecules on the surface compared to imDCs (Fig. 3). Anti-CD40 mAb-treated DCs, as a positive control for mature DCs, expressed the highest levels of those molecules. It is thus suggested that eckol may convert viable DCs into a certain status of mature DCs.

The increased alloproliferation of $CD4^+$ T lymphocyte activated eckol-treated DCs. As a crucial role in immune system, DCs have the capability to initiate the activation of naïve T lymphocytes. We measured the stimulatory ability of eckol-treated DCs for alloproliferation, which was quantified by ^{3}H-thymidine incorporation (Fig. 4). Eckol-treated DCs significantly enhanced the alloproliferation at a ratio of DC (5×10^3 cells/well) and $CD4^+$ T lymphocytes (1×10^5 cells/well). Anti-CD40 mAb-treated DCs, as a positive control for mature

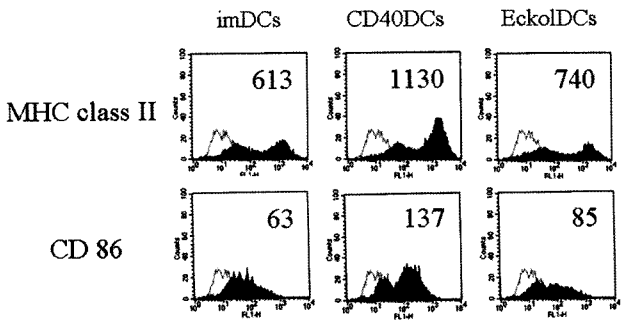

Figure 3. Enhanced expression of maturation markers on the surface of DCs treated by eckol. DCs were seeded and treated as Fig. 2B. Anti-ouse I-A^b and anti-mouse CD86 mAb were used for the surface staining of MHC class II and CD86, respectively. In flow cytometric analysis, 1×10^4 cells were acquired and viable cells were gated based on cell size. Number of histograms indicates mean fluorescence intensity (MFI) of viable DCs.

DCs, markedly stimulated the alloproliferation of $CD4^+$ T lymphocytes. It is suggested that eckol may enhance the stimulatory capability of DCs for allo-

stimulation, which is recognized a role of mature DCs.

IL-4 production was enhanced from CD4$^+$ T lymphocytes by eckol-treated DCs. In the co-culture of CD4$^+$ T lymphocytes and DCs, we measured the levels of cytokines (Fig. 5), IFN-gamma and IL-4, a representative cytokine of Th1 and Th2 respectively. In the interest of accuracy, we performed the quantification of both IFN-gamma and IL-4 in same samples. Eckol-treated DCs significantly enhanced the production of IL-4 from CD4+ T lymphocytes compared to imDCs. And also, the level of IFN-gamma was marginally increased in same samples.

Discussion

Eckol is a pure compound originated from one of algae, *Ecklonia cava.* It has been studied for many years and elucidated to have its anti-oxidative (1,3) and inhibitory activity for enzymes, which are critical for a certain condition or pathogenic virus (2,10). Most studies have been focused on established cell lines, but primary cultured cells were used in our study.

It was demonstrated in our study that eckol-treated DCs increased the maturation markers on the surface of DCs, but did not enhance the production of IL-12 compared to imDCs (medium alone-treated DCs). Thus, we do not exclude the possibility which eckol-treated DCs may generate some anergic CD4$^+$ T lymphocytes. Based on our data, it is likely that eckol-treated DCs may stimulate CD4$^+$ T lymphocytes via cell-to-cell contact.

The eckol-treated DCs significantly enhanced the proliferation of allogeneic CD4$^+$ T lymphocytes compared to imDCs. And also, the activated CD4$^+$ T lymphocytes by eckol-treated DCs produced more IFN-γ and IL-4 compared to those cells by imDCs. These data suggest that eckol may at least have some maturing effects on DCs. Although we tried to investigate Th1 or Th2 shift of CD4$^+$ T lymphocytes activated by eckol-treated DCs, we observed a similar pattern of the production of two cytokines to those cells by anti-CD40 mAb-treated DCs from ELISA assay. It was suggested that the difference of cytokine amount from activated CD4$^+$ T lymphocytes by eckol-treated DCs and anti-CD40 mAb-treated DCs might be attributed from the proliferation rate of activated CD4$^+$ T lymphocytes (Fig. 4). The effects of eckol on immune system in vivo and specific immune cells in diseases or pathologic conditions were needed to be studied in future.

Taken together, we demonstrated in this study that eckol modulated immune responses via changing the phenotype and function of DCs. The effects of eckol

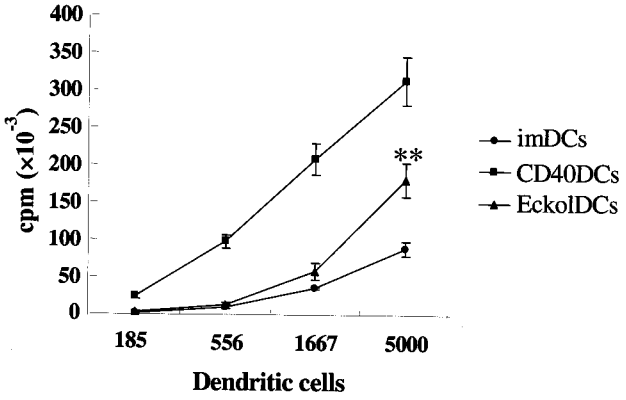

Figure 4. Eckol-treated DCs increased the alloproliferation of CD4$^+$ T lymphocytes. DCs were harvested and treated as Fig. 2B. And then, DCs were washed with HBSS twice and co-cultured with allogeneic CD4$^+$ T lymphocytes in 96-well culture plates. Incorporated ^{3}H-thymidine in cells was measured as counts per minute (cpm) by a scintillation counter. Data represent mean ±SD of cpm and one of three independent similar results. **indicate $p<0.01$.

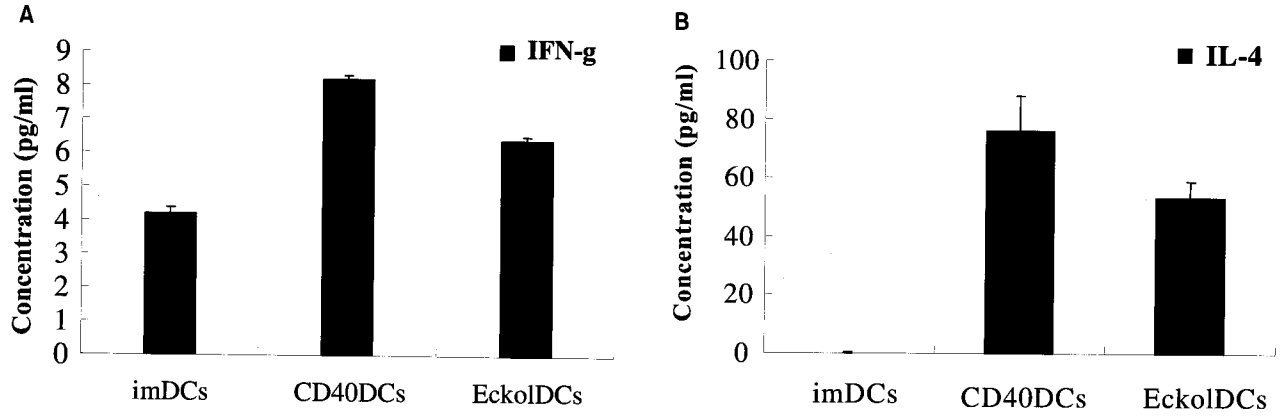

Figure 5. IL-4 production of CD4$^+$ T lymphocytes was enhanced by eckol-treated DCs. In same experiments as Fig. 4, the supernatants were harvested from the co-culture of 5×10^3 cells/well of DCs and 1×10^5 cells/well of CD4$^+$ T lymphocytes, and used for the determination of IFN-γ (A) and IL-4 (B) using ELISA. Data represent mean±SD and one of two independent similar results.

on immune cells were first studied in our knowledge. Especially, since DCs are very specialized antigen-presenting cells, playing a critical role in cellular and humoral immune systems, our study may provide the information about eckol with new aspects for further use.

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