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Immunomodulatory effects of *Ecklonia cava*

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GRADUATE SCHOOL

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국문 초록

제주도에서 서식하고 있는 해조류는 다양한 색소 성분들을 함유하고 있고, 그 특이적인 색소 성분의 함유 정도에 따라 갈조류, 홍조류, 녹조류로 나누어지게 된다. 이러한 해조류의 생리활성 성분으로는 polysaccharide, fucoidan, polyphenolic compounds (phlorotannins), xanthopophyll, fucoxathin, alginate, vitamins, carotene, thiamin, ascorbic acid, fucans, laminarans 등이 있다. 게다가 동의보감에서는 *Ecklonia cava* (감태)를 포함하는 갈조류가 방광염, 대장암, 편도염, 안검염, 고환염, 방광염, 결핵, 자궁내막염, 피부염증 (항종 및 등창) 등의 염증성 질환과 아토피 등 면역성 질환에 효능을 보이고 또한 피부면역증강 등의 면역 활성을 가진다고 보고 되어져왔다. 특히, 감태의 생리활성기능으로는 항산화, 항암, 세포 손상에 대한 억제, 항응고, ACE 저해, 지질 과산화 억제, 항균 활성과 MMP 억제 활성 등이 있으나, 면역 반응에 따른 구체적인 연구는 극히 드물다.

따라서 이 연구에서는 갈조류의 한 종류인 *Ecklonia cava*로부터 단백질 분해효소인 kojizyme에 의한 효소적 가수분해방법을 통해 추출물 (ECK) 을 분리하였고, 그 추출물이 *in vitro* 와 *in vivo* 상에서 가지는 면역활성을 확인하기 위해 실험을 수행하였다. 그 결과, ECK는 마우스로부터 얻은 primary splenocytes에서 고농도 (500ug/ml) 에서도 세포독성을 보이지 않았고, ^{3}H-thymidine incorporation assay를 통해 ECK를 처리하지 않은 세포에 비하여 저농도 (3ug/ml)에서도 세포증식능을 가지고, 또한 농도 의존적으로 splenocyte의 증식을 유도함을 알 수 있었다. 그리고 증식된 세포의 표면분자를 이용하여 phenotype을 확인한 결과, ECK를 처리하였을 경우, $CD4^{+}$ cytotoxic T cells은 14.5% (약 1.4배), $CD8^{+}$ cytolytic T cells은 8.4% (약 2배), 그리고 $CD45R/B220^{+}$ pan B cells은 12.1% (약 1.4배)만큼 증가된 것을 확인하였다. 또한 각각의 표면분자를 발현하고 있는 세포의 증식 및 분화 능력을 확인하기 위해 CFSE assay를 수행한 결과, ECK는 CFSE-labeled $CD4^{+}$ T cells과 $CD8^{+}$ T cells은 다소 증가하는 경향이었고, 특히 CFSE-labeled $CD45R/B220^{+}$ B cells이 유의성

있게 증가되었다. 이렇게 ECK에 의해 분화, 증식된 세포에서 사이토카인의 mRNA 발현과 단백질 생성 정도를 확인한 결과에서 우리는 ECK에 의해 Th1 type 사이토카인의 일종인 IL-1β mRNA의 발현양은 다소 감소하였고, TNF-α와 IFN-γ mRNA 발현은 유의성 있게 감소하였다는 것을 확인했다. 한편, Th2 type 사이토카인의 일종인 IL-4와 IL-10의 mRNA 발현은 ECK에 의해 증가되었다. 또한 ECK는 비특이적 T cell 자극자인 ConA에 의해 자극된 분화, 증식세포에서 분비되는 TNF-α와 IFN-γ 의 생산을 유의성 있게 감소시켰으며, IL-4와 IL-10의 생산은 유의성 있게 증가시켰다. 이상의 결과를 통해, ECK가 세포를 활성화시켜 증식을 유도하고, 염증성 사이토카인과 항염증성 사이토카인의 mRNA 발현과 단백질 생산을 조절하는 면역조절 효과를 가진다는 것을 알 수 있었다. 이에 따른, ECK에 의한 T, B cell의 활성 기전을 알고자 림프구의 분화, 증식, 활성 및 사이토카인 생성 등에 관여하는 것으로 알려진 NFκB signal pathway를 확인하였다. 그 결과, ECK는 short term과 long term에서 모두 NFκB의 발현을 증가시킴과 동시에 NFκB의 inhibitory protein인 IκB의 인산화를 유도하여 p-IκB의 발현양도 증가시켰다. 반면 NFκB와 p-IκB의 발현양이 증가되는 시간에서는 IκB는 분해되어 발현양이 감소되었다. 게다가 EMSA assay를 통해 ECK는 NFκB DNA binding 활성도 증가시킴을 알 수 있었다. 이상의 결과는 ECK에 의해 유도된 T, B cell의 분화, 증식 및 사이토카인의 분비의 조절이 NFκB pathway에 의한 것임을 제시하였다. 마지막으로 TPA에 의해 마우스의 귀에 유발시킨 피부염증 모델인 mouse ear edema model을 이용하여 ECK가 생체 내에서 항염증 효과를 가지는지를 확인하였다. ECK는 TPA에 의해 유도된 부종과 염증반응을 억제시켜 외이의 피부조직 부종의 범위를 유의성 있게 감소시켰고 (p <0.05), 이들 피부조직에 대한 H&E 염색을 통한 조직학적 소견에서는 현저하게 부종이 감소되고, 피부의 상피 조직에 중성호성 백혈구와 림프구 등의 염증세포의 침윤이 감소함을 알 수 있었다. 또한 외이 조직에서 사이토카인의 생성정도를 관찰한 결과 IL-1β, TNF-α와 IFN-γ 등의 Th1 type cytokine과 염증 매개성 분자인 COX2의 mRNA발현은 감소시켰다. 따라서, 이 연구를 통해 ECK가 Th1 type

cytokine과 염증 매개성 분자의 조절을 통해 항염증 효과를 가진다는 것을 확인하였다.

지금까지의 결과를 종합해 볼 때, ECK는 T cell과 B cell에서 NFκB를 활성화시킴으로써 세포의 증식과 활성화, 사이토카인의 분비를 유도하고, 나아가 Th1 & Th2 type 사이토카인의 생성을 효율적으로 조절함으로써 면역조절 활성효과를 가진다고 할 수 있다. 또한 이 실험에서 사용된 ECK는 해조류로부터 얻은 독성작용이 없는 수용성의 천연물질이기에 면역 반응과 관련된 질병 치료를 위한 시약으로써 사용될 수 있을 것이다.

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*, p <0.01.

Fig. 1-16. Effect of ECK on Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokine production by Con A-stimulated blastogenic cells. (A) IFN-γ (B) TNF-α (C) IL-4 (D) IL-10. Splenocytes were stimulated with Con-A (1μg/ml), and these stimulated blastogenic cells were treated or not with ECK (100 μg/ml) for 72 h. The culture supernatants were collected, and concentrations of IL-1β, TNF-α, IL-4, and IL-10 were measured using commercially available ELISA kits. Spontaneous cytokine release: IFN-γ, 66 ± 21 pg/ml; TNF-α, 38± 21 pg/ml: IL-4, undetectable; IL-10, undetectable. Data are means ± S.D. of three experiments. Statistical evaluation was performed to compare the Con A-treated cells and ECK-treated blastogenic cells with corresponding untreated cells, respectively. *, $p < 0.01$, **, $p < 0.05$.

Fig. 2-1. The effect of ECK on the viability of splenocytes. Spleen cells were cultured in the absence or presence of ECK at the concentrations indicated (3 - 100 μg/ml) for 48 h. Their viability was measured by MTT assay. Experiments were performed in triplicates, and data are expressed as average percent change from control ± S.D.

Fig. 2-2. The effect of ECK on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (50 - 500 μg/ml). After incubation with ECK for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$.

Fig. 2-3. Expressions of phosphor-IκBα, IκBα, NFκB p65 and β-actin of ECK in short term. The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with 50 ug/ml of ECK for 0 min, 15 min, 30 min and 60 min. The 60 ug/ml of cytoplasmic and 40 ug/ml of nuclear proteins were applied into gel, respectively. The blot was blocked in 5% non-fat milk. All antibodies were diluted into 1: 500 with

2% milk in TBST. Statistical evaluation was performed to compare ECK-treated cells and vehicle-treated cells, respectively.

Fig. 2-4. Expression of phosphor-IκBα, IκBα, NFκB p65 and β-actin of ECK in lomg term. The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with 50 ug/ml of ECK for 0 h, 3 h, 24 h and 48 h. The 60 ug/ml of cytoplasmic and 40 ug/ml of nuclear proteins were applied into gel, respectively. The blot was blocked in 5% non-fat milk. All antibodies were diluted into 1: 500 with 2% milk in TBST. Statistical evaluation was performed to compare ECK-treated cells and vehicle-treated cells, respectively.

Fig. 2-5. Effect of ECK on NFκB DNA binding activity by EMSA. Nuclear protein was obtained from splenocytes incubated with ECK for 0.25 h. 0.5 h and 48 h. NFκB specific oligonucleotide–protein complexes were detected by electrophoresis mobility shift assays. The values represent the normalized intensities of the NFκB band against intensities of control band measured by bio-image analyzer system.

Fig. 3-1. Histological analysis of vertical sections in TPA-induced mouse ears model. Histology of vertical sections of ECK in TPA-induced mouse ears stained by haematoxylin and eosin were evaluated. A; Ears treated with TPA (2.5 ug/ear), B; Ears treated with TPA plus dexamethasone (50 ug/ear) and C; Ears treated with TPA plus ECK (100 ug/ear).

Fig. 3-2. Effect of ECK on the mRNA expression level of cytokines on TPA-induced ear edema model. After incubation for 48 h, RNA extracted from mouse ear with TPA plus ECK or TPA was used for cDNA synthesis. The cDNA (1 ug) was amplified by PCR using gene specific primers. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three separate experiments.

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INTRODUCTION

Seaweeds that are a widely available source of biomass as over two million tones are either harvested from the oceans or cultured annually for food or phycocolloid production. They are rich in vitamins, minerals, natural bioactive compounds, and various functional polysaccharides. These seaweeds can be divided into three basic types such as brown (Phaeophyta), red (Phodophyta), and green (Chlorophyta) seaweed by the dominance of a kind of pigment among various pigments such as xanthophylls, fucoxanthin, phycoerythrin, and chlorophyll a and b (Hashim et al., 2004). Among them, brown seaweeds have the brown color resulted from the dominance of the fucoxanthin pigment and various polysaccharides, such as and possess many kinds of bioactive properties (Ahn et al., 2002; Siriwardhana et al., 2004; Lahaye et al., 1997; Ruperez et al., 2001). *Ecklonia cava* is a kind of brown seaweed that has lots of xanthopophyll, fucoxathin, vitamins, vitamin precursors such as α-tocopherol, β-carotene, niacin, thiamin and ascorbic acid, and polysaccharides such as alginates, fucans and laminarans which are water-soluble dietary fibers and phycocoloids and is plentifully found in Jeju Island of Korea (Guiry et al., 1991). It has been reported that total polyphenolic compounds in *E. cava* are richer than in other brown seaweeds (Heo et al., 2003a, b, 2005a). These polyphenolic compounds of brown seaweeds have been called as phlorotannins. And the phlorotannin components of *E. cava* that are phenolic secondary metabolites such as eckol (a closed-chain trimer of phloroglucinol), 6,6'-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) and triphlorethol-A have been known to be related to the biological activities (Kang et al., 2005a and b). Recently, it has been reported that *Ecklonia* species exhibit radical scavenging activity (Kang et al., 2003 and 2004), anti-plasmin inhibiting activity (Fukuyama et al., 1989 and 1990), antimutagenic activity (Lee et al., 1998), bactericidal activity (Nagayama et al., 2002), HIV-1 reverse transcriptase and protease inhibition activity (Ahn et al., 2004), tyrosinase inhibitory activity (Kang et al., 2004) and cell damage inhibition activities (Kang et al., 2005). Also,

our previous studies demonstrated that the enzymatic extracts of *E. cava* have the antioxidative effects *in vitro,* anticancer activities, anticoagulant activities and matrix metalloproteinase inhibitory activity for many years (Heo et al., 2005; Athukorala et al., 2006; Kim et al., 2006; Kim et al., 2006). However, no other studies have reported the immune activities of extracts from *E. cava* and furthermore, the biological mechanisms for these activities in vitro and in vivo have not been elucidated.

The previous study suggested that in order to survive, the environment rapidly and effectively changes of all organisms might include externally increased concentrations of signaling molecules (e.g. hormones, cytokines or growth factors). And, the relatively few transcription factors that regulate inducible gene expression can be the targets for many distinct signal transduction pathways triggered by a wide variety of stimuli. In addition, the researchers suggested that one important and widely used transcription factor that plays a pivotal role in many cellular responses to environmental changes is NF-κB (May et al., 1998). NFκB is known as the Rel family that exist in the cytoplasm of the majority of cell types as homo- or heterodimers including proto-oncogene *c-rel,* p50/p105 (NFkB1), p65 (RelA), p52/p100 (NFκB2), and RelB (Baldwin, 1996; Kopp, 1995; Baeuerle et al., 1994; Kopp et al., 1994; May et al., 1998). Normally, NFκB proteins are sequestered in the cytoplasm through physical interaction with inhibitors of the IκB (inhibitor of κB) family. Following several extracellular signals, IκB kinase (IKK), a cytoplasmic kinase complex, becomes activated and phosphorylates the IκB molecules, leading to their degradation through the ubiquitin-proteasome pathway (Israël, 2000). Then, NFκB dimers translocate to the nucleus and activate their target genes such as cytokines including IL-2. Also, although NFκB was initially discovered and characterized as a transcription factor required for B-cell-specific gene expression, subsequent studies demonstrated that it is ubiquitously expressed and regulates the inducible expression of various cellular genes, including cytokines, cytokine receptors, and stress proteins and the replication of viruses in the immune, inflammatory and anti-apoptotic responses (Sen et al., 1986a and b; Baeuerle et al., 1994; Kopp et al., 1994; Weil et al., 2004). Recently,

Weil et al. has reported that NFκB pathway is divided into two types; the alternative pathway which is initiated by complexes of NFκB2 and RelB cause NFκB activation in response to a specific set of stimuli and the classical pathway which is initiated by complexes of NFκB1 and RelA and involved in the response of multiple cell types to pro-inflammatory cytokines and phathogen-associated molecular patterns. In addition, the previous study has reported that activation of RelA that has a C-terminal transcriptional activation domain in classical pathway leads to the cytokines production such as IL-2 and lymphocytes activation, differentiation and proliferation for immune activation (Weil et al., 2004; Ishimaru et al., 2006; Beg et al., 1993; Ruben et al., 1992; Ballard et al., 1992).

Anti-inflammatory effects are related to inhibit the inflammatory response pathways or specifically with certain components of the pathway, such as release of pro-inflammatory mediators, migration of leukocytes under inflammatory stimulus with consequent release of the cytoplasmic contents at inflammation sites, activation of complement sequence, etc. (Okoli et al., 2003). Normally, the inflammatory response is a defense mechanism evoked by body tissues in response to injury or microbial invasion and a usual accompaniment of most disease processes, and can on its own constitute a source of discomfort thus requiring treatment. Many researchers have investigated natural products for the treatment of the disorders associated with the inflammatory response or control of the inflammatory aspects of diseases (Okoli et al., 2004; Perez e., 1996; Pereira et al., 2001; Koo et al., 2004).

In the present study, we have used the enzymatic extraction technique that help releasing various bioactive compounds released from the seaweeds by breaking down their cell walls which consist of carboxyhydrates, proteins, glycoproteins and lipoproteins, and investigated the immune effects and NFκB signal pathway on splenocytes *in vitro* and anti-inflammatory effects *in vivo* of *E. cava* enzymatic extract (ECK).

Part 1.

Immunomodulatory effects of *Ecklonia cava* on splenocytes *in vitro*

Part 1

Immunomodulatory effects of *Ecklonia cava* on splenocytes *in vitro*

1. ABSTRACT

Ecklonia cava used in this study is a kind of brown seaweeds. In this study, we investigated whether *E. cava* extract by Kojizyme (ECK) known as a kind of protease has the immunological effects in splenocytes *in vitro*. ECK enhanced the proliferation ability of the splenocytes in ^{3}H-thymidine incorporation assay. In detail, to identify the specific cell phenotype of proliferated lymphocytes we performed flow cytometry assay. ECK significantly increased the number of $CD4^{+}$ T cells, $CD8^{+}$ T cells, $CD19^{+}$ B cells and $CD45R/B220^{+}$ B cells compared to non-treated controls. In addition, the mRNA expression level of IL-4 and IL-10 known as Th2 type cytokines was increased compared with that of non-treated cells. On the other hand, the level of TNF-α and IFN-γ known as Th1 type cytokine was significantly decreased. In parallel with these results, the production level of Th1 type cytokine was down-regulated, whereas that of Th2 type cytokines was up-regulated by ECK. Taken together, the number of lymphocytes dramatically increased, indicating proliferation activity and the production of Th1 type cytokines as well as Th2 type cytokines can be regulated by ECK in immune cells. These results suggested that ECK has the immunomodulatory effects activating the anti-inflammatory response and/or suppressing the pro-inflammatory response and can be used for therapy of immune-related diseases.

2. MATERIALS AND METHODS

2. 1. Mice

All experiments were performed with ICR mice, aged 8 to 9 weeks, purchased from Japan SLC, Inc.. The animals were housed, five to a cage, in conventional animal facilities with NIH-07 diet and water *ad libitum* under constant temperature (23±1°C) according to the internationally accepted guideline.

2. 2. Preparation of enzymatic extract from *Ecklonia cava* (ECK)

E. cava was collected from the coastal waters of Jeju Island, South Korea, washed with fresh water and dried. The enzymatic extract was prepared in accordance with the method developed by Heo *et al* (Heo et al., 2003). Briefly, the *E. cava* samples were freeze-dried then pulverized into powder with a grinder. One gram of dried *E. cava* was homogenized with 100 ml of buffer (pH 6.0) and mixed with 100 μl of kojizyme (Navo Co., Novozyme Nordisk, Bagsvaed, Denmark). The pH of sample was adjusted to within the optimal range of the kojizyme, and the enzymatic reaction was conducted at 40℃ for 12 h. Following digestion, the digest was boiled for 10 min at 100℃ to inactivate the enzymes. Finally, the sample was clarified via 20 min of centrifugation at 3000 × g to remove any unhydrolyzed residue (Fig. 1-1). The resulting enzymatic extract (ECK) obtained after filtration of the supernatant was adjusted to pH 7.0 hereafter and then stored for use in experiments.

2. 3. Molecular weight fractionation of enzymatic extract

ECK was fractionated with Millipore's Labscale TFF system (Millipore, Billerica, USA) with ultrafiltration membranes (5, 10 and 30 kDa) (Fig. 1-2). The resultant fractions were then collected according to molecular weights (<5, 5–10, 10–30 and 30 kDa<) and stored at -20°C for use in further experiments.

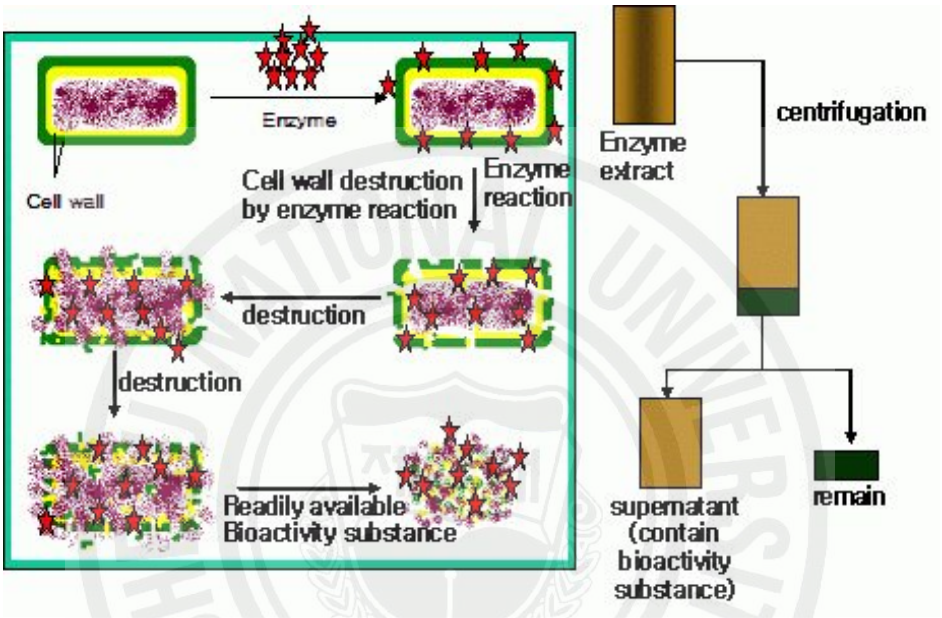

Fig. 1-1. Preparation of enzymatic extract.

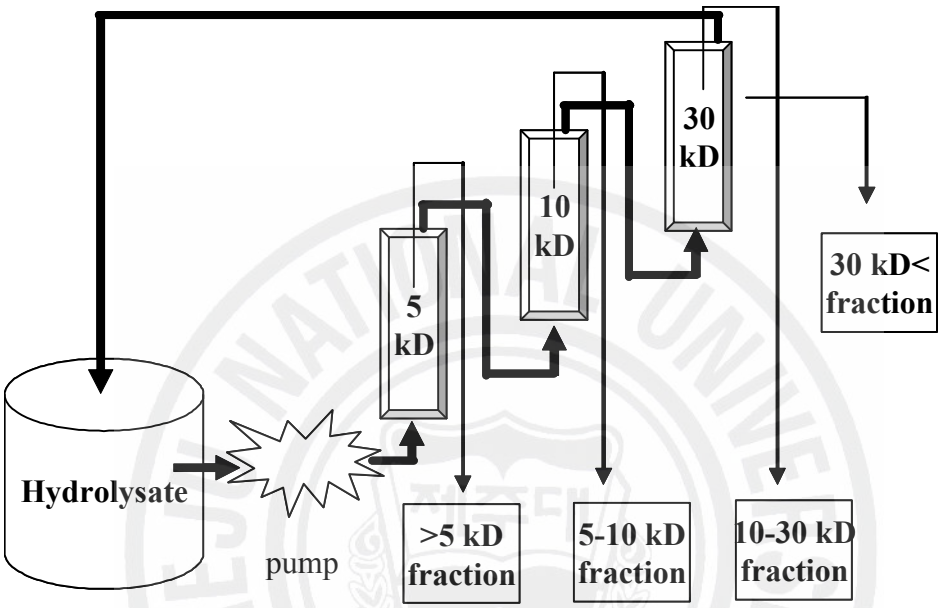

Fig. 1-2. Ultrafiltration membrane system for fraction of *E. cava* enzymatic extract according to the molecular weight of constituents.

2. 4. Isolation of sulfated polysaccharide from ECK

The 240 ml of ECK was mixed well with 480 ml of 99.5% ethanol and allowed to stand for 30 min at room temperature. The crude polysaccharides were collected by centrifugation at 10,000g for 20 min at 4°C (Matsubara et al., 2000 and Kuda et al., 2002). Hereafter, crude polysaccharide fraction obtained was concentrated separately under vacuum at 40°C and removed all ethanol. Then samples were dissolved in water for further experiments.

2. 5. T cell culture medium (TCM)

Cells were suspended in DMEM supplemented with 1% (v/v) minimum essential medium (Gibco BRL, Life Technologies, Paisley, UK), 2 mM glutamine (Flow Laboratories, Irvine, UK), 50 IU/ml penicillin and 50 mg/ml streptomycin and 10% (v/v) FCS.

2. 6. Preparation of spleen cells

Spleens were removed aseptically from ICR mice, and single splenocytes were suspended in TCM containing 10% fetal bovine serum, 1% antibiotic (100 U/ml penicillin and 100 mg/ml streptomycin) then separated. Red blood cells were lysed with ACK lysis buffer (BD Biosciences Pharmingen, San Jose, CA, USA) at room temperature for 10 min. After washing with DPBS, the purified cells were obtained and used directly for experiments in which they were treated with EKC or a vehicle, hereafter called "untreated cells".

2. 7. MTT assay

To identify the cell viability of various compounds on splenocytes, we performed MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. In addition, it is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann er al., 1983). The

2×10^4 cells were incubated in 96-well plates with ECK, ECK fractions, sulfated polysaccharides, phloroglucinol (Sigma-Aldrich, Steinheim, Germany and Fig. 1-3A) or fucoidan (Sigma-Aldrich, Steinheim, Germany and Fig. 1-3B) at from 1 ug/ml to 100 ug/ml of various concentrations. After 48 h, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium-bromide) (Sigma, Saint Louis, USA) stock solution (50 μl; 2 mg/ml) was then applied to each of the wells and incubated at 37°C for 4 h. The plates were centrifuged for 10 min at 800 × *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2. 8. ^{3}H-thymidine incorporation assay

The proliferative ability of T cells was tested by the standard assay based on the principle that the thymidine base of DNA sequences in these cells is replaced with radioactive ^{3}H-thymidine (Amersham, Arlington Heights, IL). For this assay, the 4×10^6 cells were treated with ECK, ECK fractions, phloroglucinol, fucoidan or sulfated polysaccharides in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). After incubation for 3 days at 37°C, 95% humidity, and 5% CO_2, 1 μCi of ^{3}H-thymidine (specific activity 42 Ci/mmol) was added to the cells, and the plates were incubated for additional 18 h. The cells were then harvested onto glass fiber filters by an automatic cell harvester. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

2. 9. Flow cytometry assay

Flow cytometry assay simultaneously measures multiple characteristics of single cells at a rapid rate, in our experiment, to detect accessory molecule expression on spleen cells after fluorescence activated cell sorting (FACS). Briefly, spleen cells were harvested and washed with 1×DPBS distilled. The cells were blocked with anti-mouse IgG solution in

A B

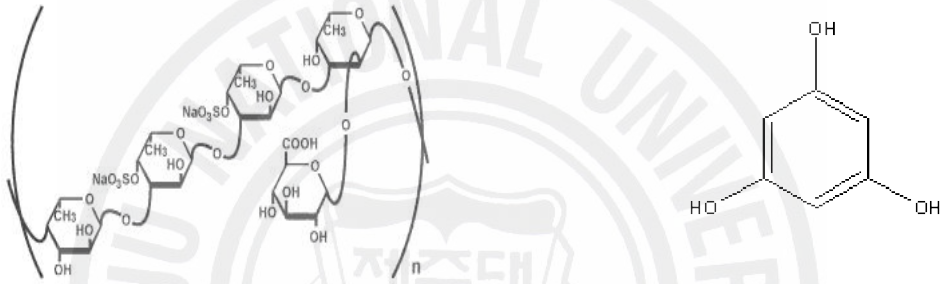

Fig. 1-3. Structure of fucoidan (A) and phloroglucinol (B).

PBS for 15 min at 4°C to inhibit nonspecific staining, and then stained with fluorescently labeled monoclonal antibodies (Abs) to the mouse antigens of interest at optimal concentration for an additional 15 min at 4°C. Abs were from BD Biosciences, San Jose, CA, USA. Abs directly labeled with the following fluorescent tags; FITC- or PE- were CD4 (H129.19), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3) and CD45R/B220 (RA3-6B2). Those used in these experiments were CD4-FITC as a specific marker for T helper lymphocytes, CD8-PE as a specific marker for cytolytic T lymphocytes, CD11b-FITC as a specific marker for granulocytes and Mϕ, CD11c-FITC as a specific marker for NK cells and DC cells, CD19-FITC as a specific marker for specific B lymphocytes and CD45R/B220-FITC as a specific marker for pan B lymphocytes. Appropriate isotype controls were always included. After centrifugation, DPBS was added to the cells, and twenty thousand viable cells per treatment (as determined by light scatter profiles) were analyzed using a BD FACSCalibur™ flow cytometer and CellQuest software (BD Biosciences).

2. 10. CFSE-labeled cells proliferation assay

To identify the proliferation and differentiation of ECK on splenocytes, we performed CFSE (Carboxy fluorescein diacetate succinimidyl ester) assay. The 60×10^6 cells of splenocytes were suspended in warm RPMI without FBS and mixed with a working solution of 50 uM CFSE-FITC (final concentration 0.5 μM). After incubation in the dark at 37°C for 10 min, 50 mL of cold RPMI containing 1% FBS was added to stop the uptake of the CFSE dye. The CFSE-labeled cells were washed cold RPMI and resuspended in RPMI 1640 media containing 10% fetal bovine serum, 1% antibiotic. The 3×10^6 cells of CFSE-labeled cells were incubated with 100 ug/ml of ECK or vehicle at 37°C for 3 days or 5 days and the medium was replaced with new fresh medium containing 10% fetal bovine serum, 1% antibiotic at 3 days. After 3 days or 5 days, the CFSE-labeled cells were harvested and stained with monoclonal antibodies such as R-PE-anti-CD4 (H129.19) which is a surface marker of cytotoxic T cell, R-PE anti-CD8 (53-

6.7) which is a surface marker of cytolytic T cells and CD45R/B220 (RA3-6B2) which is a surface marker of pan B cells. Data were collected on 50,000 cells and analyzed using a BD FACSCalibur^{TM} flow cytometer and CellQuest software (BD Biosciences, San Jose, CA). Proliferating lymphocyte subsets (CD4, CD8 and CD45R/B20) were distinguished by multi-parameter flow cytometry.

2. 11. RNA preparation

Spleen cells were incubated with 100 ug/ml of ECK for 48 h before RNA extraction. After incubation, the cells were collected and mixed with trizol (Molecular Research Center, Inc., Cincinnati, Ohio, USA). The addition of chloroform (Sigma, St Louis, MS, USA) and incubation for 5 min at 4°C followed. The supernatants obtained after centrifugation were treated with isopropanol (Sigma), and the resulting RNA pellets were washed and stored at -20°C until use.

2. 12. RT–PCR

The cDNA was synthesized with RNA purified from spleen cells by using a Promega A3500 kit, according to manufacturer's instructions (Promega, San Luis, CA, USA). PCR of this cDNA and the primer (Bioneer, Daejeon, South Korea) displayed in Table 1 was performed for 40 cycles with a 5 min denaturing step at 94°C, a one min annealing step at 55 to 60°C and a 20 min extension phase at 72°C using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were run on a 1.5% EtBr/agarose gel and visualized by UV transillumination.

2. 13. Enzyme linked immunoabsorbant (ELISA) assay

To identify the effects of ECK on the production of interferon (INF)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4 and IL-10 in ConA-activated splenocytes, blastogenic cells, splenocytes were incubated with 100 ug/ml ECK and/or ConA, after which the supernatants were collected from the cell suspensions. Then, these supernatants were analyzed according to the manufacturer's recommendations with mouse cytokine-specific

Table 1-1. The sequences and expected size of cytokine primers for RT-PCR in ECK-activated splenocytes

Oligonucleotide		Sequence	Expected size (bp)
IL-1β	5'-primer	5'-GCT ACC TGT GTC TTT CCC GTC G-3'	291
	3'-primer	5'-TTG TCG TTG CTT GGT TCT CCT TG-3'	
IFN-γ	5'-primer	5'-AGG TCA ACA ACC CAC AGG TCC A-3'	397
	3'-primer	5'-CCA GAT ACA CCG CAA TCA C-3'	
TNF-α	5'-primer	5'-GGC AGC TTC TGT CCC TTT CAC TC-3'	366
	3'-primer	5'-CAC TTG GTG GTT TGC TAC GAC G-3'	
IL-4	5'-primer	5'-ACG GAG ATG GAT GTG CCA AAC GTC-3'	361
	3'-primer	5'-CGA GTC ATC CAT TTG CAT GAT GC	
IL-10	5'-primer	5'-CAC TGC TAT GCT GCC TGC TCT T-3'	417
	3'-primer	5'-TCT TCA CCT GCT CCA CTG CCT T-3'	
GADPH	5'-primer	5'-AAC GAC CCC TTC ATT GAC C-3'	701
	3'-primer	5'-TCA GAT GCC TGC TTC ACC-3'	

ELISA kits (Biosource International, Camarillo, CA, USA). Cytokine levels were calculated with standard curves using recombinant murine cytokines.

2. 14. Statistical analysis

Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as means ± standard error (SE). A *p*-value of less than 0.05 was considered significant.

3. RESULTS

3. 1. ECK has the cell viability of splenocytes

The viability of splenocytes incubated for 48 h with ECK was quantified by assessing the MTT assay. As shown in Fig. 1-4A, ECK did not show the cytotoxicity on splenocytes at all concentrations. Also, we identified that the various fractions (5 KD>, 5-10 KD, 10-30 KD, 30 KD<) obtained from ECK (Fig. 1-4B), 30 KD<, sulfated polysaccharides, fucoidan and phloroglucinol considered as active compounds of *Ecklonia cava* have the cell viabilities without cytotoxicity at all tested concentrations (Fig. 1-5 and Fig. 1-6). These results indicate the beneficial effect of ECK on the survival of immune cells.

3. 2. ECK stimulates the proliferation of splenocytes

To confirm that ECK similarly benefits splenocytes' proliferative ability, we examined such cells in a ^{3}H-thymidine incorporation assay. Accordingly, the proliferation of these ECK-treated cells was significantly higher than that of untreated cells and increased with rising concentrations of ECK from 50 μg/ml to 500 μg/ml (p <0.01) (Fig. 1-7). In addition, all fractions (p <0.01 and p <0.05) (Fig. 1-8), sulfated polysaccharides (p <0.05) (Fig. 1-9), fucoidan (p <0.01) (Fig. 1-10A) and phloroglucinol (p <0.01) (Fig. 1-10B) considered as active compounds of *E. cava* stimulated and increased the proliferation of splenocytes at all tested concentrations. Especially, 30KD< fraction and fucoidan.

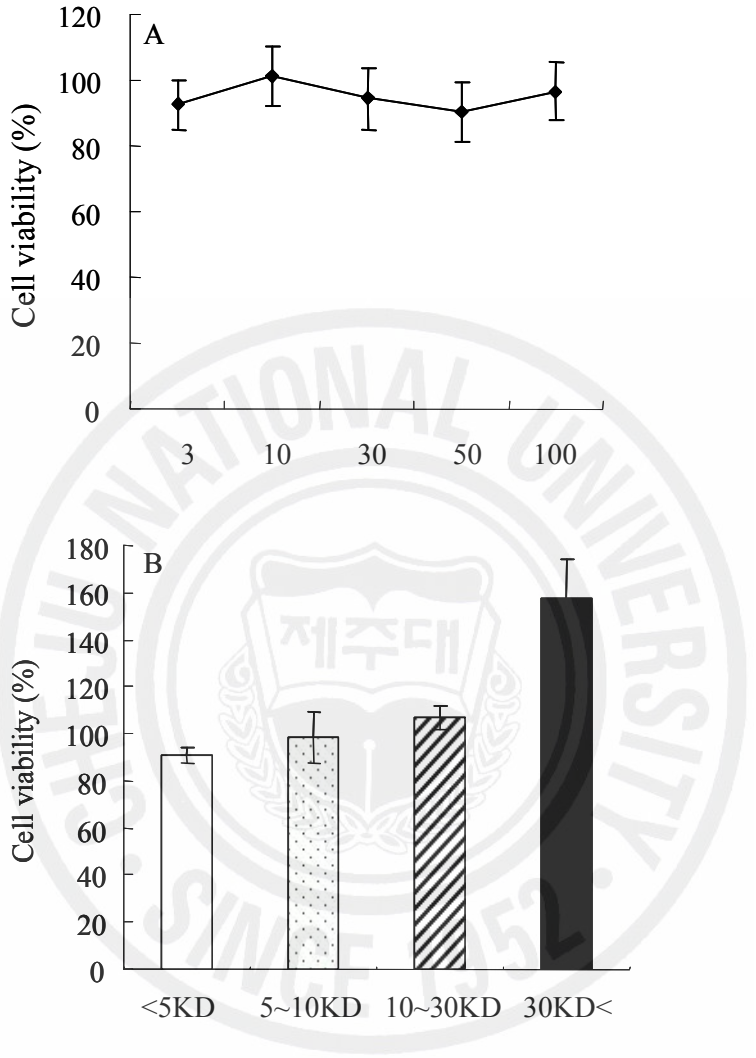

Fig. 1-4. The effect of ECK (A) and ECK fractions (B) on the viability of splenocytes. Spleen cells were cultured in the absence or presence of ECK at the concentrations indicated (3 - 100 μg/ml and 50 ug/ml) for 48 h. Their viabilities were measured by MTT assay. Experiments were performed in triplicates, and data are expressed as average percent change from control ± S.D.

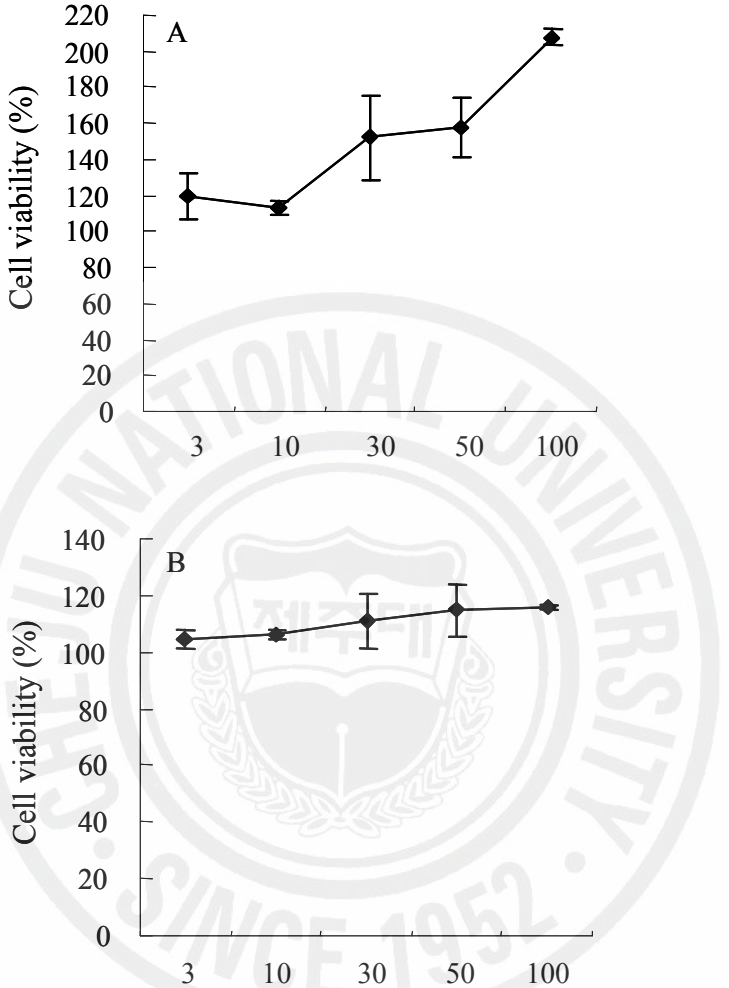

Fig. 1-5. The effect of 30KD< fractions (A) and sulfated polysaccharide (B) on the viability of splenocytes. Spleen cells were cultured in the absence or presence of 30KD< fractions and sulfated polysaccharide at the concentrations indicated (3 - 100 μg/ml) for 48 h. Their viabilities were measured by MTT assay. Experiments were performed in triplicates, and data are expressed as average percent change from control ± S.D.

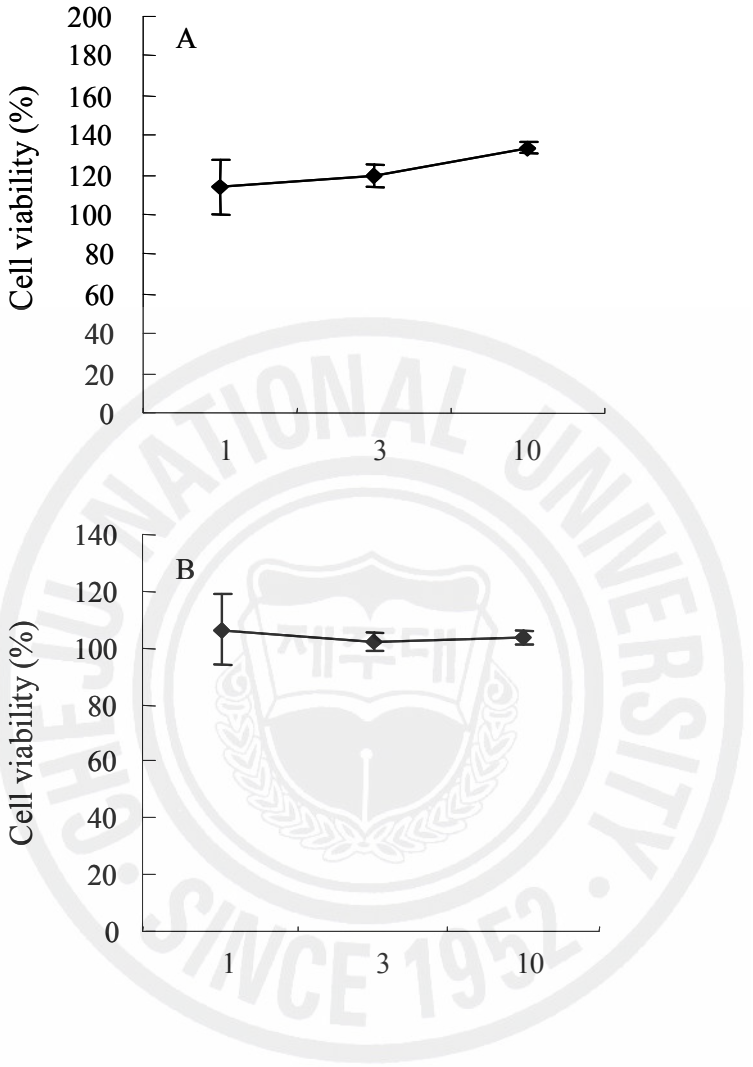

Fig. 1-6. The effect of phloroglucinol (A) and fucoidan (B) on the viability of splenocytes. Spleen cells were cultured in the absence or presence of phloroglucinol and fucoidan at the concentrations indicated (1 - 30 μg/ml) for 48 h. Their viability was measured by MTT assay. Experiments were performed in triplicates, and data are expressed as average percent change from control ± S.D.

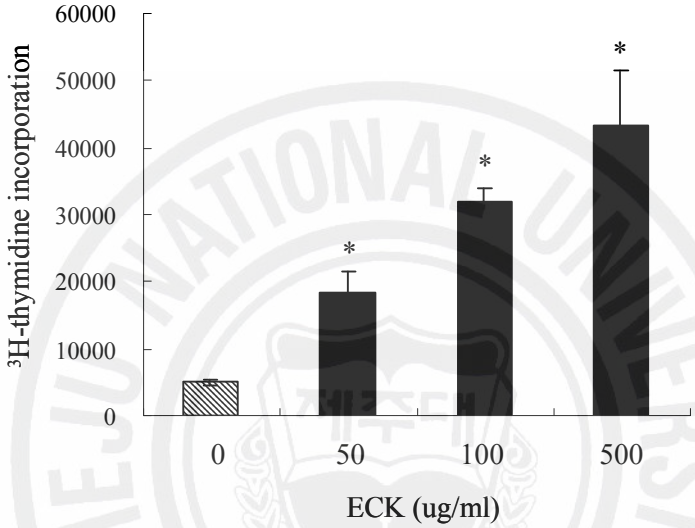

Fig. 1-7. The effect of ECK on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (50 - 500 μg/ml). After incubation with ECK for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$.

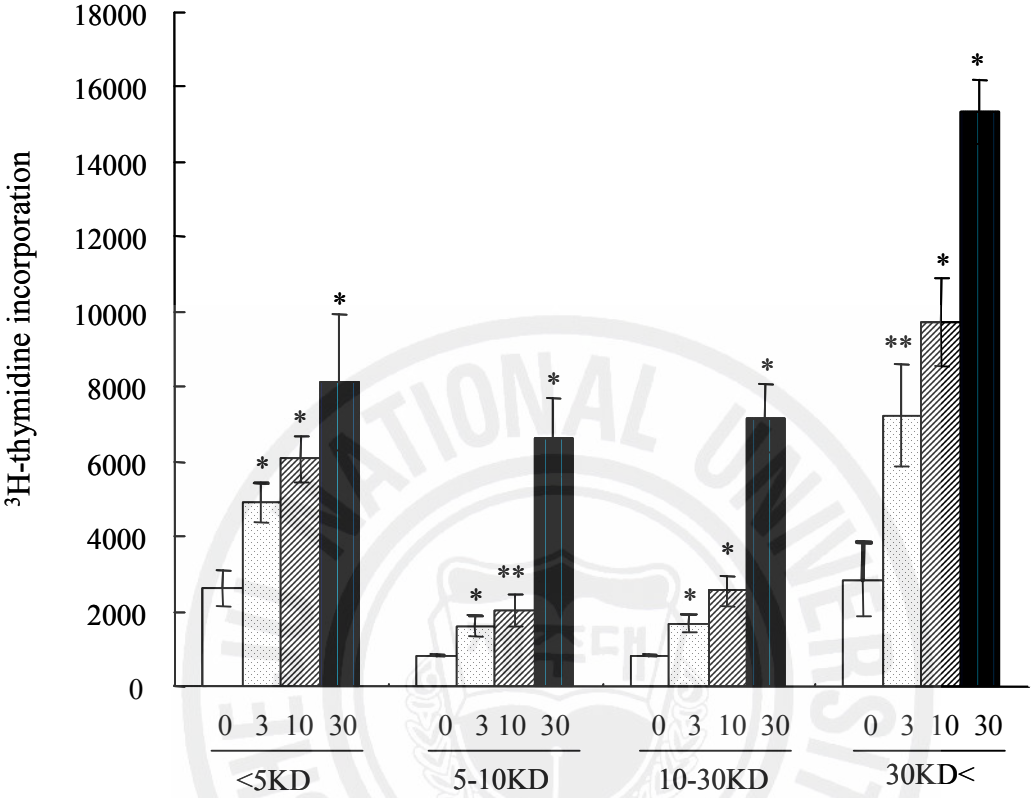

Fig. 1-8. The effect of ECK fractions on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (0 - 30 μg/ml). After incubation with ECK fractions for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$, **, $p < 0.05$.

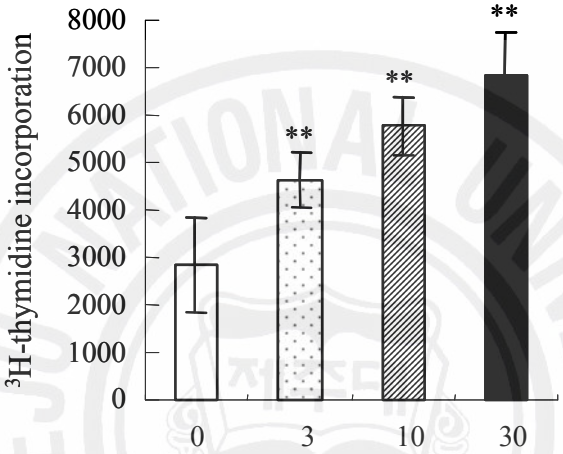

Fig. 1-9. The effect of sulfated polysaccharide on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (0 - 30 μg/ml). After incubation with sulfated polysaccharide for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. **, $p < 0.05$.

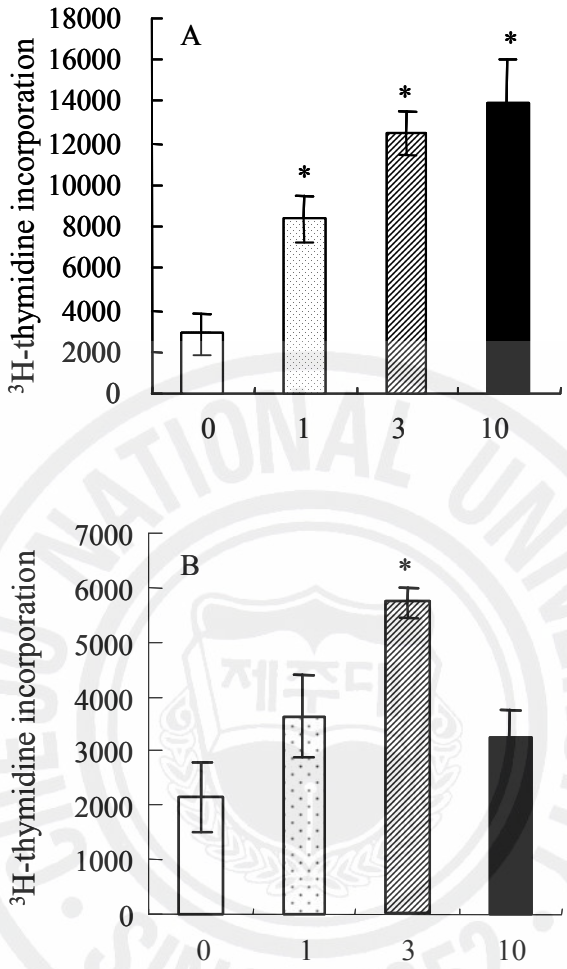

Fig. 1-10. The effect of fucoidan (A) and phloroglucinol (B) on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (0 - 30 μg/ml). After incubation with fucoidan and phloroglucinol for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$.

significantly increased the proliferation of splenocytes with rising concentrations. According to these results, we identified that splenocytes are strongly stimulated and proliferated by ECK or various active compounds of *E. cava* such as ECK fractions, sulfated polysaccharide, phloroglucinol and fucoidan without cytotoxicity. Presumably, then, treatment with ECK may lead to an enhancement of mitogenic or antigenic stimulation.

3. 3. ECK enhances the populations of T and B cells

To further define the specific cell population stimulated by ECK, the spleen cells' ability to interact with antibody markers was tested and subjected to FACS analysis. The splenocytes were incubated with ECK for 48 h and stained with FITC-conjugated anti-CD4, CD19, CD11b, CD11c and CD45R/B220 or PE-conjugated anti-CD8 antibody. We identified that ECK increased the numbers of lymphocytes, granulocytes and monocytes compare to untreated cells during incubation. Especially, the number of lymphocytes gated was significantly increased by ECK with the progress of incubation (Fig. 1-11). Also, although the numbers of $CD11b^+$ DC cells and Mϕ and $CD11c^+$ NK cells do not have significance (data not shown), the numbers of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD45R/B220^+$ pan B cells were markedly up-regulated by ECK compared with untreated cells (Fig. 1-12 and Fig. 1-13). In particular, the number of $CD8^+$ cytolytic T cells was elevated two fold above that of control cells. Moreover, the specific populations of $CD4^+$ cytotoxic T cells, $CD19^+$ B cells and $CD45R/B220^+$ pan B cells were larger by about 13.5%, 14.2% and 13.1%, respectively. These results indicated that ECK not only stimulates lymphocytes in general but also increases the quantities of specific cell phenotypes.

3. 4. ECK enhances the proliferation and differentiation of CFSE-labeled T and B lymphocytes

To confirm whether ECK enhances the differentiation and proliferation of lymphocytes or not, we performed to measure the progressive decrease in intensity of the CFSE dye in

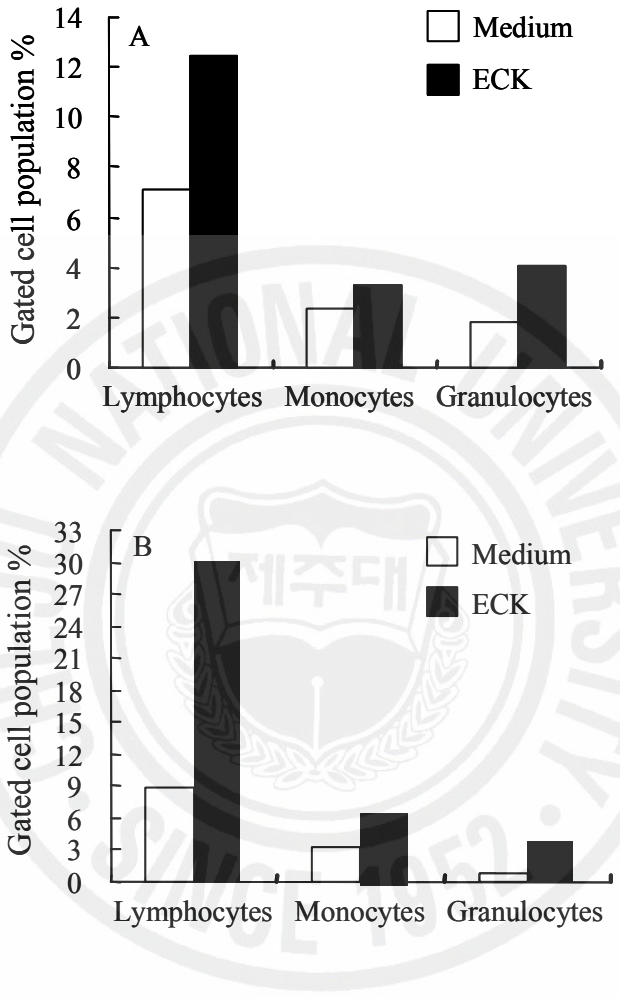

Fig. 1-11. Gated cell polulation analysis of proliferating splenocytes incubated with ECK for 3 days (A) and 5 days (B). The cells were cultured in the absence or presence of ECK (100 μg/ml) for 3 days and 5 days. After incubation, the cell population was measured by FACS analysis. Experiments were performed in triplicate.

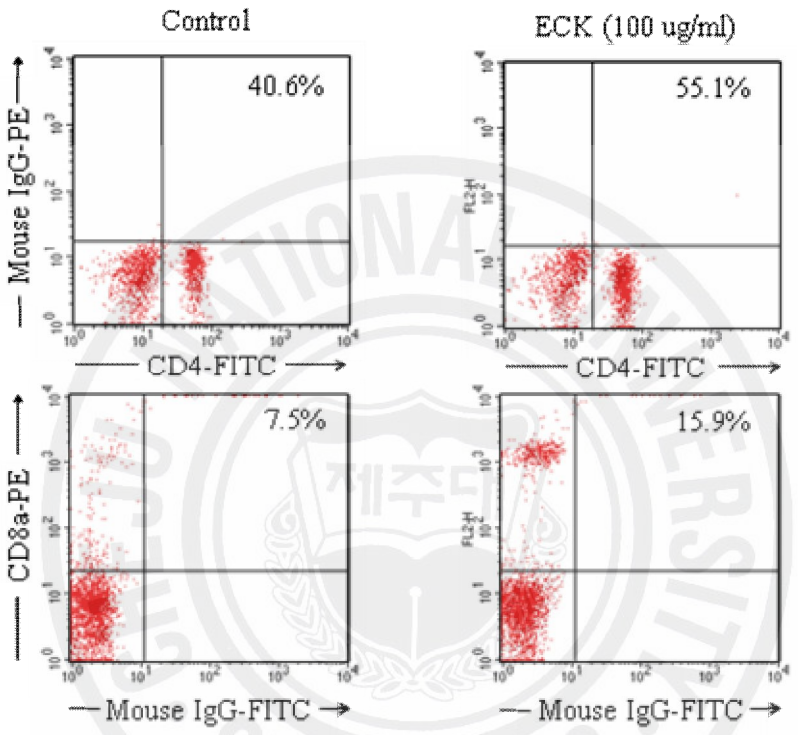

Fig. 1-12. Phenotypic analysis of proliferating splenocytes after ECK treatment. After incubation for 48 h, the expression level of each cell type was measured by FACS analysis using anti-CD4 and CD8 mAbs. The cells were cultured in the absence or presence of ECK (100 μg/ml) for 48 h. Then, the cells were blocked by anti-mouse IgG and stained by the mAbs as indicated. Experiments were performed in triplicate.

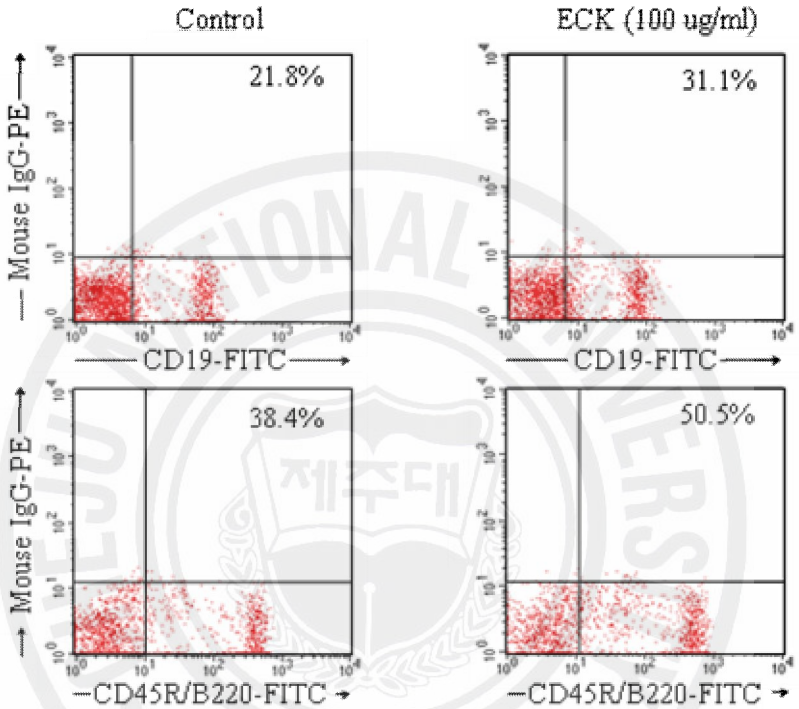

Fig. 1-13. Phenotypic analysis of proliferating splenocytes after ECK treatment. After incubation for 48 h, the expression level of each cell type was measured by FACS analysis using anti-CD19 and CD45R/B220 mAbs. The cells were cultured in the absence or presence of ECK (100 μg/ml) for 48 h. Then, the cells were blocked by anti-mouse IgG and stained by the mAbs as indicated. Experiments were performed in triplicate.

each succeeding lymphocyte generation. Normally, CFSE-FITC is transferred from parent cells to daughter cells. As shown in Fig. 1-14, the proliferation of CFSE-labeled $CD4^+$ cytotoxic T cells, $CD8^+$ cytolytic T cells and $CD45R/B220^+$ pan B cells was increased by ECK compare with untreated CFSE-labeled cells. Especially, the number of CFSE-labeled $CD45R/B220^+$ pan B cells was larger about 20% than that of untreated cells. These results indicated that ECK not only increases the population of specific cell phenotypes in general but also induces succeeding lymphocyte generation.

3. 5. ECK regulates the expression level of mRNA between Th1- and Th2-type cytokines

The specific cytokines produced by polarized Th1 (helper) and Th2 cells are the primary effectors that promote the differentiation of precursor Th cells, but these cytokines also cross-regulate the other subset's functional activity. Presumably, if ECK contributes to the proliferation and changes the phenotype of immune cells, ECK can then alter and even enhance their immunological functions. Therefore, we determined what effect ECK has on cytokine mRNA in these numerically upregulated splenocytes. As shown in Fig. 1-15, in fact, the mRNA expression levels of the IL-1β, IFN-γ and TNF-α, all Th1-type cytokines, as well as IL-4 and IL-10, the Th2-type cytokines, were changed by ECK compared to that expressed by the untreated cells. Furthermore, although IL-1β mRNA expression was not significantly induced in ECK-treated cells, IFN-γ and TNF-α mRNA expression was significantly decreased by ECK (p <0.01). On the other hand, the levels of IL-4 and IL-10 mRNA expression increased significantly after ECK exposure compared to that of untreated cells (p <0.01). The expression levels of GAPDH mRNA used as an internal control on ECK-treated lymphocytes were identical with those of untreated cells. Then, the expression levels of various target cytokines/GAPDH mRNA were calculated to normalize the level of target cytokines to GAPDH mRNA (Fig. 1-15B). Together, these results suggest that ECK regulates the release of Th2- and also Th1-type cytokines, showing immunomodulatory capacity of ECK.

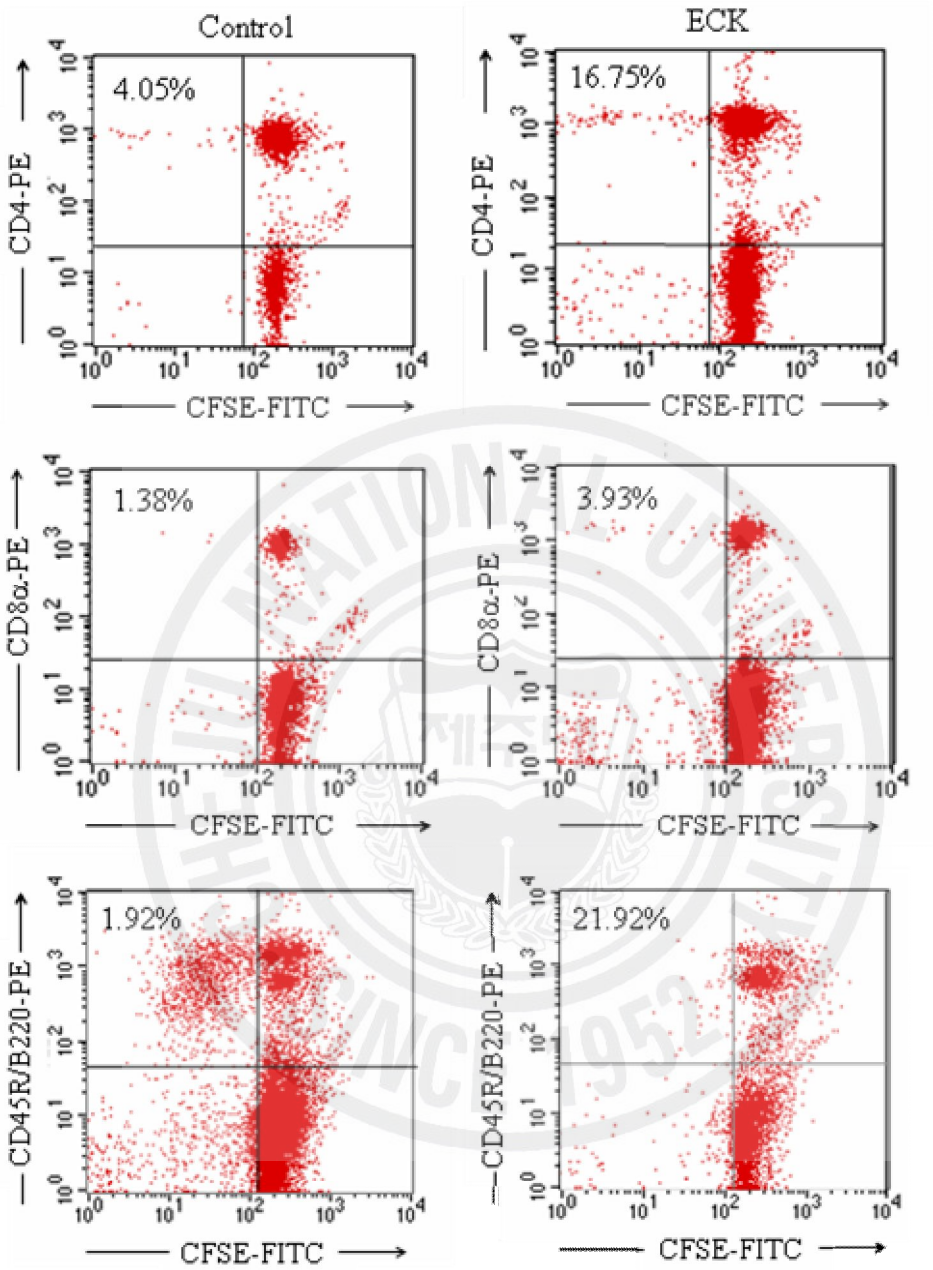

Fig. 1-14. Proliferation analysis in CFSE-labeled cells after ECK treatment. After incubation for 5 days, the expression level was measured by FACS analysis using anti-CD4, CD8 and CD45R/B220 mAbs. The CFSE-labeled cells were cultured in the absence or presence of ECK (100 ug/ml) for 5 days. Then, the cells were blocked by anti-mouse IgG and stained by the mAbs as indicated. Experiments were performed in triplicates.

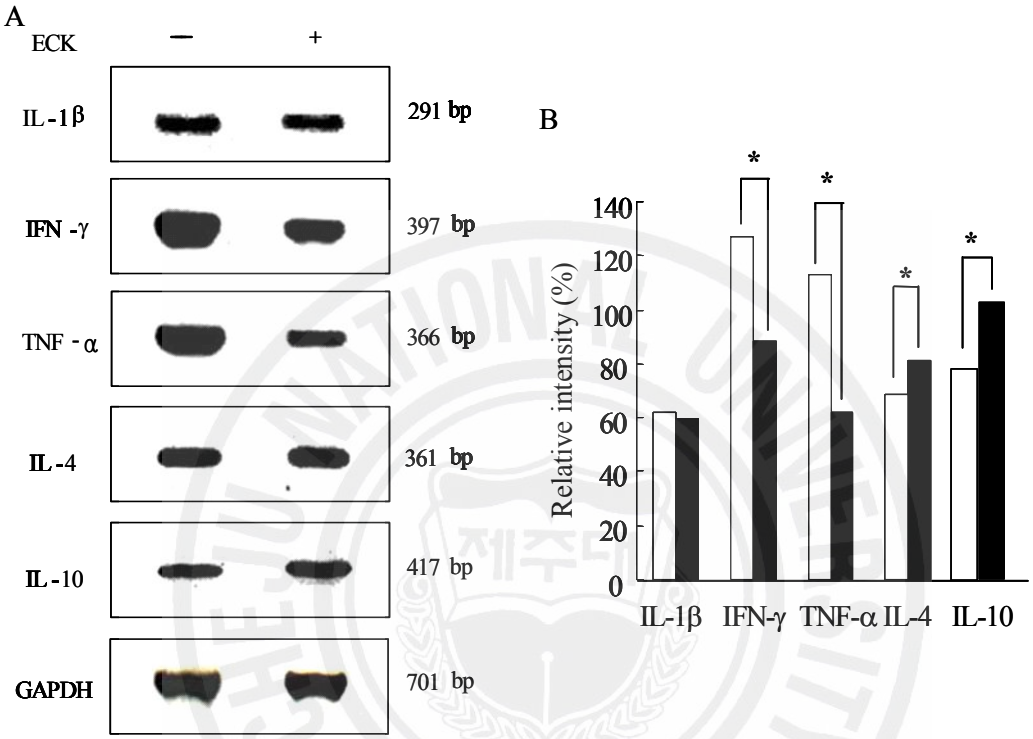

Fig. 1-15. Effect of ECK on the mRNA expression of cytokines by proliferating splenocytes (A); relative intensity of GAPDH expression indicating cytokine levels (B). After incubation for 48 h, RNA from the cultured cells treated or not with ECK (100 μg/ml) was used for cDNA synthesis. The cDNA (1μg) was amplified by PCR using gene-specific primers. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The expression levels of various target cytokines/GAPDH mRNA were calculated to normalize the level of target cytokines to GAPDH mRNA. Similar results were obtained in three separate experiments.
*, p <0.01.

3. 6. ECK regulates the production of Th1- and Th2-type cytokines

To assess whether ECK affected production of the cytokines IFN-γ, TNF-α, IL-4, and IL-10 in Con A-stimulated blastogenic cells, we quantified these cytokines by using ELISA. Because supernatants from the splenocytes tested produced very low levels of cytokines without specific mitogen stimulation, Con A was used to promote T lymphocyte proliferation. As Figure 1-16 depicts, IFN-γ and TNF-α production was markedly increased by Con A, whereas ECK-treatment significantly reduced the production of these two cytokines in blastogenic cells (p <0.01). In particular, IFN-γ production decreased two fold in ECK-treated blastogenic cells. In contrast, the levels of IL-4 and IL-10 produced in ECK-treated blastogenic cells were significantly higher than that of untreated cells (p <0.01 and p <0.05, respectively). However, the levels of IL-4 and IL-10 production increased only slightly in ECK-treated cells above that by untreated cells and not to a significant extent (data not shown). Collectively, ECK up-regulated the production of Th2-type cytokines yet down-regulated the production of Th1-type cytokines in Con A-stimulated blastogenic cells. This immunomodulatory effect of ECK in Con A-stimulated blastogenic cells may be associated with reduced production of IFN-γ and TNF-α and enhanced production of IL-4 and IL-10.

4. DISCUSSION

In the present study, we demonstrated that ECK, a protease extract from brown seaweed, dramatically strengthened the survival and proliferation of splenocytes. In addition, functional subsets of both T and B cells were markedly increased by ECK treatment. This outcome supports the stimulatory effect of ECK on lymphocytes, particularly on the subsets of T and B cells involved in antibody synthesis (Benacerraf et al., 1978; Dean et al., 1979; Luster et al., 1982), e.g., the number of $CD4^+$ (helper) T cells was increased by ECK. Although the exact mechanism by which ECK induces the proliferation of splenocytes is not known, it may involve the nuclear factor kappa B

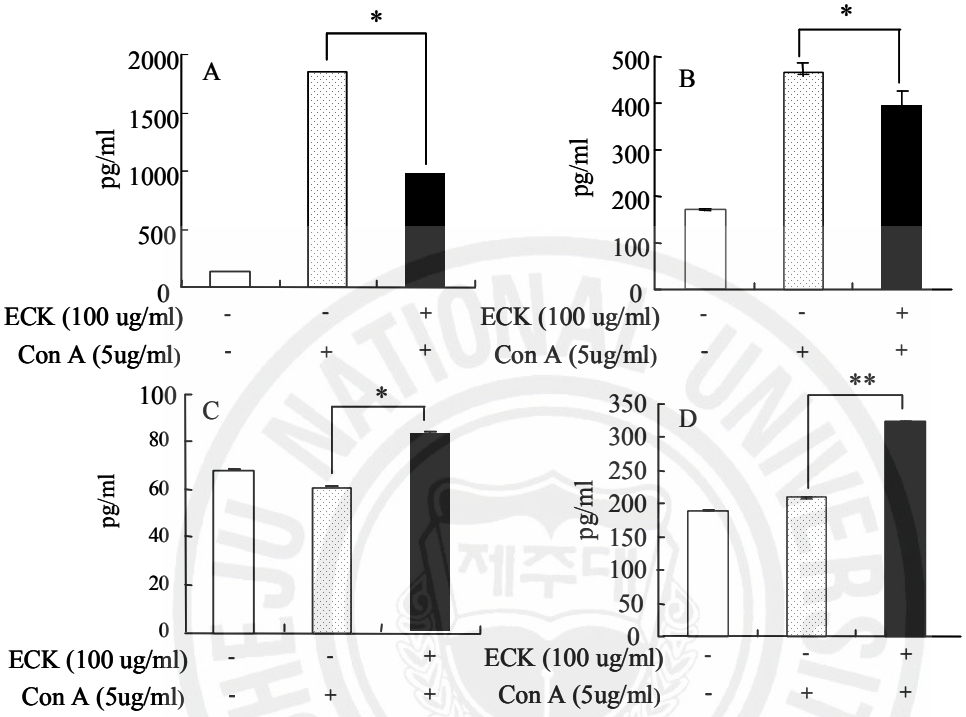

Fig. 1-16. Effect of ECK on Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokine production by Con A-stimulated blastogenic cells. (A) IFN-γ (B) TNF-α (C) IL-4 (D) IL-10. Splenocytes were stimulated with Con-A (1μg/ml), and these stimulated blastogenic cells were treated or not with ECK (100 μg/ml) for 72 h. The culture supernatants were collected, and concentrations of IL-1β, TNF-α, IL-4, and IL-10 were measured using commercially available ELISA kits. Spontaneous cytokine release: IFN-γ, 66 ± 21 pg/ml; TNF-α, 38± 21 pg/ml: IL-4, undetectable; IL-10, undetectable. Data are means ± S.D. of three experiments. Statistical evaluation was performed to compare the Con A-treated cells and ECK-treated blastogenic cells with corresponding untreated cells, respectively. *, $p < 0.01$, **, $p < 0.05$.

(NFκB) signaling pathway. Indeed, the component of ECK that increased NFκB could be eckol, since a transcription molecular mechanistic study revealed that eckol increased phosphorylation of extracellular signal-regulated kinase and the activity of NFκB (Kang et al, 2006b).

NFκB plays a critical role in the transcription of several genes involved in immune and inflammatory responses, cell proliferation/differentiation and cell transformation. The possible involvement of the NFκB signal pathway and/or other pathways related to this molecule offers opportunities for future exploration. Also, the mechanism of ECK's influence on signals related to modulation of immune responses needs clarification. Although the literature contains a multitude of research reports on lymphocyte subpopulations and cytokines, the basic mechanisms underlying their interactions during immune reactions remain obscure (Dhuley et al., 1997; Davis et al., 1999).

Still, distinctive cytokines released by Th1- and Th2- type cells clearly regulate specific immune responses. For example, Th1 cells have inflammatory functions and the ability to activate cytotoxicity by producing such pro-inflammatory cytokines as, IL-2, IFN-γ and TNF-α. Th2 cells can function to encourage antibody production, particularly IgE, and eosinophil proliferation/function and also produce such anti-inflammatory cytokines as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann et al., 1989 and 1996). The distinctive and opposing effector functions of these cellular subsets are well known as the fact that extremes of either type can lead to disease. $CD4^+$ T cells can be divided into Th1 and Th2 effector subsets and are defined as based on the cytokines they secrete (Mosmann et al., 1986). Moreover, any factor that affects the balance of either Th1/Th2 cell cytokines or antibody isotypes may play arole in the development or resolution of autoimmune disease (Cole et al., 1998; Schifitto et al., 2000). The outcome of vaccine administration to humans is similarly affected (Maisel, 1994). The environmental milieu regulates the ensuing $CD4^+$ T lymphocyte differentiation into a Th1 or a Th2 phenotype (Rao et al., 2000). Further, Th1 and Th2 cells have been found to reciprocally inhibit each other's differentiation pathways. IFN-γ can inhibit Th2 development (Gajewski et al., 1988), and

IL-4 has been found to inhibit Th1 development (Hsieh et al., 1992). Now, our results have shown that, after exposure to ECK, mRNA expression levels of the Th1-type cytokines IL-1β, IFN-γ and TNF-α decreased markedly, whereas mRNA levels of the Th2-type cytokines IL-4 and IL-10 significantly increased as compared to those of untreated controls. ECK also increased the production of IL-4 and IL-10 but suppressed the production of IFN-γ and TNF-α in Con A-stimulated blastogenic cells. These results suggest that the activation of $CD4^+$ T cells by ECK specifically regulates the expression of Th1- and Th2-type cytokine mRNAs, thereby inhibiting the development of Th1 cells via IL-10 and stimulating the differentiation of Th2 cells via IL-4. The apparent ability of ECK to modulate these two cytokines, as shown here, would be an important factor in regulating immune responses. The ECK extract used in this study has numerous advantages such as its properties as a natural product, water solubility, great variation of constituents, multiple biological activities, high extraction efficiency, low cost and minimal environmental pollution and toxicity compared to solvent extracts (Heo et al., 2003). We previously reported that many components from seaweeds isolated with the enzymatic extraction technique used here act as antioxidants (Ahn et al., 2004; Heo et al., 2003 and 2005; Park et al., 2005), anticoagulants, (Athukorala et al., 2006a) and anticancer agents (Athukorala et al., 2006b). To the contrary, solvent extracts sometimes suffer from extremely low recovery rates, strict regulations for use in the food industry and limited yield in recovery of water-soluble components in water extractions. Therefore, ECK is a superior product for nutritional and therapeutic applications.

In the latter regard, researchers have indicated that optimal immunotherapy should restore or maintain a well-balanced Th1 and Th2 response (Mosmann et al., 1989; Patwardhan et al., 2005). Our results demonstrated that ECK has ideal immunostimulatory characteristics, in that it activates T cells and B cells, and also modulatory characteristics evident here in production of Th1- as well as Th2-type cytokines. ECK also seems to be a suitable agent for selectively modulating either Th1 or Th2 responses and, thereby, may provide means of achieving T cell homeostasis.

Therefore, ECK has the advantage of being a natural product that may be a useful drug for the therapy of immune-related diseases.

Part 2.

The classical pathway of NFκB activation induced by ECK; production T and B cell proliferation

Part 2

The classical pathway of NFκB activation induced by ECK; production T and B cell proliferation

1. ABSTRACT

Nuclear factor-κB (NFκB) is the generic name of a family of transcription factors that function as dimers and regulate genes involved in the immune and inflammatory response. Activated NFκB normally lead to activation, differentiation and proliferation of lymphocytes and secretion of cytokines. In this study, we investigated whether ECK extracted by kojizyme from *Ecklonia cava* is related to NFκB pathway in activated lymphocytes. To identify the activation of NFκB by ECK, we performed western blot. The results showed that ECK induced the translocation of NFκB into nuclear in both short term and long term of the classical pathway in proliferated cells. In addition, the expression of nuclear NFκB p65 protein and phospho-IκBα protein was significantly increased from 0.25 h to 0.5 h and markedly with progressing of incubation times from 3 h to 48 h in ECK-treated cells. In contrast, the degradation of IκBα protein was induced by ECK. In addition, we performed EMSA to investigate nuclear NFκB DNA binding activity by ECK and identified NFκB DNA binding activities were increased by ECK at 0.25 h and 0.5 h compare to control. These results suggested that ECK activates proliferation, differentiation of lymphocyte and secretion of cytokines by NFκB pathway due to enhance the expression level and NFκB DNA binding activity in classical pathway.

2. MATERIALS AND METHODS

2. 1. Mice

All experiments were performed with ICR mice, aged 8 to 9 weeks, purchased from Japan SLC, Inc.. The animals were housed, five to a cage, in conventional animal facilities with NIH-07 diet and water *ad libitum* under constant temperature (23±1°C) according to the internationally accepted guideline.

2. 2. Preparation of enzymatic extract from *Ecklonia cava* (ECK)

E. cava was collected from the coastal waters of Jeju Island, South Korea, washed with fresh water and dried. The enzymatic extract was prepared in accordance with the method developed by Heo *et al* (Heo et al., 2003). Briefly, the *E. cava* samples were freeze-dried then pulverized into powder with a grinder. One gram of dried *E. cava* was homogenized with 100 ml of buffer (pH 6.0) and mixed with 100 μl of Kojizyme (Navo Co., Novozyme Nordisk, Bagsvaed, Denmark). The sample was adjusted to within the optimal range of the kojizyme, and the enzymatic reaction was conducted at 40℃ for 12 h. Following digestion, the digest was boiled for 10 min at 100℃ to inactivate the enzymes. Finally, the sample was clarified via 20 min of centrifugation at 3000 × g to remove any unhydrolyzed residue. The resulting enzymatic extract (ECK) obtained after filtration of the supernatant was adjusted to pH 7.0 hereafter and then stored for use in experiments.

2. 3. T cell culture medium (TCM)

Cells were suspended in DMEM supplemented with 1% (v/v) minimum essential medium (Gibco BRL, Life Technologies, Paisley, UK), 2 mM glutamine (Flow Laboratories, Irvine, UK), 50 IU/ml penicillin and 50 mg/ml streptomycin and 10% (v/v) FCS.

2. 4. Preparation of spleen cells

Spleens were removed aseptically from ICR mice, and single splenocytes were suspended in TCM containing 10% fetal bovine serum, 1% antibiotic (100 U/ml penicillin and 100 mg/ml streptomycin) then separated. Red blood cells were lysed with ACK lysis buffer (BD Biosciences Pharmingen, San Jose, CA, USA) at room temperature for 10 min. After washing with DPBS, the purified cells were obtained and used directly for experiments in which they were treated with EKC or a vehicle, hereafter called "untreated cells."

2. 5. MTT assay

To identify the cell viability of ECK on splenocytes, we performed MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. In addition, it is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann er al., 1983). The 2×10^4 cells were incubated in 96-well plates with ECK at various concentrations such as from 3 ug/ml to 100 ug/ml. After 48 h, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium-bromide) (Sigma, Saint Louis, USA) stock solution (50 μl; 2 mg/ml) was then applied to each of the wells and incubated at 37℃ for 4 h. The plates were centrifuged for 10 min at 800 × *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2. 6. ^{3}H-thymidine incorporation assay

The proliferative ability of T cells was tested by the standard assay based on the principle that the thymidine base of DNA sequences in these cells is replaced with radioactive ^{3}H-thymidine (Amersham, Arlington Heights, IL). For this assay, the 4×10^{6} cells were treated with ECK in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). After incubation for 3 days at 37°C, 95% humidity, and 5% CO_2, 1 μCi of ^{3}H-thymidine (specific activity 42 Ci/mmol) was added to the cells, and the plates were incubated for additional 18 h. The cells were then harvested onto glass fiber filters by an automatic cell harvester. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

2. 7. Preparation of cytoplasmic and nuclear extract

Splenocytes were seeded in RPMI 1640 media containing 10% fetal bovine serum, 1% antibiotic and incubated with the 100 ug/ml of ECK or vehicle at 37°C for 0 h, 0.25 h, 0.5 h, 1 h, 3 h, 24 h and 48 h, respectively. At the time point, the cell harvested were washed with DPBS and stored at -20°C until use for experiment. The cells were lysed by TNN buffer consisting of 40 mM Tris-HCl (pH 7.4), 120 mM NaCl containing the protease inhibitors 1 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml aprotinin, and 2 mM Na_3VO_4 on ice. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was used as the cytoplasmic protein and the pellet was used for nuclear protein extract. The pellet were washed in buffer A consisting of 10 mM HEPES (pH 7.9), 10mM KCl, 1.5 mM $MgCl_2$ and 1.0% NP-40 containing the protease inhibitors 0.5 mM DTT and 0.1 mM PMSF on ice. After centrifugation at 12,000 rpm for 30 sec at 4°C, the pellet was lysed by buffer B consisting of 20 mM HEPES, 20% glycerol, 0.42 mM NaCl and 1 mM EDTA containing the protease inhibitors 0.5 mM DTT and 0.1 mM PMSF and homogenized for 30 min on ice. Finally, nuclear extracts were obtained by centrifugation at 12,000 rpm for 15 min. The protein extracts were used for the western blot analysis and EMSA. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

2. 8. Western blot analysis

To identify whether ECK is related with NFκB pathway in proliferated and activated lymphocytes, we performed western blot analysis. The cytoplasmic (60 ug/well) and nuclear protein (35 ug/well) were loaded in each lane and electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH). After blocking with 5% nonfat milk for 1 h, the blots were incubated with NFκB p65, phospho-IκBα and IκBα (1:500 dilution, Santa Cruz Biotechnology, CA) for 60 min followed by incubation with HRP-conjugated anti-mouse IgG or rabbit IgG (1:500, Santa Cruz Biotechnology, Inc) for 45 min, respectively. Visualization was achieved using ECL reagents (Amersham Life Science, Buckinghamshire, UK).

2. 9. Electrophoretic mobility shift assay

To identify whether ECK has the NFκB DNA binding activity, we performed electrophoretic mobility shift assay (EMSA). DNA-oligonucleotide complex containing NFκB consensus sequence were annealed, labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and used as probes. The probes (about 40,000 cpm) were incubated with 10 μg of the nuclear extracts at 4°C for 30 min in a final volume of 20 μl containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris–HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly (dI–dC). Binding products were resolved on 5% polyacrylamide gel and the bands were visualized by autoradiography (Kim et al., 1998).

2. 10. Statistical analysis

Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as means ± standard error (SE). A *p*-value of less than 0.05 was considered significant.

3. RESULTS

3. 1. ECK has the cell viability and stimulates the proliferation of splenocytes

The viability of splenocytes incubated for 48 h with ECK was quantified by assessing the MTT assay. As shown in Figure 2-1, we identified that ECK has the cell viability without cytotoxicity at all concentrations.

Next, to confirm that ECK similarly benefits splenocytes' proliferative ability, we examined such cells in a ^{3}H-thymidine incorporation assay. Accordingly, the proliferation of these ECK-treated cells was significantly higher than that of untreated cells and increased with rising concentrations of ECK from 50 μg/ml to 500 μg/ml (p <0.01) (Fig. 2-2). Presumably, then, treatment with ECK may lead to an enhancement of mitogenic or antigenic stimulation.

3. 2. ECK induces the NFκB activation and IκBα degradation in both short term and long term

To confirm whether the differentiation, proliferation and activation of lymphocytes induced by ECK are related to NFκB signal pathway, we performed western blot analysis. When IκB-NFκB complex is degraded by IκB kinase, NFκB isolated is translocated into nucleus and lead to translocation of cytokine gene such as IL-2. As shown in Fig. 2-3 and Fig. 2-4, ECK induced the translocation of NFκB into nuclear at both short term and long term of the classical pathway. In addition, the expression of nuclear NFκB p65 protein and phospho-IκBα protein was significantly increased from 0.25 h to 0.5 h and markedly with progressing of incubation times from 3 h to 48 h in ECK-treated cells. In contrast, the degradation of IκBα protein was induced by ECK. Although the degradation of IκBα protein was induced at 0.25 h and gradually decreased by ECK, the degradation was significantly increased as progressing incubation. These results suggested that ECK stimulate the lymphocytes and induce differentiation and activation of lymphocytes by translocation of nuclear NFκB at both short term and long term in classical pathway.

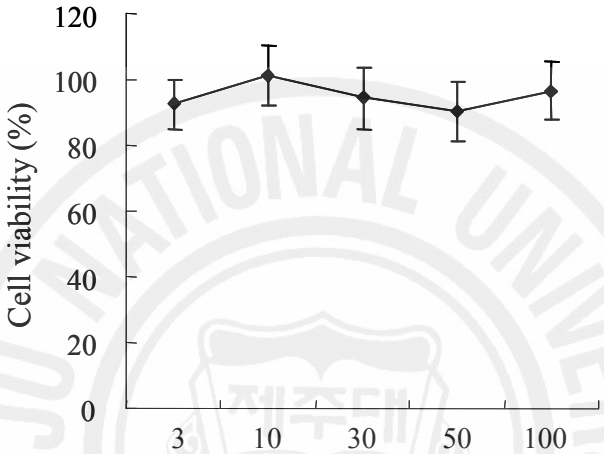

Fig. 2-1. The effect of ECK on the viability of splenocytes. Spleen cells were cultured in the absence or presence of ECK at the concentrations indicated (3 - 100 μg/ml) for 48 h. Their viability was measured by MTT assay. Experiments were performed in triplicates, and data are expressed as average percent change from control ± S.D.

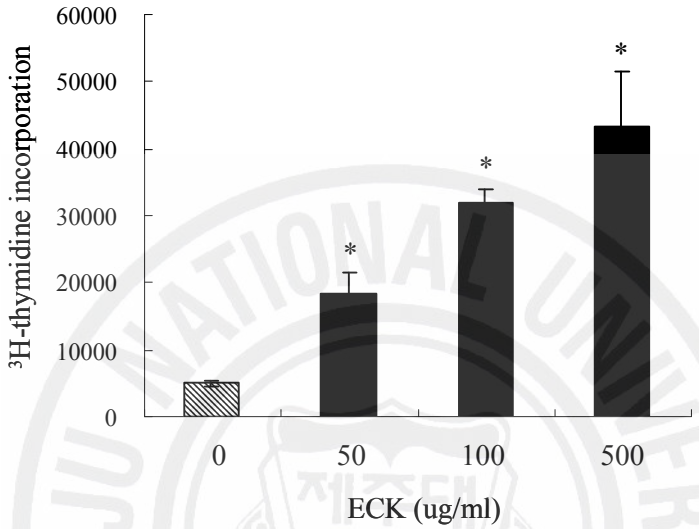

Fig. 2-2. The effect of ECK on the proliferation of splenocytes. 4 X 10^5 viable cells from each culture were transferred to wells of a 96-well microtiter tissue culture plate in triplicate at the concentrations indicated (50 - 500 μg/ml). After incubation with ECK for 72 h, proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.D. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$.

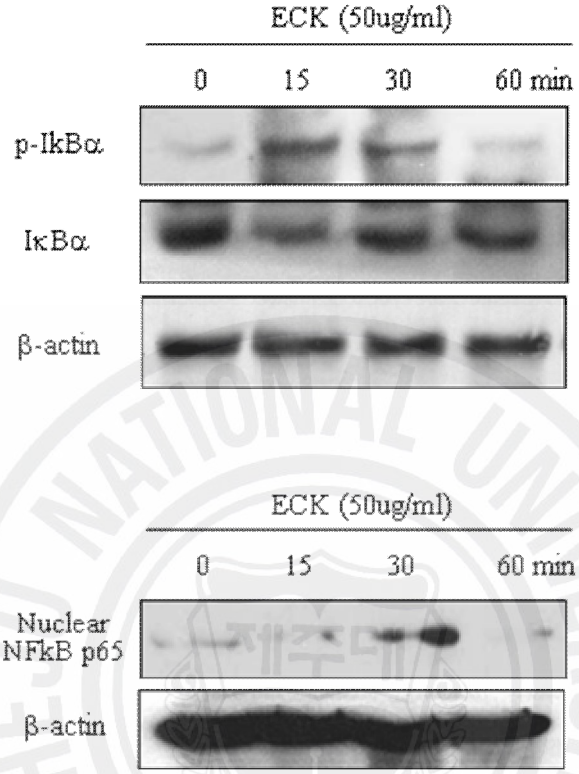

Fig. 2-3. Expressions of phosphor-IκBα, IκBα, NFκB p65 and β-actin of ECK in short term. The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with 50 ug/ml of ECK for 0 min, 15 min, 30 min and 60 min. The 60 ug/ml of cytoplasmic and 40 ug/ml of nuclear proteins were applied into gel, respectively. The blot was blocked in 5% non-fat milk. All antibodies were diluted into 1: 500 with 2% milk in TBST. Statistical evaluation was performed to compare ECK-treated cells and vehicle-treated cells, respectively.

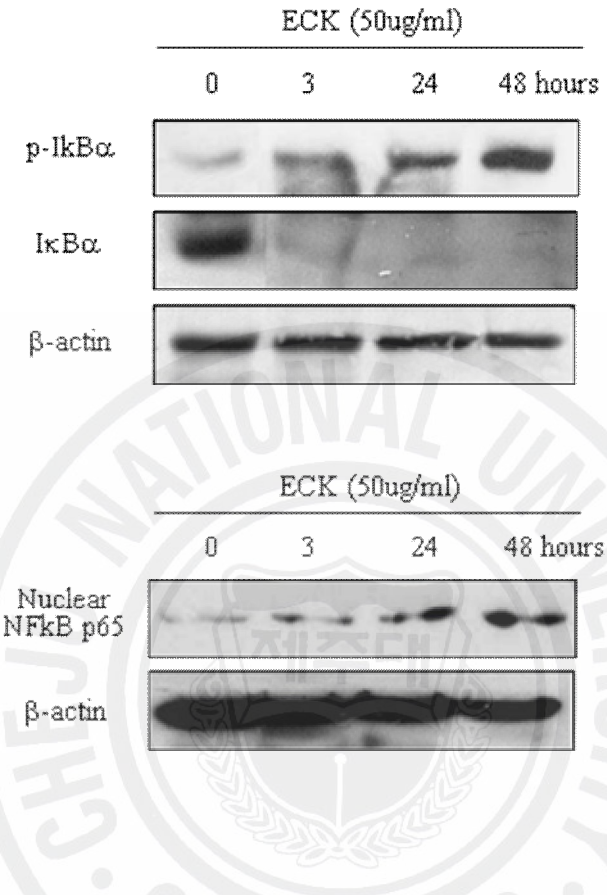

Fig. 2-4. Expression of phosphor-IκBα, IκBα, NFκB p65 and β-actin of ECK in lomg term. The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with 50 ug/ml of ECK for 0 h, 3 h, 24 h and 48 h. The 60 ug/ml of cytoplasmic and 40 ug/ml of nuclear proteins were applied into gel, respectively. The blot was blocked in 5% non-fat milk. All antibodies were diluted into 1: 500 with 2% milk in TBST. Statistical evaluation was performed to compare ECK-treated cells and vehicle-treated cells, respectively.

3. 3. ECK induces NFκB DNA binding activity

If ECK induces NFκB DNA binding activity in splenocytes, ECK might be a inducer for activation of NFκB pathway and proliferation of lymphocytes. Therefore, to identify whether the NFκB DNA binding activity can be changed by ECK, we performed EMSA (electrophoretic mobility shift assay). We identified that the expression of NFκB DNA complex was slightly increased at 0.15 h and 0.5 h and decreased at 48 h of incubation time by ECK compare to that of untreated lymphocytes (Fig. 2-5). These results suggest that ECK leads to differentiation and proliferation of lymphocytes by inducing the NFκB DNA complex and increasing NFκB DNA binding activity.

4. DISCUSSION

Many researchers have reported that T cells have multiple signaling pathways that affect cell proliferation, survival and apoptosis to maintain homeostasis in regulating the immune response (Ashkenazi et al., 1999; Chan et al., 2000). T cells require appropriate signals through the T cell receptor with appropriate co-receptors to become activated and to proliferate (Tomlinson et al., 1999). NFκB pathway is a important mediator in the control of the immune response such as apoptosis, B-lymphocyte survival, mitogen-dependent cell proliferation, and isotype switching, which lead to the differentiation of B lymphocytes into plasma cells (Yamamoto et al., 2001; Barkett et al., 1999). Additionally, the activation of NFκB leads to the induction of multiple genes that regulate the immune and the inflammatory response such as IL-2 production, which increases the proliferation and differentiation of T lymphocytes (Yamamoto et al., 2001; Gerondakis et al., 1998; Weil et al., 2004).

In the present study, we demonstrated that ECK, a protease extract from *Ecklonia cava*, dramatically strengthened the survival and proliferation of splenocytes. Although the exact mechanism by which ECK induces the proliferation of splenocytes is not known, it may involve the nuclear factor kappa B (NFκB) signaling pathway. So, we confirmed by

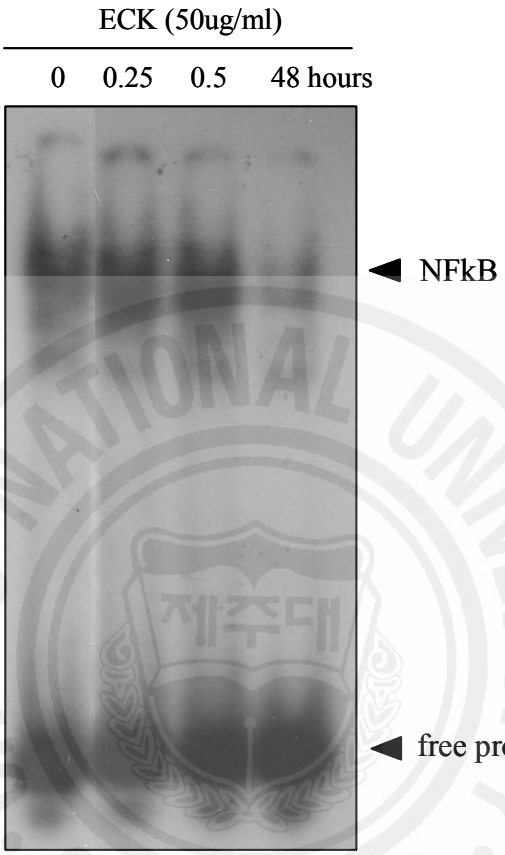

Fig. 2-5. Effect of ECK on NFκB DNA binding activity by EMSA. Nuclear protein was obtained from splenocytes incubated with ECK for 0.25 h. 0.5 h and 48 h. NFκB specific oligonucleotide–protein complexes were detected by electrophoresis mobility shift assays. The values represent the normalized intensities of the NFκB band against intensities of control band measured by bio-image analyzer system.

western blot whether the proliferation of lymphocytes induced by ECK without cytotoxicity is related with nuclear NFκB p65 signaling pathway. Our results showed that ECK stimulates lymphocytes and induces differentiation and activation of lymphocytes by translocation of nuclear NFκB p65 compared to non-treated lymphocytes at both short term and long term of classical pathway. In addition, we identified that ECK increase NFκB DNA binding activity in activated lymphocytes by EMSA. The previous study has reported that the stimulation of cells with agents that can lead to the appearance of NFκB in the nucleus and the preferential loss of p65 involved in classical NFkB binding form is significant since the p65 subunit of NFκB is necessary for transcriptional activation of antiapoptotic genes and important for inducing factors that inhibits Fas-madiated death (Hayden et al., 2004; Aggarwal et al., 2004; Silverman et al., 2001; Beg et al., 2000). In addition, a number of recent studies lend support to the proposal that p65 phosphorylation may be necessary for transcriptional competence of nuclear NFκB (Bird et al., 1997; Madrid et al., 2000 and 2001, Wang et al., 2000). Also, NFκB p65 are posttranslationally regulated came from the work of Scheidereit and colleagues who demonstrated inducible phosphorylation of p65 following cellular stimulation and DNA binding activity was inhibited by activation of IkBα (Naumann et al., 1994 and 1995). This finding indicates that ECK increase nucleus NFκB activation by inducing the degradation of IkBα as cellular stimulator and might play as a inhibitor of Fas-madiated death or apoptosis in activated lymphocytes.

Additionally, the previous study indicated that nuclear NFκB causes the up-regulation of transcription of the IκBα gene, and translation of the newly transcribed IκBα mRNA results in the rapid replenishment of the depleted pool of IκBα protein (Brown et al., 1993; Cordle et al., 1993; Henkel et al., 1993; Rice et al., 1993; Sun et al., 1993 and 1994). Also, Suyang et al. reported that the newly synthesized, unphosphorylated IκBβ plays an active, chaperone-like role in transporting a portion of newly made NFκB to the nucleus (Suyang et al., 1996). This suggests that the activation of nucleus NFκB p65 induced by ECK might be related to IκBα rapid replenishment in short term and induce

the persistent activation of nucleus NFκB p65 to due to sustained lowering IκBβ levels in long term.

Thus, the previous study has reported that eckol increased phosphorylation of extracellular signal-regulated kinase and the activity of NFκB in the previous study (Kang et al, 2006b). Indeed, our results suggested that the component of ECK used in this study activated nucleus NFκB p65 might to relate with eckol known as a kind of phlorotannins, since a transcription molecular mechanistic study revealed.

According to these results, this study suggests that ECK can be used as a stimulator for activation of immune related cells due to activation of NFκB p65 pathway such as increasing the accumulation of NFκB p65 in the nucleus and NFκB DNA binding activity. Further, we need to investigate whether NFκB activation induced by ECK in long term is related to the expression level of IκBβ in activated lymphocytes.

Part 3.

The inhibition effects of ECK in TPA-induced mouse ear edema *in vivo*

Part 3

The inhibition effects of ECK in TPA-induced mouse ear edema *in vivo*

1. ABSTRACT

Ecklonia cava used in this study is a kind of brown seaweeds. In this study, we investigated whether kojizyme extract from *E. cava* (ECK) has the anti-inflammatory effects *in vivo*. The application of 12-*O*-tetradecanoylphorbol acetate (TPA) known as a topical activator induces a long-lasting inflammatory response and offers a skin inflammation model appropriate for evaluating anti-inflammatory agents. We used TPA-induced mouse ear edema model known as anti-inflammatory model to investigate the anti-inflammatory effects of ECK *in vivo*. Also, to identify whether ECK reduce the histological inflammation responses, we performed haematoxylin and eosin (H&E) staining procedure with ear samples. We identified that ECK reduce histological skin inflammation responses such as decreasing the number of the inflammation cells and inhibiting ear edema similar to dexamethasone used as positive control. Interestingly, the histology evaluation showed the correlation with the inhibition activity of ECK and dexamethasone (about 42% and 48 %) on TPA-induced inflammation. In addition, to identify mRNA expression of the cytokines and inducible molecules from TPA-induced ear edema model, we performed RT-PCR and identified that although the mRNA expression level of IL-1β was not significance, the mRNA expression level of IFN-γ and TNF-α, a kind of Th1 type cytokines, and COX2, a kind of inducible molecules, was decreased by ECK as compared to the only TPA-treated ear. These results suggested that ECK has anti-inflammation effects *in vivo* due to inhibit histological skin inflammation responses and the expression level of Th1 type cytokine and inducible molecules and its usefulness as therapy for diseases of the immune system.

2. MATERIALS AND METHODS

2. 1. Mice

All experiments were performed with ICR mice, aged 8 to 9 weeks, purchased from Japan SLC, Inc.. The animals were housed, five to a cage, in conventional animal facilities with NIH-07 diet and water *ad libitum* under constant temperature (23±1°C) according to the internationally accepted guideline.

2. 2. Preparation of enzymatic extract from *Ecklonia cava* (ECK)

E. cava was collected from the coastal waters of Jeju Island, South Korea, washed with fresh water and dried. The enzymatic extract was prepared in accordance with the method developed by Heo *et al* (Heo et al., 2003). Briefly, the *E. cava* samples were freeze-dried then pulverized into powder with a grinder. One gram of dried *E. cava* was homogenized with 100 ml of buffer (pH 6.0) and mixed with 100 μl of kojizyme (Navo Co., Novozyme Nordisk, Bagsvaed, Denmark). The pH of sample was adjusted to within the optimal range of the kojizyme, and the enzymatic reaction was conducted at 40℃ for 12 h. Following digestion, the digest was boiled for 10 min at 100℃ to inactivate the enzymes. Finally, the sample was clarified via 20 min of centrifugation at 3000 × g to remove any unhydrolyzed residue. The resulting enzymatic extract (ECK) obtained after filtration of the supernatant was adjusted to pH 7.0 hereafter and then stored for use in experiments.

2. 3. Mouse ear edema induced by TPA

To evaluate whether ECK has the topical anti-inflammatory activity, we performed mouse ear edema model induced by TPA (Sigma Aldrich Inc., Saint Louis, USA). We applied ECK (100 ug) dissolved in ethanol (20 ul) into the surface of mouse ear and used dexamethasone (50 ug) that is a topical inhibitor of inflammatory activity as positive control (Sigma Aldrich Inc., Saint Louis, USA). After 1 h, we measured the thickness of mouse ear treated ECK or dexamethasone (a). Then, TPA (2.5 ug) in ethanol (20 ul) was

applied into the same surface of ECK- and dexamethasone-treated mouse ear. After 6 h, we measured the thickness of TPA-treated ear (b) and TPA plus ECK- or dexamethasone-treated ear of mice group (b′), respectively. The inhibition activities on ear edema induced by TPA were calculated:

Edema A is induced by TPA alone (b-a).

Edema B is induced by TPA plus sample (b′-a).

Inhibitory activity (%) = [(Edema A-Edema B)/Edema A] × 100.

Each value was the mean of individual determinations from three mice and statistical analysis was carried out Student's t-test.

2. 4. Histological analysis

We performed histological analysis with the ear samples obtained from TPA-induced mouse ear model. The samples fixed in 10% formalin were re-fixed in 10% neutral formalin for 1 day. After washing with tap water for 5 h, the samples were applied into tissue processing machine. After 18 h, the ear samples were embedded in paraffin and sectioned on 3-aminopropyl triethoxy silane-coated slides. And then, the slides were dried at about 40℃ for 12 h and stored at room temperature until use for haematoxylin and eosin (H&E) stain.

2. 5. Haematoxylin and Eosin (H&E) staining procedure

To evaluate whether ECK has the histological anti-inflammatory effects in TPA-induced mouse ear edema, we performed haematoxylin and eosin (H&E) staining procedure. The paraffin on slides were removed and dehydrated in alcohol. Dehydrated slides were replaced by tap water and applied into haematoxylin stain to stain nuclear for 5 min. Then, the slides were applied into eosin stain for cytoplasm and dehydrated with alcohol. A representative area was selected for qualitative light microscope analysis.

2. 6. RNA preparation

The ear samples were treated with trizol reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) and vortexed vigorously for 30 min on ice. The addition of chloroform (Sigma, Saint Louis, MS, USA) and incubation for 5 min at 4°C followed. The supernatants obtained after centrifugation were treated with isopropanol (Sigma), and the resulting RNA pellets were washed and stored at -20°C until use.

2. 7. RT–PCR

The cDNA was synthesized with RNA purified from spleen cells by using a Promega A3500 kit, according to manufacturer's instructions (Promega, San Luis, CA, USA). PCR of this cDNA and the primer (Bioneer, Daejeon, South Korea) displayed in Table 3-1 was performed for 40 to 45 cycles with a 5 min denaturing step at 94°C, a 1 min annealing step at 53 to 60°C and a 20 min extension phase at 72°C using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were run on a 1.5% EtBr/agarose gel and visualized by UV transillumination.

3. RESULTS

3. 1. ECK reduces the inflammation response in TPA-induced mouse ear edema model

We accessed to identify topical anti-inflammatory activity of ECK in vivo in the mouse ear model, inducing edema with 12-*O*-tetradecanoylphorbol acetate (TPA, 2.5 μg/ear). As shown in Fig. 3-1, ECK showed anti-inflammatory effects such as decreasing the number of the inflammation cells and inhibiting ear edema induced by TPA in histological evaluation. Indeed, ECK showed the reduction of the ear thickness and significantly the inhibition activity on TPA-induced ear edema (42%) and similar to dexamethasone (48%) (Table. 3-2). This result suggested that ECK has anti-inflammatory activity due to the inhibition effects on TPA-induced inflammation response.

Table 3-1. The sequences and expected size of oligonucleotides for RT-PCR in TPA-induced mouse ear model.

Oligonucleotide		Sequence	Expected size (bp)
IL-1β	5'-primer	5'-GCT ACC TGT GTC TTT CCC GTC G-3'	291
	3'-primer	5'-TTG TCG TTG CTT GGT TCT CCT TG-3'	
IFN-γ	5'-primer	5'-AGG TCA ACA ACC CAC AGG TCC A-3'	397
	3'-primer	5'-CCA GAT ACA CCG CAA TCA C-3'	
TNF-α	5'-primer	5'-GGC AGC TTC TGT CCC TTT CAC TC-3'	366
	3'-primer	5'-CAC TTG GTG GTT TGC TAC GAC G-3'	
IL-4	5'-primer	5'-ACG GAG ATG GAT GTG CCA AAC GTC-3'	361
	3'-primer	5'-CGA GTC ATC CAT TTG CAT GAT GC	
COX-2	5'-primer	5'-GCA AAT CCT TGC TGT TCC AAT C-3'	335
	3'-primer	5'-GGA GAA GGC TTC CCA GCT TTT G-3'	
GADPH	5'-primer	5'-AAC GAC CCC TTC ATT GAC C-3'	701
	3'-primer	5'-TCA GAT GCC TGC TTC ACC-3'	

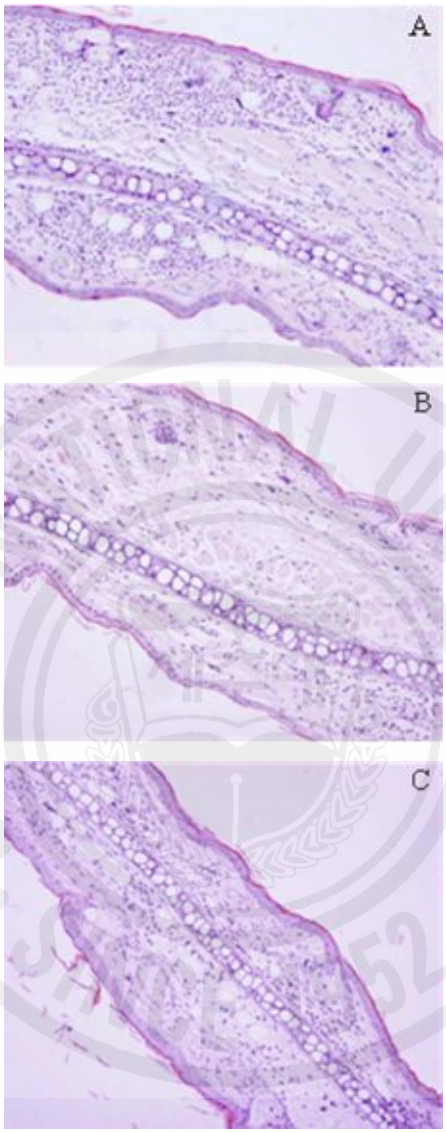

Fig. 3-1. Histological analysis of vertical sections in TPA-induced mouse ears model. Histology of vertical sections of ECK in TPA-induced mouse ears stained by haematoxylin and eosin were evaluated. A; Ears treated with TPA (2.5 ug/ear), B; Ears treated with TPA plus dexamethasone (50 ug/ear) and C; Ears treated with TPA plus ECK (100 ug/ear).

Table 3-2. The mouse ear thickness and inhibition activity of ECK in TPA-induced ear edema.

	TPA	TPA+Dexamethasone	TPA+ECK
Ear thickness (mm)	0.25±0.028	0.145±0.007	0.13±0.085
Inhibition activity (%)	-	42	48

3. 2. ECK reduces the mRNA expression level of Th1 cytokines and COX2 in TPA-induced mouse ear edema model

The cytokines produced from Th cells plays as important factors to promote the differentiation of Th cells that have Th (helper) 1 and Th2 cells. But these cytokines also cross-regulate the other subset's functional activity. If ECK induces to anti-inflammation activities, ECK is able to alternation for inhibiting the inflammation response. To determine this possibility, we examined whether ECK has the anti-inflammation effects on the mRNA expression of cytokine with RNA obtained from TPA-induced mouse ear edema model treated ECK or not. As shown in Fig. 3-2, although the mRNA expression level of IL-1β was not significance, the mRNA expression level of IFN-γ and TNF-α, a kind of Th1 type cytokines, and COX2, a kind of inducible molecules, was decreased by ECK as compared to that of the only TPA-treated ear. In addition, the mRNA expression level of IL-4, a kind of Th2 type was slightly increased by ECK. Taken together, these results suggested that ECK has anti-inflammation effects by decreasing the mRNA expression of Th1 type cytokine and inducible molecules.

4. DISCUSSION

The previous study has reported that topical application of phorbol ester (TPA) induce a long-lasting inflammatory response and offers a skin inflammation model appropriate for evaluating anti-inflammatory agents (Young et al., 2006). Thus, many researchers have reported that TPA-induced edema lead to oedema formation, the migration of polymorphonuclear leukocytes, and increase in tissue IL-1β and TNF-α levels (Michel et al., 2005; Masao et al., 2006) and skin leukocyte accumulation induced by TPA that the marked inhibition induced by ECK of ear oedema in response to topical application of TPA may be related to their ability to inhibit the release of the pro-inflammatory cytokine interleukin-1β, TNF-α and IFN-γ and inducible molecule such as COX2. In fact, a marked difference exists in the mechanisms of the inflammatory response induced by

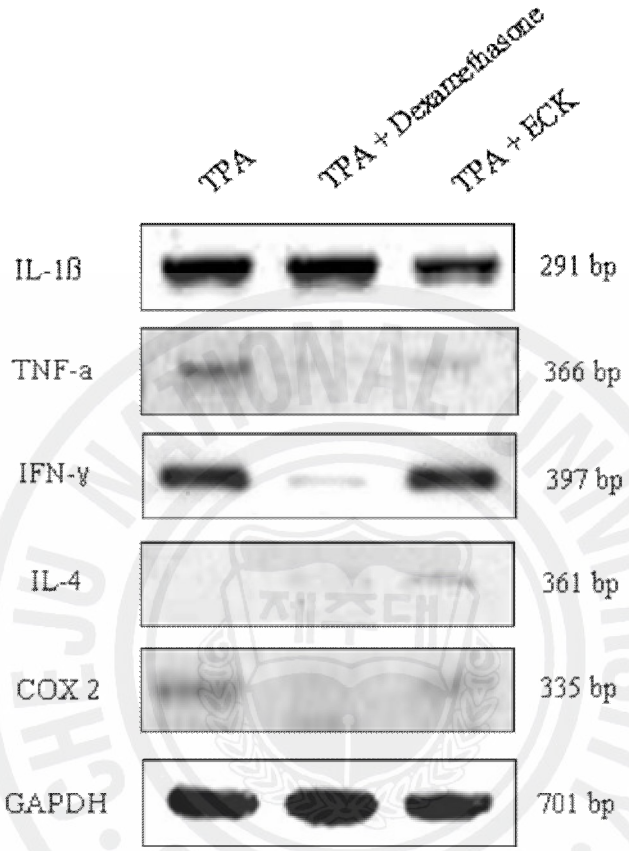

Fig. 3-2. Effect of ECK on the mRNA expression level of cytokines on TPA-induced ear edema model. After incubation for 48 h, RNA extracted from mouse ear with TPA plus ECK or TPA was used for cDNA synthesis. The cDNA (1 ug) was amplified by PCR using gene specific primers. PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three separate experiments.

topical application of TPA in the mouse. In the previous study, researchers have reported that cyclooxygenase inhibitors have highly effective against the inflammation caused by TPA (Kwan et al., 2005). This finding indicates that the ECK is of potential interest for the development of low-molecular-weight agent easily permeably through the skin and of interest in the treatment of skin inflammatory states. One or more targets could be involved including proteins that play a role in the genesis of the inflammatory response, namely cyclooxygenase-1 and cyclooxygenase-2, as well as an interaction with the nuclear transcription factor κB (Calixto et al., 2003 and 2004).

In conclusion, our data indicates that ECK has anti-inflammation effects due to the inhibition ability on TNF-α and IFN-γ which is a Th1 cytokines and COX2 which is inducible molecule in TPA-induced skin inflammation model. Additionally, this study suggests that ECK has the advantage of being a natural product that may be a useful drug for the therapy of inflammation-related diseases.

SUMMARY

Ecklonia cava used in this study is a kind of brown seaweeds plentifully found in Jeju Island of Korea. We investigated whether *E. cava* extract by kojizyme (ECK) has immunological effects on splenocytes *in vitro*. Here, ECK enhanced the proliferative ability of splenocytes in ^{3}H-thymidine incorporation assay. To identify in detail the specific phenotypes of these proliferating cells, we performed flow cytometry assays and CFSE assay. As a result of ECK treatment, the numbers of $CD4^+$ T cells, $CD8^+$ T cells and $CD45R/B220^+$ cells and the numbers of CFSE-labeled $CD4^+$ T cells, $CD8^+$ T cells and $CD45R/B220^+$ cells increased significantly compared to those in untreated control cultures. In addition, the mRNA expression level of the Th2-type cytokines IL-4 and IL-10, increased compared with that of untreated cells. On the other hand, ECK significantly decreased the level of TNF-α and IFN-γ, i.e., the Th1-type cytokines. In parallel with these results, the production level of Th1-type cytokine was down-regulated, whereas that of Th2-type cytokines was up-regulated by ECK. Overall, this dramatic increase in splenocyte numbers indicated that ECK can induce these cells to proliferate, and can regulate the production of Th1- as well as Th2-type cytokines in immune cells. These results suggest that ECK has the immunomodulatory ability to activate the anti-inflammatory response and/or suppress the pro-inflammatory response. Furthermore, we wanted to identify whether ECK is related to NFκB signal pathway in activated lymphocytes *in vitro* and has the anti-inflammatory effects *in vivo*.

So, first, we performed western blot and EMSA to identify the nuclear NFκB activation and nuclear NFκB DNA binding activity by ECK. Normally, nuclear factor-κB (NFκB) known as the generic name of a family of transcription factors that function as dimers and regulate genes involved in the immune and inflammatory response. Also, activated NFκB leads to activation, differentiation and proliferation of lymphocytes and secretion of cytokines. Our results showed that ECK induced the translocation of NFκB into nuclear and increased NFκB DNA binding activities in both short term and long term of the

classical pathway. These results suggested that ECK activates proliferation, differentiation of lymphocyte and secretion of cytokines by NFκB pathway due to enhance the expression level and NFκB DNA binding activity in classical pathway.

As the final study, we used TPA-induced mouse ear edema model known as anti-inflammatory model to investigate the anti-inflammatory effects of ECK *in vivo*. The application of 12-*O*-tetradecanoylphorbol acetate (TPA) known as a topical activator induces a long-lasting inflammatory response and offers a skin inflammation model appropriate for evaluating anti-inflammatory agents. We performed haematoxylin and eosin (H&E) staining procedure with ear samples to identify whether ECK reduce the histological inflammation responses. Our results showed that ECK reduce histological skin inflammation responses such as decreasing the number of the inflammation cells and inhibiting ear edema similar to dexamethasone used as positive control. In addition, we identified that the mRNA expression level of IFN-γ and TNF-α, a kind of Th1 type cytokines, and COX2, a kind of inducible molecules, was decreased by ECK as compared to the only TPA-treated ear in RT-PCR performed to identify mRNA expression. These results suggested that ECK has anti-inflammation effects *in vivo* due to inhibit histological skin inflammation responses and the expression level of Th1 type cytokine and inducible molecules.

According to these results, this study suggests that ECK has the immunomodulatory effects such as immune activation by increasing stimulation, differentiation and proliferation of lymphocytes due to the activation of NFκB translocation into nuclear and NFκB DNA binding and anti-inflammation effects by decreasing the mRNA and protein expression level of Th1 type cytokines *in vitro* and histological inflammation response *in vivo*. So, ECK can be used as a natural agent for therapy of diseases related to immune system.

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저에게는 2년이라는 짧지만 길었던 석사과정의 시간 동안 언제나 든든한 버팀목이 되어주신 분들이 계십니다. 저의 버팀목이 되어주신 분들의 많은 관심과 사랑에 힘입어 제가 지금까지의 석사과정을 무사히 마칠 수 있었고, 지금 이 자리에 설 수 있었다고 생각됩니다. 그 분들 중, 특히, 아직 확실하지 않은 미래에 대한 설계를 그릴 수 있도록, 제가 가는 길 위에서 어떻게 나아가야 바른 길이며 어떻게 그 길을 찾을 수 있는지.. 그 길을 저에게 가르쳐 주신 저의 꾸준한 스승님이신 전유진 교수님, 저를 안지 10개월이 채 되지 않으셨지만, 저에게 많은 가르침과 관심을 나눠주신, 많은 것을 가지고 계시기에 그것을 통해 제가 그렇게 되어야 한다는 생각을 갖게 해주신 저의 스승님 수의학과의 지영흔 교수님, 언제나 인자하시고 고요하게 그림자처럼 저의 뒤에서 많은 관심과 배움을 주심으로써 저의 버팀목이 되어 주신 이기완 교수님께 진심으로 감사하고 사랑한다는 말을 전합니다. 또한, 저의 학부시절부터 지금까지 저에게 많은 관심과 사랑으로 많은 배움을 주셨던 해양생물공학과의 송춘복 교수님, 허문수 교수님, 이제희 교수님, 여인규 교수님, 증식학과의 최광식 교수님, 이영돈 교수님, 이경준 교수님, 김기영 교수님, 식품공학과의 김수현 교수님, 하진환 교수님, 화학과의 이남호 교수님께도 정말 감사드립니다.

그리고, 제가 바쁜 2년 동안 많은 관심을 주지 못해 미안한 사랑하는 저의 실험실 사람들.. 언제나 한결 같은 용기를 준 수진오빠, 실험 가르쳐 주느라 고생했던 길남오빠, 따뜻한 말 한마디 아끼지 않던 영빈오빠, 언제나 뒤에서 소리 없이 응원해준 실험실장 승홍오빠, 항상 많은 도움을 준 야산타, 마힌다 지금까지 쭉 저의 동기가 된 선희, 그리고 착한 동기이자 친구로 응원을 아끼지 않던 성명이, 석천이, 사랑하는 후배인 대승이, 실험실 막내 이쁜 아름이, 지금은 없지만 저의 영원한 실험실 멤버인 인선이, 진희, 인식이, 지혁이, 은산이, 로한, 날린에게 고맙고 사랑한다는 말 전합니다. 그리고 항상 격려를 아끼지 않으시는 양현필 선배님, 영건오빠, 만철오빠, 문휴오빠,

용욱오빠, 철홍오빠, 맹진오빠, 상규오빠, 태형오빠, 현실언니, 차밀라니, 마하나마, 왕닝, 현성오빠, 학부 선배님들인 태원오빠, 주상오빠, 송헌오빠, 경주오빠 동기인 민주, 미란이, 후배인 혜영, 봉근, 완창에게도 그 동안 저에게 보내준 응원과 격려에 감사드립니다. 그리고 저의 사랑하는 친구이자 곁에서 언제나 마음 깊이 챙겨준 경임이와 석재, 친언니 같은 지은언니, 효림언니, 호숙언니, 사랑하는 친구들 수연이, 봉규, 승호, 종수, 영득이, 기정이, 현아, 사랑하는 동생들인 진성이, 성이, 소영이, 태경이, 인천에 있는 사랑하는 친구들인 이쁜 수연이, 일본에 있어 보고 싶은 미옥이, 열심히 공부 중인 미영이와 미경이, 천사 같은 간호사 지은이, 사랑하는 선아, 순정이, 영주, 도현이 엄마 선경이, 나에게 웃음을 주는 재선이와 진이, 언제나 사랑하는 정수, 윤미에게 감사한 마음을 전합니다. 또 많은 도움을 준 수의학과의 착하고 이쁜 인선언니, 해진언니, 은진이, 의과대학의 경아언니, 부산에 계시는 정원교 선배님과 김원석 선배님, 아쿠아 그린텍㈜의 제종선 전무님, 이정석 박사님에게도 그 마음을 전합니다.

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